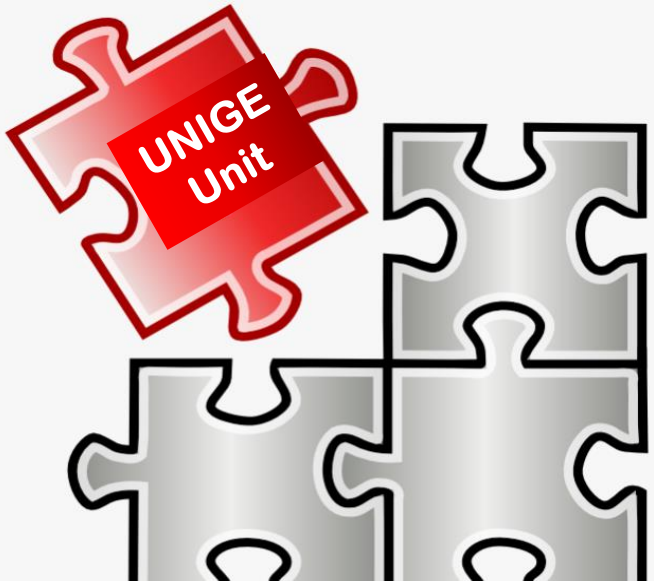
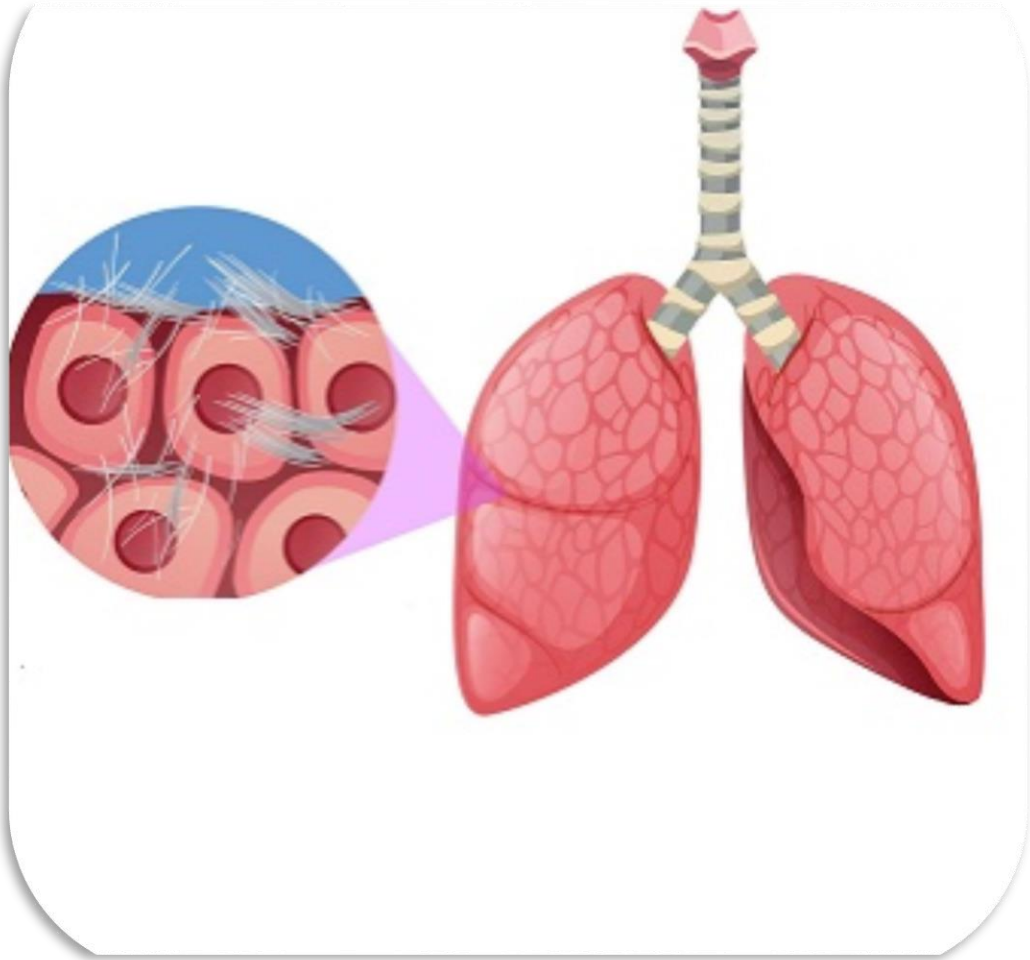
UNIVERSITÀ DEGLI STUDI
DI GENOVA



Progetto PRIN 20173X8WA4

FIBRES: a multidisciplinar minerealogic, crystal-chemical and biological project to amend the paradigm of toxicity and cancerogenicity of mineral fibres.

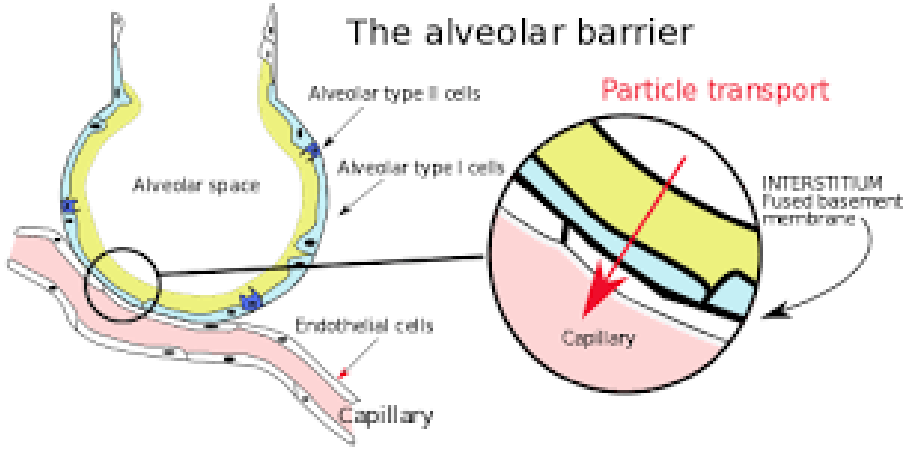


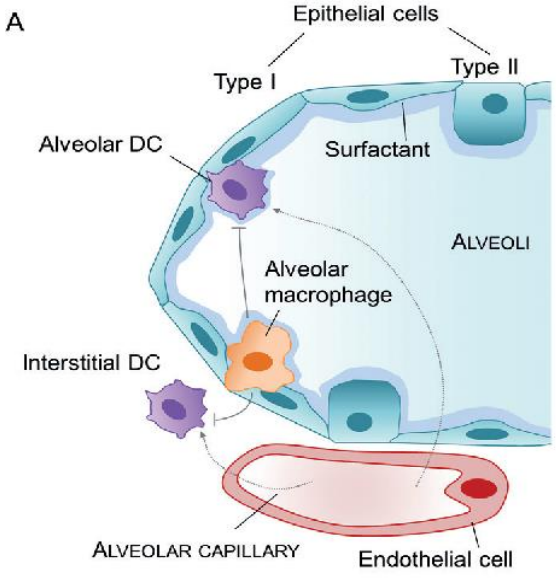
- **Experimental approaches 2019-2020**

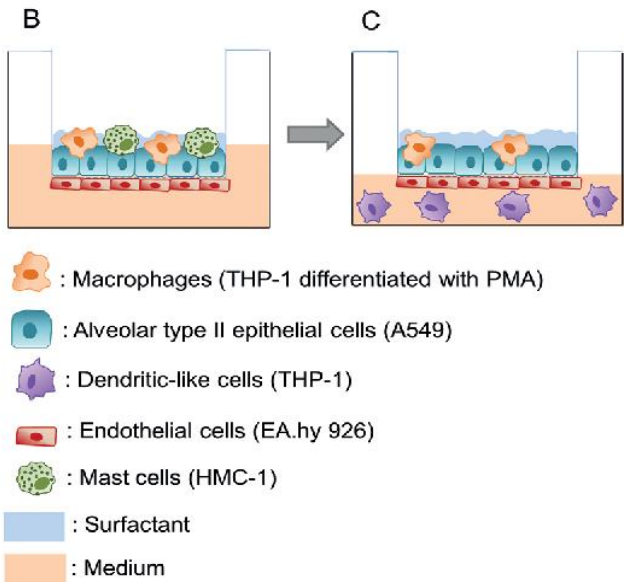
  Set-up of an *in vitro* 3D innovative lung model

  Preliminary evaluation of crocidolite, chrysotile and erionite fiber toxicity on human alveolar and monocyte cells

Proposed model: Co-culture of different phenotypic cells as representative of pulmonary model







"An *In Vitro* Coculture System for the Detection of Sensitization Following Aerosol Exposure" *(Chary et al. 2019)*

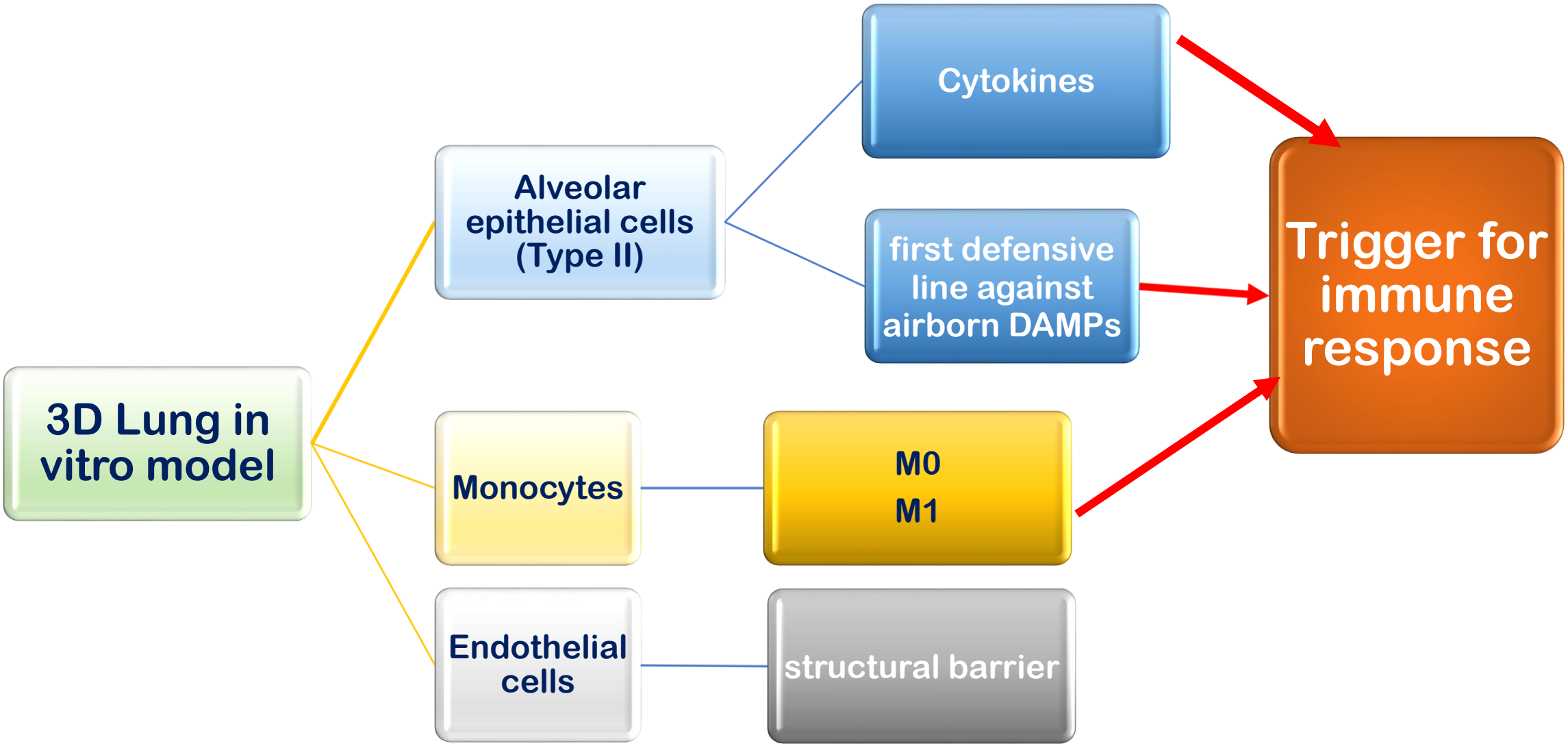
4 cell lines:

I. A549 human alveolar type II cells on the upper side of the membrane;

II. THP-1-macrophage differentiated cell associated with lung alveolar monolayer;

III. HECV human endothelial cell line on the bottom side of the membrane;

IV. THP-1 undifferentiated cells (suspension culture condition) in the basolateral chamber;


3D Lung in vitro model
Alveolar epithelial cells (Type II)
Cytokines
first defensive line against airborn DAMPs
Monocytes
M0
M1
Endothelial cells
structural barrier
Trigger for immune response

Set up of 3D advanced lung model

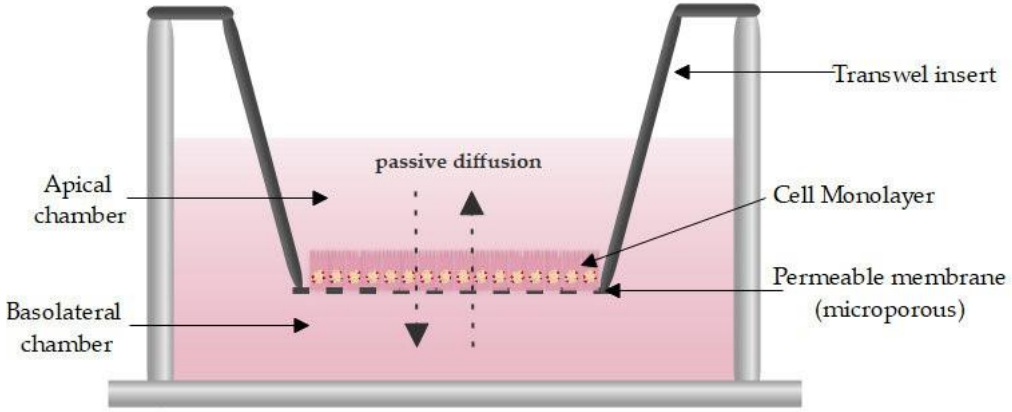
2D co-culture

(alveolar cells, endothelial cells and macrophages MØ)

on Transwell© insert

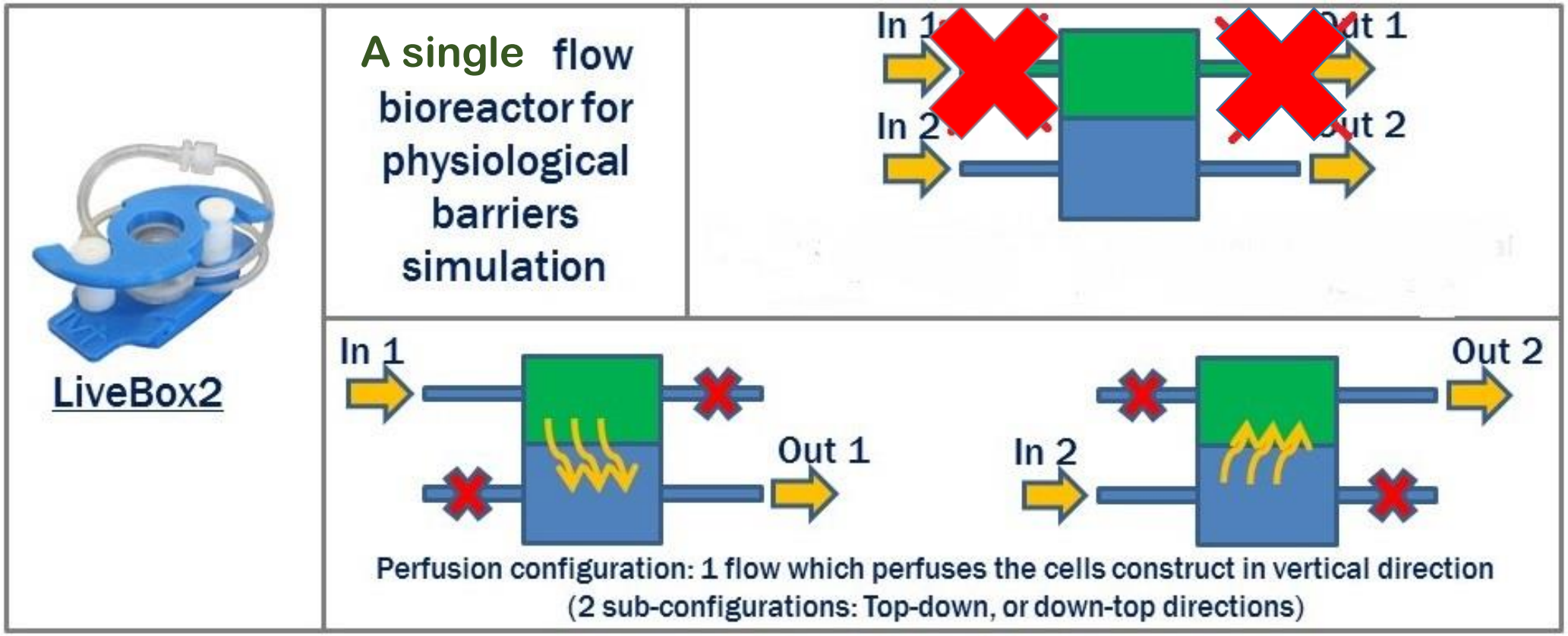
Medium flow in basolateral chamber (by peristaltic pump)

3D dynamic model



IvTech Bioreactors (Live Box 2)

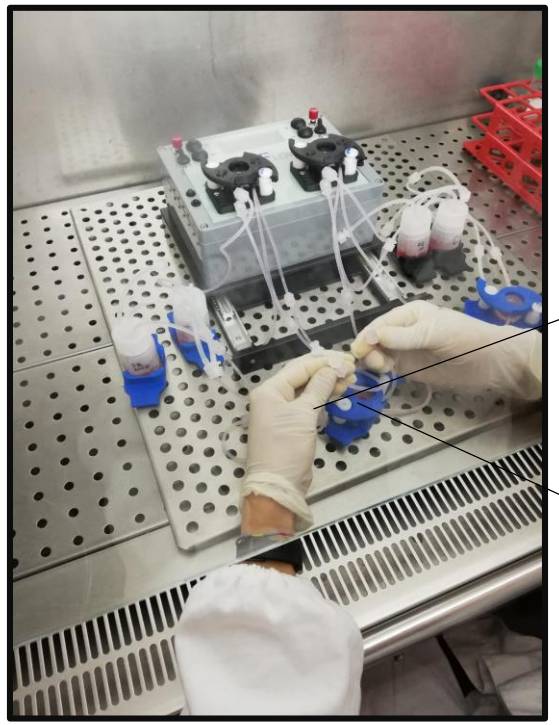
The flow in the apical chamber is not applied in the lung in vitro model, the bioreactor has an upper access which places the cells in direct contact with the air.



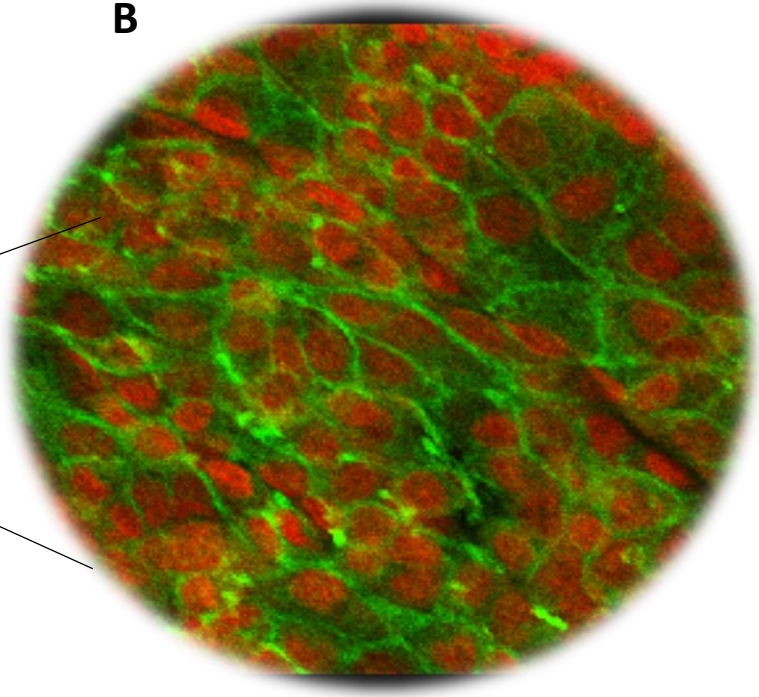
1st esperimental approach

Human endothelial cells were seeded in Matrigel™ Matrix™ on PET membrane placed in a LiveBox2 bioreactor

A



B



(A) the LB2 biorector connected to perystaltic pump.
(B) HECV endothelial cell line fluorescently labeled with To-ProTM -3 Iodide 642/641 (Nuclei), Phalloidin Alexa Fluor 488 (actin cytoskeleton).
The fluorescence signals were captured with 20X magnification, by Leica TSC SP microscope (Leica Microsystem, Wetzlar, Germany)

- Given the complexity of the system and the need of greater intercellular communication, seeding attempts are being made on a membrane without the coating which until now had not the expected results. The material of the seeding membrane is Polycarbonate or Polyester (PET).

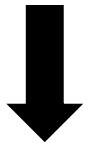
2nd experimental approach

From Lung A549 Adenocarcinoma cells to Alveolar Epithelia

Human A549 Lung Adenocarcinoma Cell Line in Air-Liquide Interface (ALI) culture shows an alveolar epithelial phenotype and several cell-specific markers.

Morphology

Cells in conventional submerged culture exhibits inerratic shapes



A549 cell cultures after 7-10 days in ALI (air liquid interface) display rough surfaces with multilamellar bodies. The cells were more compacted with clear junctions.

Differentiation cell-specific markers

Expression of cell-specific markers:

- **Aquaporin-5 protein** for Alveolar Epithelial Type I cells;
- **Surfactant Protein C** for Alveolar Epithelial Type II Cells.

Characterization of air-liquid interface culture of A549 alveolar epithelial cells, Wu et al. Braz. J Med Bio Res 2018; Air-liquid interface culture changes surface properties of A549 cells, Öhlinger et al., Toxicology in vitro, 2019;

A549 cell Morphology from conventional to ALI culture conditions

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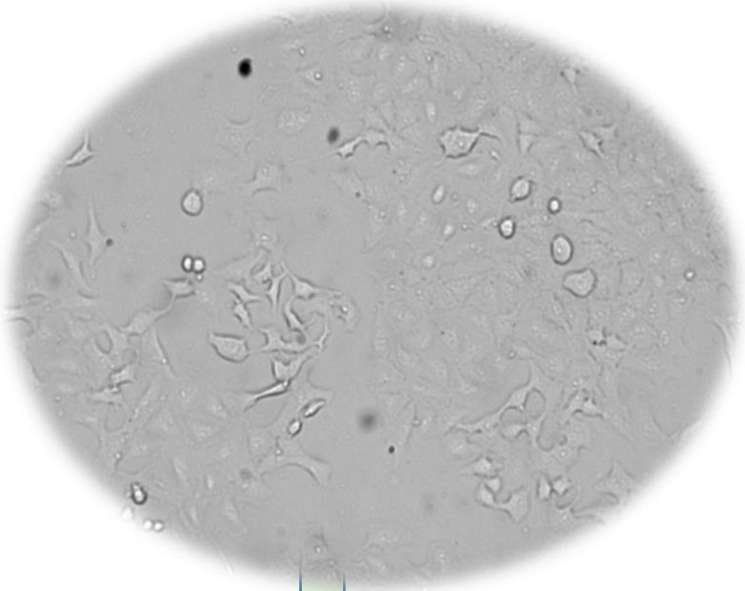
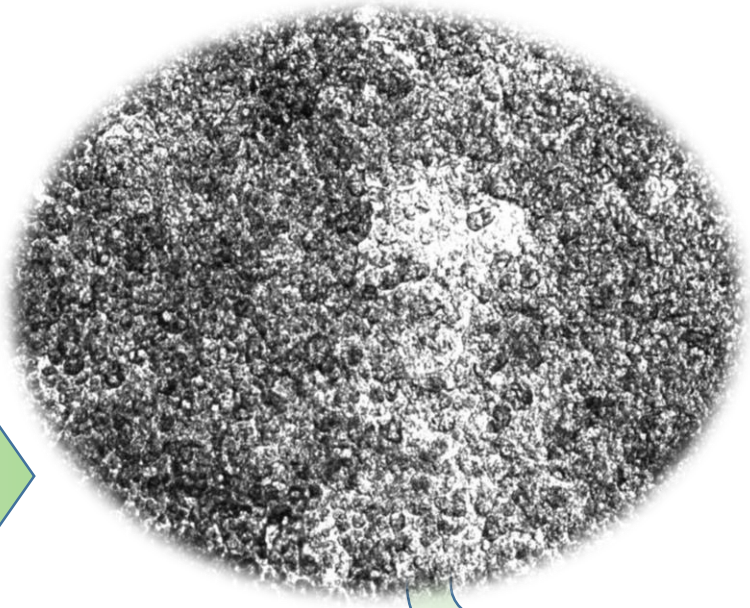
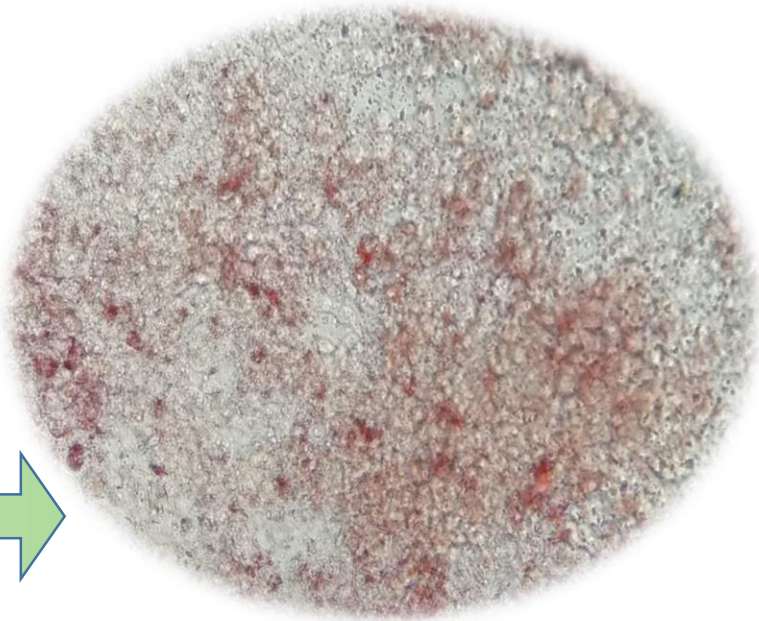


7 days ALI

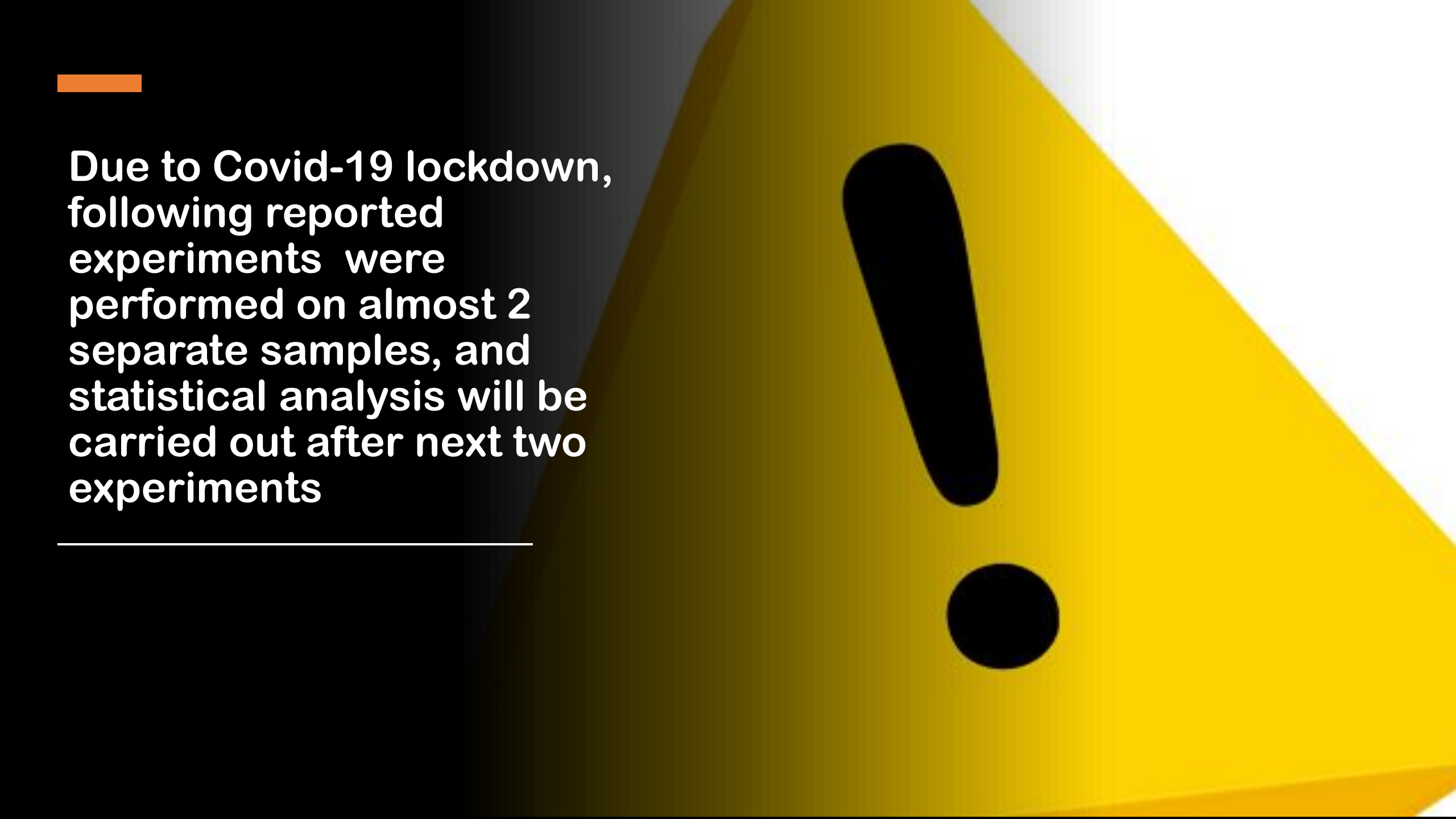


21 days ALI

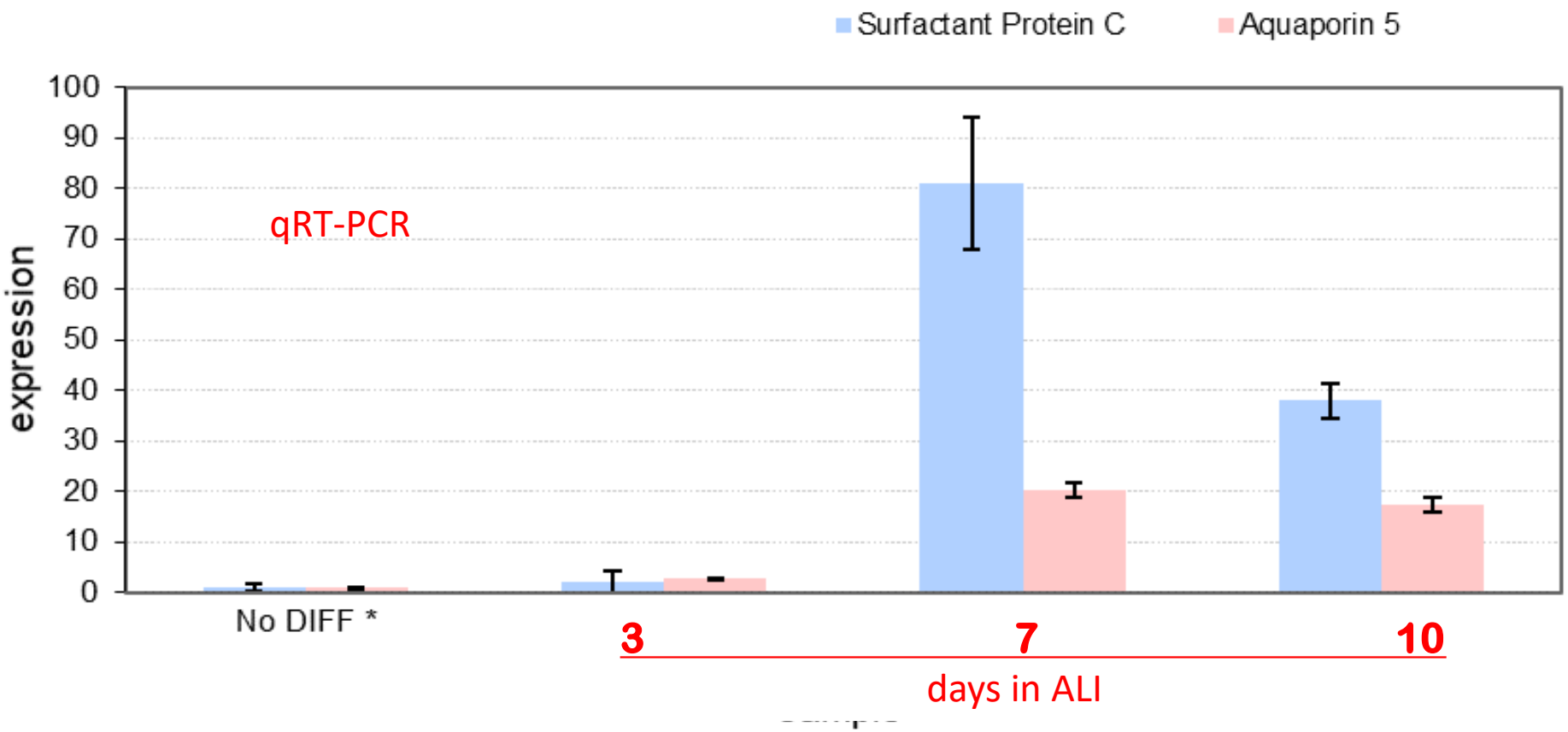


Lamellar Bodies stained in red with Oil-Red-O

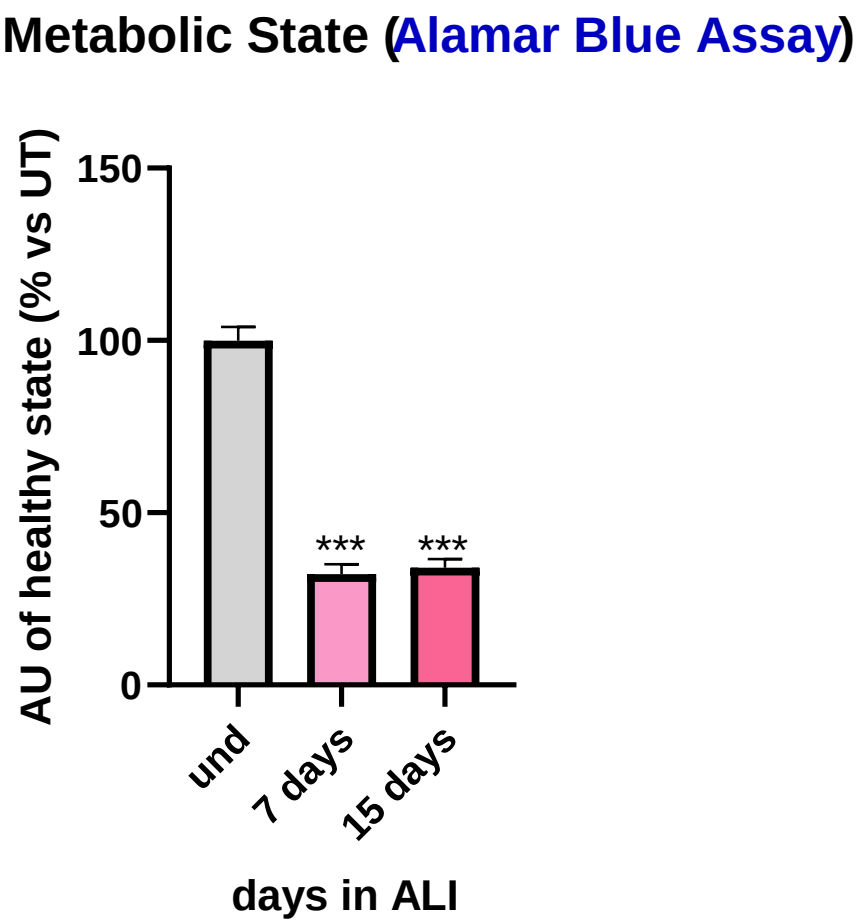
Due to Covid-19 lockdown, following reported experiments were performed on almost 2 separate samples, and statistical analysis will be carried out after next two experiments



Gene Expression specific differentiation markers in ALI culture



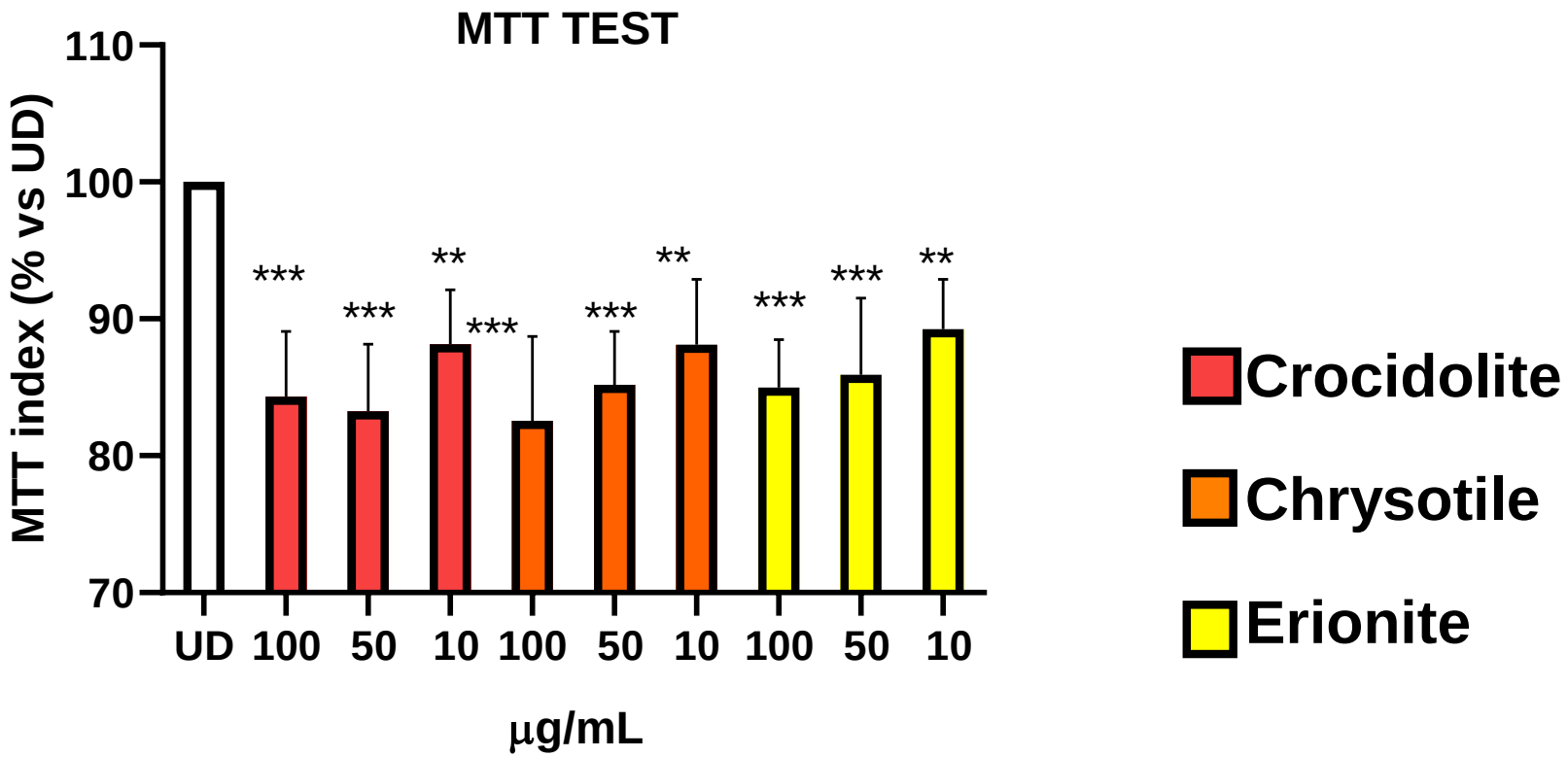
A549 Metabolic state



***p<0.0001. Ordinary One-way ANOVA

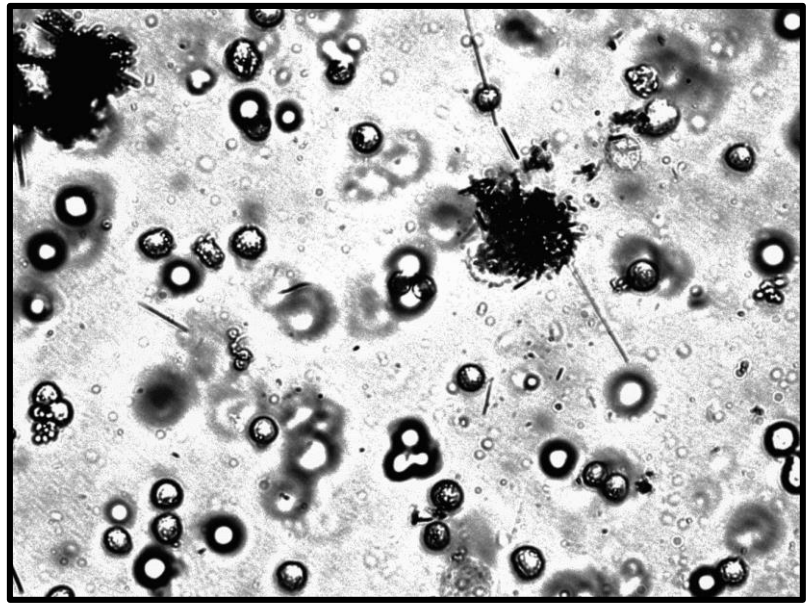
3rd experimental approach

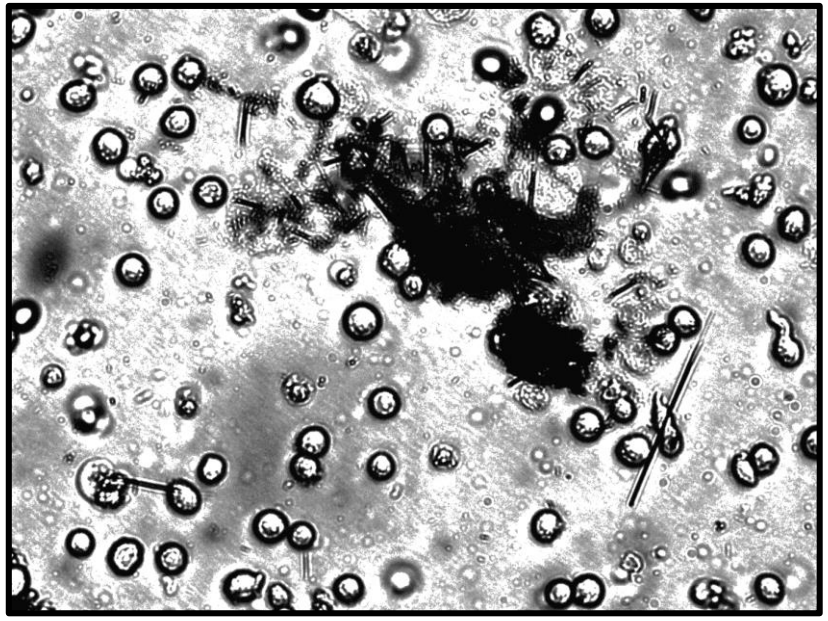
Undifferentiated A549 mitochondrial function after 24 h exposure to Crocidolite, Chrysotile and Erionite fibers (10 - 50 - 100 µg / ml)



***p<0.0001 vs respective untreated cultures (UD);
**p<0.01 vs respective untreated cultures (UD);
One-way ANOVA test

Undifferentiated THP-1 (human leukemia monocytic cell line) widely used as model of triggered activation of immune system towards exogenous agents.

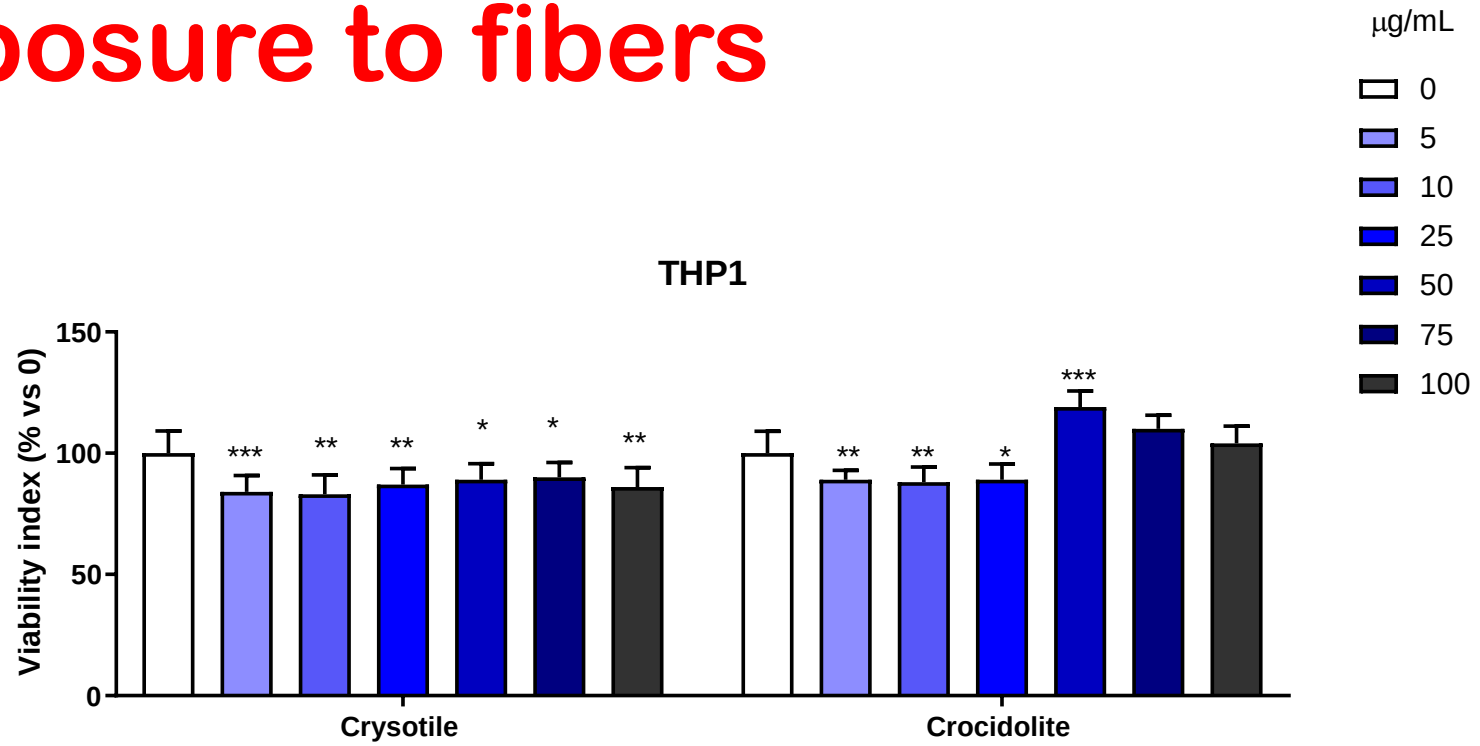
1. 

2. 

Morphology of THP-1 after 24h exposure to 100 µg/mL of crocidolite fibers. The activation and response of these cells in the presence of crocidolite fiber in the culture medium is visible after a few hours

The cells gathered pseudopodia around the fibers (visibles in photos 1 and 2), some of which are visibly larger.

Mitochondrial activity in THP-1 monocytes afte 24h exposure to fibers



***p<0.0001 vs respective untreated cultures (UT);
**p<0.01 vs respective untreated cultures (UT);
*p<0.05 vs respective untreated cultures (UT);
One-way ANOVA test

The mitochondrial activity of THP-1 was studied through the MTS test. Given the interference of the fibers with the absorbance of light used as the last step in the test, the THP-1 in suspension were placed in indirect contact with the fibers in the cell medium. The activation of the treated THP-1 was observed at higher concentrations (> 50 µg / mL), which showed higher mitochondrial activity than at lower concentrations.

Conclusion

- A549 cells were differentiated in alveolar epithelial cells after 7 days in ALI culture. In these conditions it was observed a decrease of viability/metabolic state compared to the control, and at 7° and 15° days the levels of this marker remain stable without affecting subsequent test results;

- The increase of mitochondrial activity of undifferentiated THP-1 shows an increase of MTT index, considered as energetic activity, during exposure to highest doses of fibers (50 and 100 μg/mL)
- The change of morphology, evidenced by their adhesion to plate suggests that THP1 cells are going to macrophage activated state;

Future experimetal approach

I. Protein Analysis by Western Blot to study genotoxicity of the fibers (H2AX and γ-H2AX), apoptotic cell death (PARP) and p53-mediated apoptosis (p53 phosphorylated and not);

II. Gene and Protein analysis of differentiated A549 after treatment with fibers : IL-1β, IL-8, IL-6, IL-18, TNF-α, MCP-1;

III. Gene analysis of diff. A549 in culture with conditioned medium of treated THP-1;

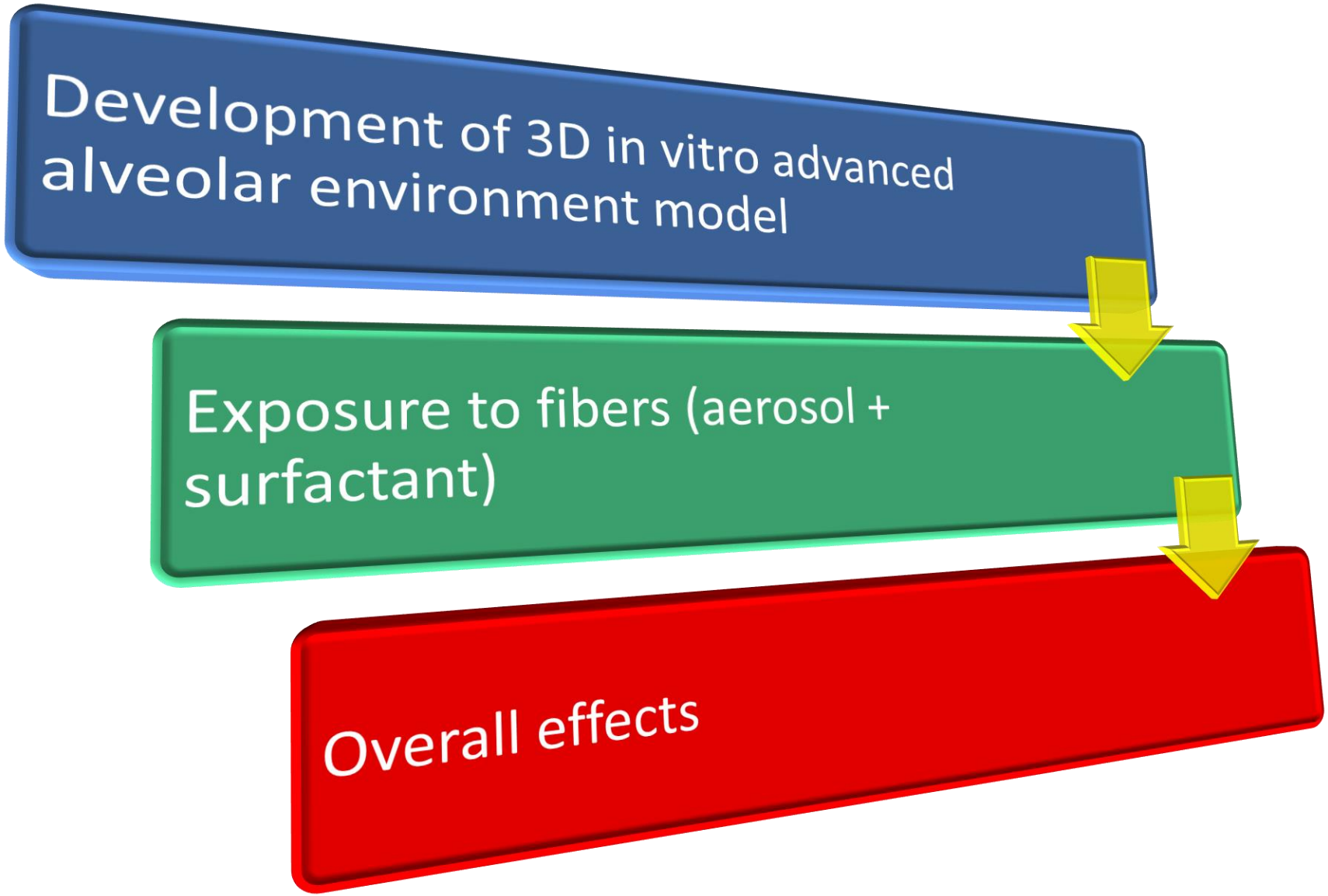
IV. Enzyme-linked immunosorbent assay (ELISA) of cell medium and Gene analysis of undifferentiated THP-1 treated with fibers;

V. New nebulizer device Aerogen Pro (Aerogen)

will be connected in the dynamic 3D lung *in vitro* model for a homogeneous and controlled distribution of the fibers on samples;

Future experimetal approaches

differentiated A-549 (D- A549)	• Toxicity of fibers : necrosis/apoptosis/ piroptosi / other ? • Analysis of pro-inflammatory Cytokine/chemokines
endothelial cells	• Toxicity of fibers : necrosis/apoptosis/ piroptosi / other ? Analysis of pro-inflammatory Cytokine/chemokines
Co-culture of D-A549 (+ fibers) and endothelial cells	• Analysis of each other's effects
Co-culture of M0, M1 M2 THP-1 (+ fibers) and endothelial cells	•Analysis of each other's effects
THP1 + artificial surfactant + fibers	• Survuval analys
Co-culture of D-A549 + M0, M1 M2 (+ fibers)	•Analysis of each other's effects


Development of 3D in vitro advanced alveolar environment model
Exposure to fibers (aerosol + surfactant)
Overall effects