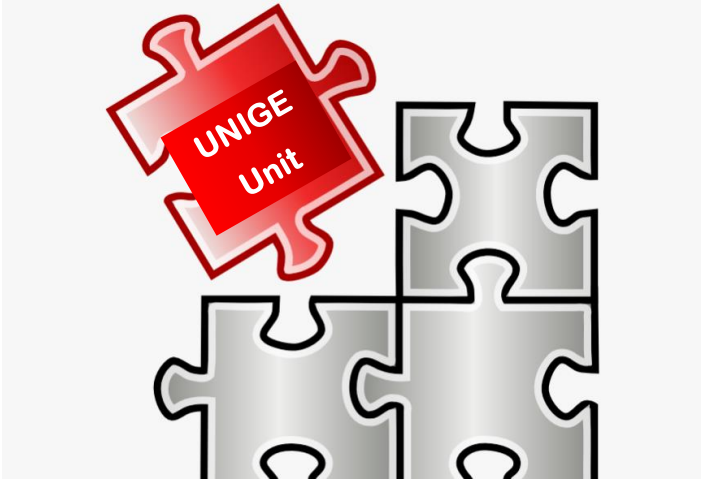


PRIN Project Prot. 20173X8WA4

FIBRES: a multidisciplinary *minerealogical*, *crystal-chemical* and *biological* project to amend the paradigm of toxicity and cancerogenicity of mineral fibers.

Mineral Fibres:

CROCIDOLITE

(UICC STANDARD)

CHRYSOTILE

(from Russia, « Yasnyi»)

WOLLASTONITE NYAD G

from Willsboro-Lewis (NY, USA)

Citotoxicity Assessment on different *in vitro* models:

➢Cell monocultures:

I. Human monocyte and differentiated macrophage cells **THP-1;**

II. Human Endothelial cells **HECV**

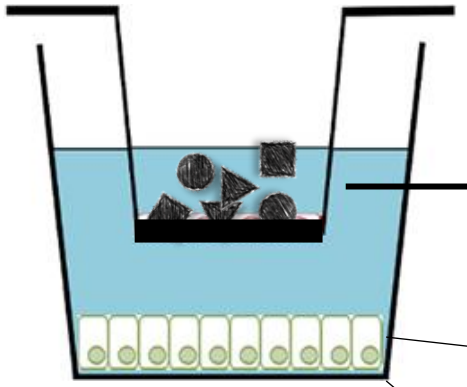
→ **Cell Coculture HECV + THP-1 M0**

III. Human Foreskin Fetal Fibroblasts **HFFF2**

➢3D Tissue Model **Epiairway™** (Mattek Corporation)

➢Permeability test on a synthetic pulmonary mucus **L-Mu³Gel** (Bac3Gel®Lda)

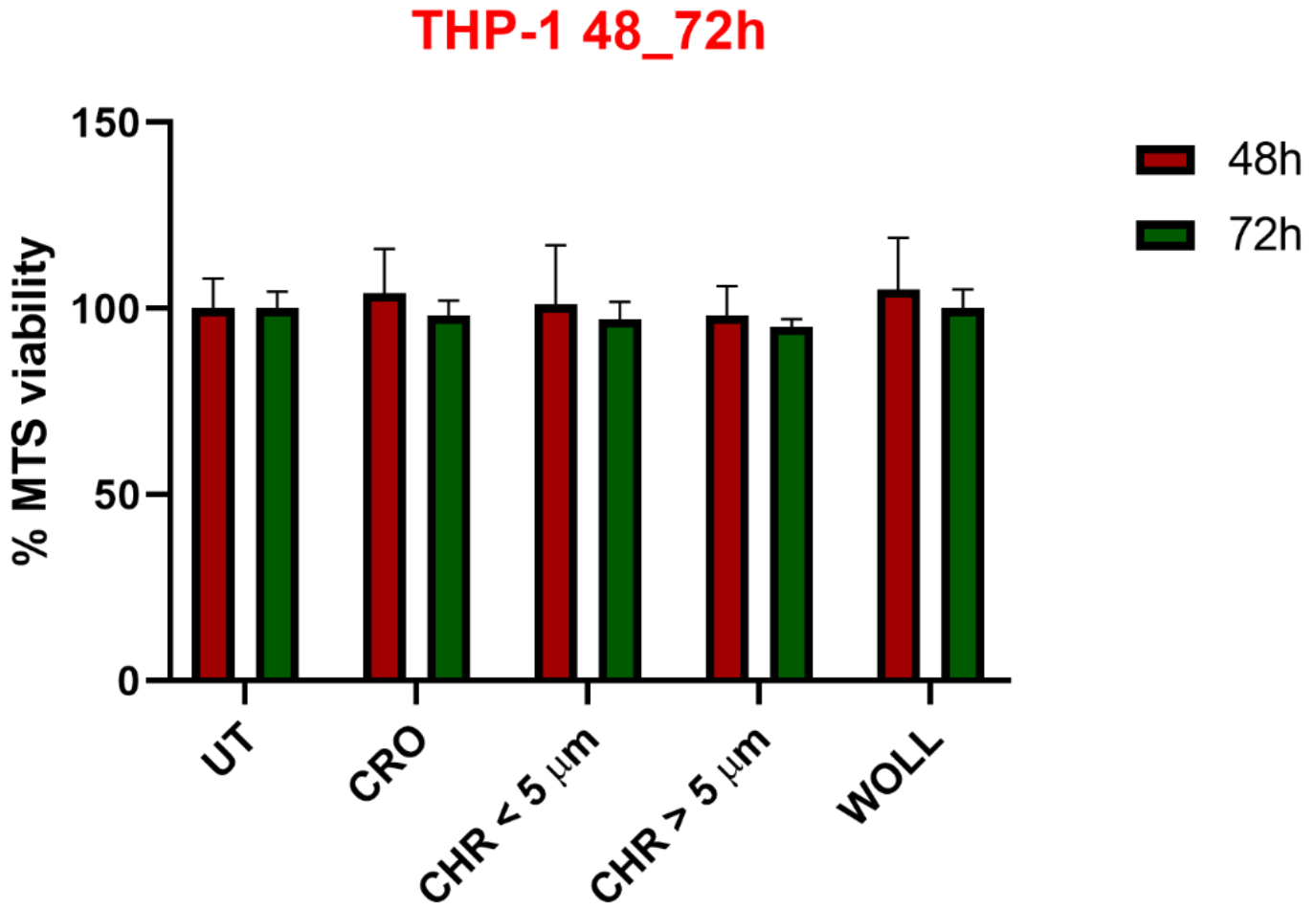
Set-up of naive **THP-1** colture in indirect exposure to fibres



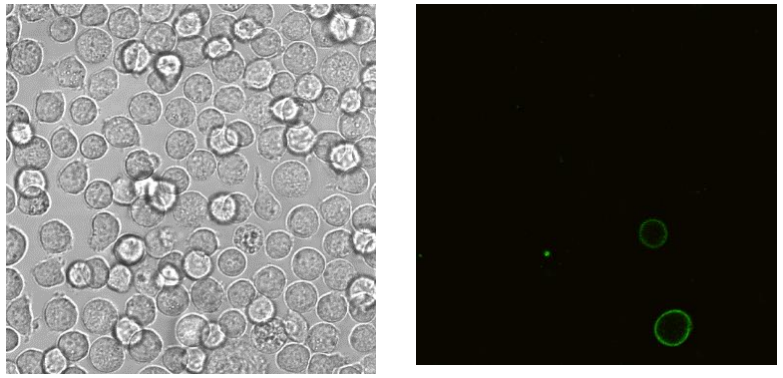
Mineral Fibers (50 μg/ml)
inside Thincert® Insert equipped with a porous membrane (0.4 μm)

THP-1 cells

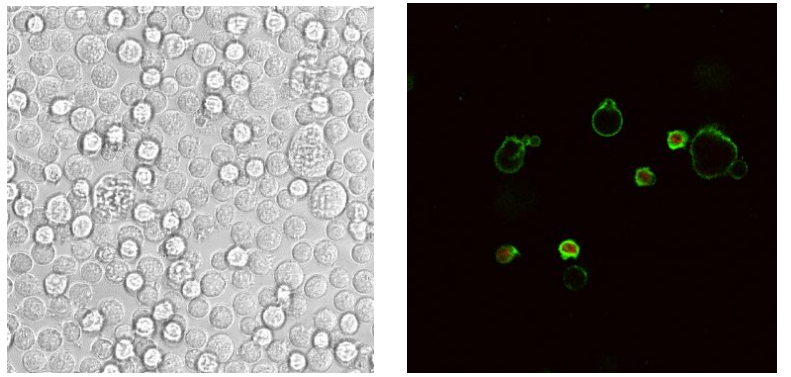
MTS viability test at 48 and 72 hrs



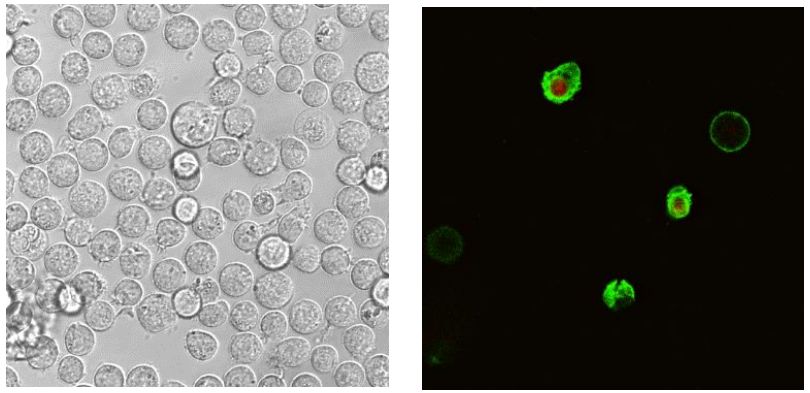
Evaluation of early and late Apoptosis Cell Death after 48 hrs of fiber treatments (50 μg/ml)



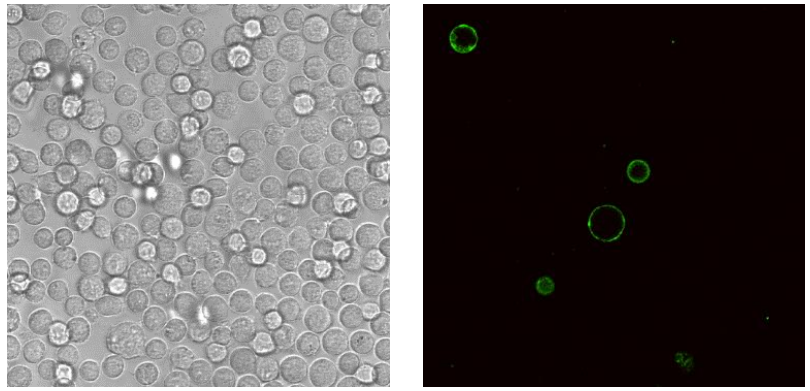
Magnification 20x; **Untreated**



Magnification 20x; **Chrysotile > 5 μm**

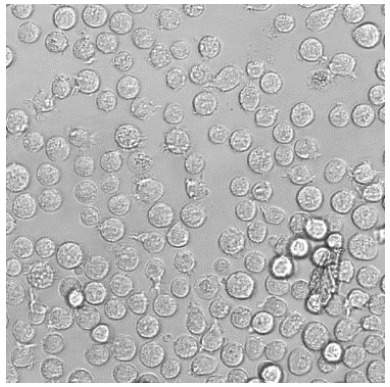


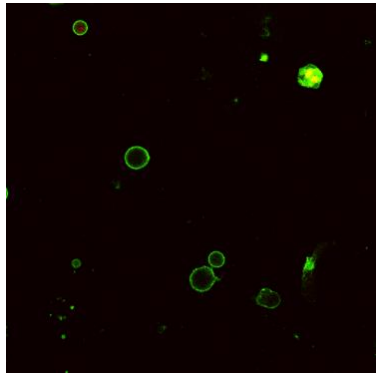
Magnification 20x; **Chrysotile < 5 μm**



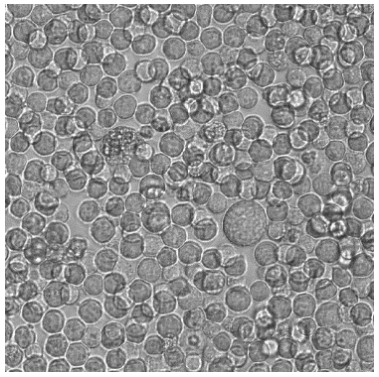
Magnification 20x; **Wollastonite**

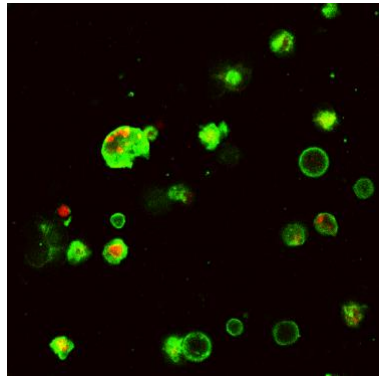
Evaluation of early and late Apoptosis Cell Death after 72 hrs of fiber treatments (50 μg/ml)



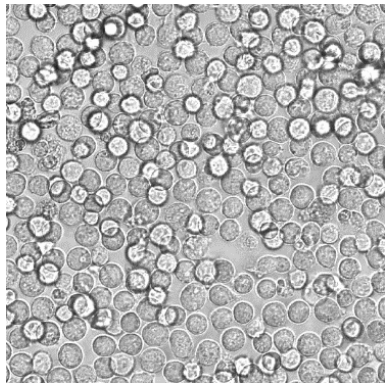


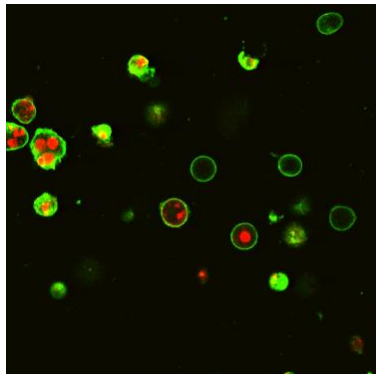
Magnification 20x; **Untreated**



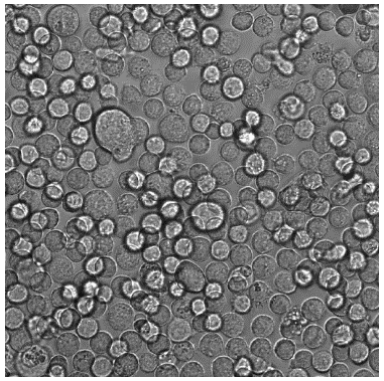


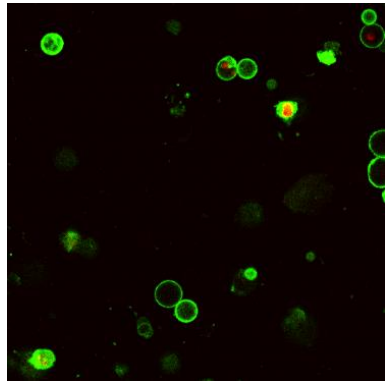
Magnification 20x; **Chrysotile > 5 μm**





Magnification 20x; **Chrysotile < 5 μm**

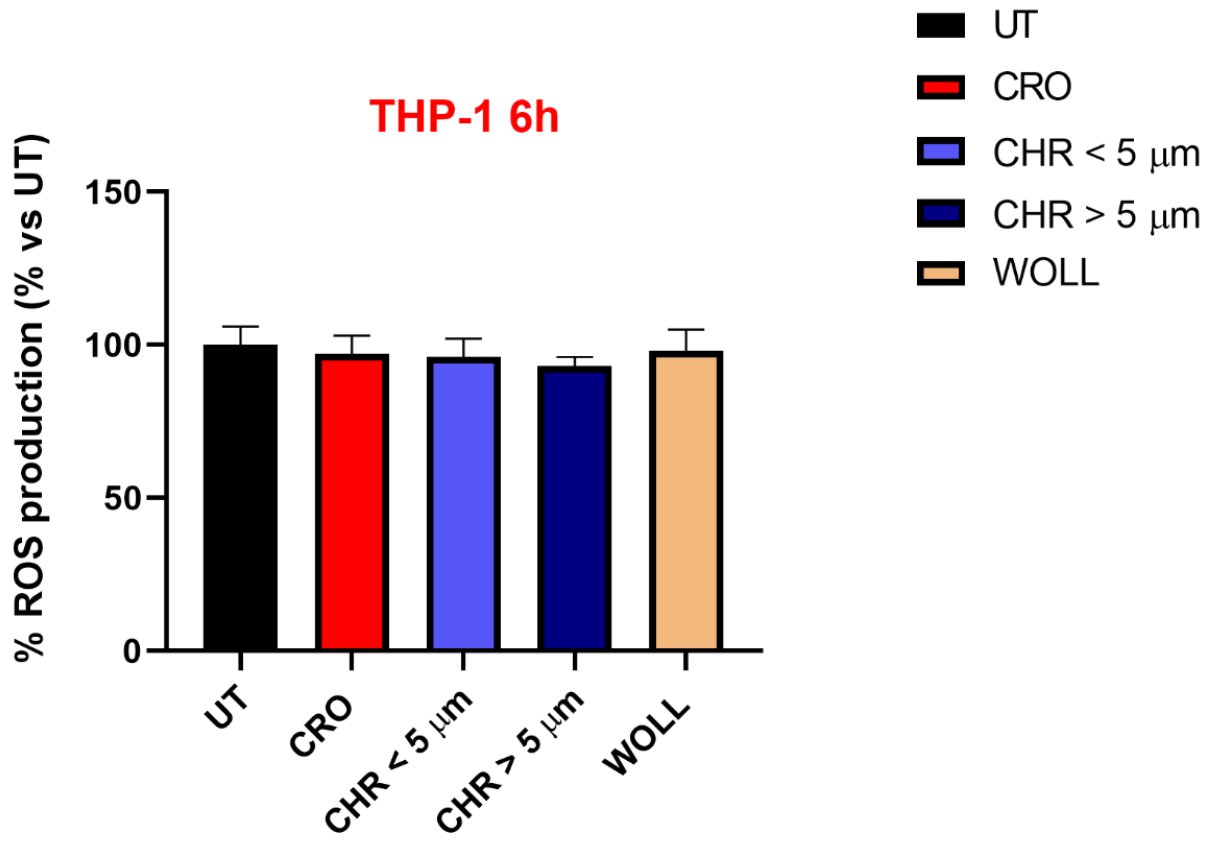




Magnification 20x; **Wollastonite**

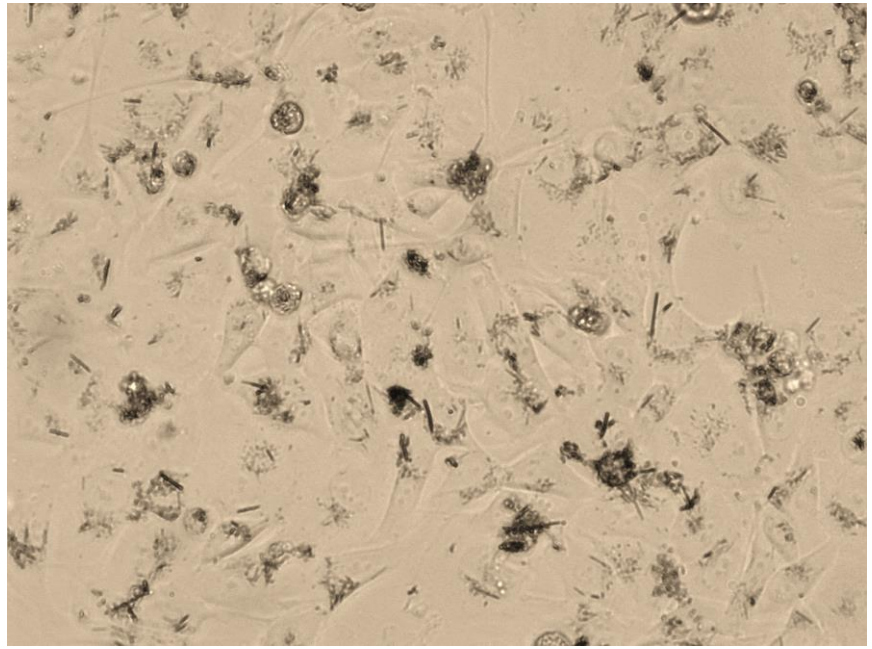
Oxidative stress Analysis

ROS levels after 6 hrs exposure at 50 μg/ml

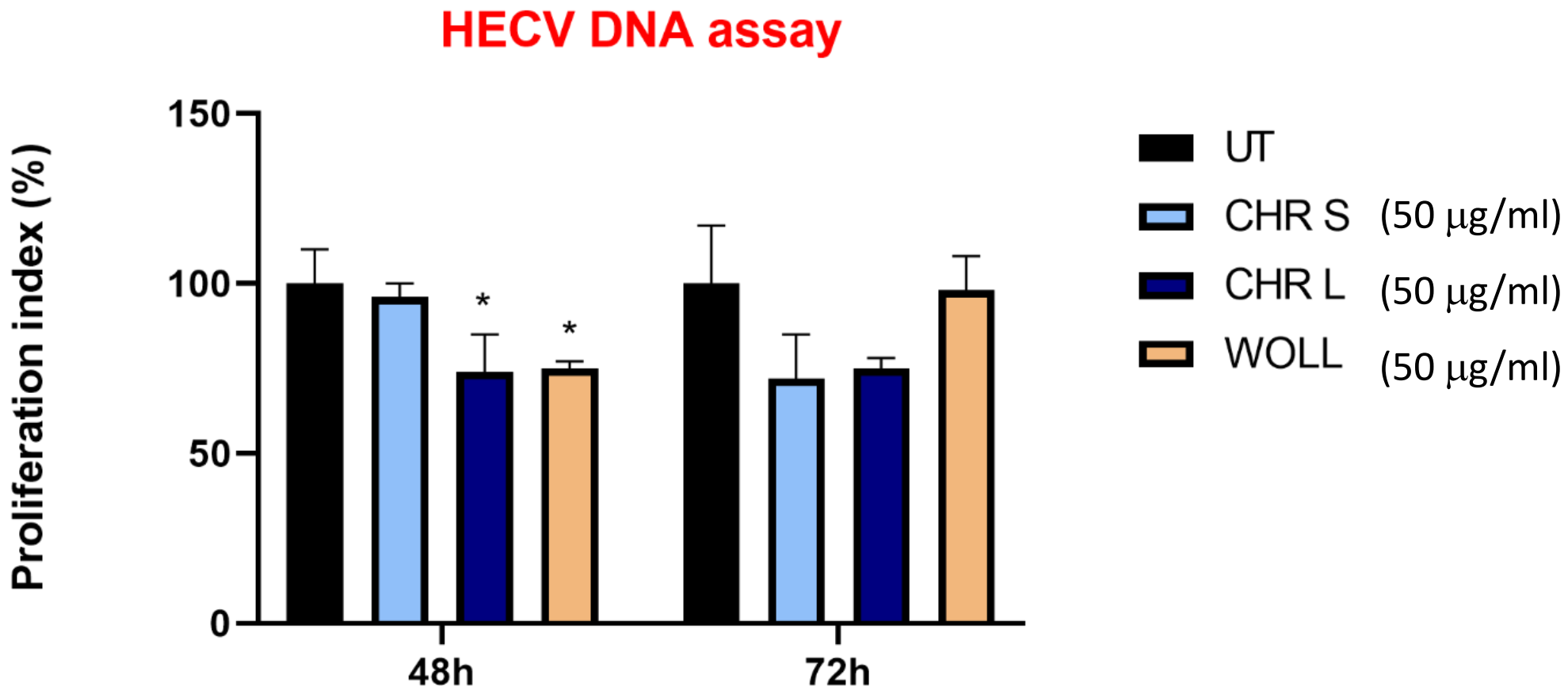


HECV

Directly exposed to the fibres

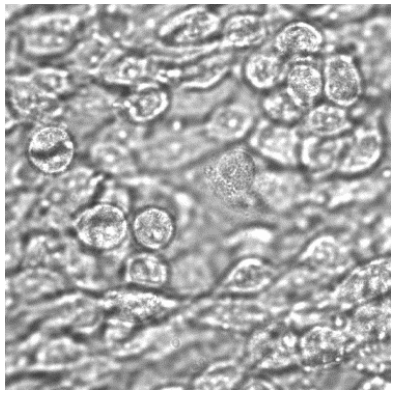


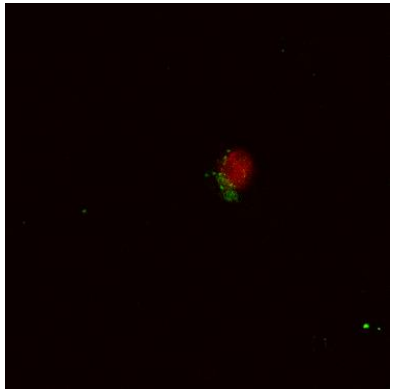
Assessment of HECV viability by DNA Assay



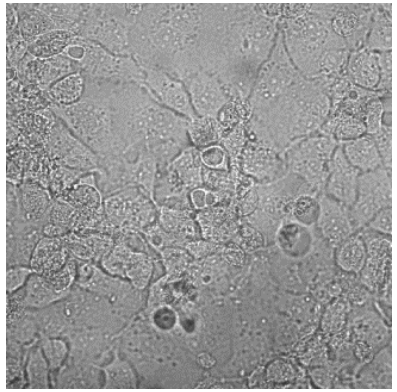
*p< 0.05 vs UT;

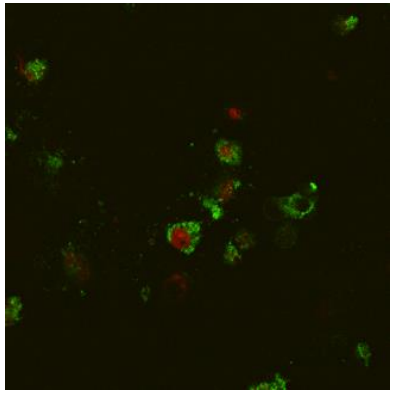
Evaluation of early and late Apoptosis Cell Death after 48 hrs of fiber treatments (50 µg/ml)



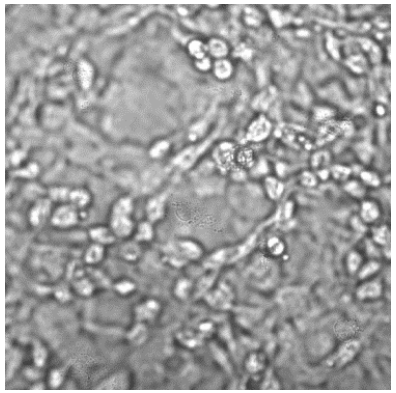


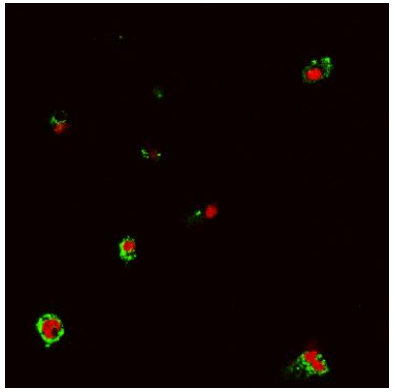
Magnification 60x; **Untreated**



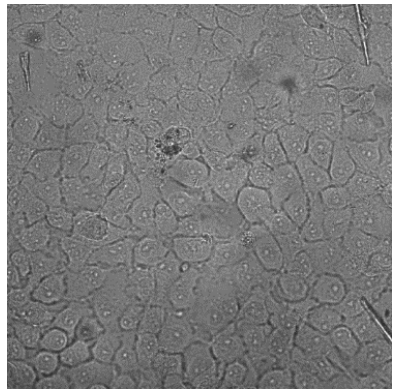


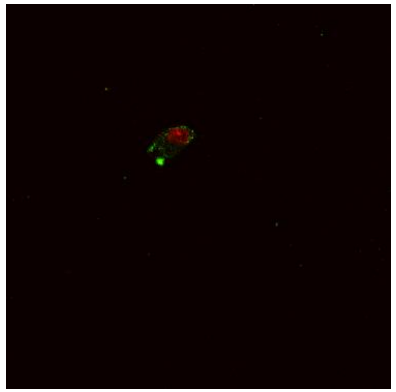
Magnification 60x; **Chrysotile > 5 µm**





Magnification 60x; **Chrysotile < 5 µm**





Magnification 60x; **Wollastonite**

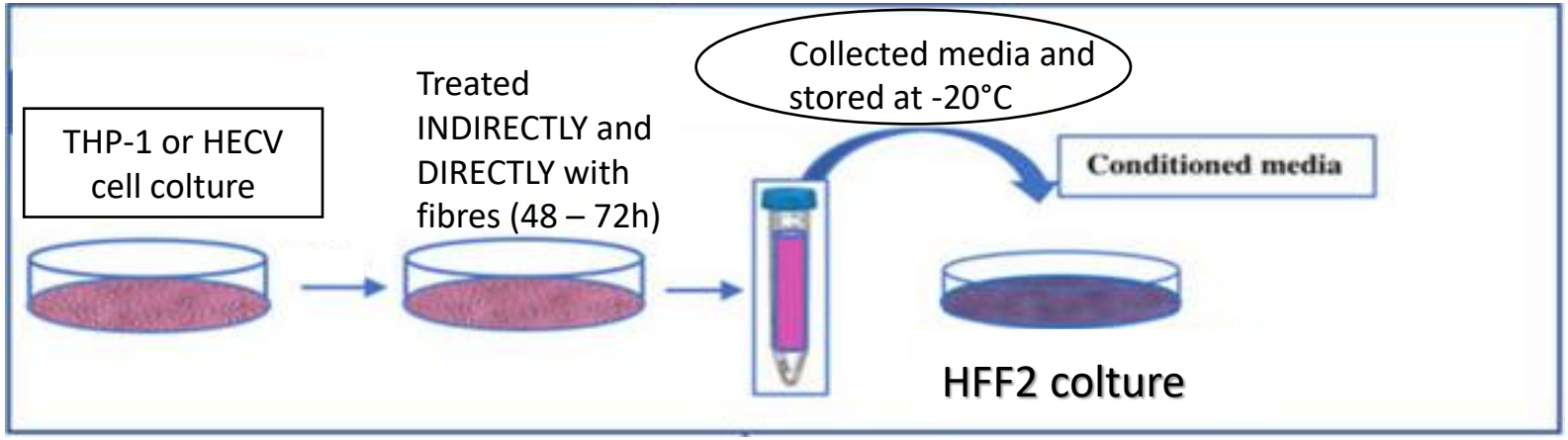
Remarks.1

I. In THP-1 cells mechanisms of programmed death have been activated after 48 and 72 hrs. Although the ROS increase is not significant, a marked signal of DNA damage caused by both CHRs (< 5 and > 5 μm) was observed.

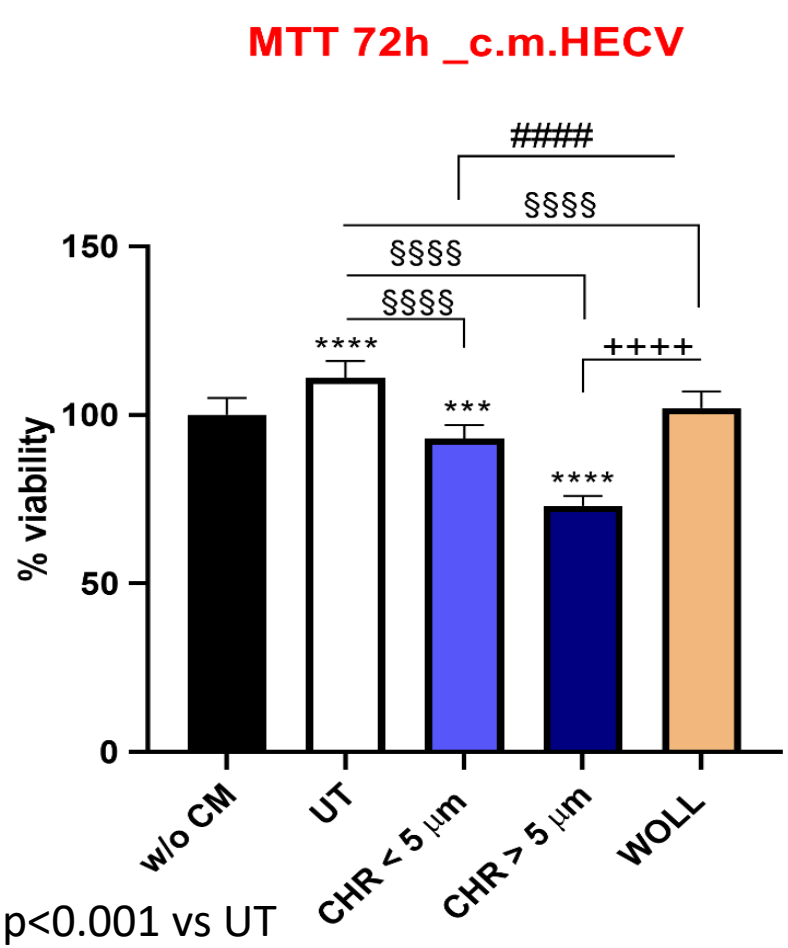
II. The HECV cell viability decreased after 24 and 48 hrs in particular those treated with CHR> 5μm. In fact, as shown by LDH and Annexin/PI tests CHR > 5 μm leads to HECV cells death after 48 hrs. The H2AX protein phosphorylation was observed after 24 hrs of CHR > 5 μm treatment related to an increase of oxidative stress.

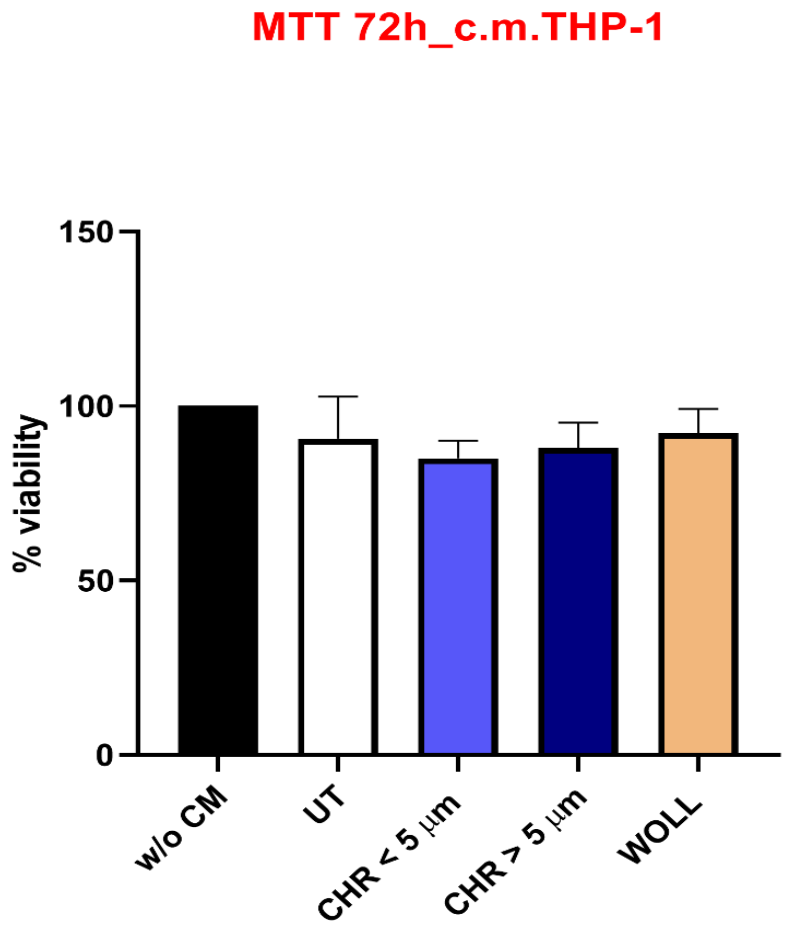
HFF2

The human fibroblast line HFF2 was treated with HECV and THP-1 conditioned medium (CM) both subjected to previous treatments with mineral fibres, in order to evaluate the potential modulation and triggering of the inflammation in the cell layers involved in the carcinogenic process.
In this regard the mitochondrial activity of fibroblasts was evaluated after 72 hrs of treatments with CM by MTT test and staining with fluorescent dye JC-1.



Evaluation of HFF2 mitochondrial activity after 72 hrs exposure to HECV and THP-1 conditioned cell medium treatment

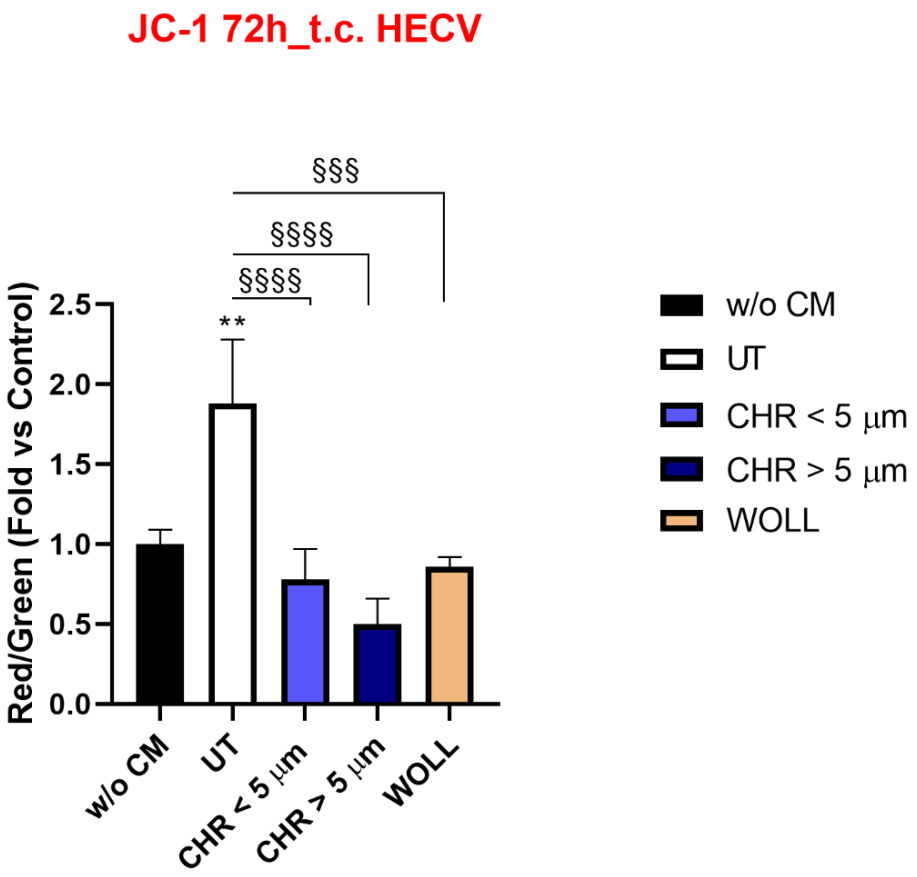


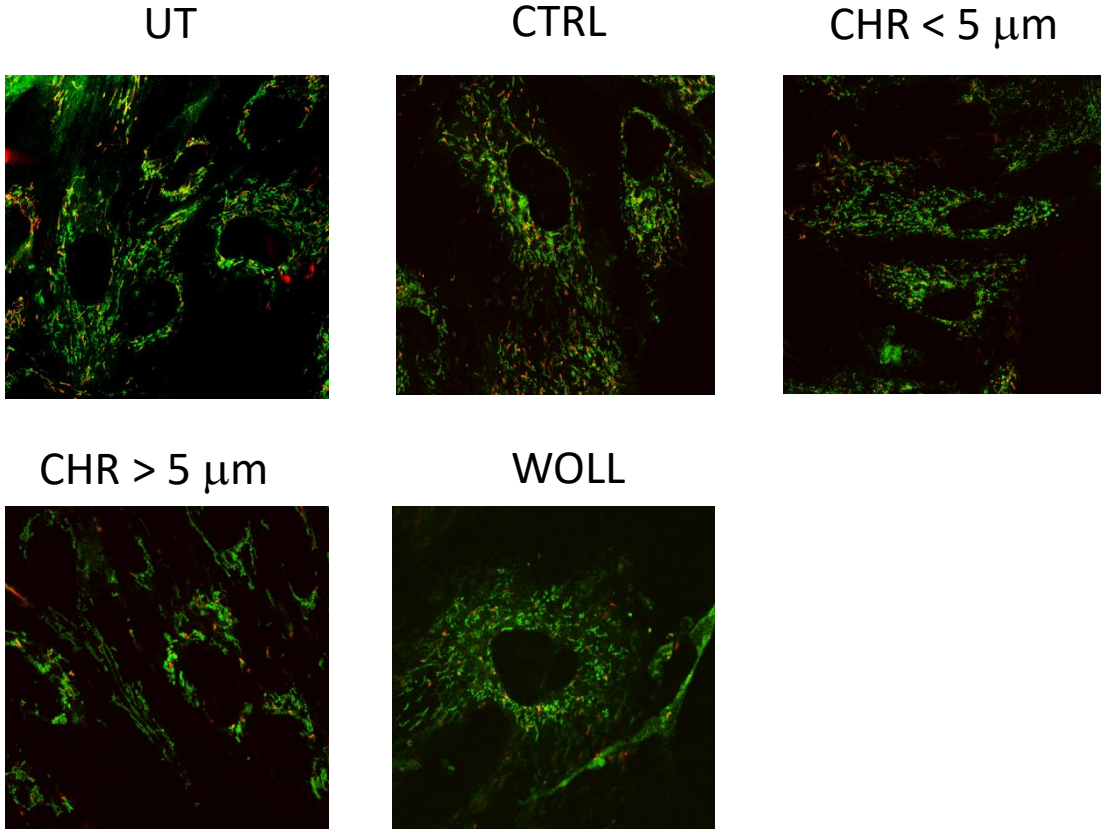


****p<0.0001, ***p<0.001 vs UT

§§§§p<0.0001, ++++p<0.0001, ####p<0.0001 vs treated cells

JC-1 Protein Analysis by Confocal Microscopy



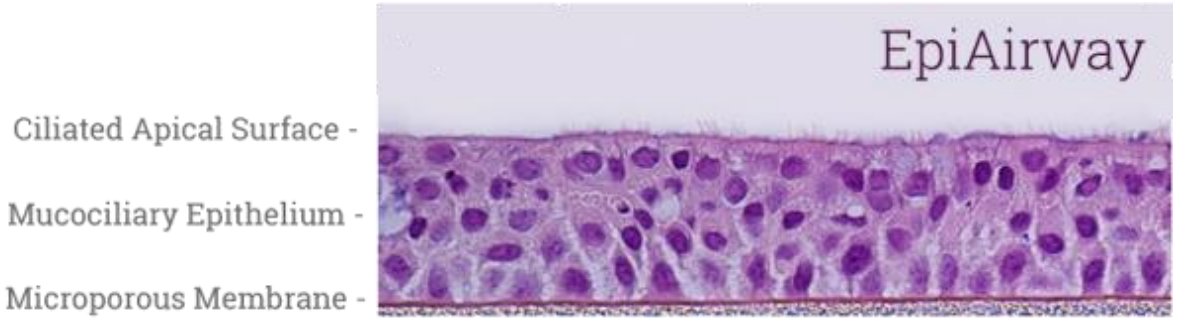


**p<0.01 vs UT; §§§§p<0.0001; §§§p<0.001 vs treated cells

Remarks.3

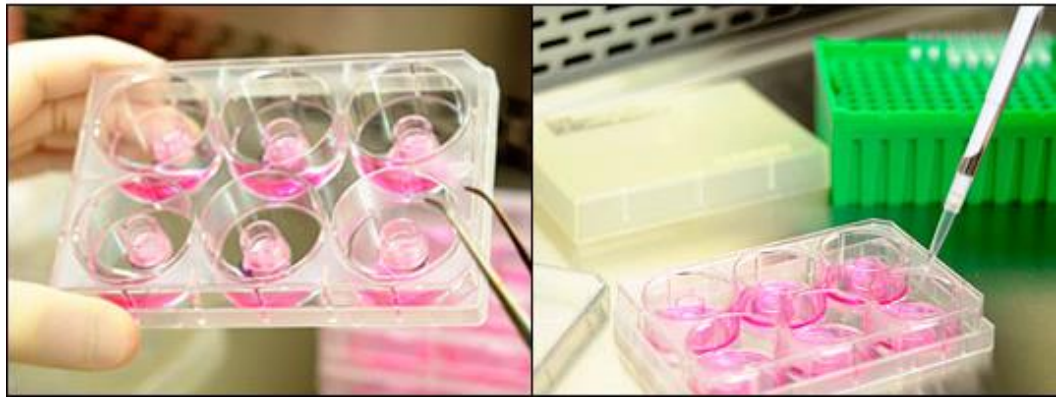
Fiber-treated HECV CM affected the HFFF2 viability and the mitochondrial health. As indicated by confocal microscopy JC-1, producing red fluorescent «J-aggregates», at lower internal mitochondrial concentrations or low membrane potential is present as monomers, i.e. green J-monomers. This phenomenon is associated with programmed cell death (apoptosis).

3D Tissue Model EpiAirway™



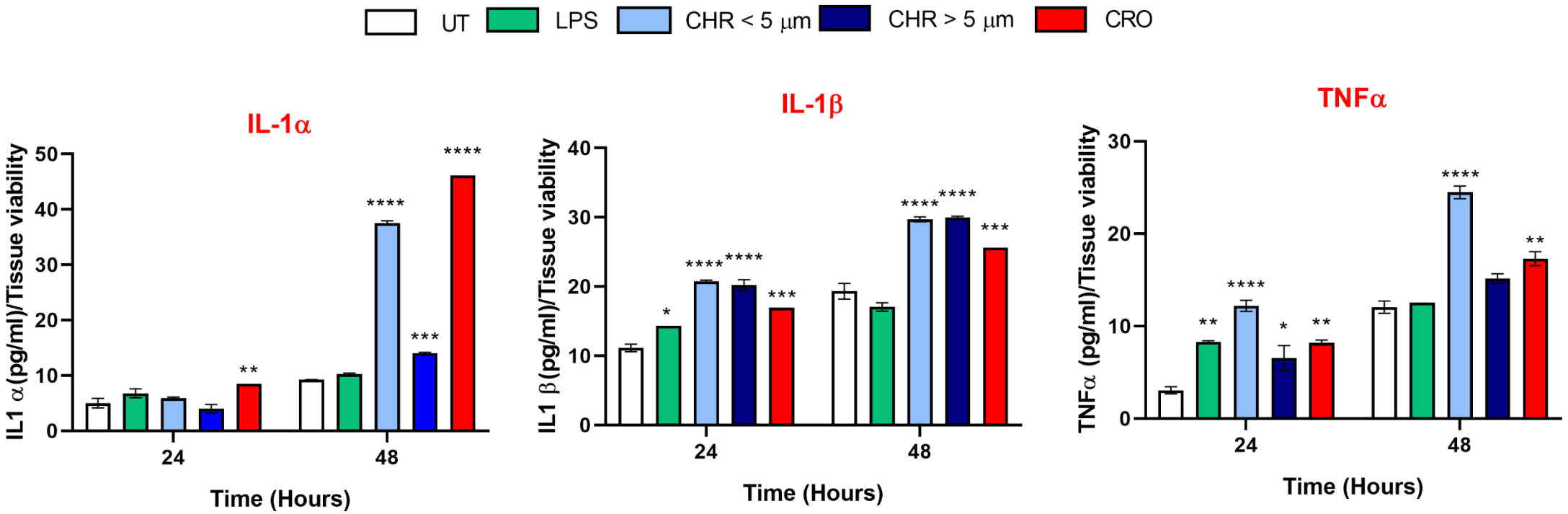
3D mucociliary tissue model consisting of normal, human-derived tracheal / bronchial epithelial cells (NHBE) from healthy / non-smoker donors.

Accumulated mucus by cells was removed from apical surface before the fiber treatments.



Cytokine Analysis of IL-1α, IL-1β, TNF-α in EpiAirway culture medium after 24 and 48 hrs

(MILLIPLEX® MAP system)

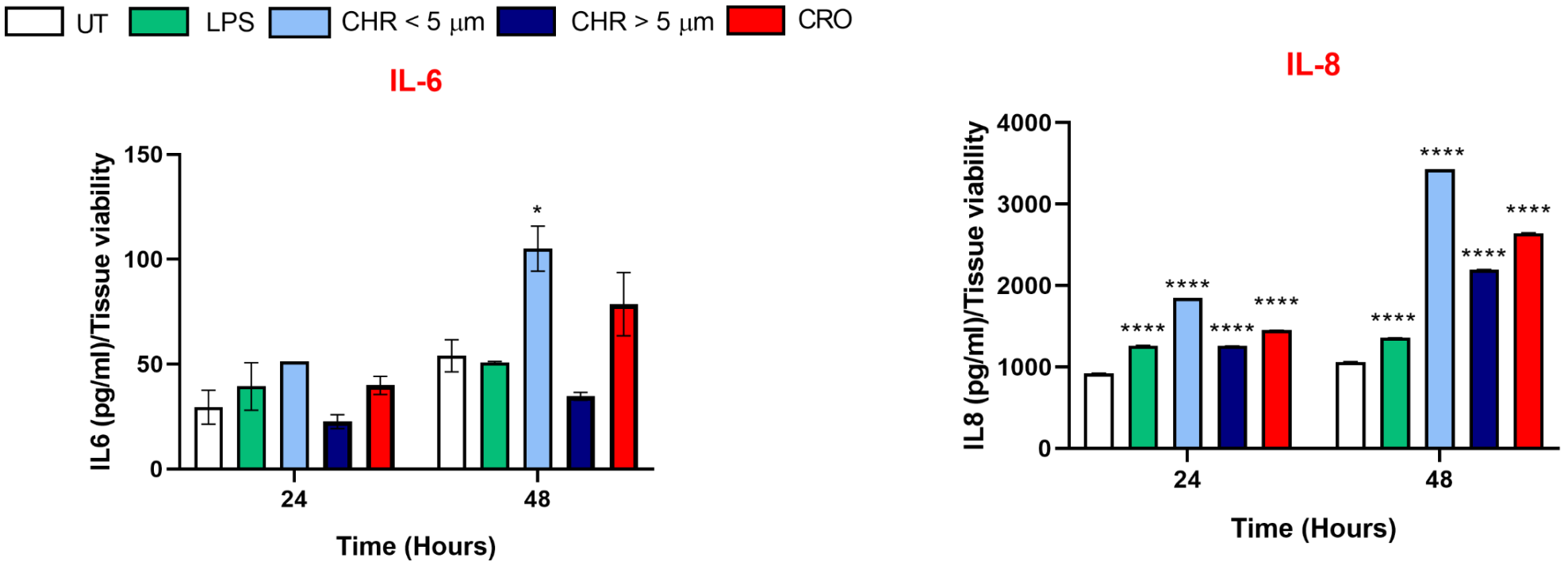


*p<0.05,**p<0.01,***p<0.001,****p<0.0001 vs UT

Cytokines were normalized to the percentage of viability measured by MTT test.

Cytokine Analysis of IL-6,IL-8 in EpiAirway colture medium after 24 and 48 hrs

(MILLIPLEX® MAP system)



*p<0.05,***p<0.001,****p<0.0001 vs UT

Cytokines were normalized to the percentage of viability measured by MTT test.

Remarks.4

Both CHRs decreased the Epiairway tissue viability after 24 hrs more than CRO. At 48 hrs CRO-treated tissue reached the same level of CHRs. As well as CHRs promoted a greater release of TNF – α, IL-1β, IL-6 and IL-8 cytokines especially after 48 hrs. A significative DNA damage was detected only in CHR > 5 – treated Epiairway.

Work in progress

Permeability test on synthetic pulmonary muci (Bac3Gel® Lda)

Mucus ~95% water

5% comprising electrolytes, lipids, DNA fragments, and proteins.

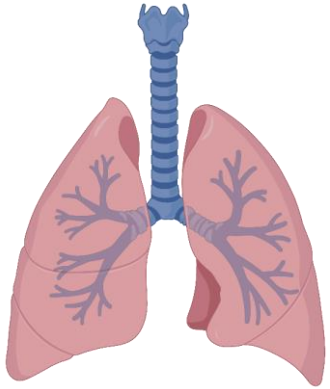
Mucins are HMW glycoproteins, consisting of a peptide backbone to which a huge amount of carbohydrate chains are attached. They permit the exchange of nutrients, water, gases, and hormones while being impermeable to most bacteria and pathogens[1].

L-Mu³Gel is a healthy lung mucus model with MUCIN capable of mimicking the steric and chemical barrier in physiologic conditions. (20 mg/ml porcine gastric mucin; 4.78 mg/ml sodium chloride)

CF-Mu³Gel is a pathologic lung mucus model with MUCIN capable of mimicking the steric and chemical barrier in pathologic conditions. (25.0 mg/ml porcine gastric mucin; 7.07 mg/ml sodium chloride)

[1] Atanasova K.R., Reznikov L.R. Strategies for measuring airway mucus and mucins; *Respiratory Research (2019) 20:261*

Mucus Mu^{3}Gel Model set-up

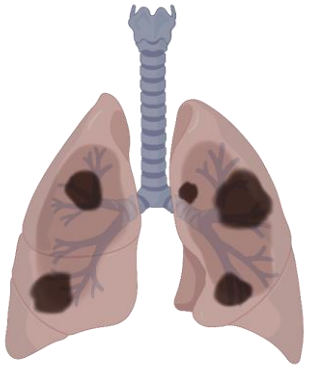


Thickness of airway mucus in **physiological** **conditions** = **10 µm = 0.010 mm**

given that the Transwell® support has a radius of 3.54 mm

Volume of L-Mu^{3}Gel to introduce on the Transwell® support = 0.390 mm^3 = 0.390 µl

We are not able to pipet this volume!

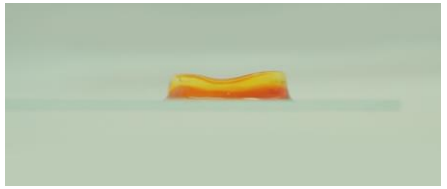


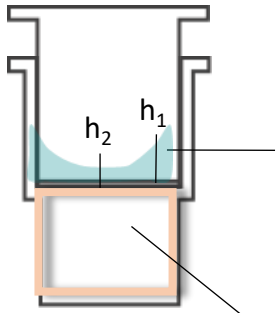
Thickness of airway mucus in **pathological** **conditions** = **220 µm = 0.220 mm**

given that the Transwell® support has a radius of 3.54 mm

Volume of Path,L-Mu^{3}Gel to introduce on the Transwell® support = 8.66 mm^3 = 8.66 µl ≅ 10 µl

In vitro set – up Mu³Gel Model





V = 30 µl

L-Mu³Gel® or

CF-Mu³Gel®

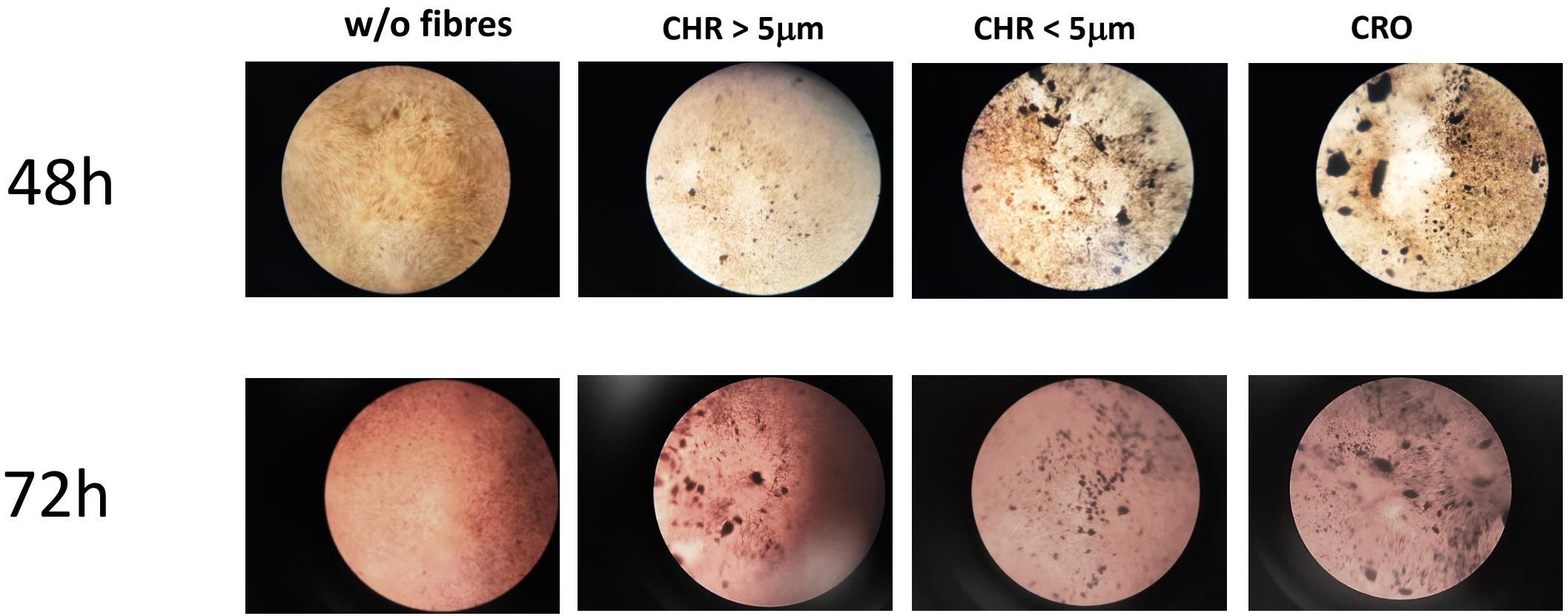
In the **absence of surface tension**, **30 µL** of L-Mu³Gel correspond to a thickness of **762 µm.**

Thincert® insert

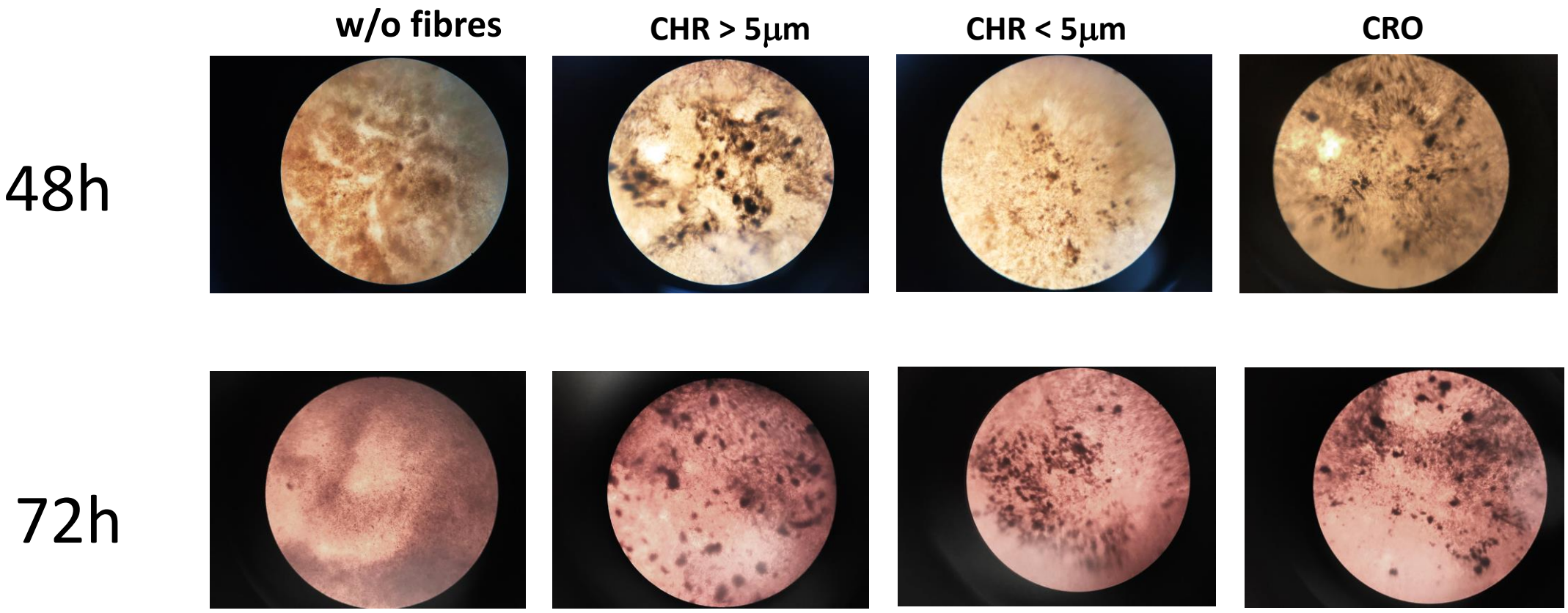
cell medium (RPMI)

The medium placed on the basolateral side of the Thincert® insert was collected and stored at -20°C to analyze the content of metal ions crossing the mucus layer by ICP-MS.

L-Mu3Gel – Healthy lung mucus



CF-Mu^{3}Gel – Pathologic lung mucus



Future plans

- To complete the analysis with the lung mucus Mu^{3}Gel:

I. Measure of metal ions content (by ICP-MS) in the collected medium by the basolateral side (*thanks to prof. Pacella, Università degli studi di Roma «La Sapienza»)*

II. Rheological Analysis by Frequency Sweep Test (a particularly useful test as it enables the viscoelastic properties of a sample to be determined as a function of timescale). Several parameters can be obtained, such as the Storage (Elastic) Modulus (G'), the Viscous (Loss) Modulus (G"), and the Complex Viscosity and stiffness (η*)
(*thanks to Daniela Pacheco, CEO of Bac3Gel*)

- Co-culture with the EpiAirway model will be set – up with naive and/or differentiated THP-1 to observe the inflammatory response in the lung tissue environment

Future plans

IARC point to be completed *by May 2023*:

- Point 6: the onset of chronic inflammation through long-term EpiAirway co-culture with monocyte / macrophage M0 THP-1, endothelium and fibroblasts
- Point 8: Analysis of receptor-signal transduction modifications. Possible targets for analysis: mesothelin, TGF-beta, HMGB1 / RAGE