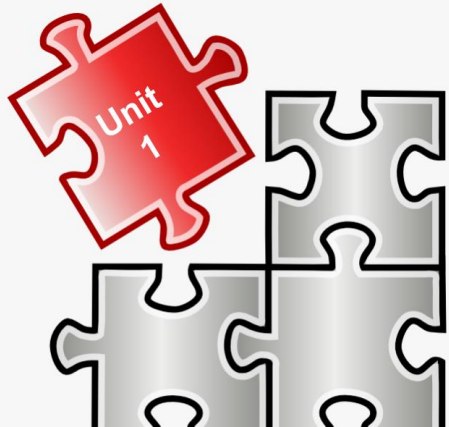


PRIN Project 20173X8WA4

FIBERS

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

Vanessa Almonti, Anna Maria Bassi, Barbara Marengo, Serena Mirata, Mario Passalacqua, Susanna Penco, Sonia Scarfì, Nicola Traverso, Stefania Vernazza



Experimental approaches 2020 -2021



CROCIDOLITE
(UICC STANDARD)



ERIONITE
(from NEVADA-USA)



CHRYSOTILE
(from Balangero, Torino, Italia)

In vitro models:

- Human monocyte – like cells THP-1 (in suspension)
- Activated monocytes (M0 – M1)
- Human Endothelial cells (HECV)
- Human adenocarcinoma alveolar epithelial cells (A549)
- Set up of in vitro models

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- **Toxicity index**

- **Proliferation index**

- **Pro-inflammatory potential:**

cytokine and chemokine gene/ protein levels

- **Genotoxic/oxidative damage:**

gene and protein levels of related biomarkers

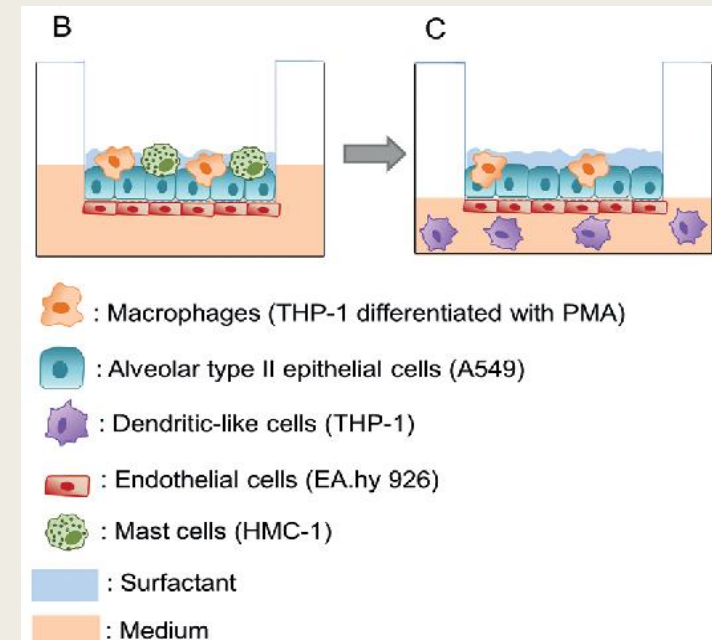
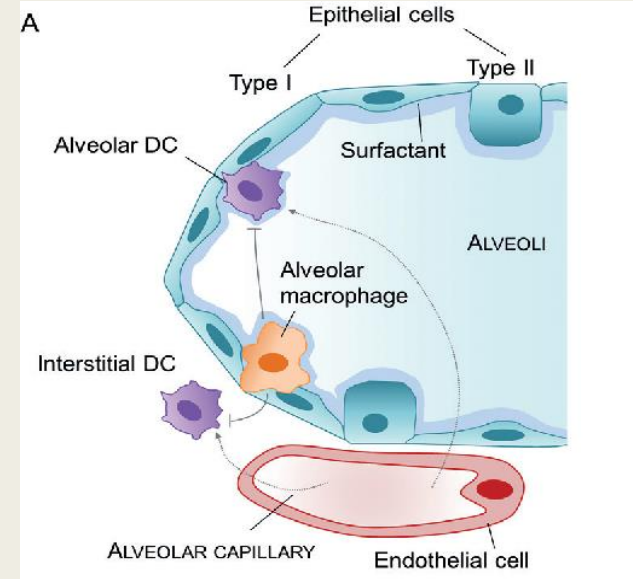
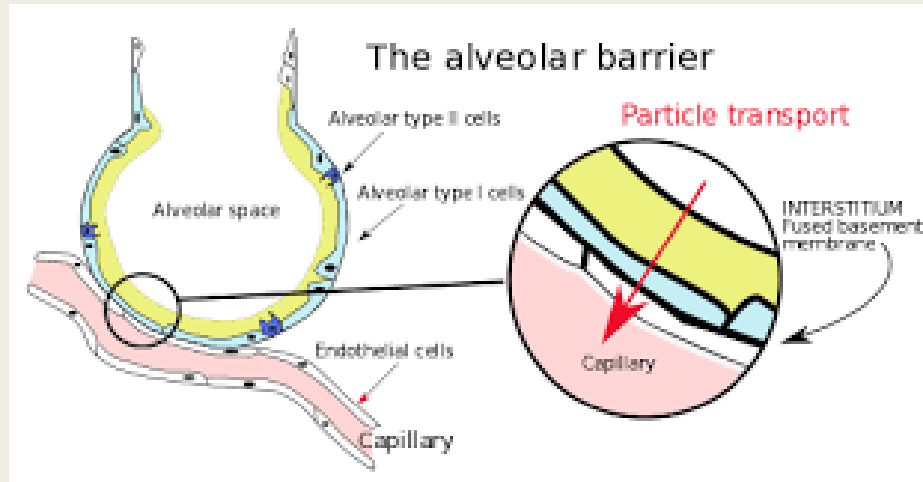
- **Cellular response to damage:**

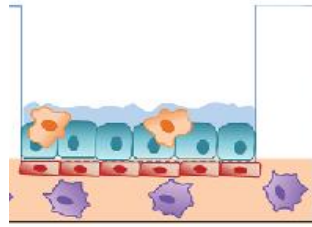
Necrosis and apoptosis biomarkers

MicroRaman analysis (coll. with University of Pisa)


Reference model: co-culture of different lung cell lines representative of alveolar barrier

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





 : Macrophages (THP-1 differentiated with PMA)

 : Alveolar type II epithelial cells (A549)

 : Dendritic-like cells (THP-1)

 : Endothelial cells (HECV)

3D Lung in vitro model

A549
Alveolar epithelial cells (Type II)

THP-1
Monocytes (suspension)

HECV
Endothelial cells

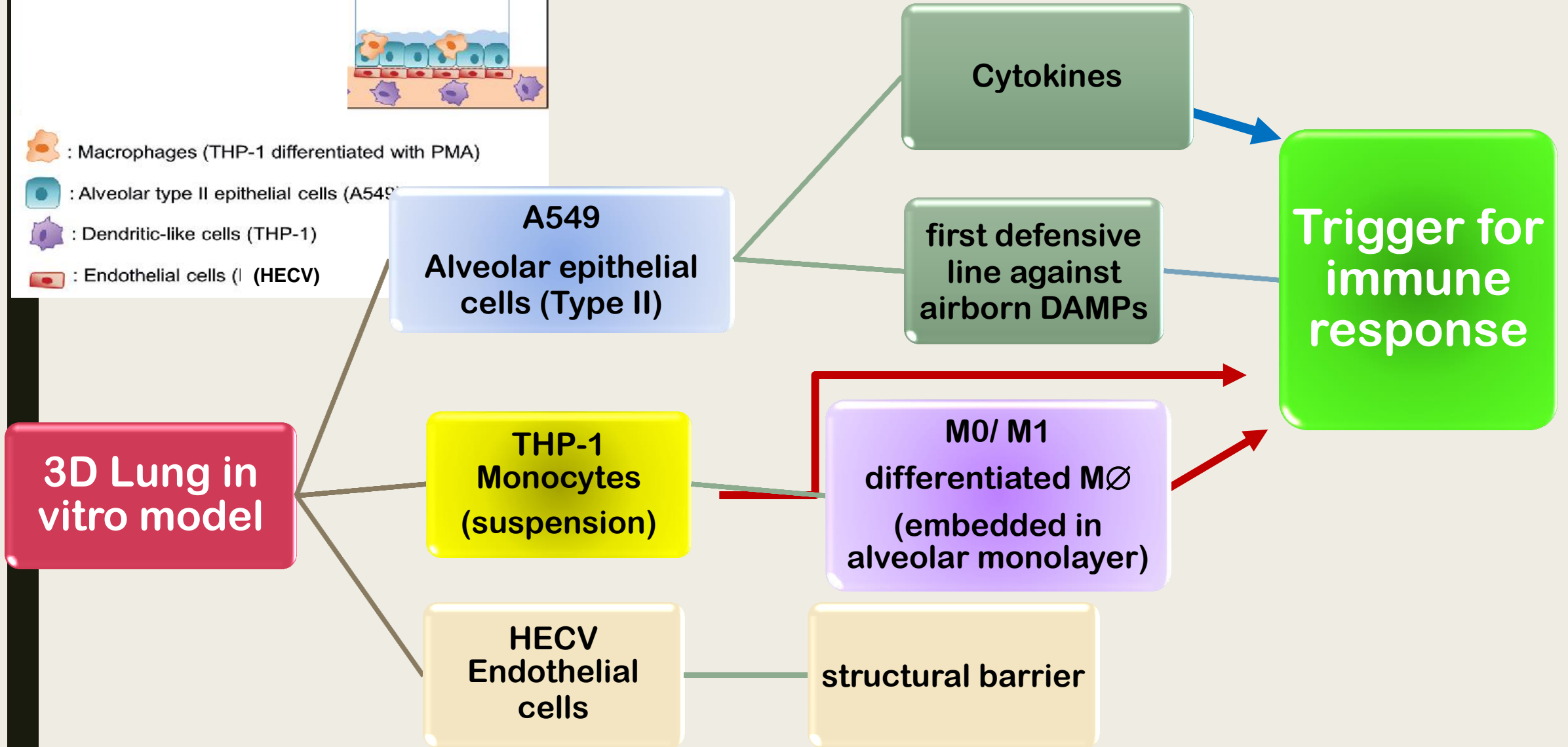
Cytokines

first defensive line against airborne DAMPs

M0/ M1
differentiated MØ
(embedded in alveolar monolayer)

structural barrier

Trigger for immune response



Set up of 3D advanced lung model

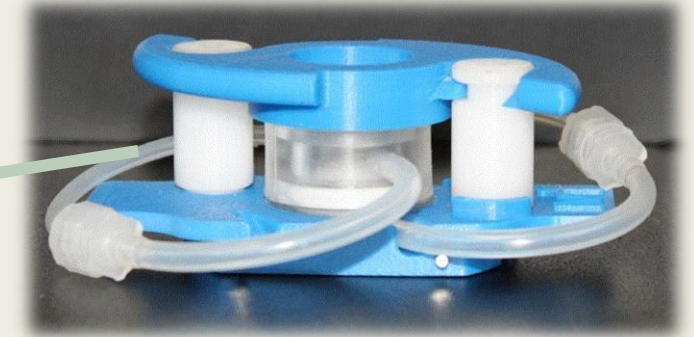
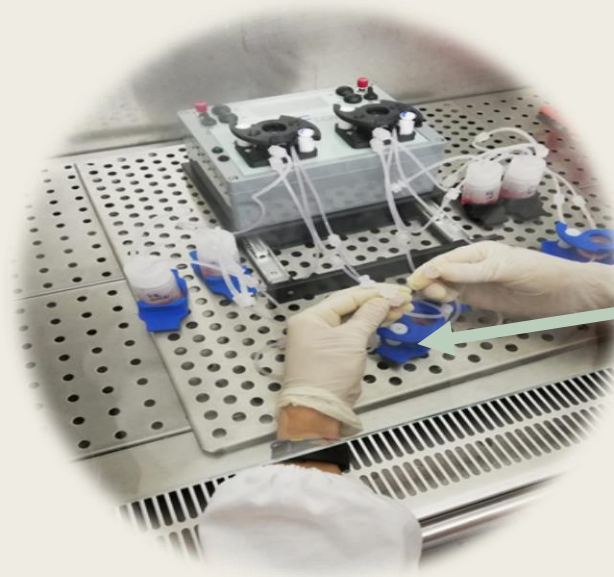
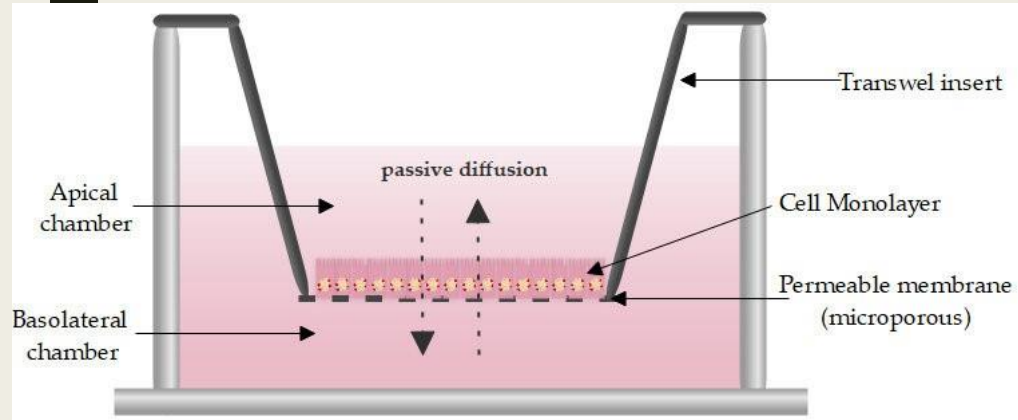
Co-culture
(alveolar, endothelial
cells and
macrophages)
on Transwell© insert



Medium flow in
basolateral chamber
(by peristaltic pump)



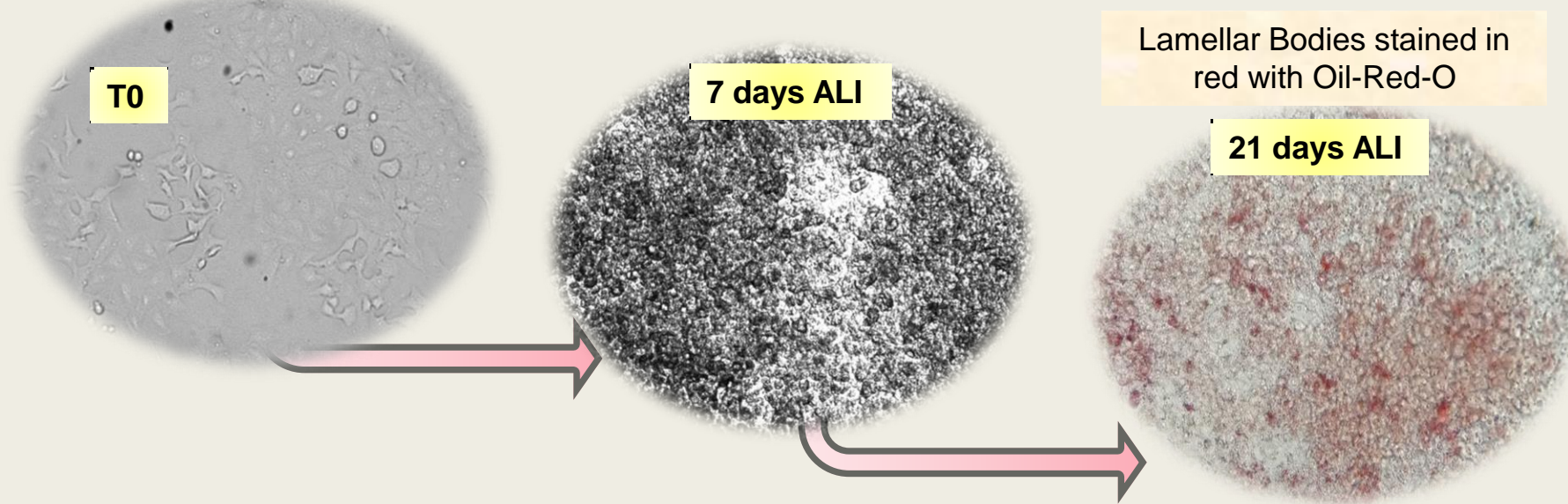
3D dynamic
model



A549 cell Morphology from conventional to ALI culture conditions

Morphology

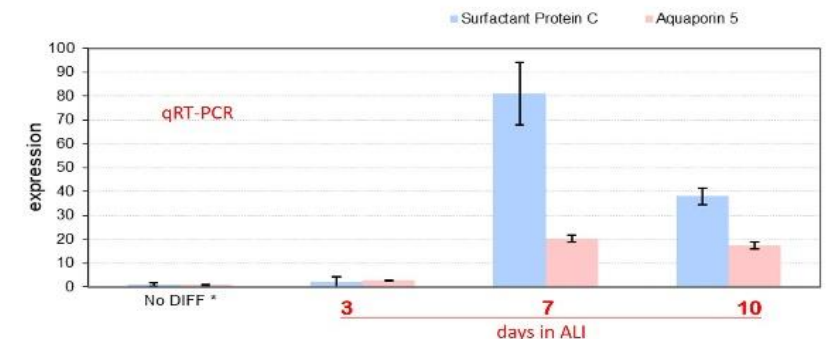
A549 Cells in conventional submerged culture exhibits inerratic shapes → after 7-10 days in **ALI** (air liquid interface) → rough surfaces with multilamellar bodies. The cells were more compacted with clear junctions.



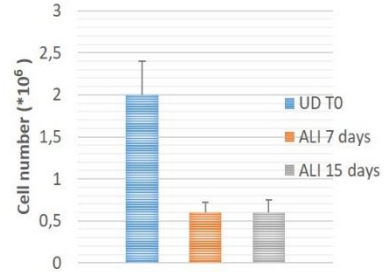
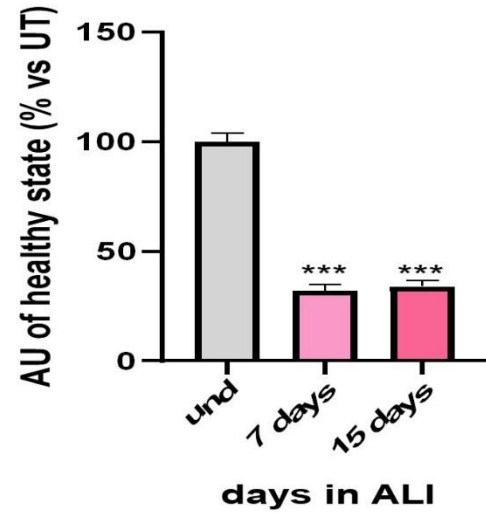
CELL DIFFERENTIATION - SPECIFIC MARKERS

Expression of cell-specific markers:

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

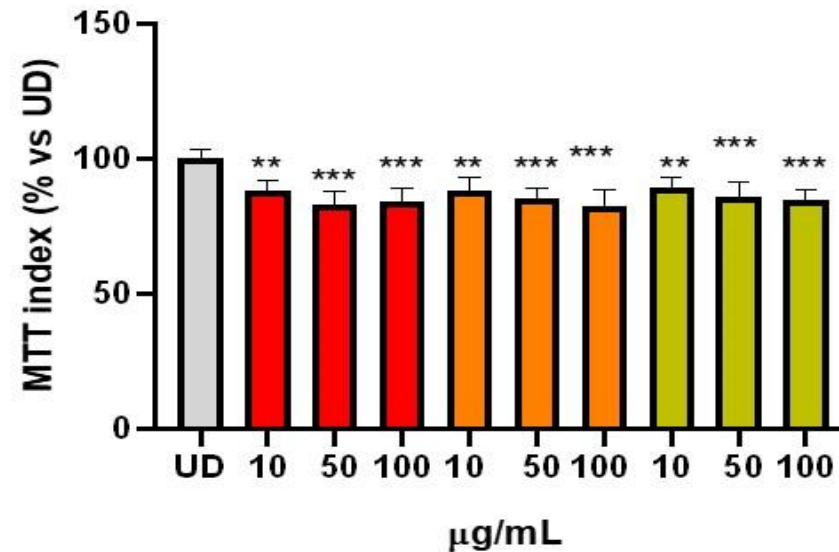


A549 Metabolic State (Alamar Blue Assay)



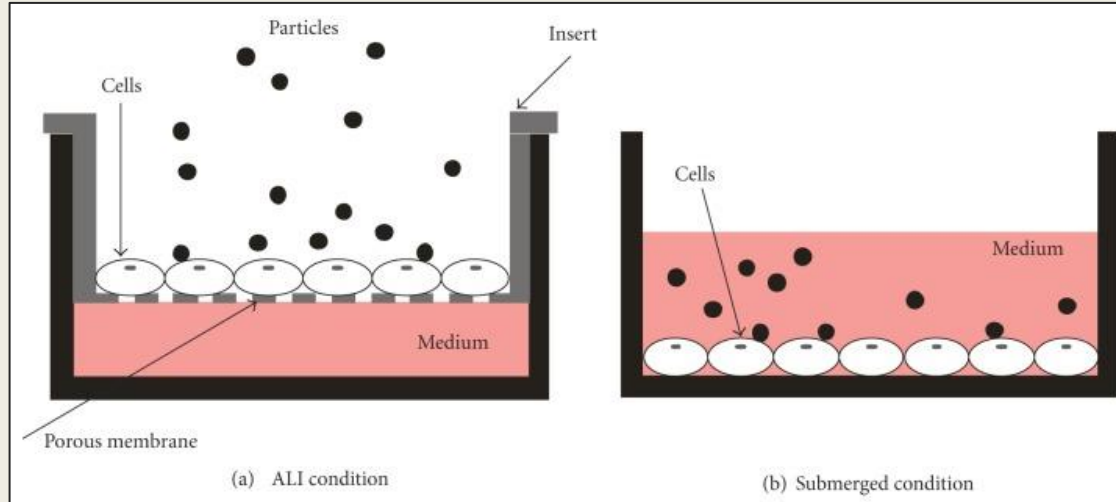
***p<0.0001. Ordinary One-way ANOVA

MTT A549-und 24h



Crocidolite
Chrysotile
Erionite

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



Exposure to fibers

some problems for exposure the differentiated A549-ALI,

→ Commitment to prepare a synthetic surfactant.

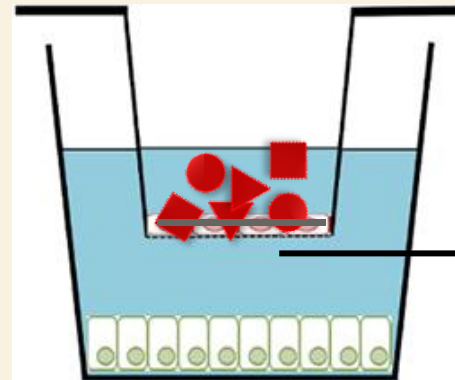


The Experiments Have Been Focused On

- naive/activated monocytes THP-1
- endothelial HECV cells

THP-1 MONOCYTE-LIKE CELL RESPONSE TO FIBER TREATMENT

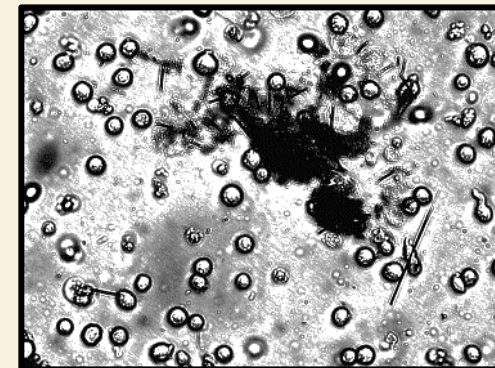
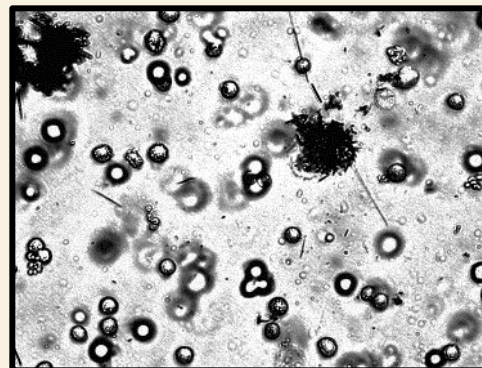
1. INDIRECT CONTACT



- Naive THP-1 standard growth in suspension
- Activated THP-1 growth attached to the support

Mineral Fibers inside Transwell Insert (w TI)

2. DIRECT CONTACT

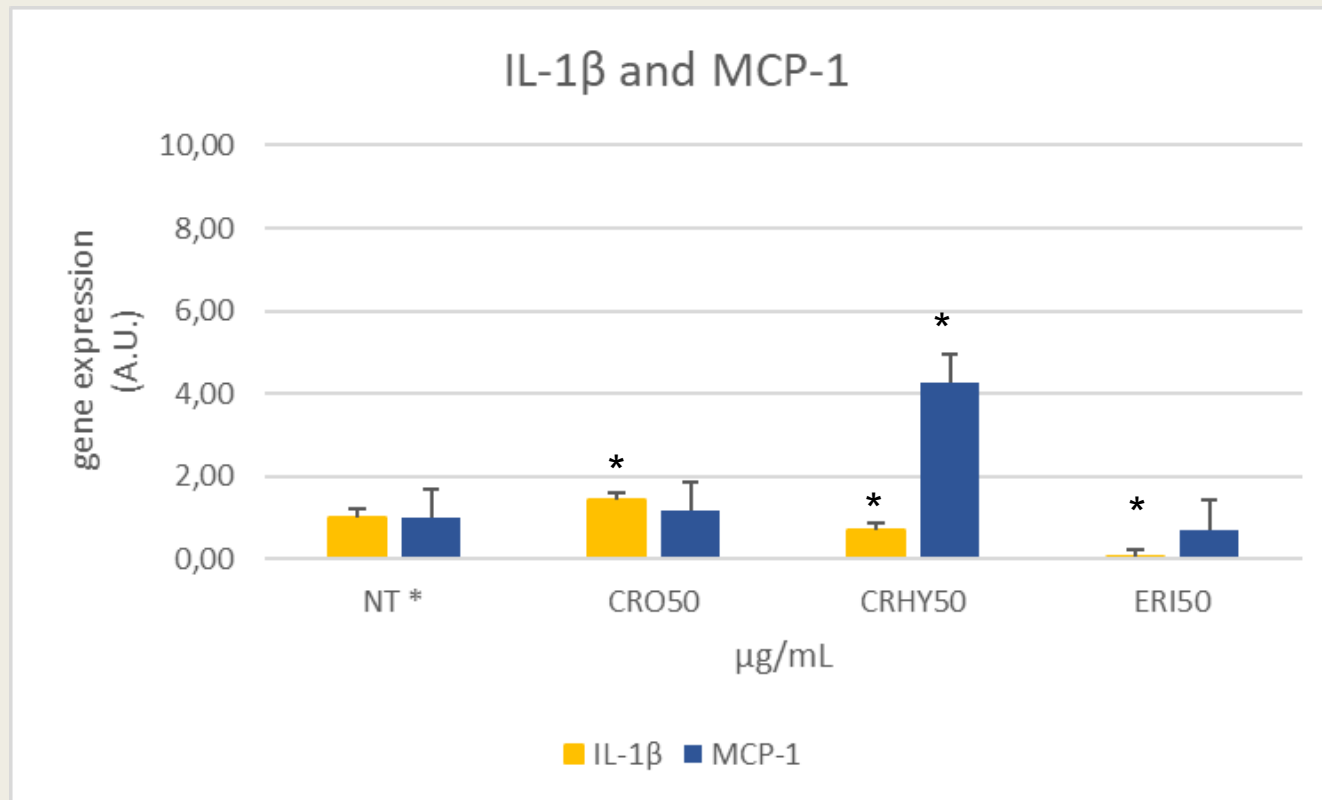


Results

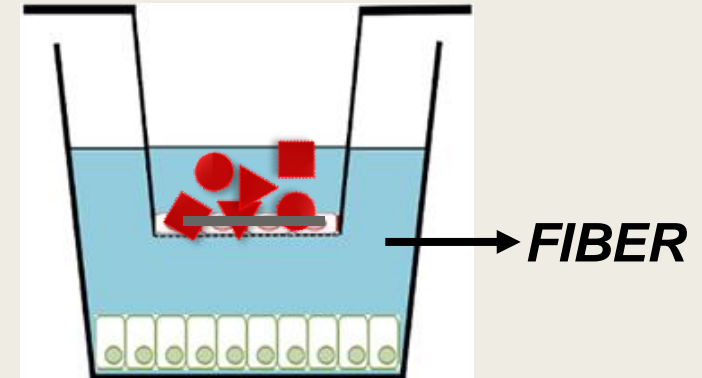
**THP-1 WITH DIRECT and INDIRECT
EXPOSURE (w TI)**

Proinflammatory potential:

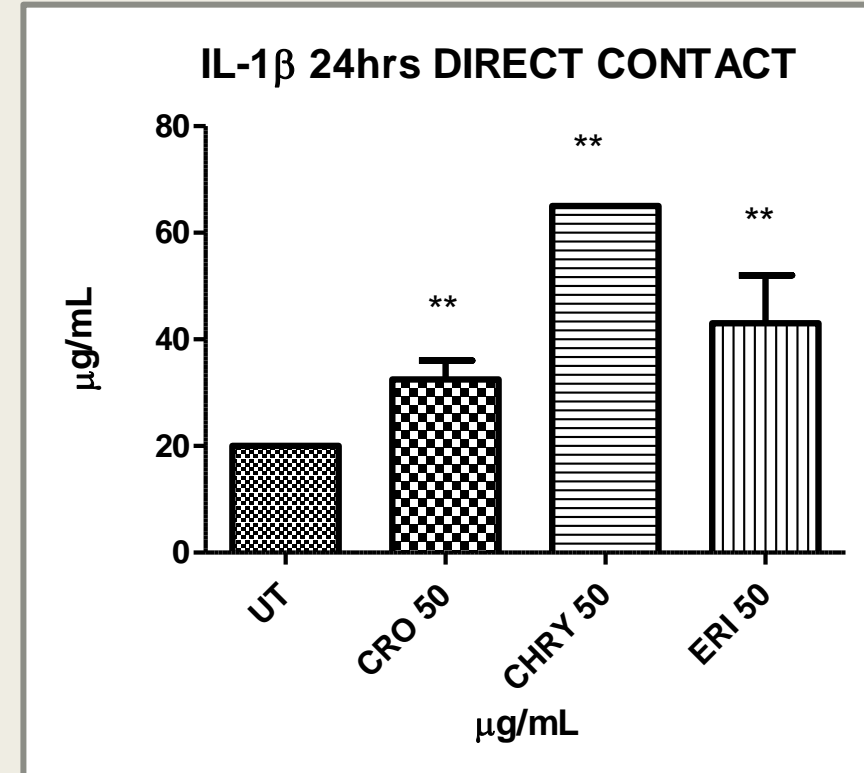
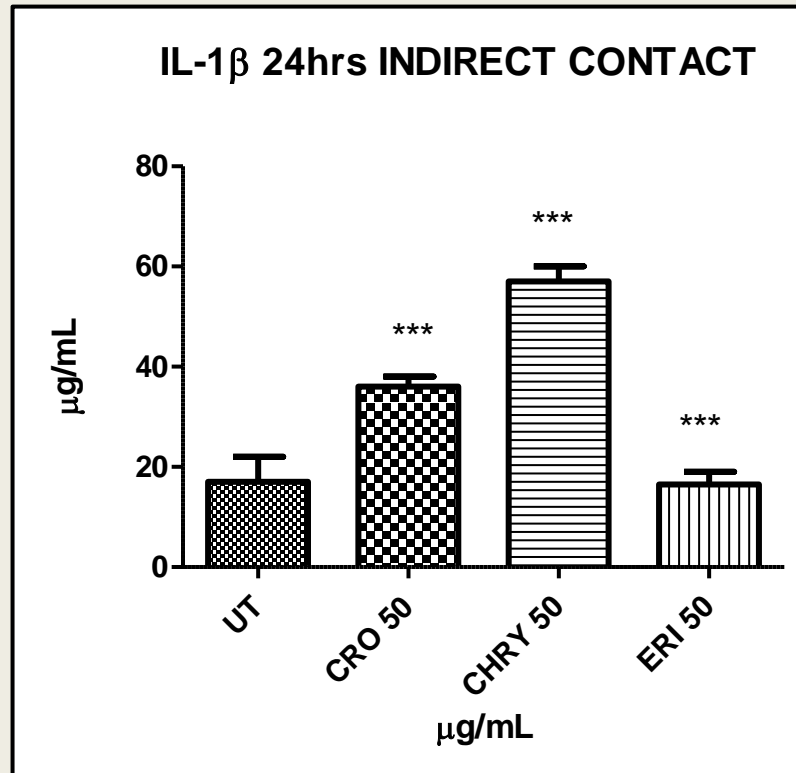
IL-1 β cytokine and MCP-1 chemokine *Gene* levels in THP-1 monocytes after fiber exposure (50 $\mu\text{g}/\text{mL}$) for 24hrs inside Transwell Inserts



$p < 0.1$



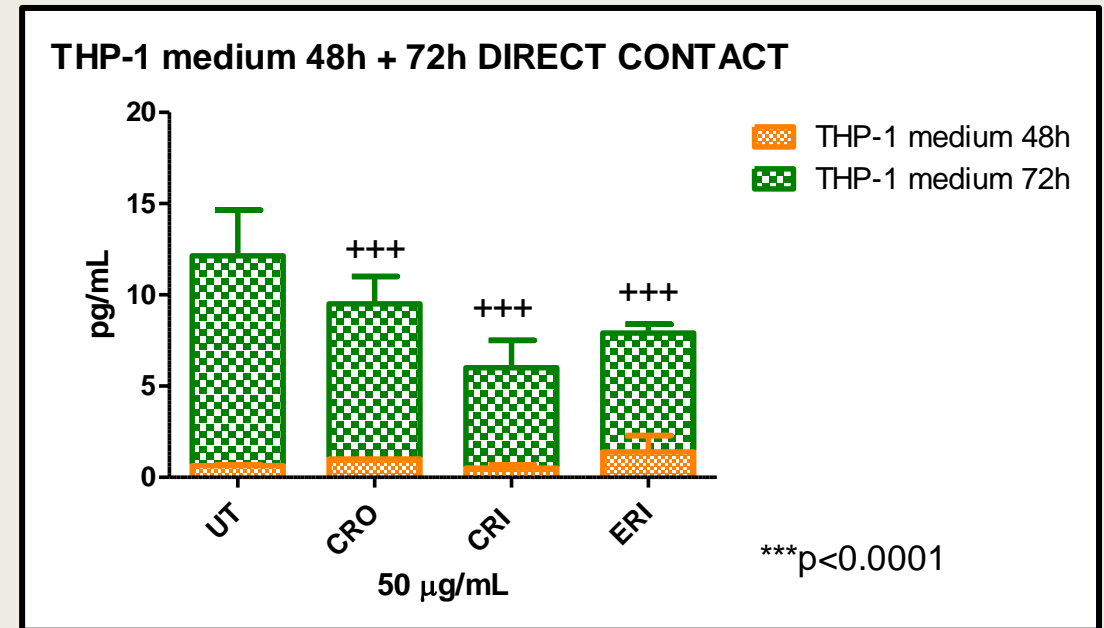
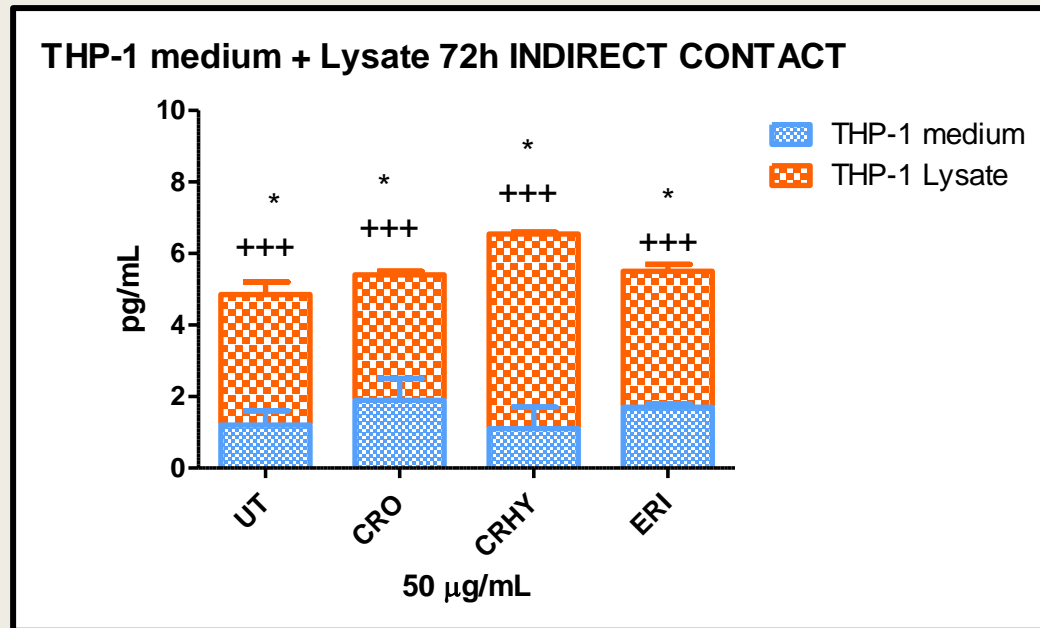
ELISA ASSAY: IL-1 β cytokine secretion



**p<0.001

***p<0.0001

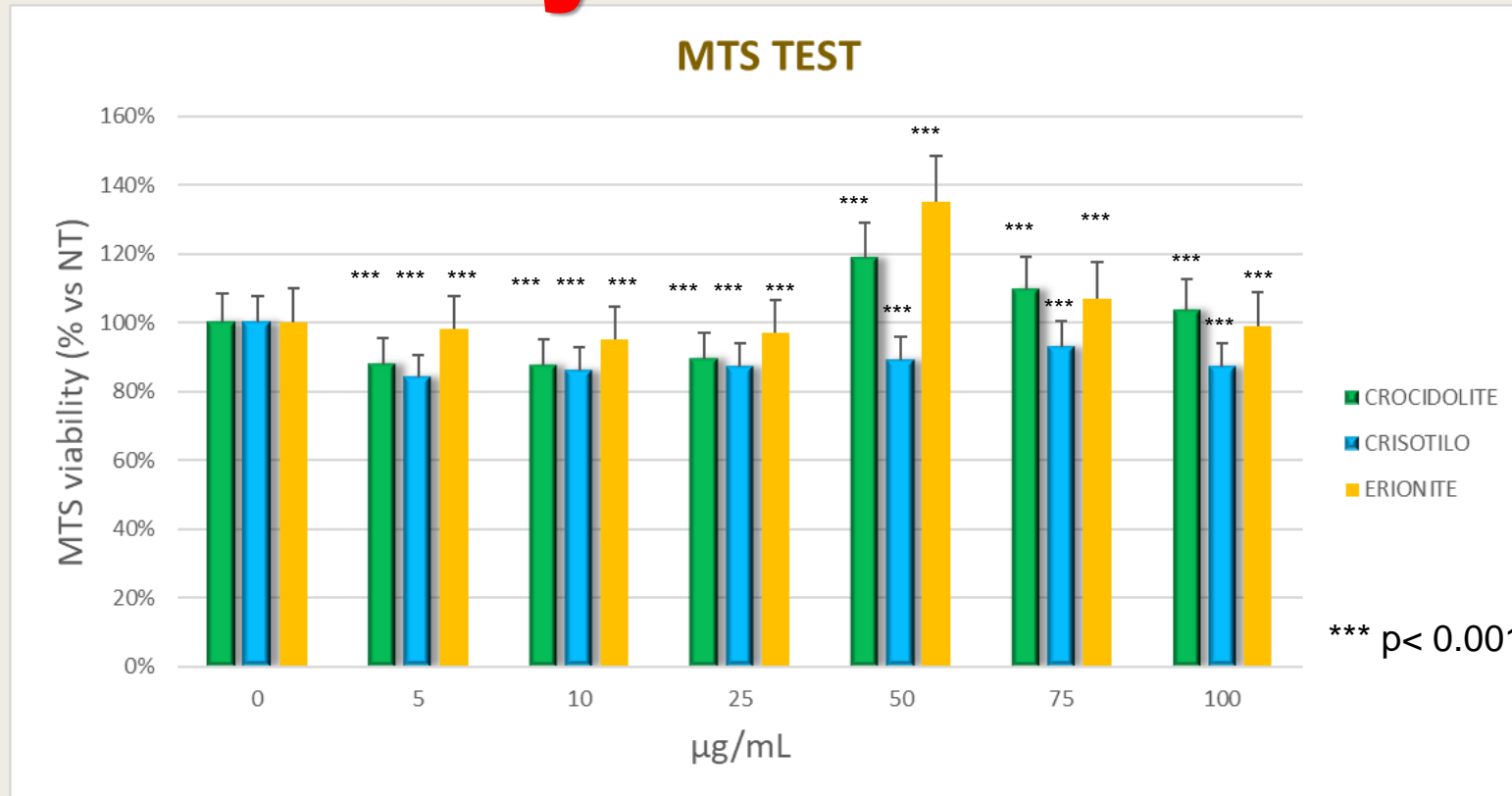
ELISA ASSAY: MCP-1 chemokine levels



*p<0.1

+++p<0.0001 column f

Viability/Mitochondrial activity of THP-1 monocytes after 24h w TI exposure to Cro, Chry and Erio fibers



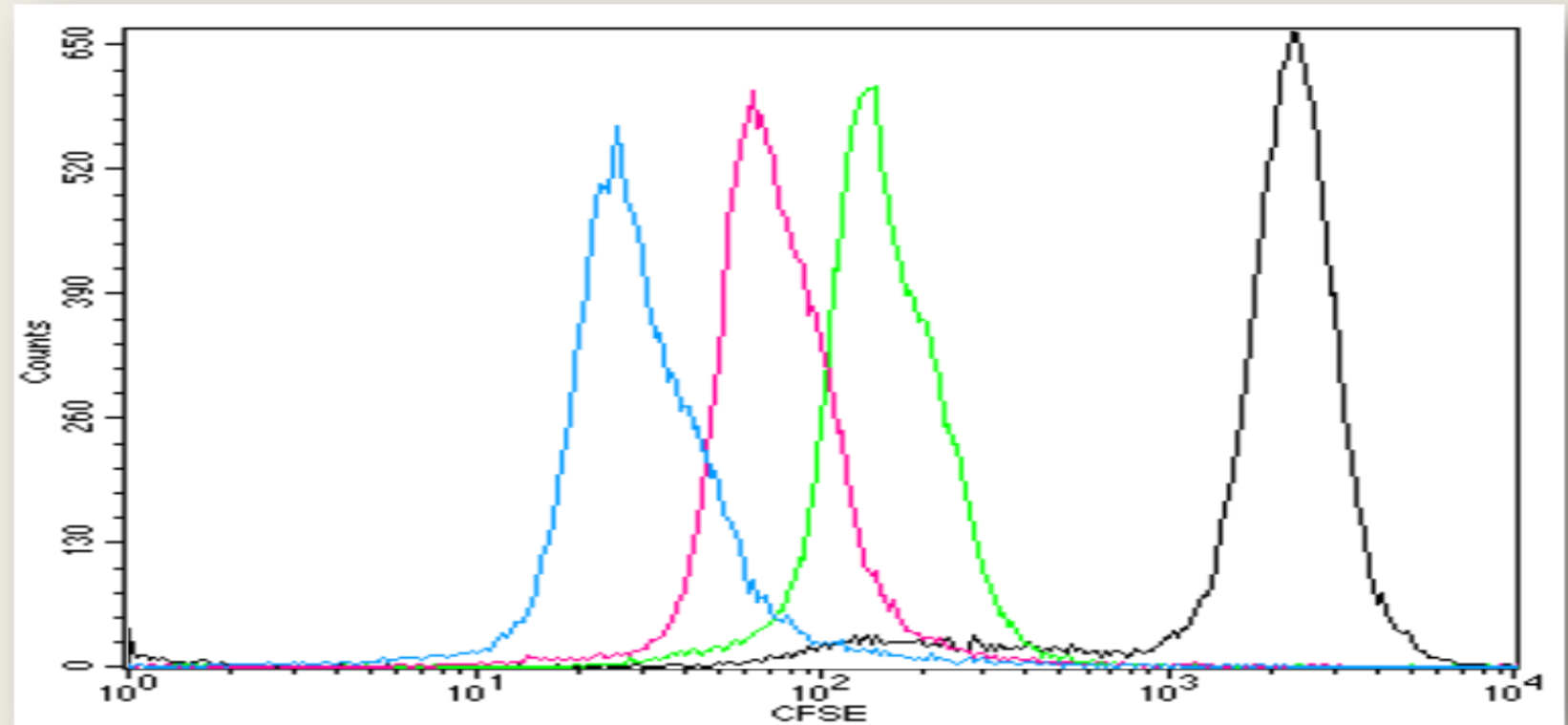
THP-1 in suspension were placed in indirect contact with the fibers (put into insert).

The activation of the treated THP-1 was observed at higher concentrations (> 50 µg / mL) of Crocidolite fibers, which showed higher mitochondrial activity than at lower concentrations.

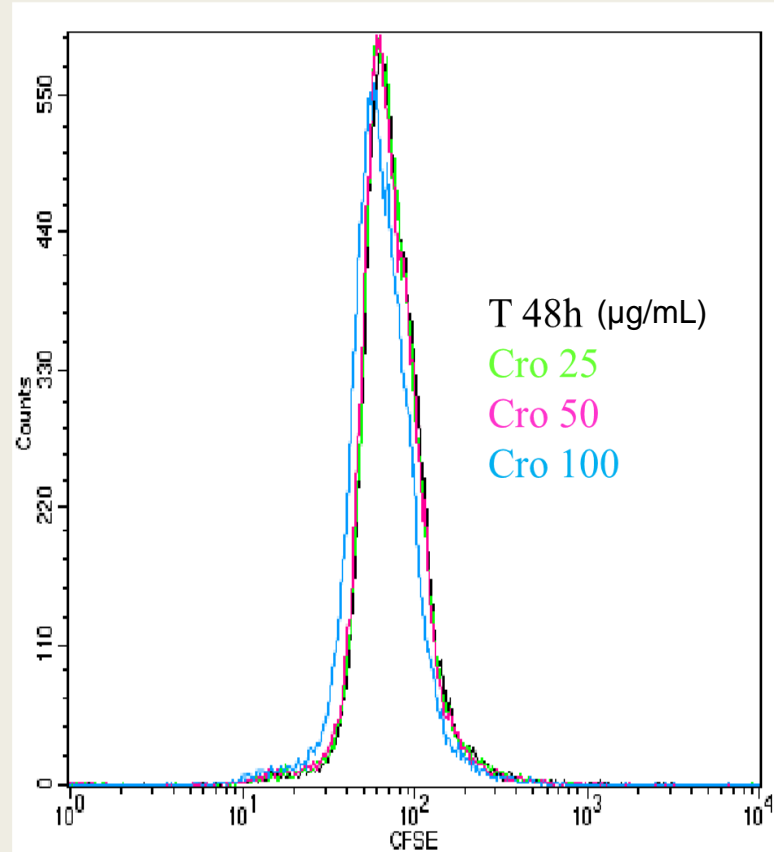
FLOW CYTOMETRY (FACS) on unstimulated THP-1 [Carboxyfluorescein succinimidyl ester CFSE]

CTRL:

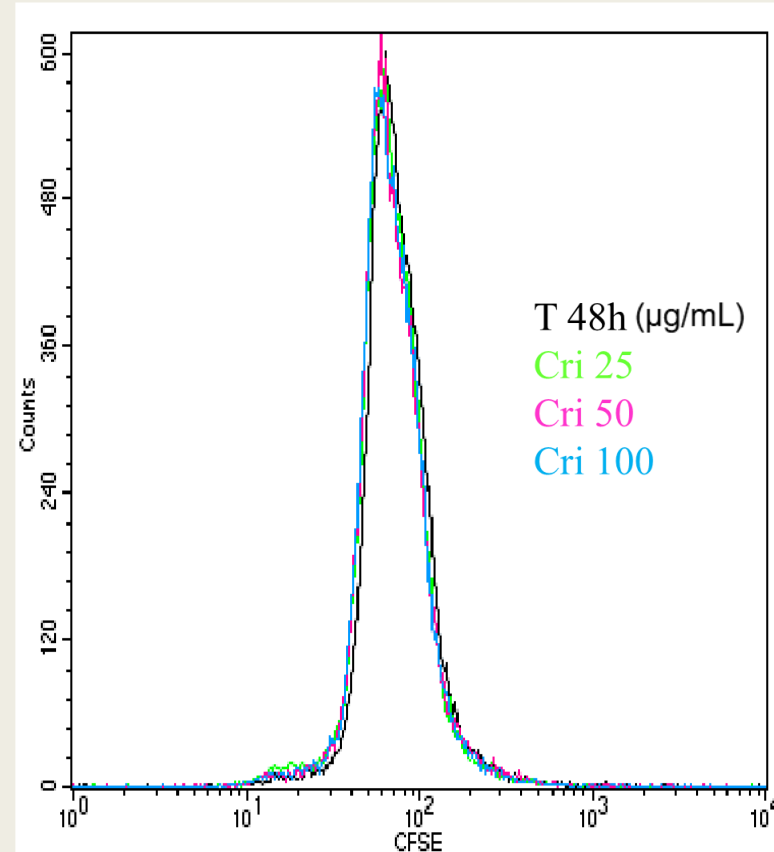
- T0
- T24h
- T48h
- T72h



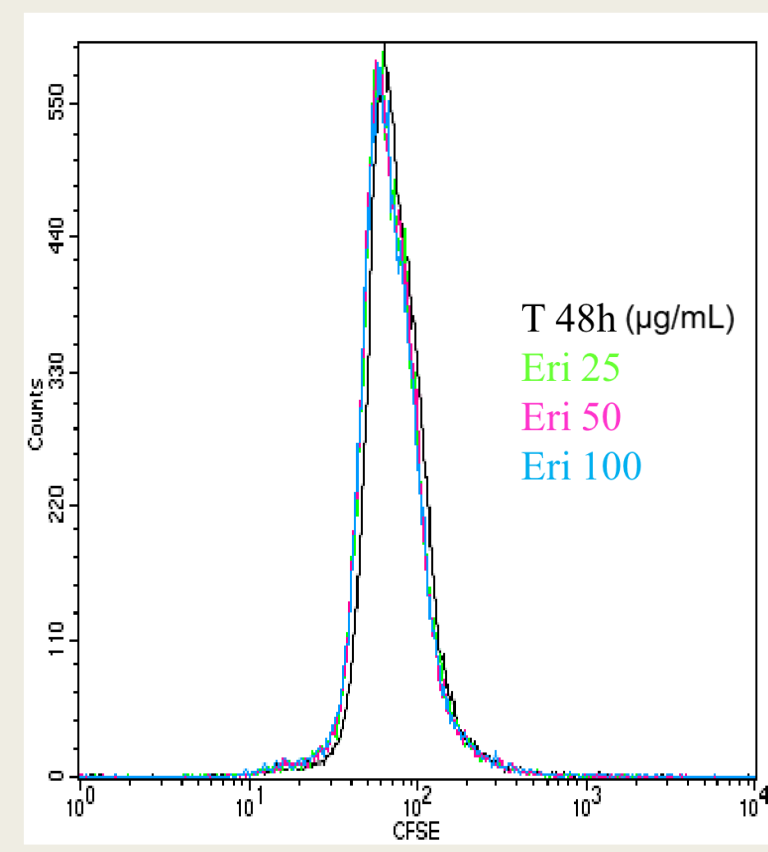
FLOW CYTOMETRY (FACS) on THP-1 treated with Crocidolite, Chrysotile and Erionite w TI for 48 hrs



Key	Name	Parameter	Gate
	THP1 CFSE CTRL T48.001	FL1-H	G1
—	THP1 CFSE CRO25 T48.001	FL1-H	G1
—	THP1 CFSE CRO50 T48.001	FL1-H	G1
—	THP1 CFSE CRO100 T48.001	FL1-H	G1

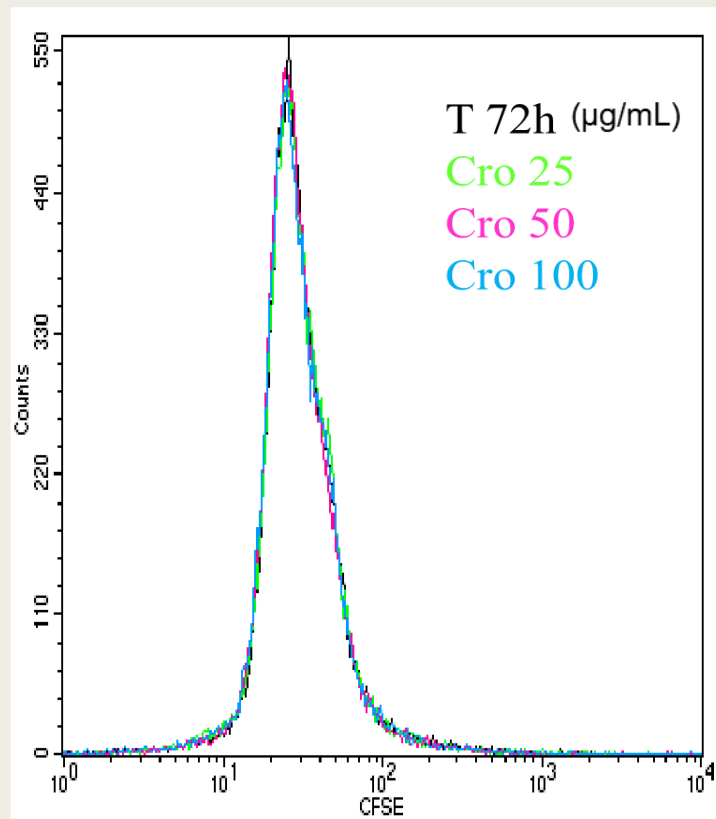


Key	Name	Parameter	Gate
	THP1 CFSE CTRL T48.001	FL1-H	G1
—	THP1 CFSE CRI25 T48.001	FL1-H	G1
—	THP1 CFSE CRI50 T48.001	FL1-H	G1
—	THP1 CFSE CRI100 T48.001	FL1-H	G1

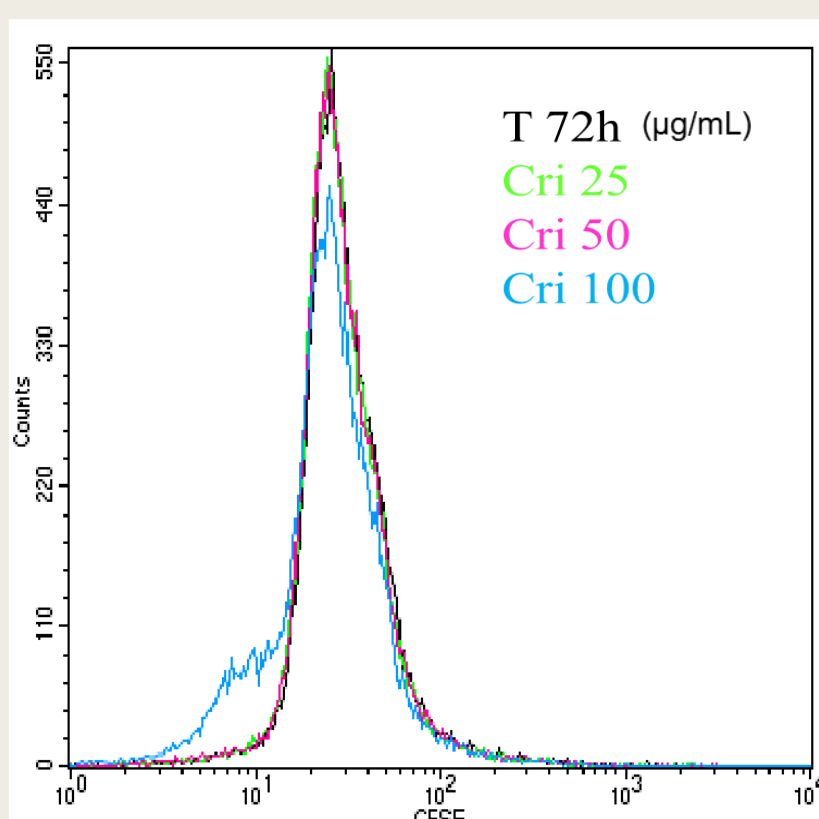


Key	Name	Parameter	Gate
	THP1 CFSE CTRL T48.001	FL1-H	G1
—	THP1 CFSE ERI25 T48.001	FL1-H	G1
—	THP1 CFSE ERI50 T48.001	FL1-H	G1
—	THP1 CFSE ERI100 T48.001	FL1-H	G1

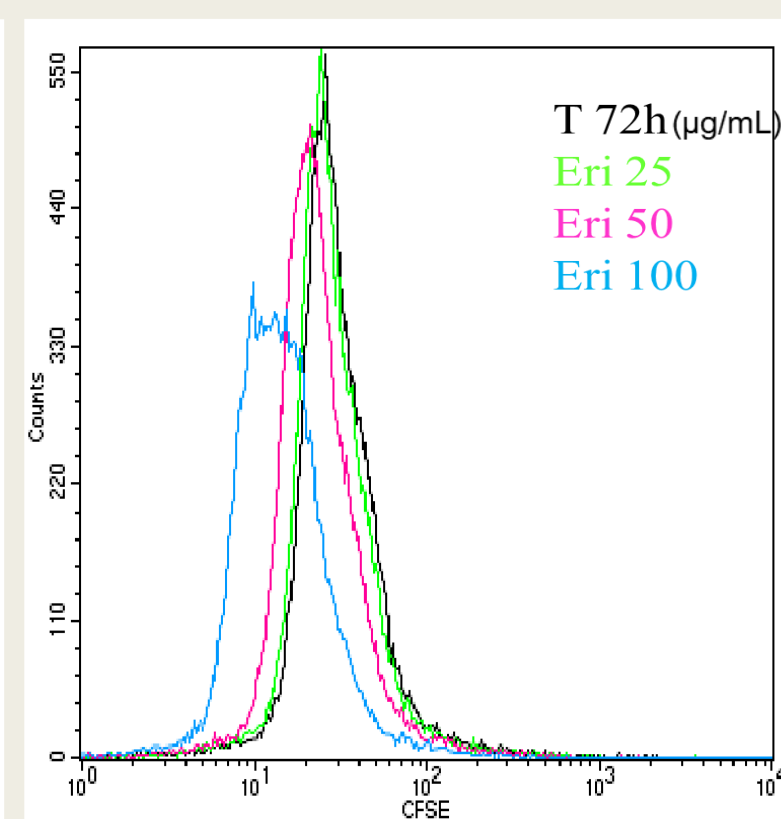
FLOW CYTOMETRY (FACS) on THP-1 treated with Crocidolite, Chrysotile and Erionite w TI for 72hrs



Key	Name	Parameter	Gate
	THP1 CFSE CTRL T72.001	FL1-H	G1
—	THP1 CFSE CRO25 T72.001	FL1-H	G1
—	THP1 CFSE CRO50 T72.001	FL1-H	G1
—	THP1 CFSE CRO100 T72.001	FL1-H	G1



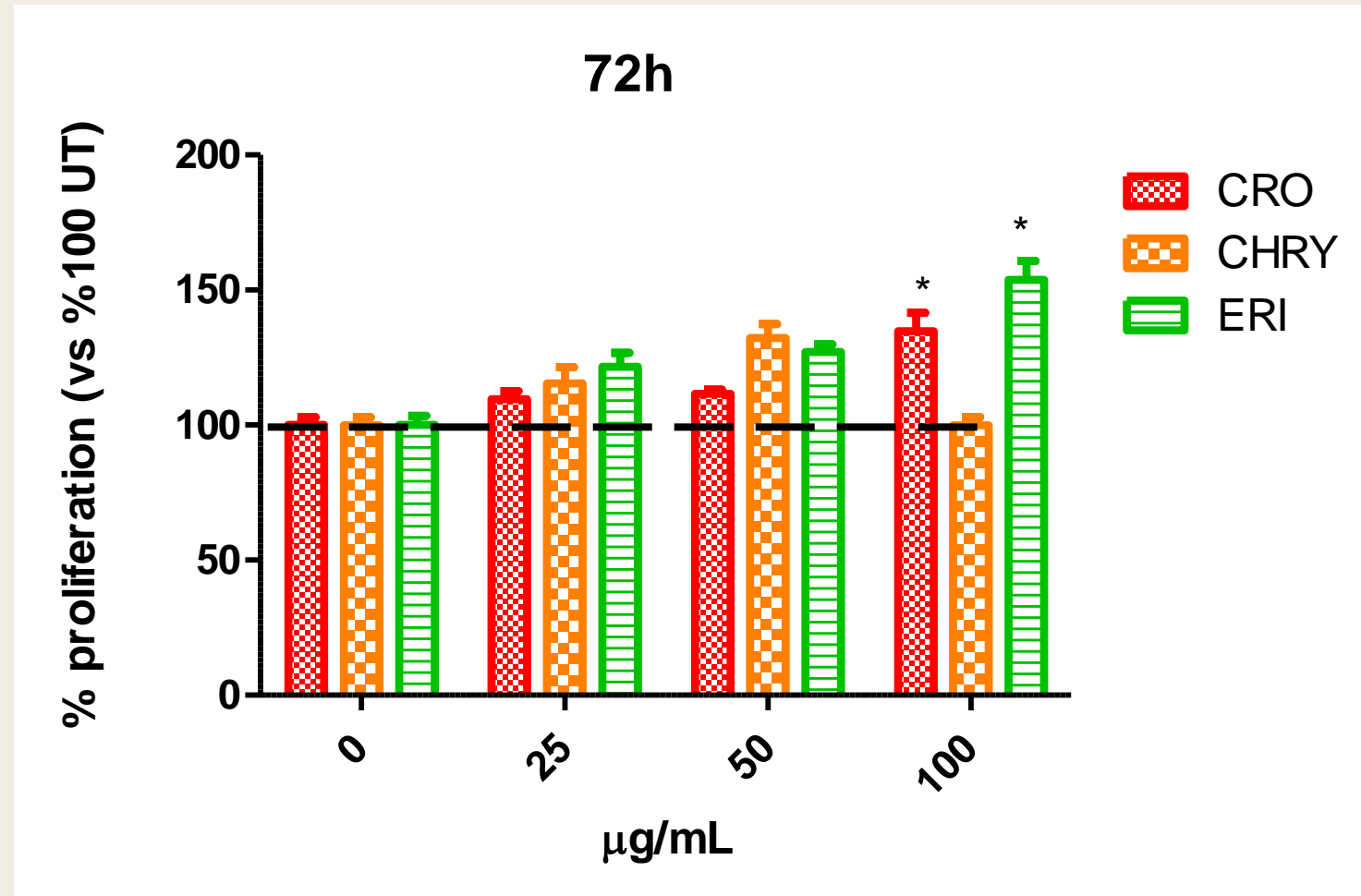
Key	Name	Parameter	Gate
	THP1 CFSE CTRL T72.001	FL1-H	G1
—	THP1 CFSE CRI25 T72.001	FL1-H	G1
—	THP1 CFSE CRI50 T72.001	FL1-H	G1
—	THP1 CFSE CRI100 T72.001	FL1-H	G1



Key	Name	Parameter	Gate
	THP1 CFSE CTRL T72.001	FL1-H	G1
—	THP1 CFSE ERI25 T72.001	FL1-H	G1
—	THP1 CFSE ERI50 T72.001	FL1-H	G1
—	THP1 CFSE ERI100 T72.001	FL1-H	G1

THP-1 cell proliferation after 72hrs of treatment with fibers in INDIRECT CONTACT by DNA ASSAY

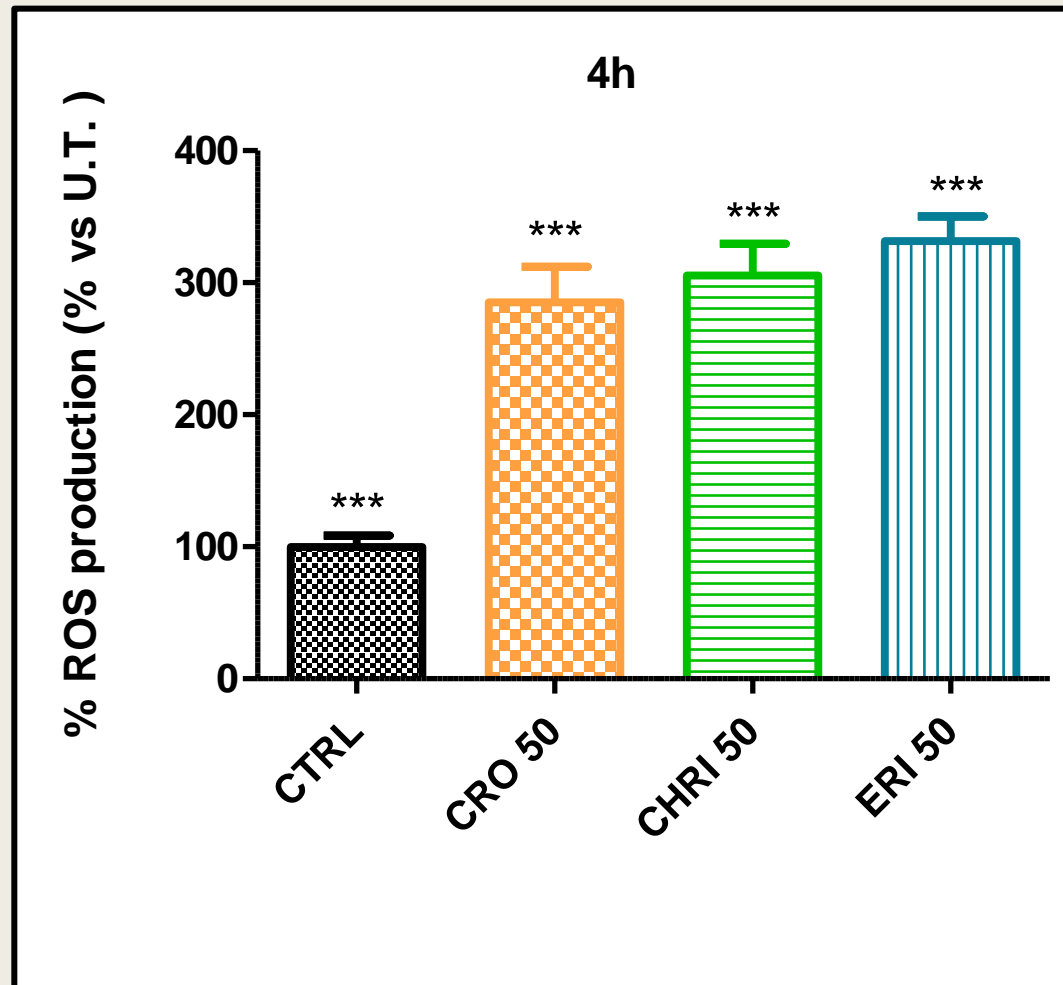
[Hoescht 33342] *(Rao J, Otto WR. Anal Biochem., 1992)*



$p < 0.5$

Oxidative stress analysis

ROS levels after 4 hrs exposure to **Crocidolite, Chrysotile and Erionite (50 µg/mL)** in THP-1 w TI



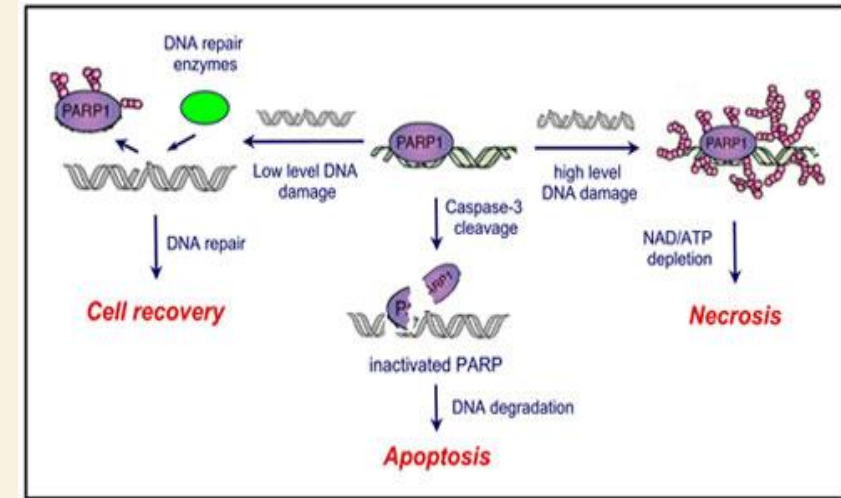
***p<0.0001

Apoptotic trigger analysis and genotoxic effects: levels of PARP-1 and H2AX / γ -H2AX

PARP – 1

Poly ADP-ribose polymerase family.

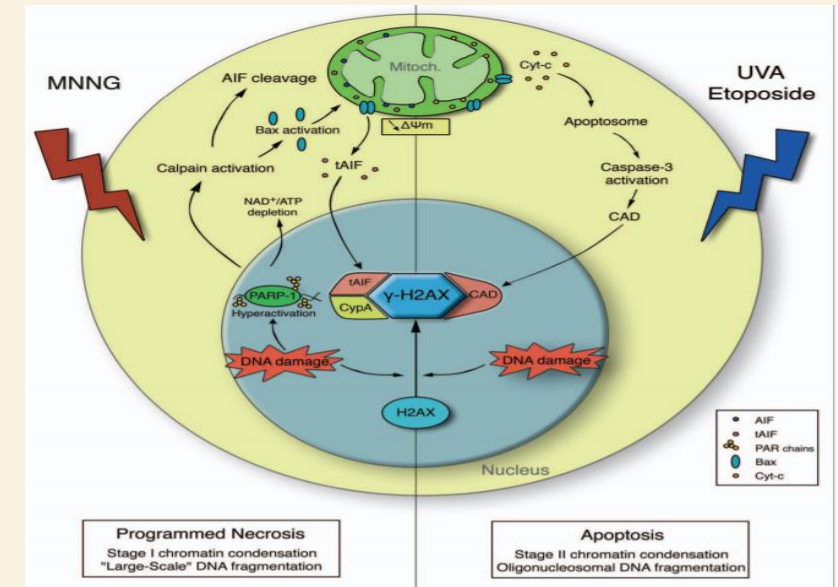
- In the nucleus \rightarrow role of identifying and signaling single-stranded DNA breaks (SSB). It allows the activation of DNA repair enzymes.
- important mediating role during the necrosis and apoptosis death.
- Apoptosis is guaranteed by the action of specific proteases (caspases) which proteolyze it, generating the 89 kDa fragment.



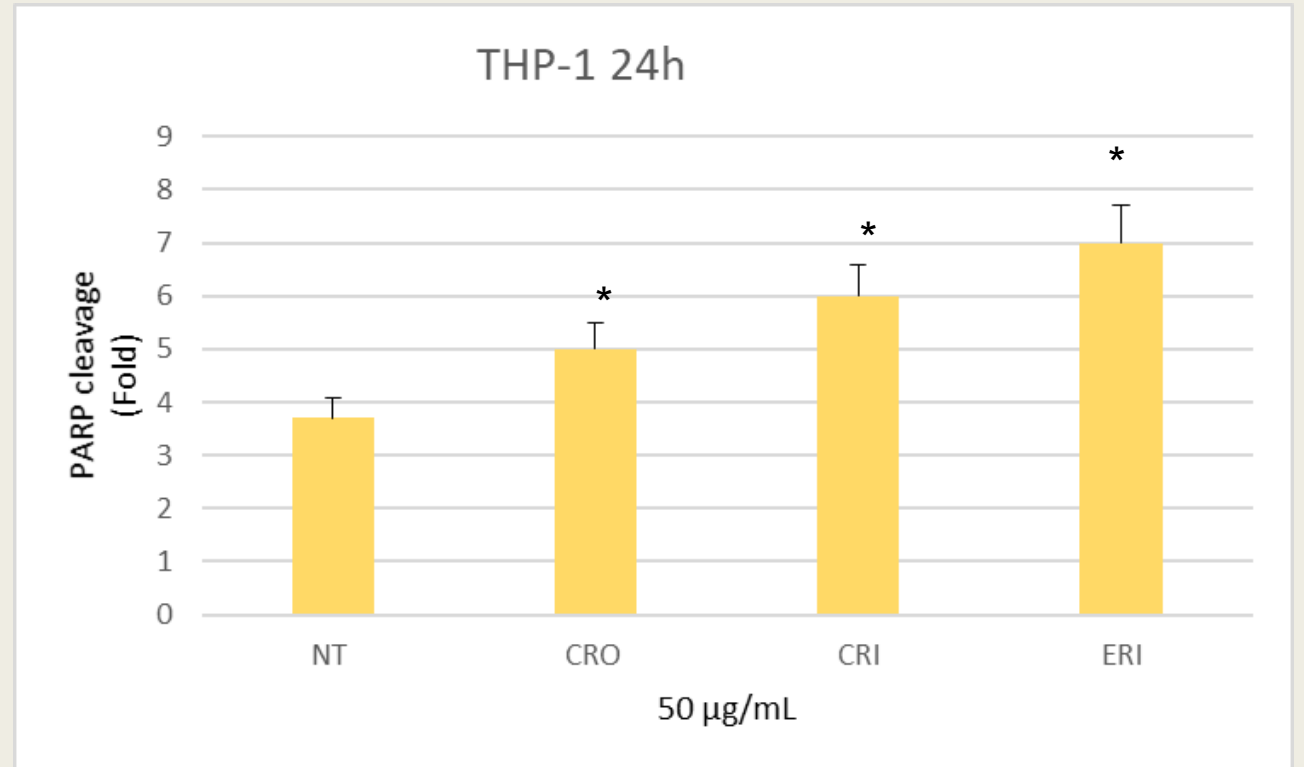
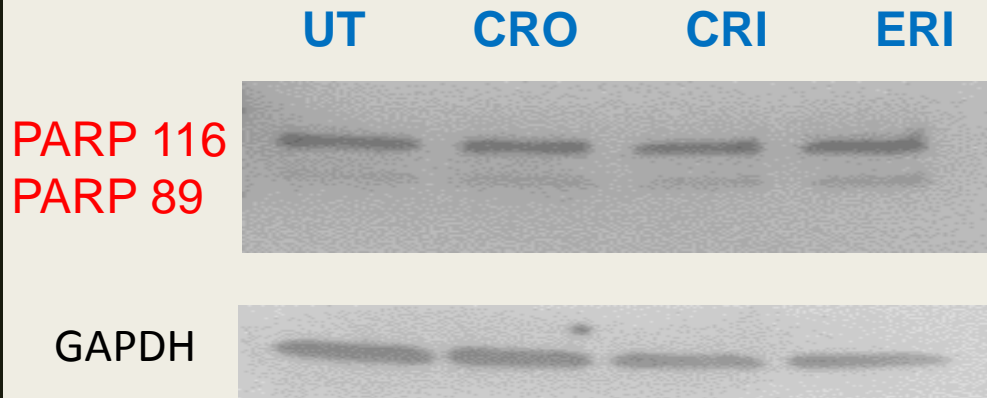
H2AX e γ -H2AX

H2AX is a histone protein belonging to the H2A family.

- The phosphorylated form γ -H2AX is present in the case of DNA double strand breaks (DSB). H2AX is phosphorylated at the serine 139 level as a reaction to DSB.
- Following phosphorylation, the DNA becomes less condensed and leads to the foci formation.
- There is a strong quantitative correlation between the foci formation and the level of DSB.

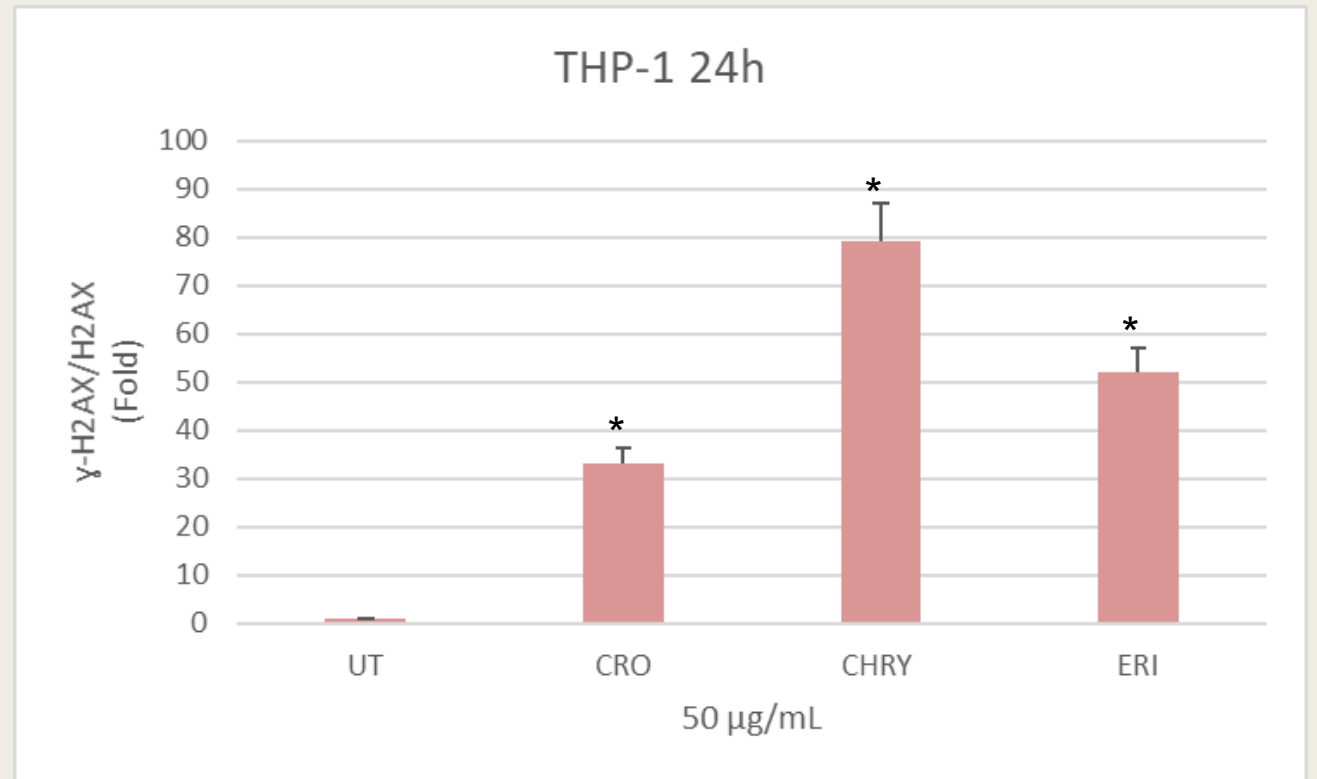
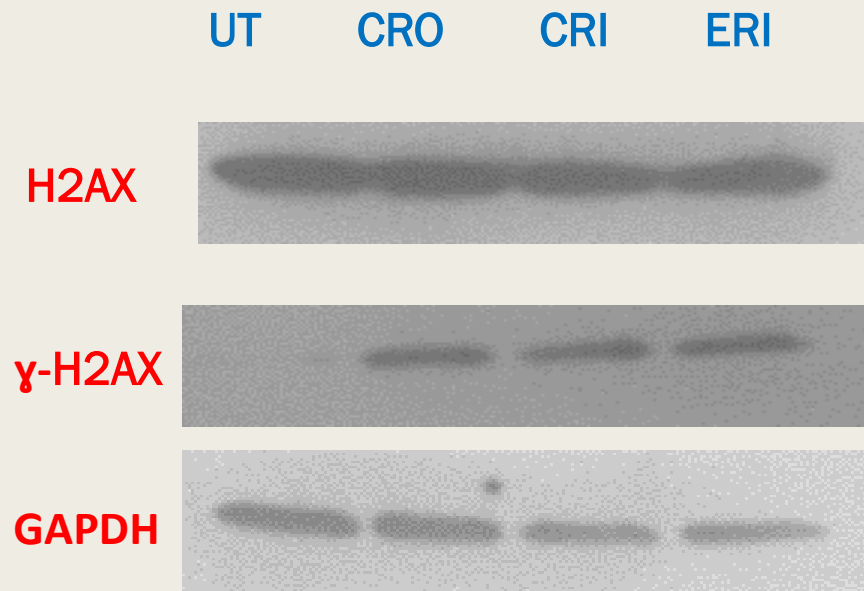


PARP-1 protein cleavage in THP-1 cells treated w TI for 24hrs with Crocidolite, Chrysotile, Erionite (50 $\mu\text{g}/\text{mL}$)



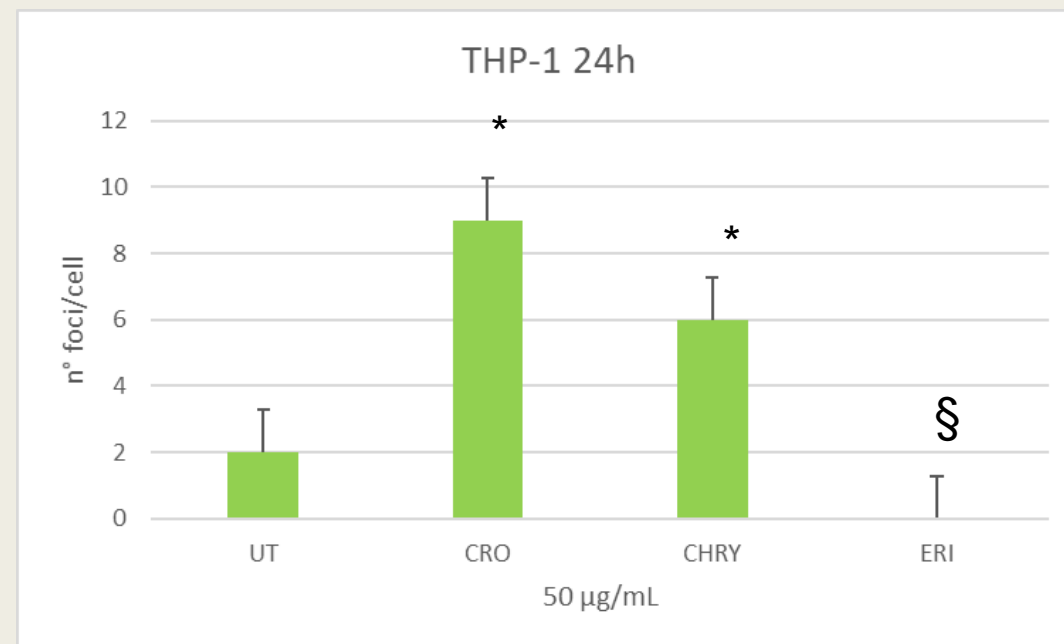
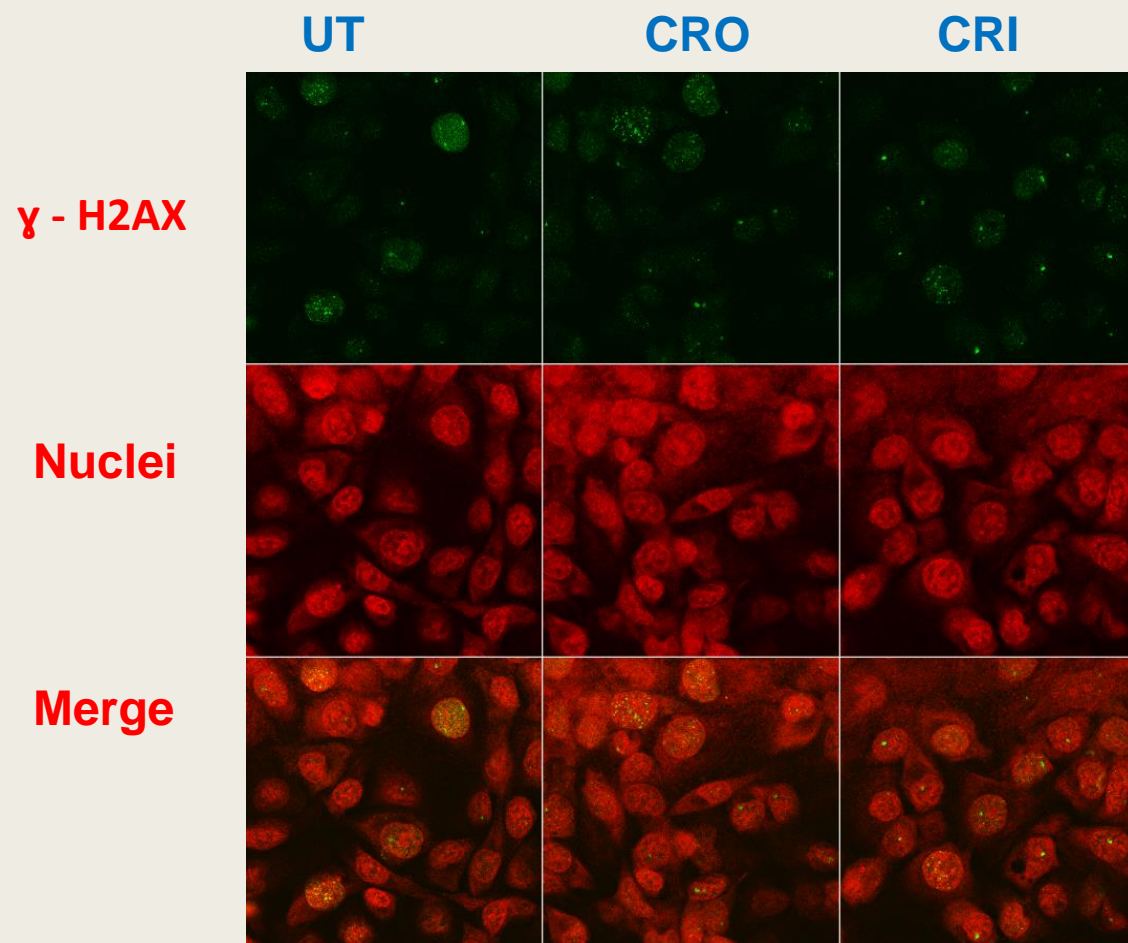
* $p < 0.5$

γ -H2AX protein expression in THP-1 cells treated w TI for 24hrs with Crocidolite, Chrysotile, Erionite (50 μ g /mL)



* $p > 0.5$

Confocal Microscopy for γ - H2AX foci in THP-1 cells w TI after 24hrs of treatment with Crocidolite, Crisotilo (50 μ g/mL)

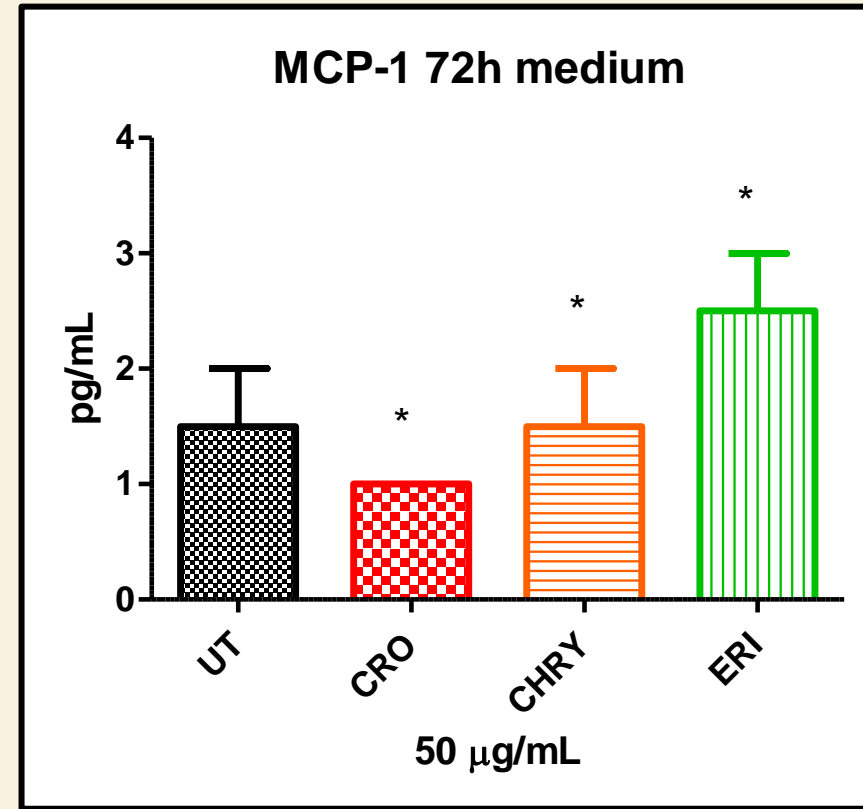
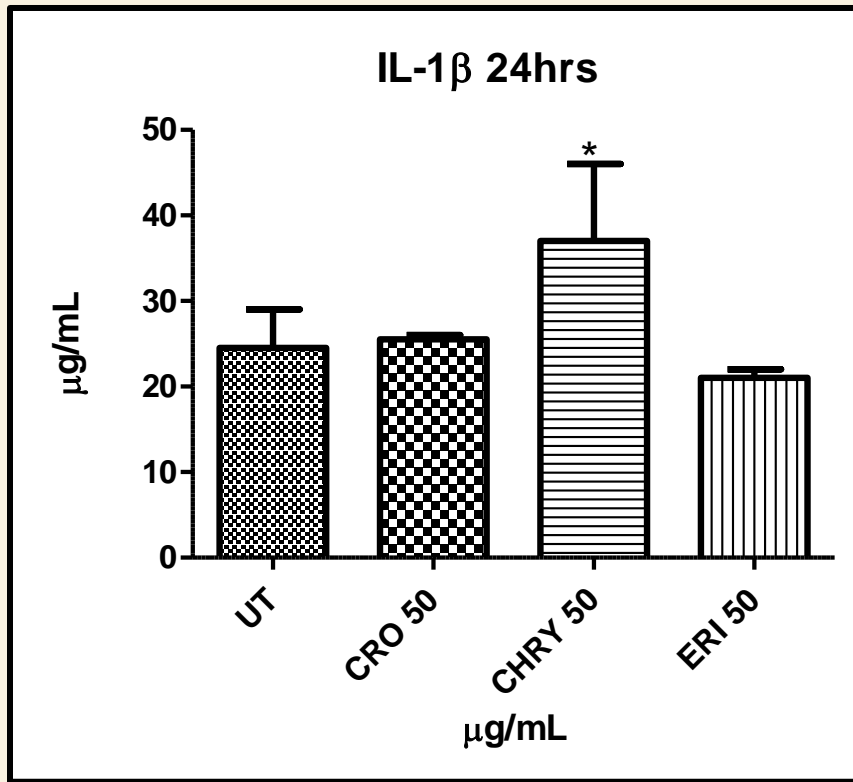


§ Dato mancante

Results

**HECV
DIRECT EXPOSURE**

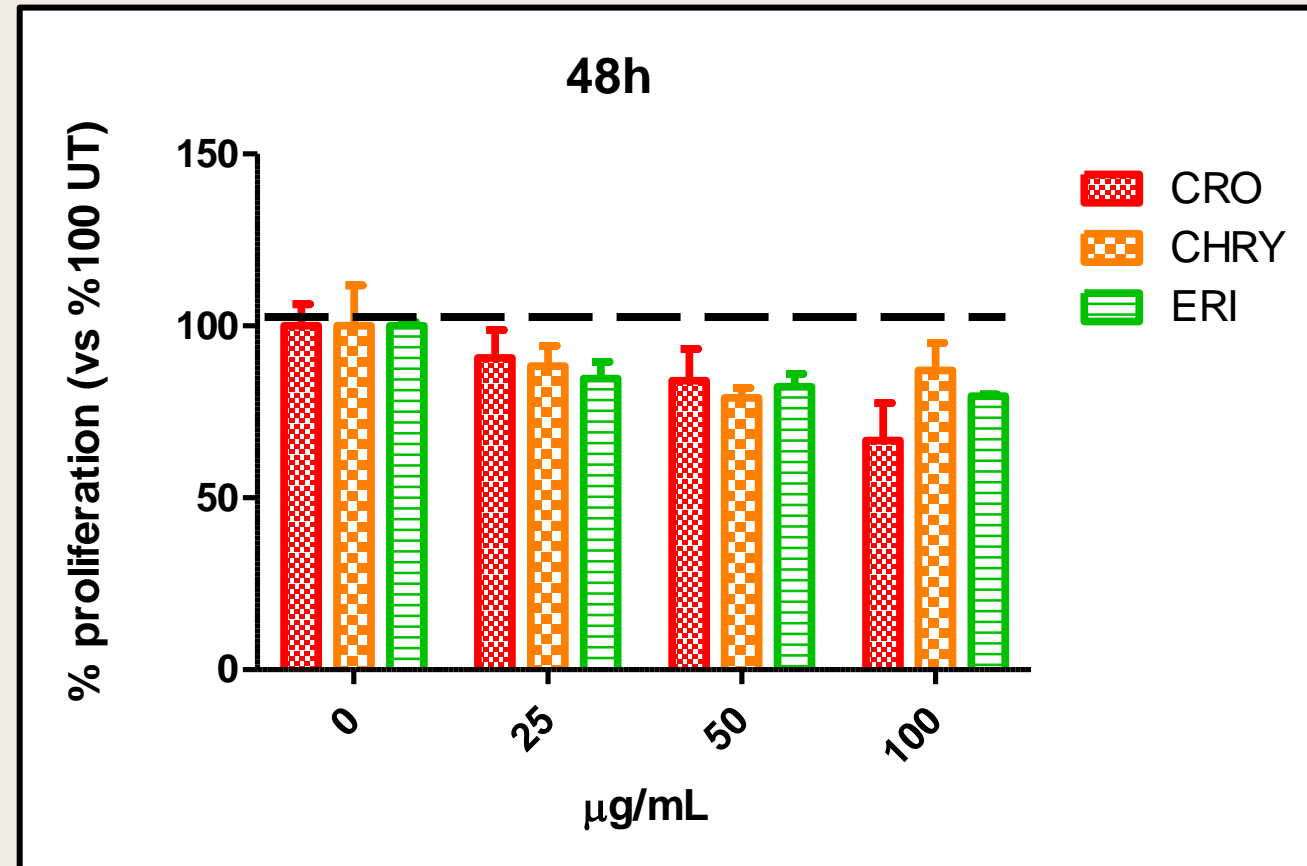
ELISA ASSAY: IL-1 β cytokine and MCP-1 chemokine level



$p < 0.5$

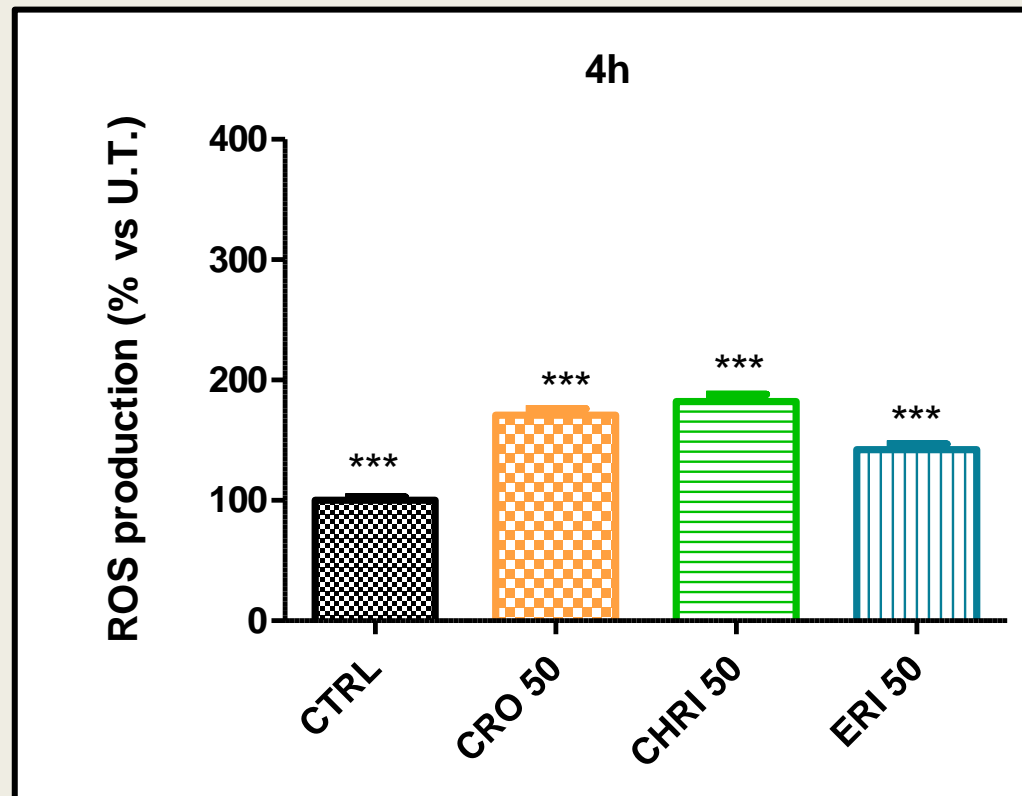
HECV cell proliferation after 48hrs of treatment with fibers by DNA ASSAY

[Hoescht 33342] *(Rao J, Otto WR. Anal Biochem., 1992)*



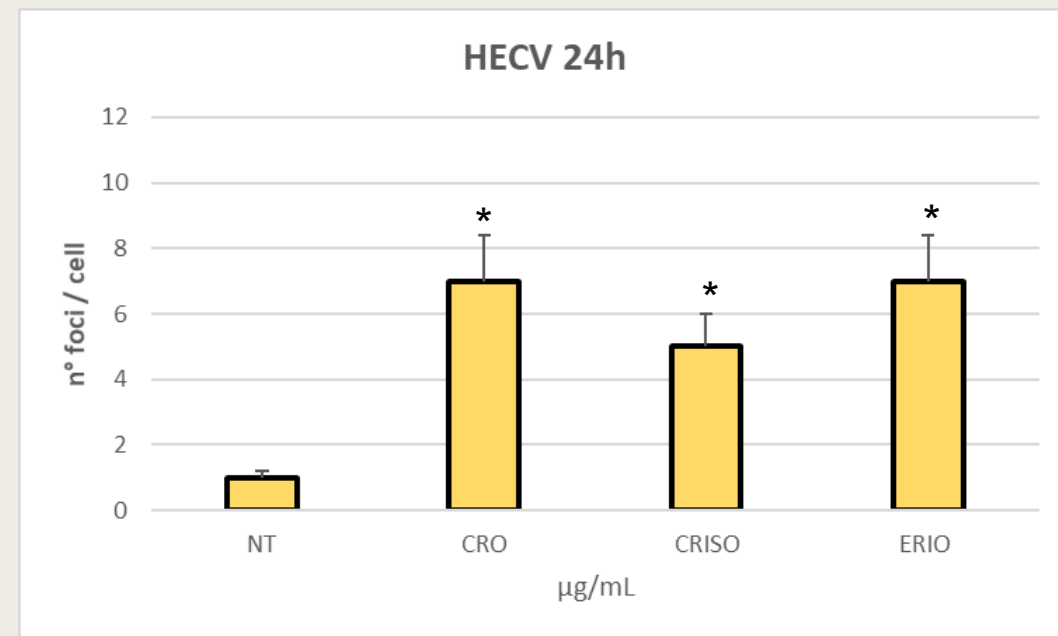
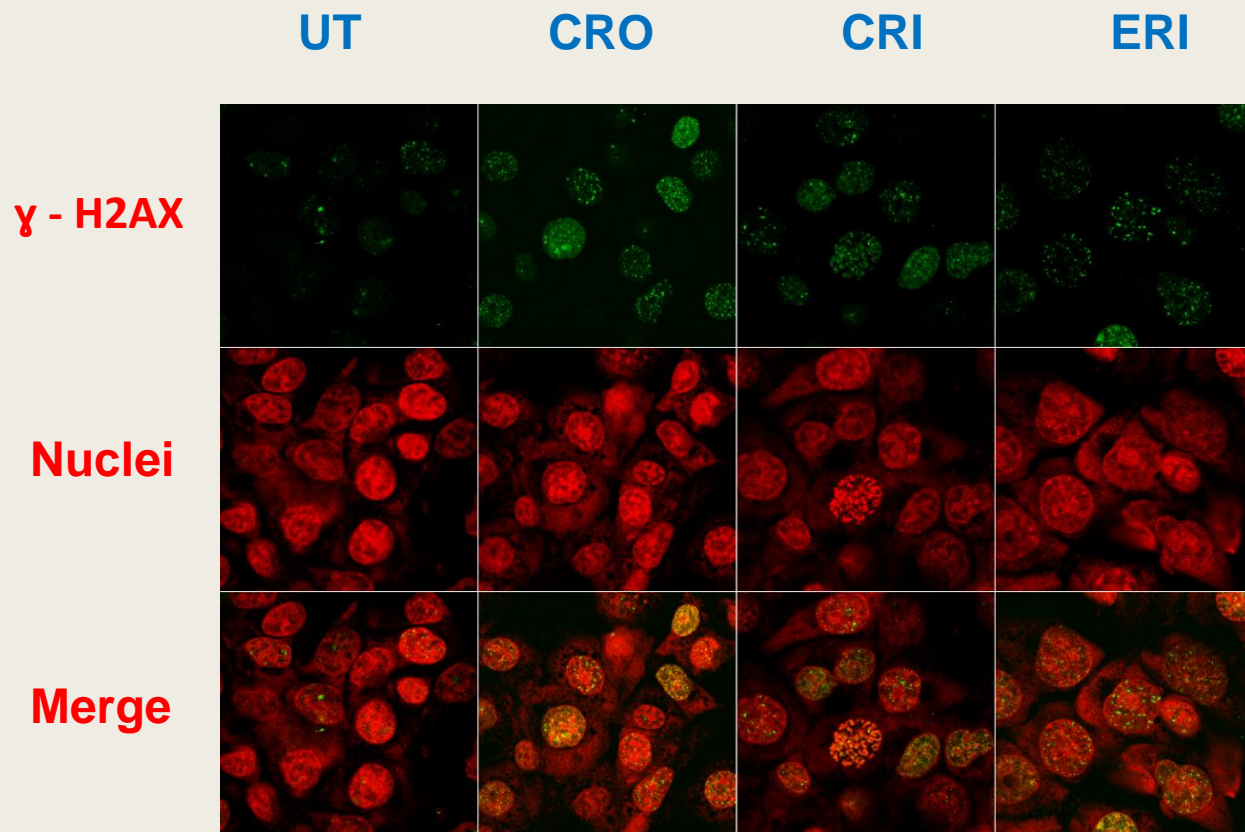
Oxidative stress analysis

ROS levels after 4 hrs exposure to **Crocidolite, Chrysotile and Erionite (50 µg/mL)** in HECV



***p<0.0001

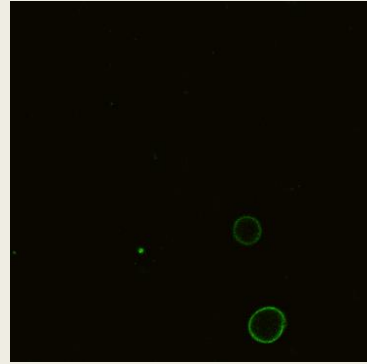
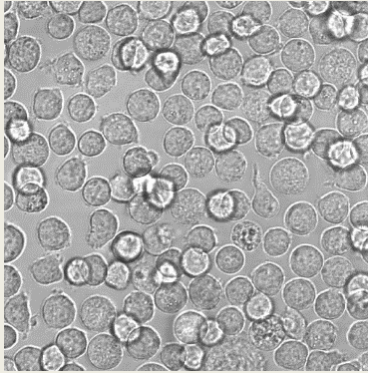
Confocal Microscopy for γ - H2AX foci in HECV cells after 24hrs of treatment with Crocidolite, Crisotilo, Erionite (50 μ g/mL)



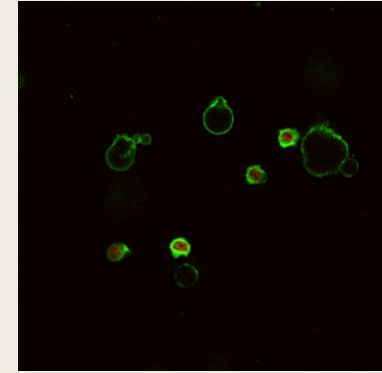
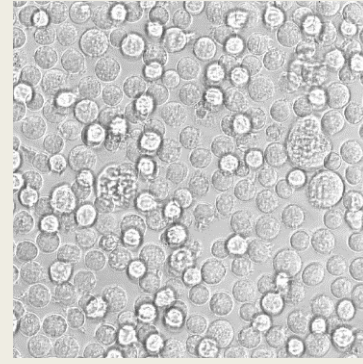
* $p < 0.1$

**First results with Russian
Chrysotile**

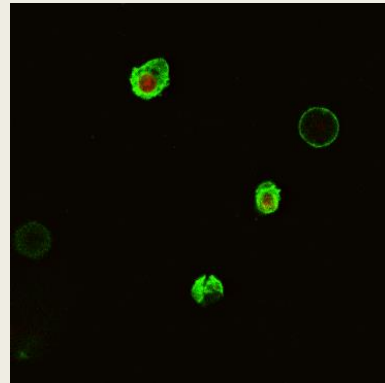
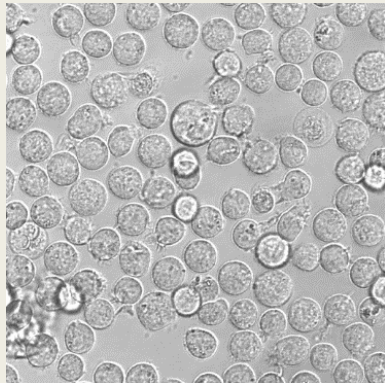
Evaluation of the **EARLY** apoptosis or **LATE** apoptosis by **Annexin** – **PI** assay in THP-1 after 48hrs exposure to Russian Chrysotile (50 $\mu\text{g}/\text{mL}$) **w Tl** (indirect contact)



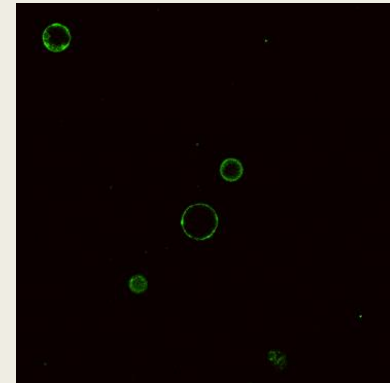
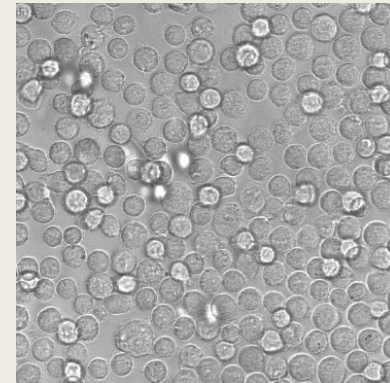
Magnification 20x; **Untreated**



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

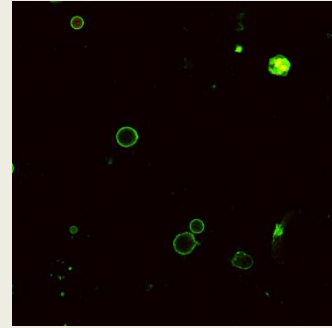
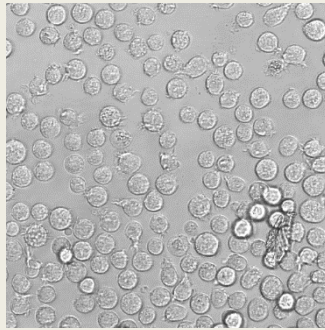


Magnification 20x; **Chrysotile < 5 μm**

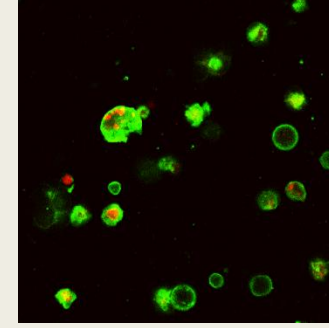
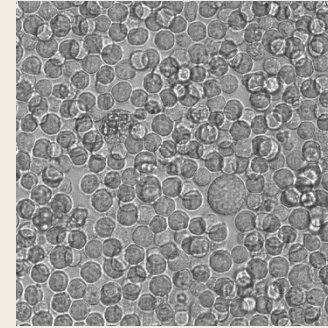


Magnification 20x; **Wollastonite**

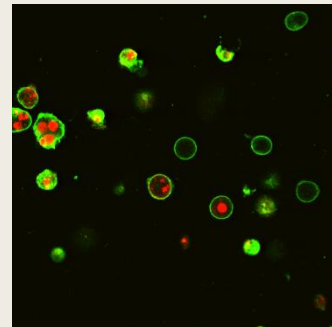
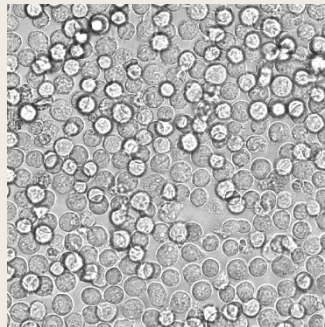
Evaluation of the **EARLY** apoptosis or **LATE** apoptosis by **Annexin** – **PI** assay in THP-1 after 72 hrs exposure to Russian Chrysotile (50 $\mu\text{g}/\text{mL}$) **W TI** (indirect contact)



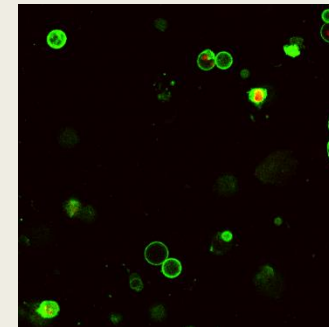
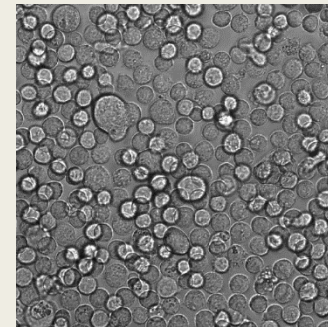
Magnification 20x; **Untreated**



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

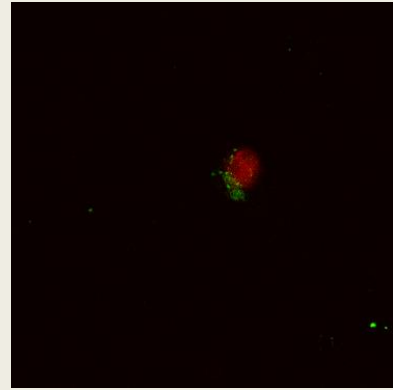
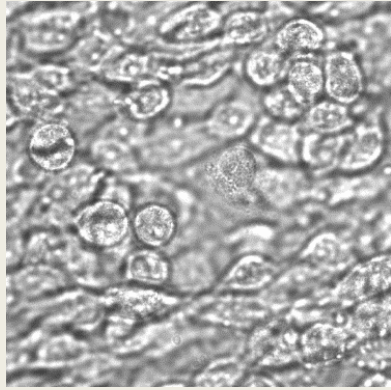


Magnification 20x; **Chrysotile < 5 μm**

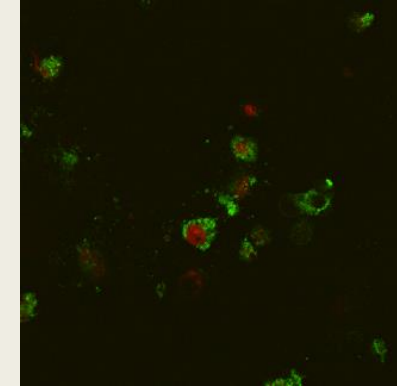
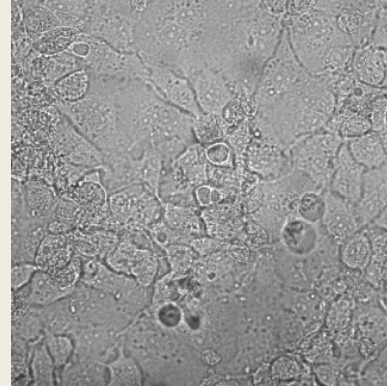


Magnification 20x; **Wollastonite**

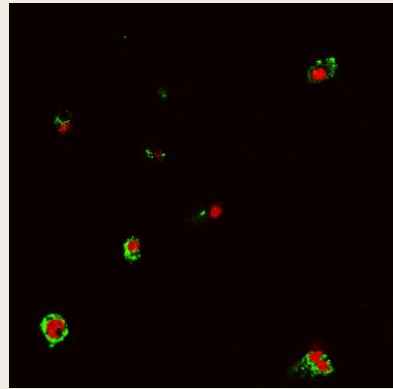
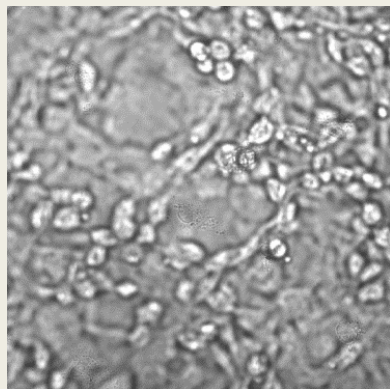
Evaluation of the **EARLY** apoptosis or **LATE** apoptosis by **Annexin – PI** assay in **HECV** after 48hrs exposure to **Russian Chrysotile** (50 $\mu\text{g}/\text{mL}$)



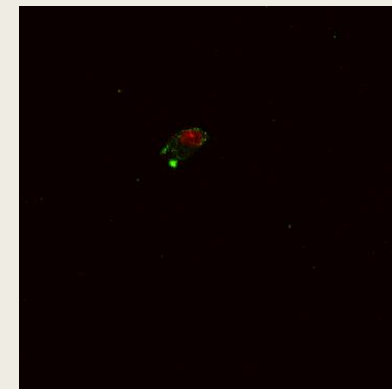
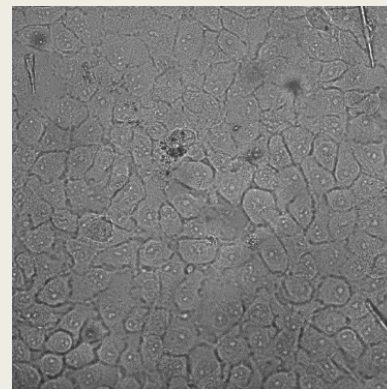
Magnification 60x; **Untreated**



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

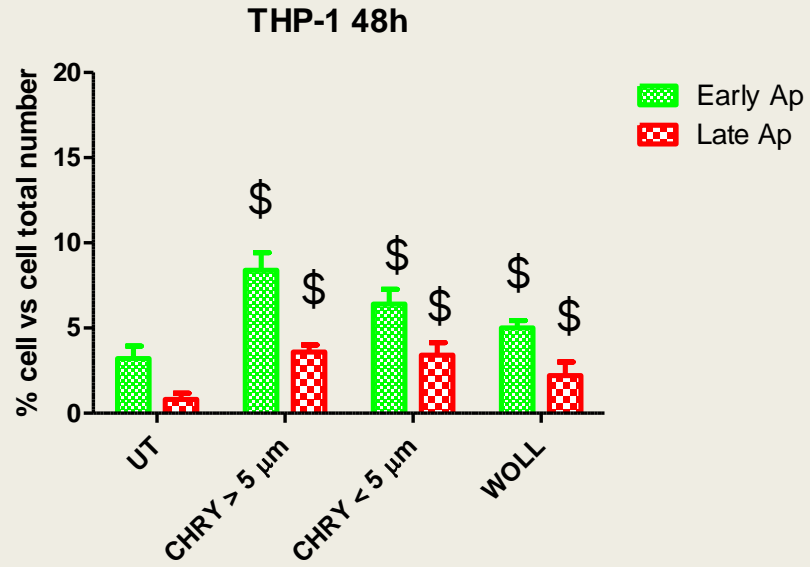


Magnification 60x; **Chrysotile < 5 μm**

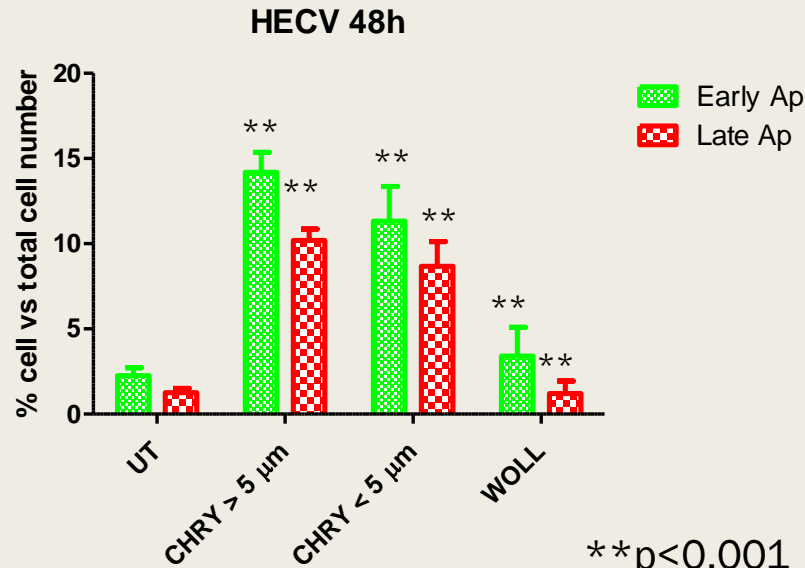
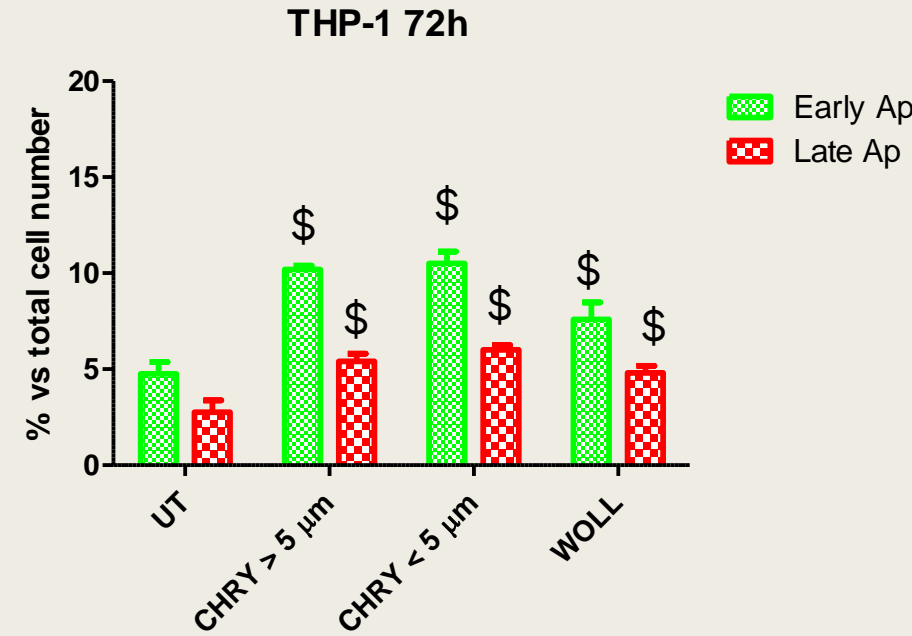


Magnification 60x; **Wollastonite**

EARLY apoptosis or **LATE** apoptosis (**Annexin** – **PI** assay) in HECV and THP-1 after exposure to Russian Chrysotile (50 µg/mL)



\$p<0.0001

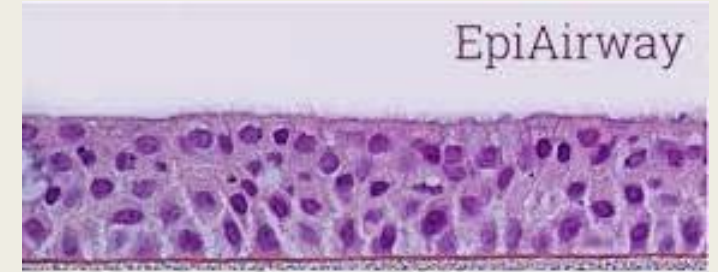


**p<0.001

WORK IN PROGRESS:

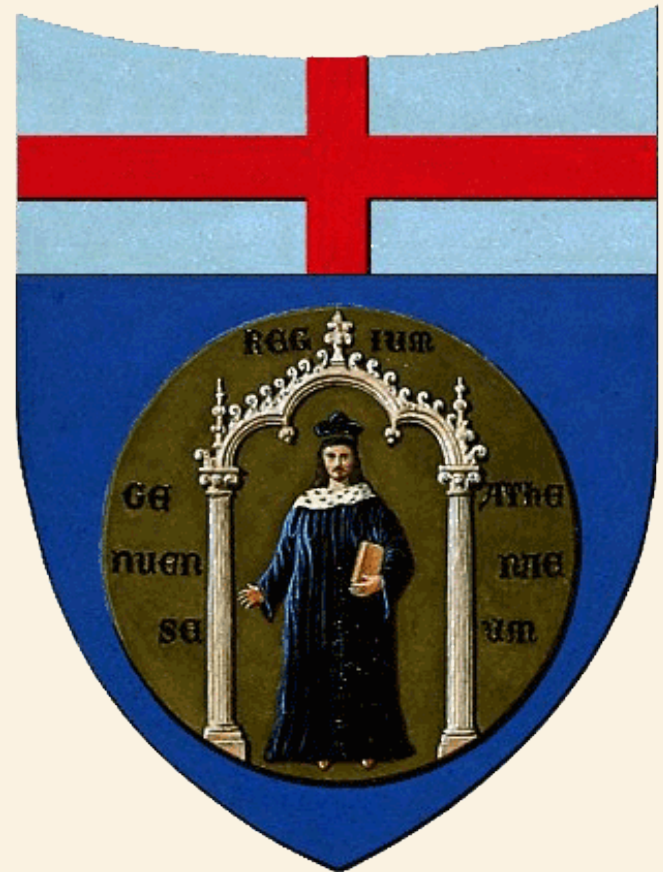
- ❑ **Commercial reconstructed model (EpiAirway, MatTek©)**
- ❑ **Synthetic Surfactant for A549 ALI exposure**
- ❑ **Analysis on human normal fibroblasts (HFF2)**
- ❑ **Coculture of THP-1 naive, M0, HECV, HFF2 and A549 ALI in bioreactors**

- ❑ **Analysis of Russian Chrysotile toxic effects**



Università degli Studi di Genova

**Valutazione del danno
citotossico e pro-
infiammatorio causato
dall'esposizione alle fibre di
amianto su modelli cellulari
umani integrati in 3D di
nuovo sviluppo**



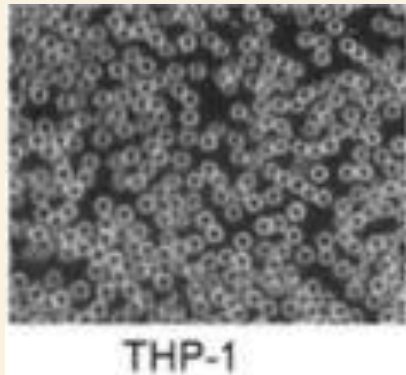
SCOPO DEL PROGETTO

Allo scopo di ottenere un **modello previsionale** per una valutazione quantitativa della tossicità, della cancerogenicità e degli effetti molecolari a lungo termine indotti dall'esposizione cronica a tali fibre minerali, ci si propone di:

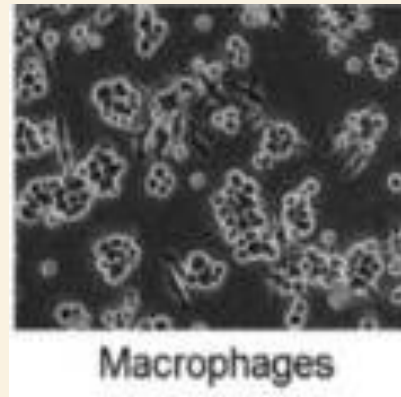
- ricostruire con modelli biodinamici in 3D i **tessuti epiteliali umani respiratori (boccale, bronchiale e alveolare)** con cui le fibre interagiscono per contatto o per inalazione, valutando alcune fondamentali risposte cellulari a dosi e tempistiche specifiche
- studiare i meccanismi infiammatori indotti dalle varie fibre minerali nelle **cellule fagocitiche**, dapprima in monoculture bidimensionali e successivamente in modelli 3D integrati con gli epitelii di cui sopra, per creare modelli *in vitro* più fedeli alle condizioni patologiche *in vivo*



MODELLO DI CELLULE FAGOCITICHE



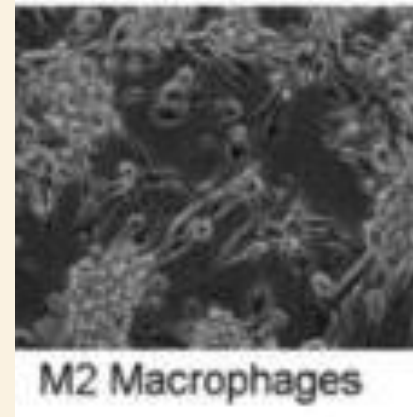
PMA →



INF- γ
e LPS ↗



IL-4 ↘



LE FIBRE MINERALI FORNITE A UNIGE

Crocidolite

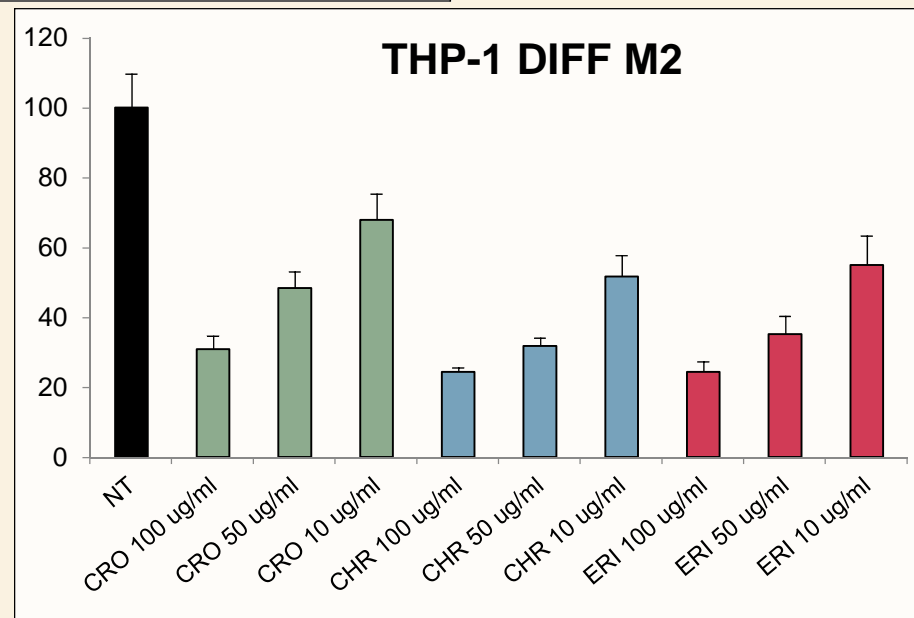
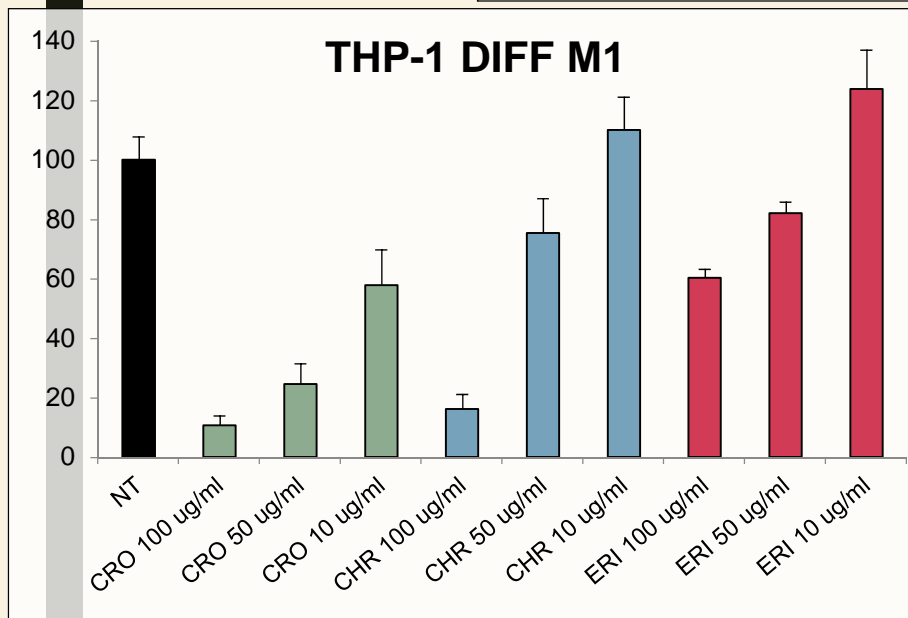
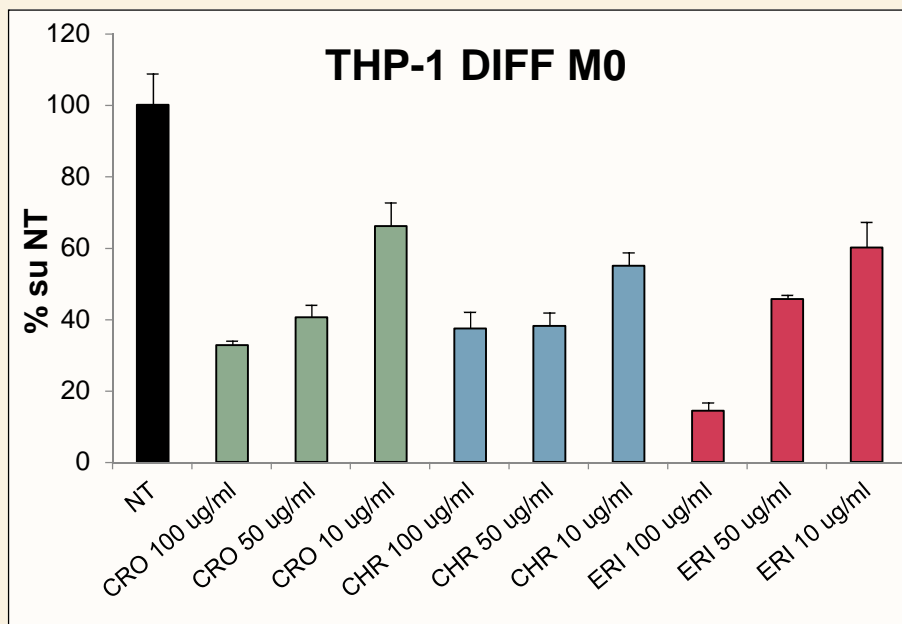


Erionite Nevada USA

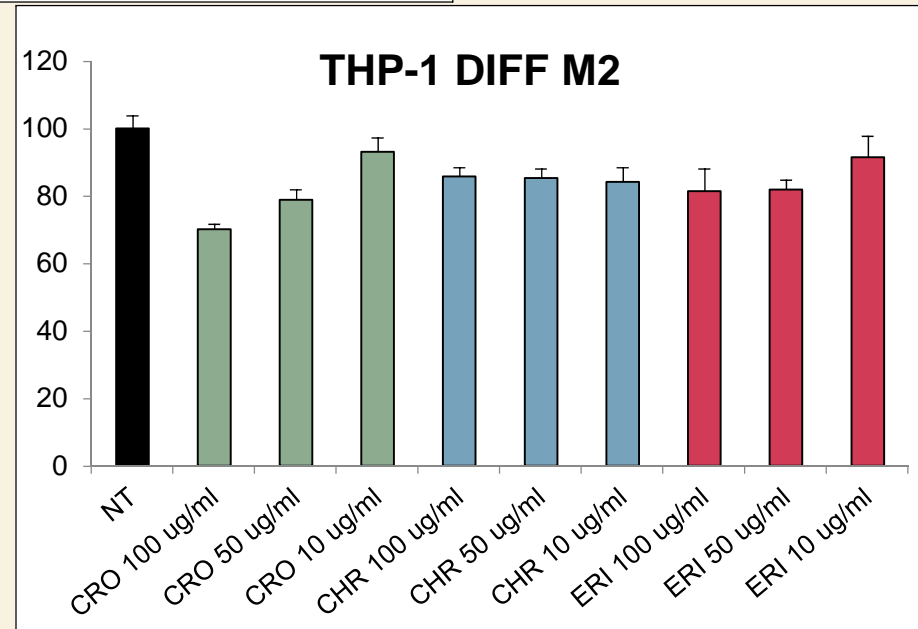
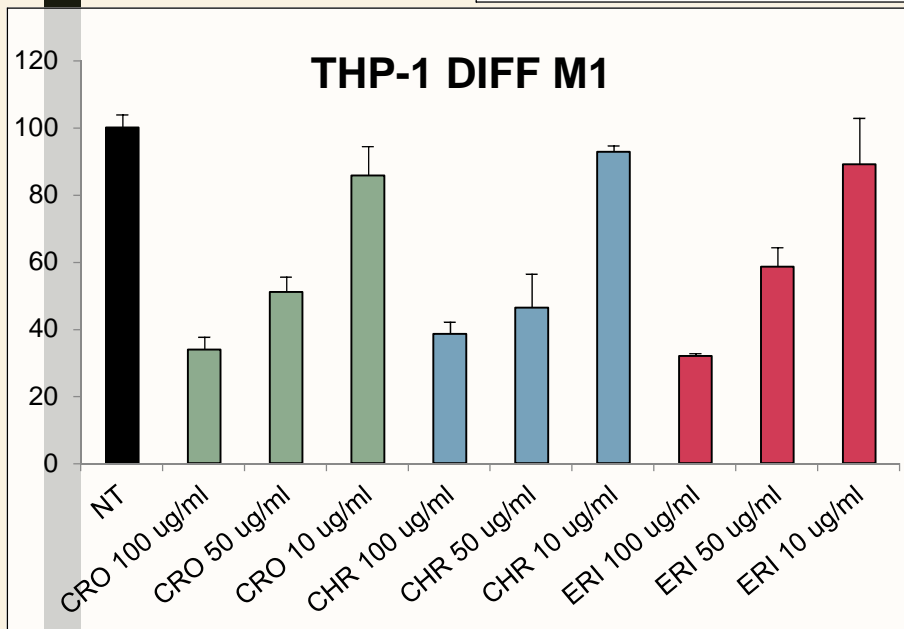
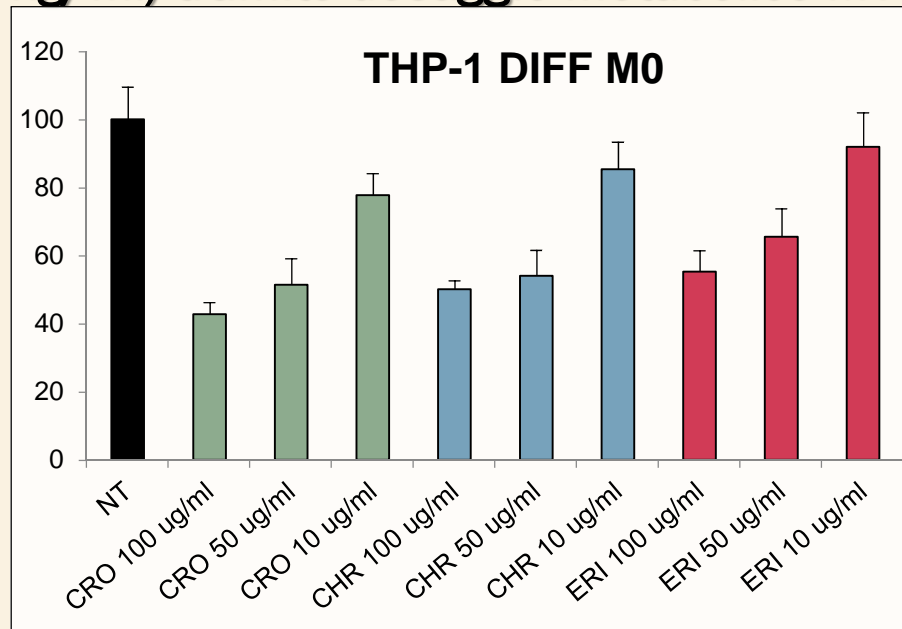


Crisotilo di Balangero

Valutazione della tossicità cellulare a 24h (diff PMA 60 ng/ml) tramite dosaggio metabolico MTT

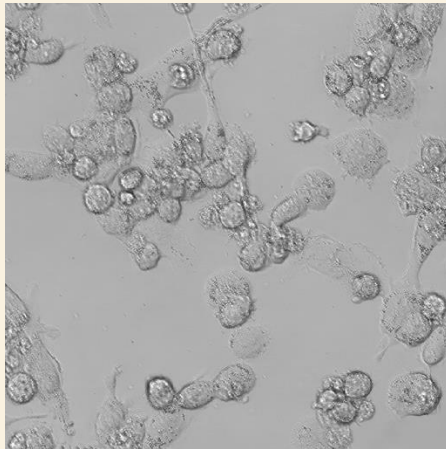
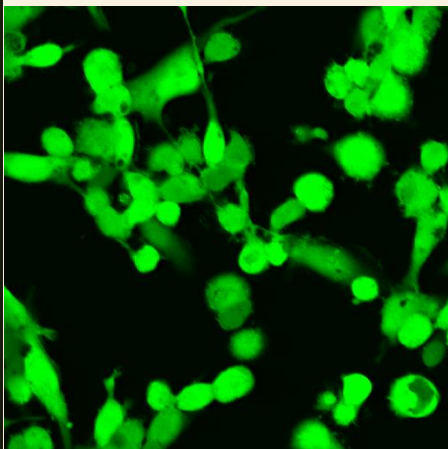


Valutazione della tossicità cellulare a 24h (diff PMA 20 ng/ml) tramite dosaggio metabolico MTT

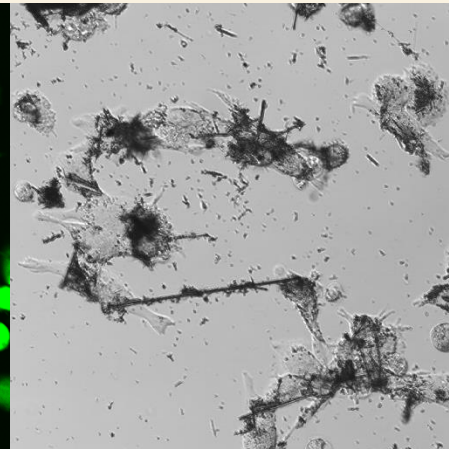
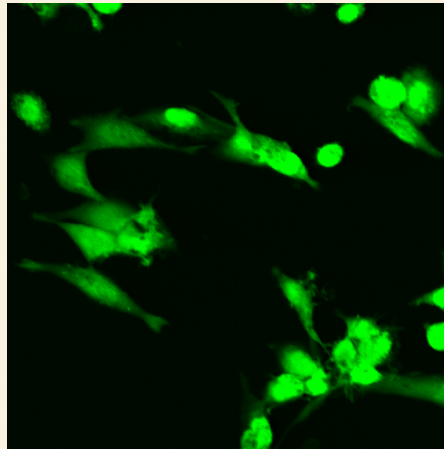


Valutazione della vitalità di M0 con fibre 50 ug/ml a tempi brevi (4h) tramite marcatura con Calcein Green (20x 2.5x)

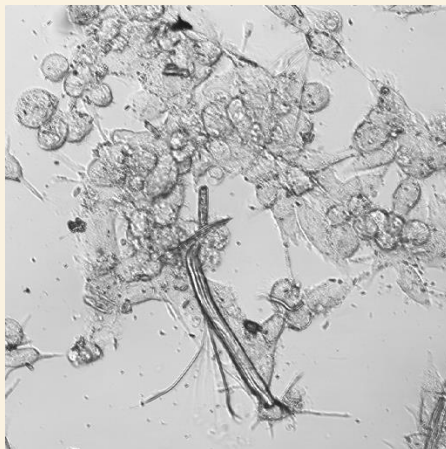
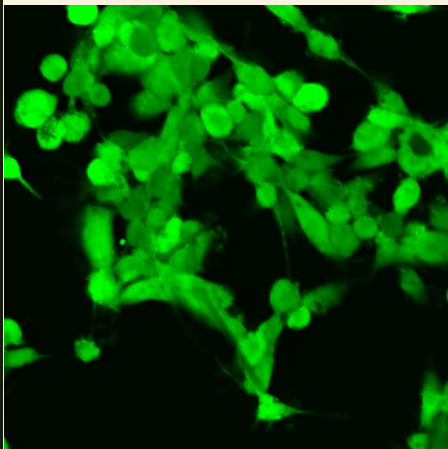
NT



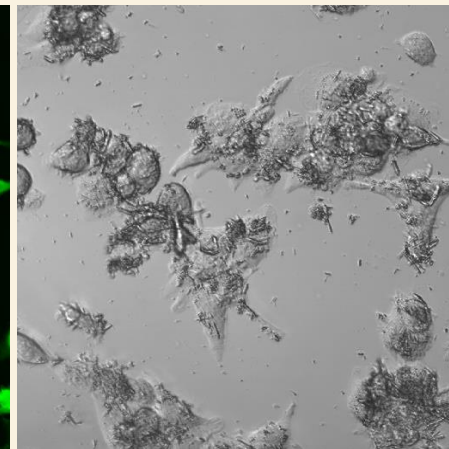
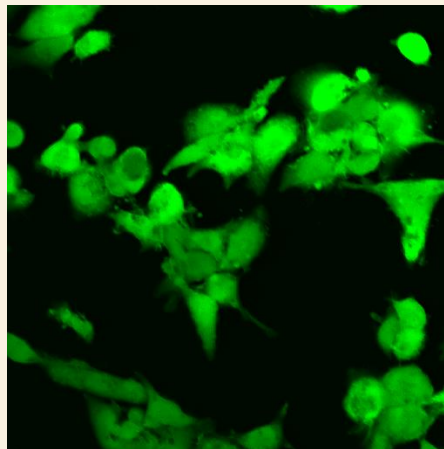
Crocidolite 50



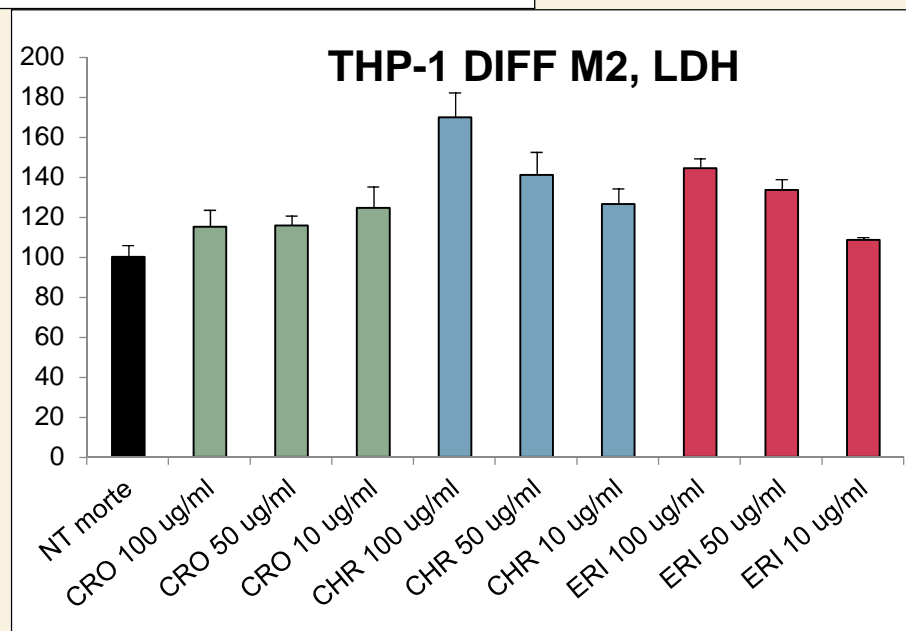
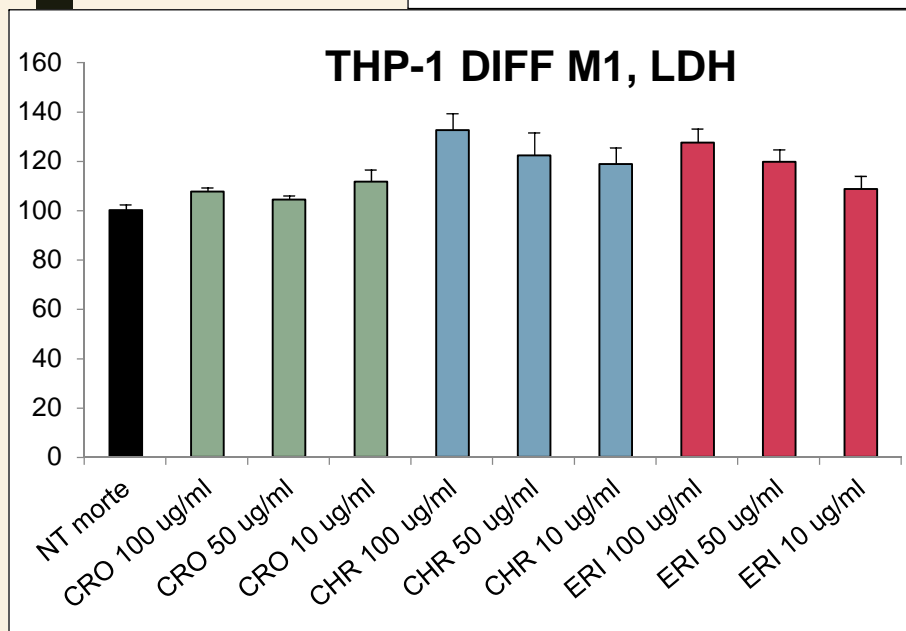
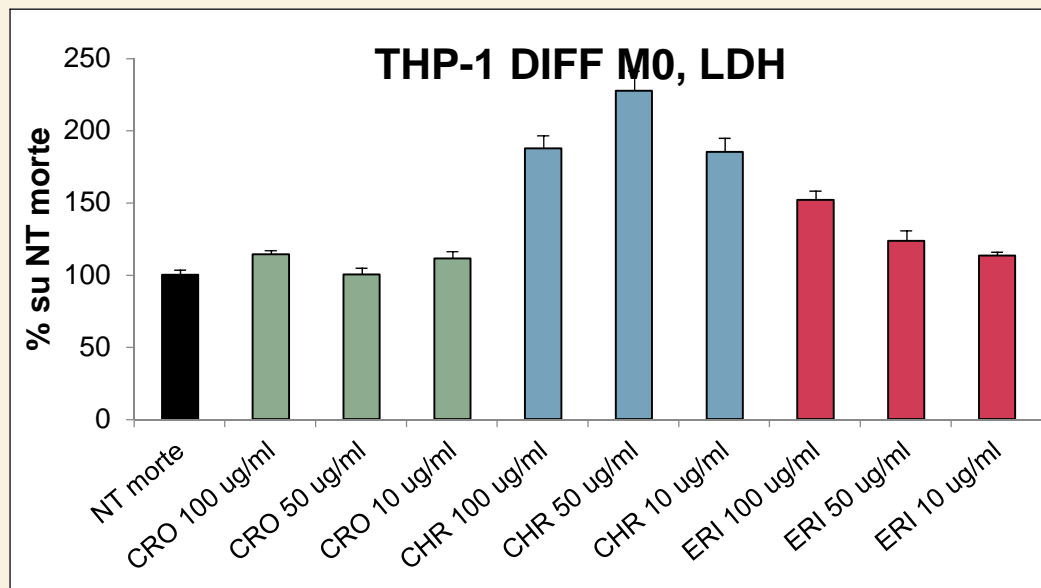
Crisotilo 50



Erionite 50

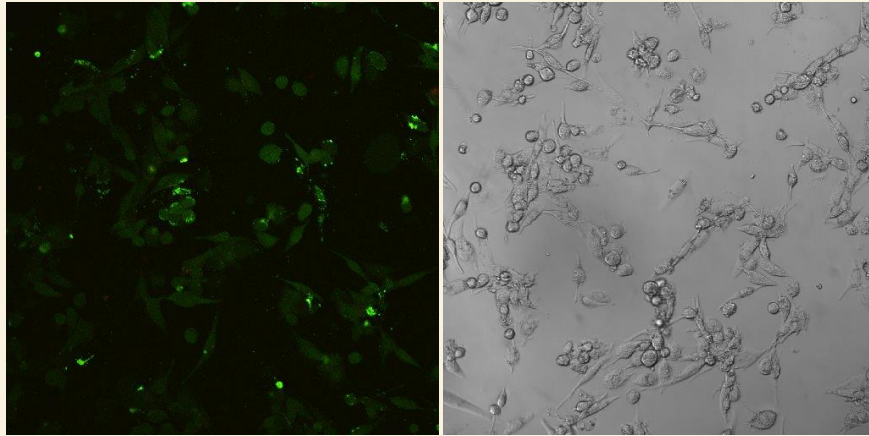


Valutazione della morte cellulare per NECROSI a 24h di M0 (PMA 20 ng/ml) con dosaggio del rilascio di LDH

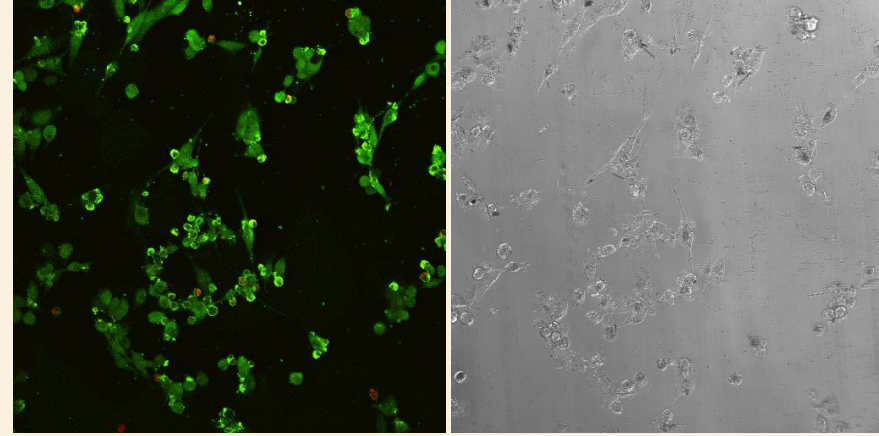


Valutazione della morte cellulare per APOPTOSI in M0 (PMA 20 ng/ml) a 24h con fibre 10 ug/ml tramite marcatura con Annessina e Ioduro di Propidio (20x)

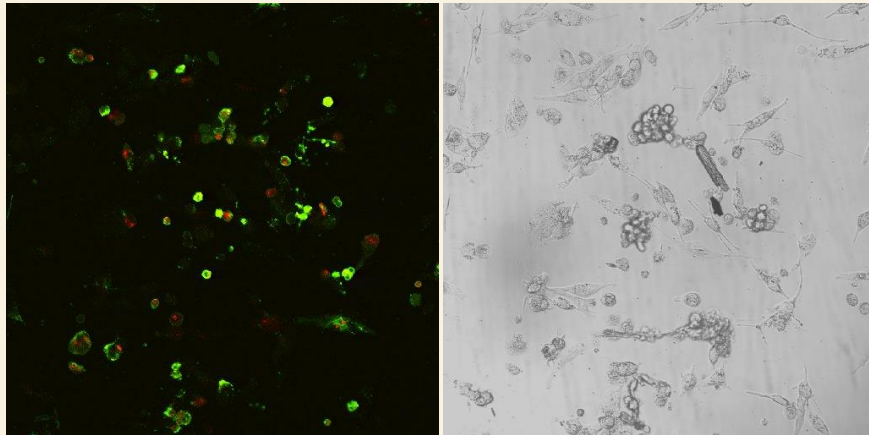
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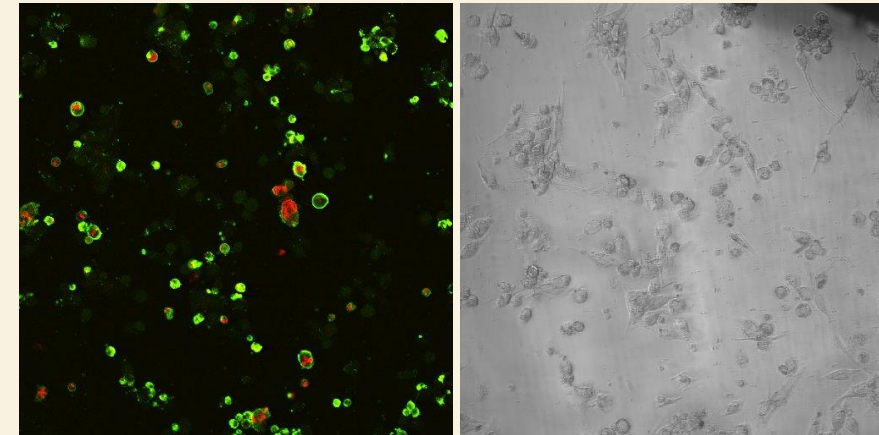
Crocidolite 10



Crisotilo 10



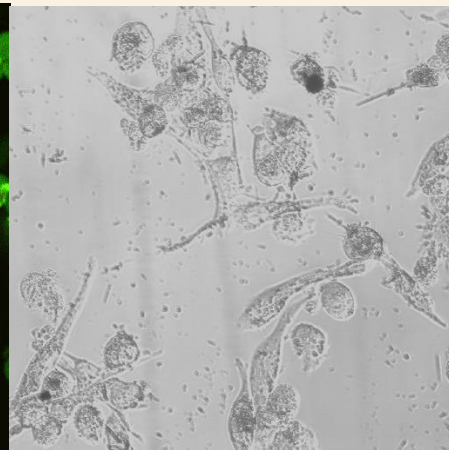
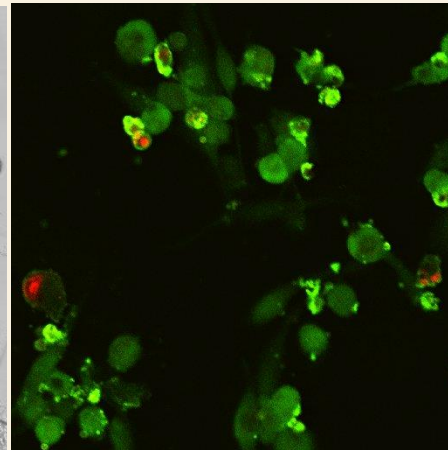
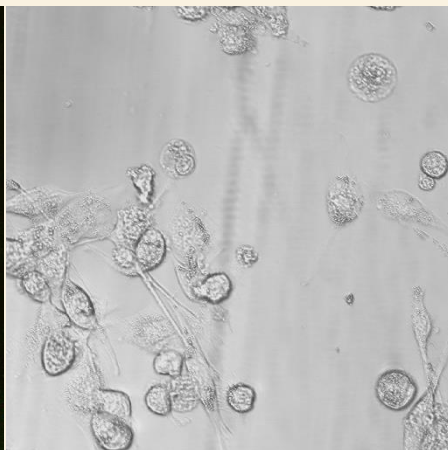
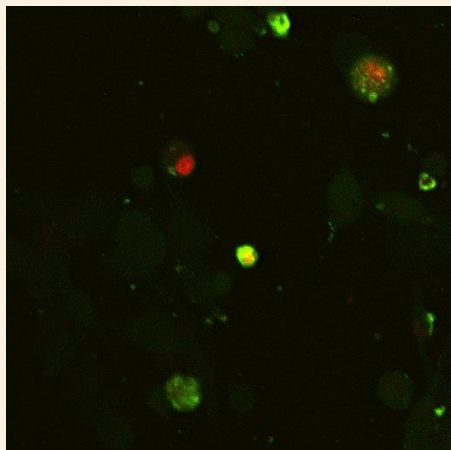
Erionite 10



Valutazione APOPTOSI in M0 a 24h con fibre 10 ug/ml con annessina e ioduro di propidio (ingrandimento)

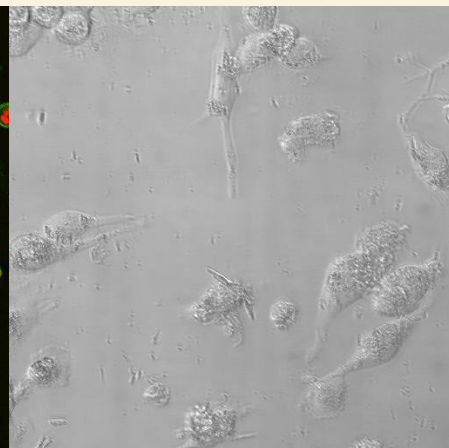
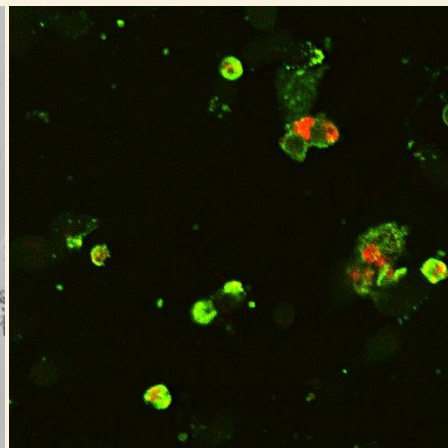
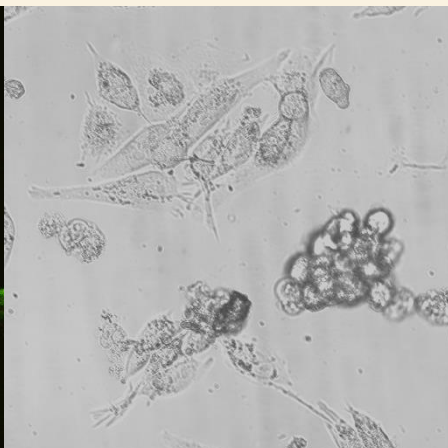
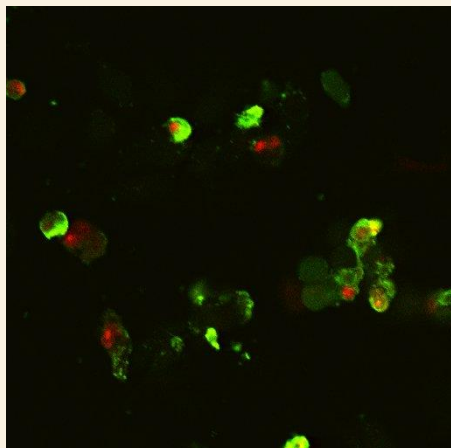
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Crocidolite 10



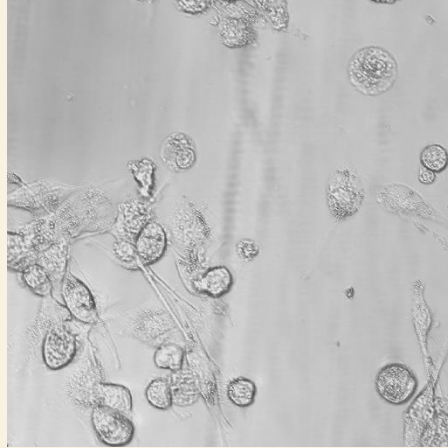
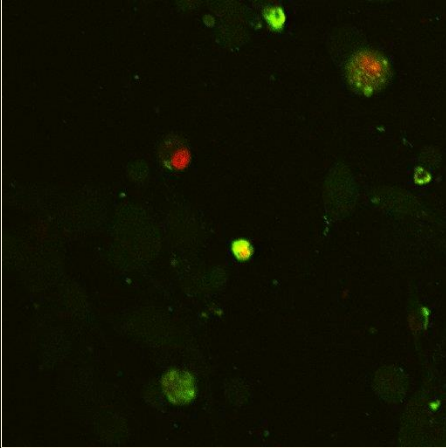
Crisotilo 10

Erionite 10

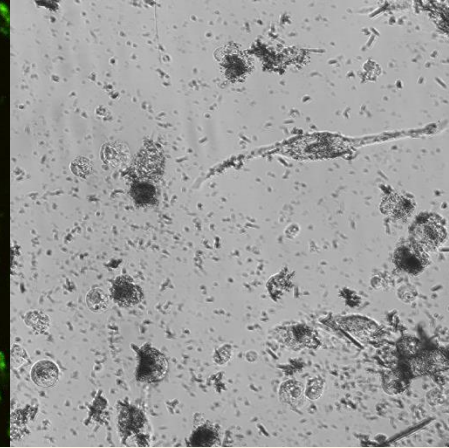
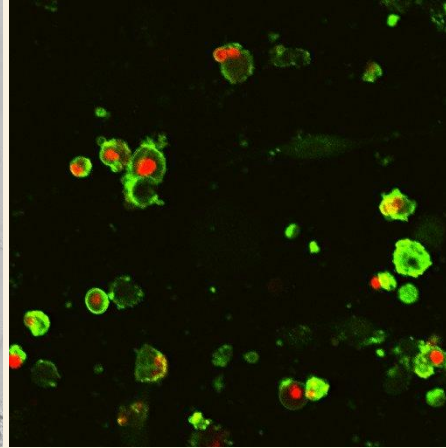


Valutazione APOPTOSI in M0 a 24h con fibre 50 ug/ml con annexina e ioduro di propidio (ingrandimento)

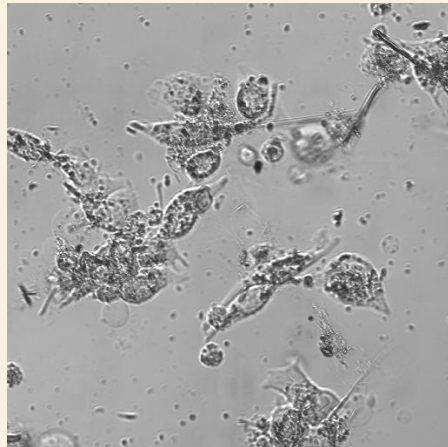
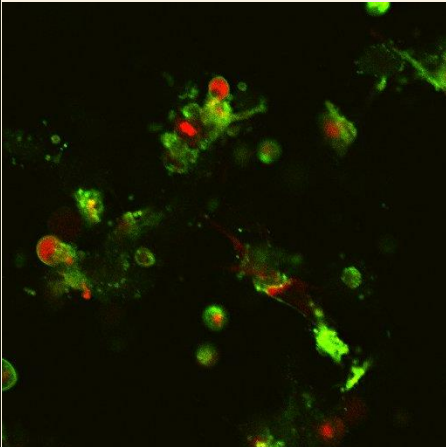
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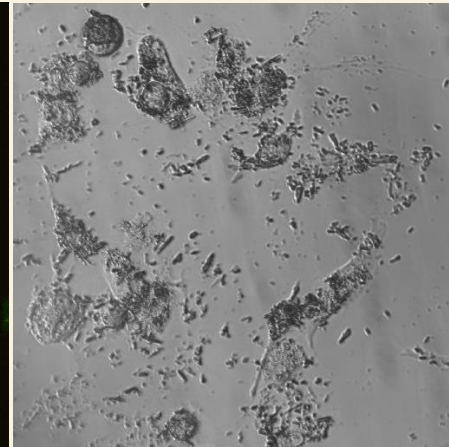
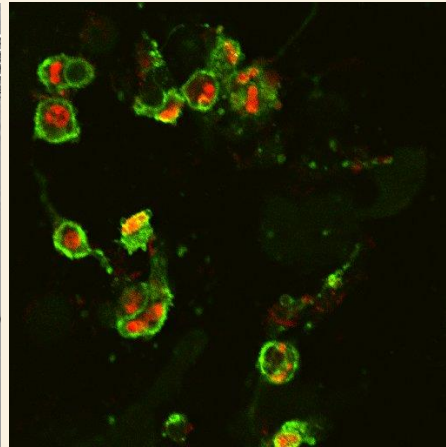
Crocidolite 50



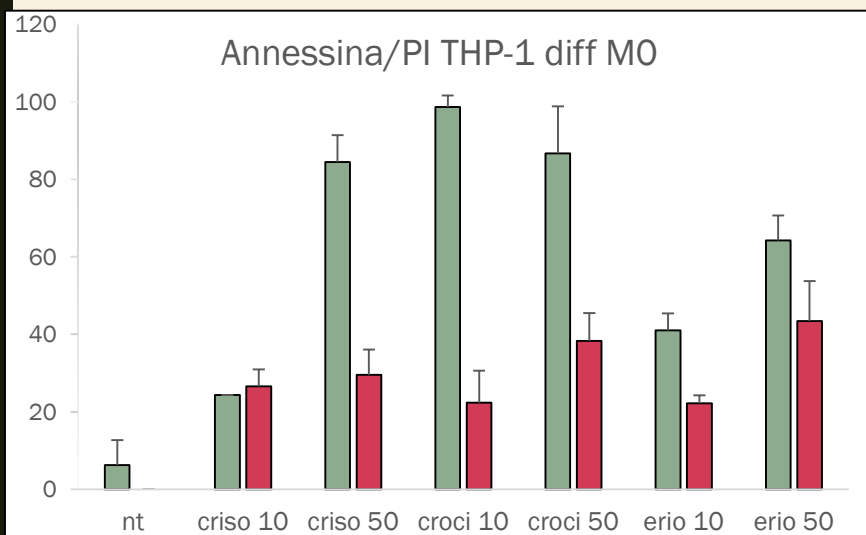
Crisotilo 50



Erionite 50

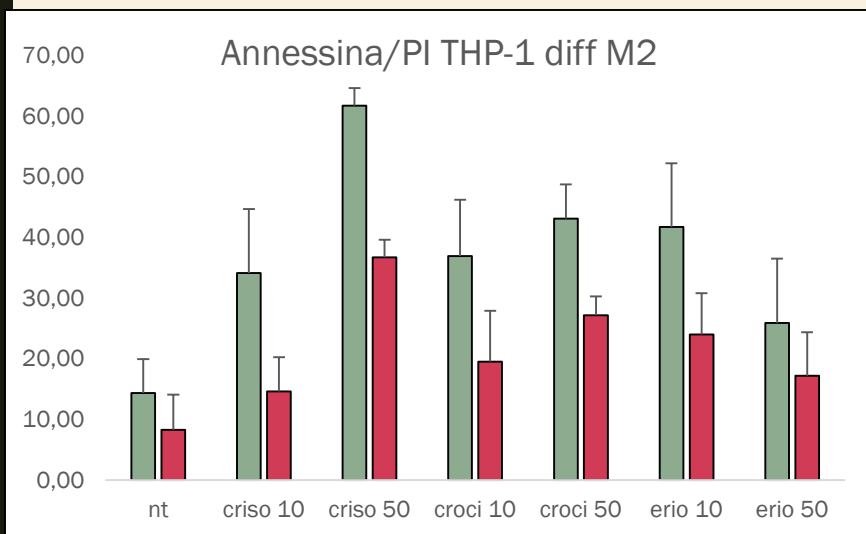
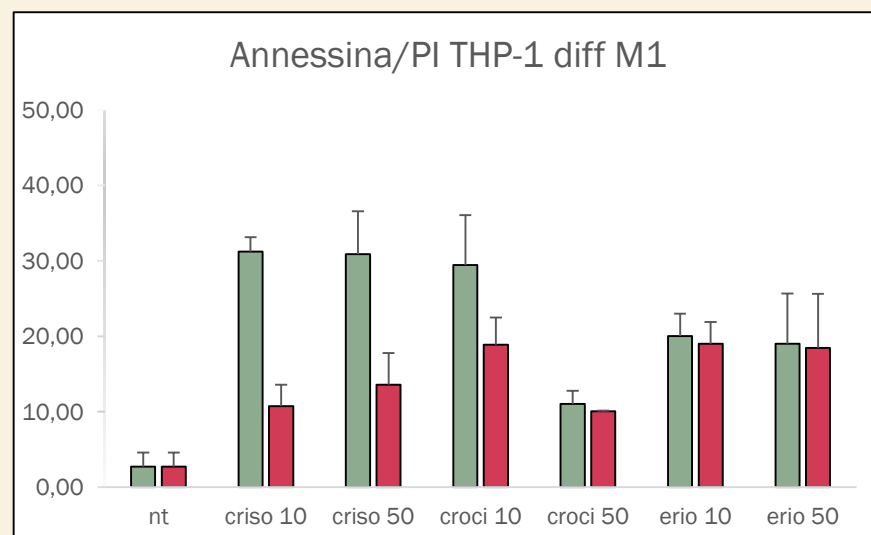


Valutazione APOPTOSI in M0, M1 e M2 a 24h con fibre 50 ug/ml e 10 ug/ml con annessina e ioduro di propidio



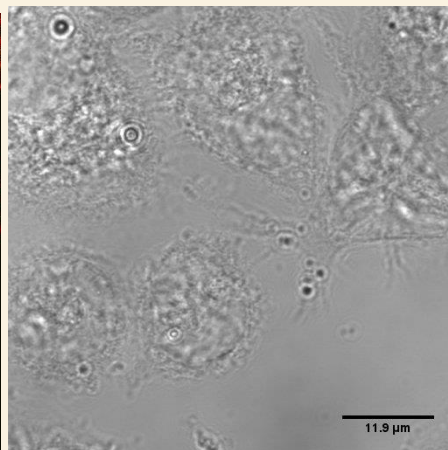
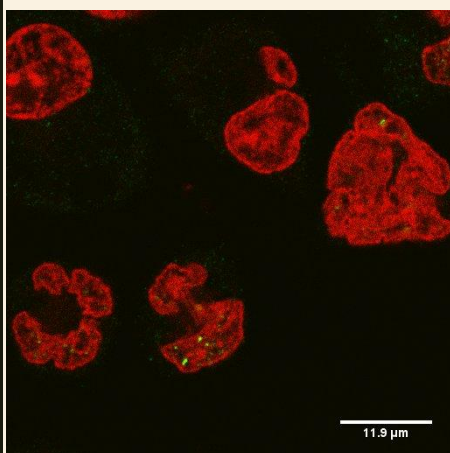
■ % early apoptotic (green)

■ % late apoptotic (green+red)

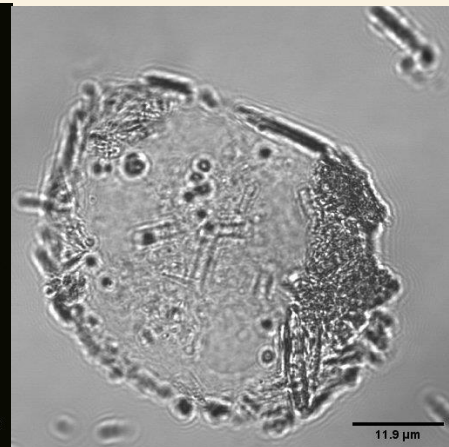
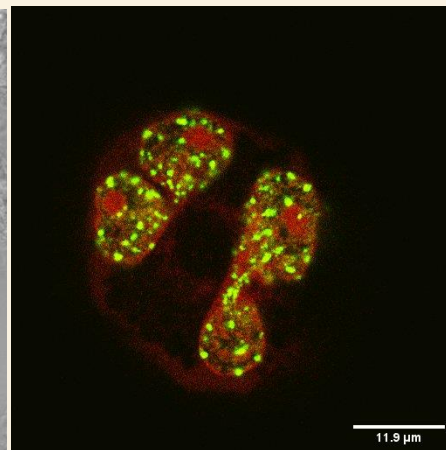


Valutazione del danno al DNA in M0 a 24h con fibre 50 ug/ml con γ -H2AX (ingrandimento 63x 4x)

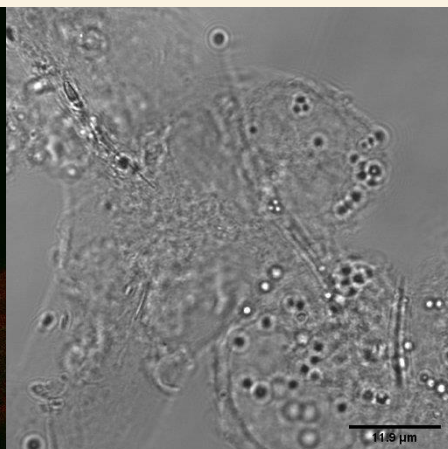
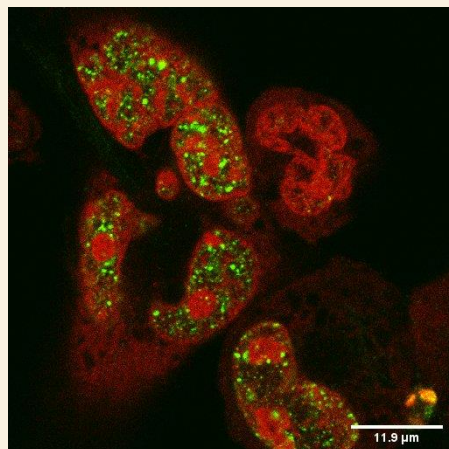
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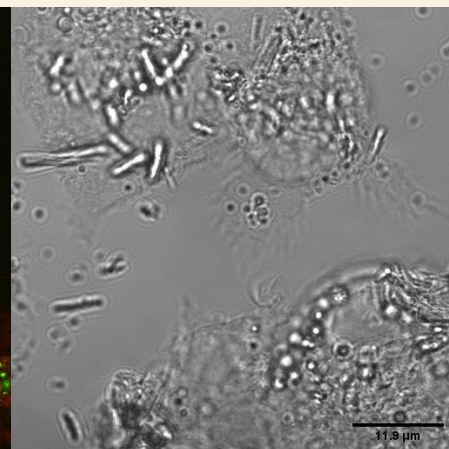
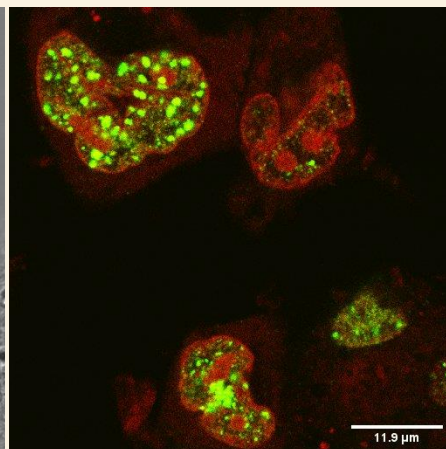
Crocidolite 50



Crisotilo 50

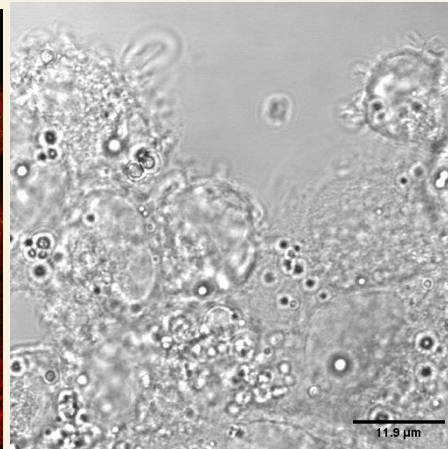
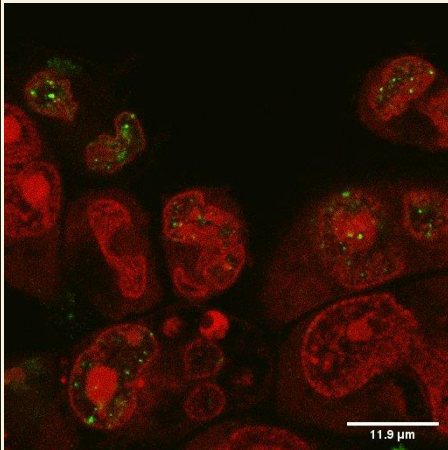


Erionite 50

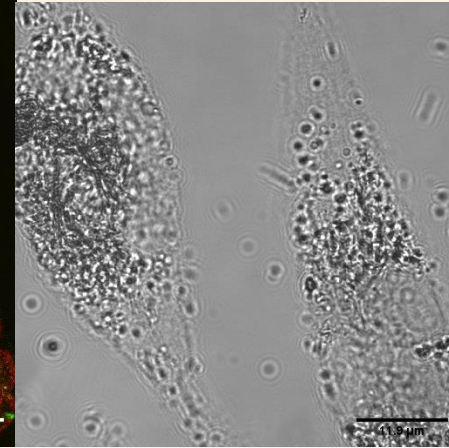
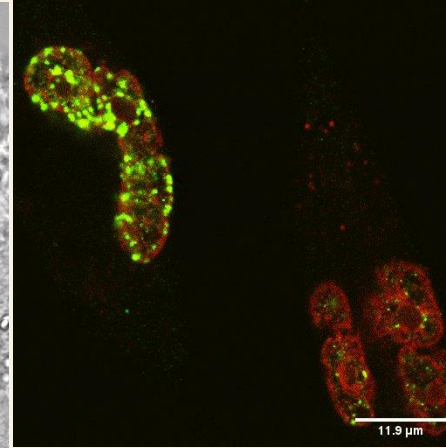


Valutazione del danno al DNA in M1 a 24h con fibre 50 ug/ml con γ -H2AX (ingrandimento 63x 4x)

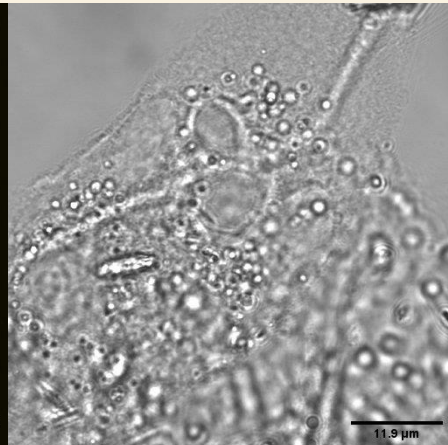
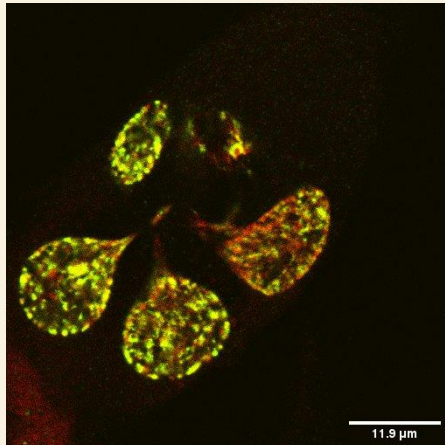
NT



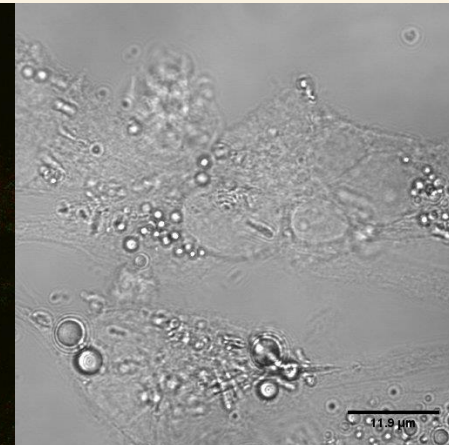
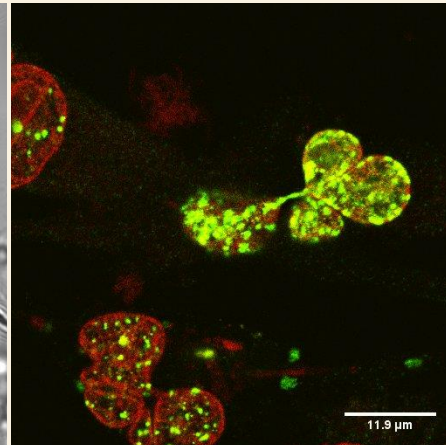
Crocidolite 50



Crisotilo 50

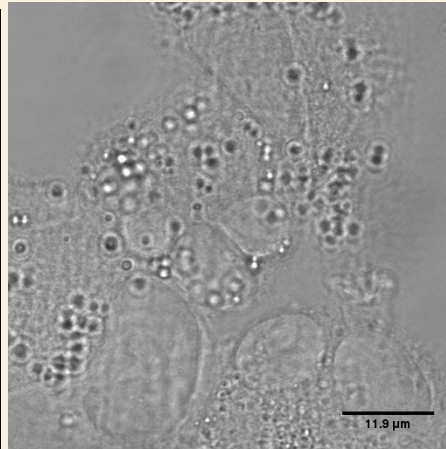
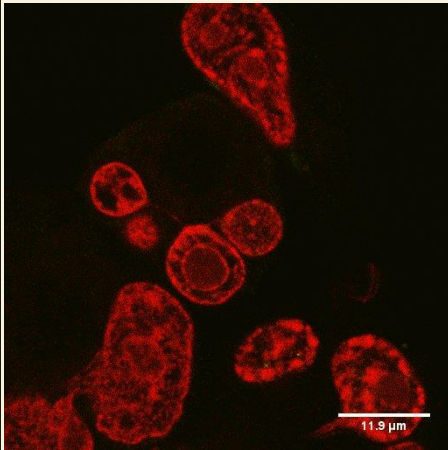


Erionite 50

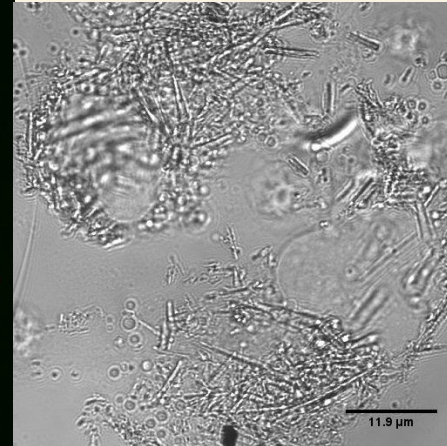
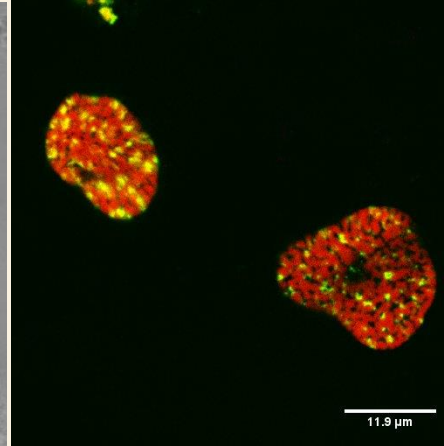


Valutazione del danno al DNA in M2 a 24h con fibre 50 ug/ml con γ -H2AX (ingrandimento 63x 4x)

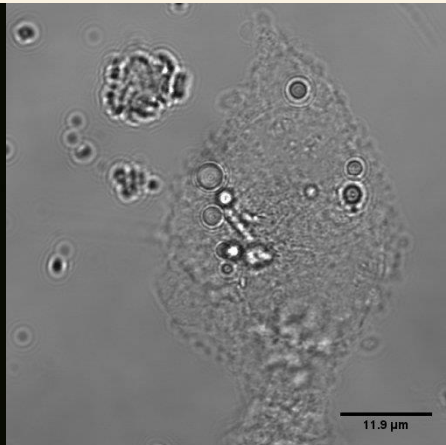
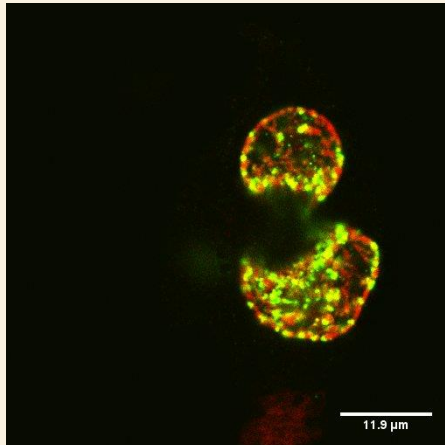
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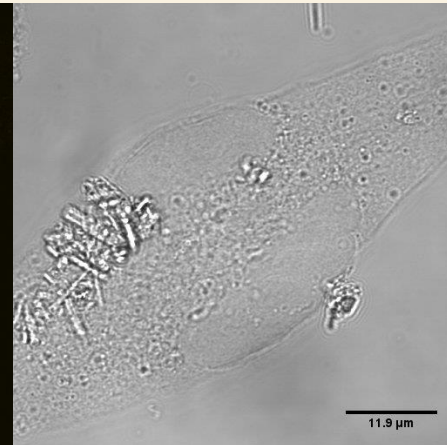
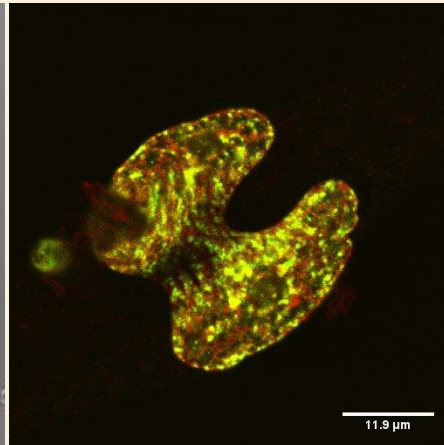
Crocidolite 50



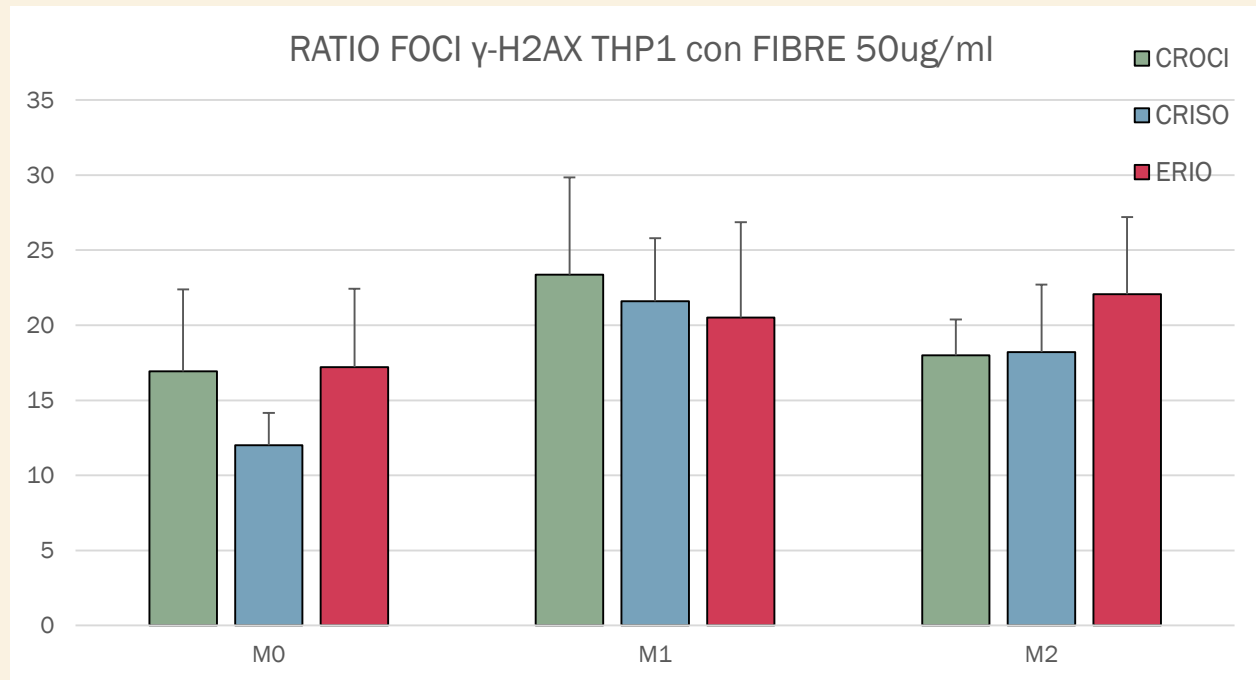
Crisotilo 50



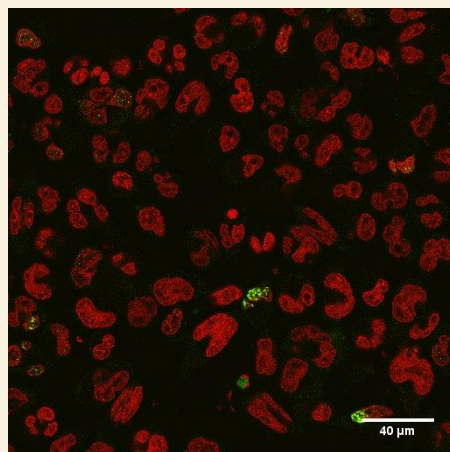
Erionite 50



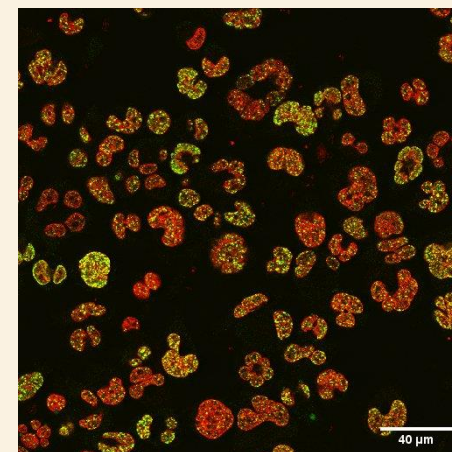
Valutazione del danno al DNA in M0, M1 e M2 a 24h con fibre 50 ug/ml sulla base dei foci evidenziati dal γ -H2AX



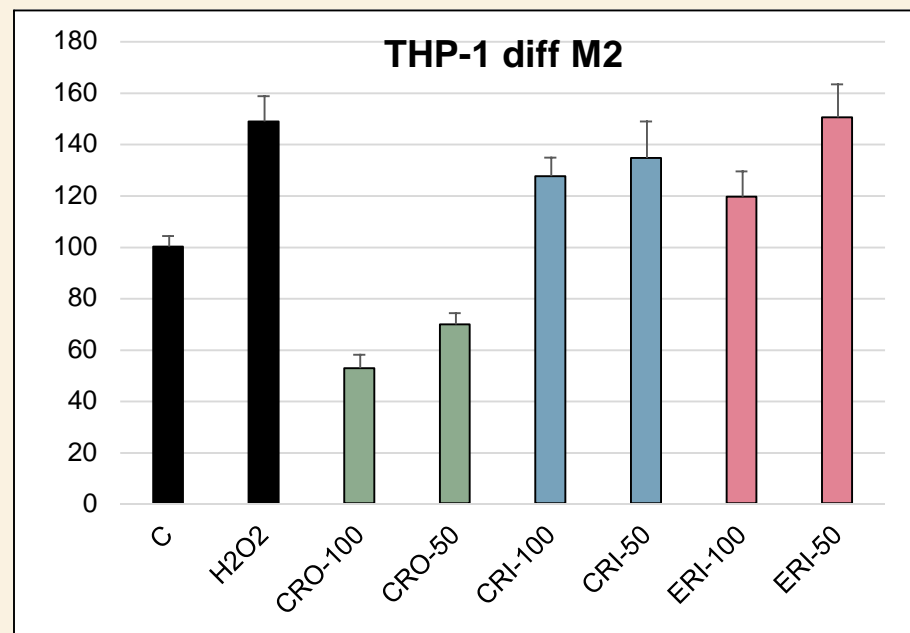
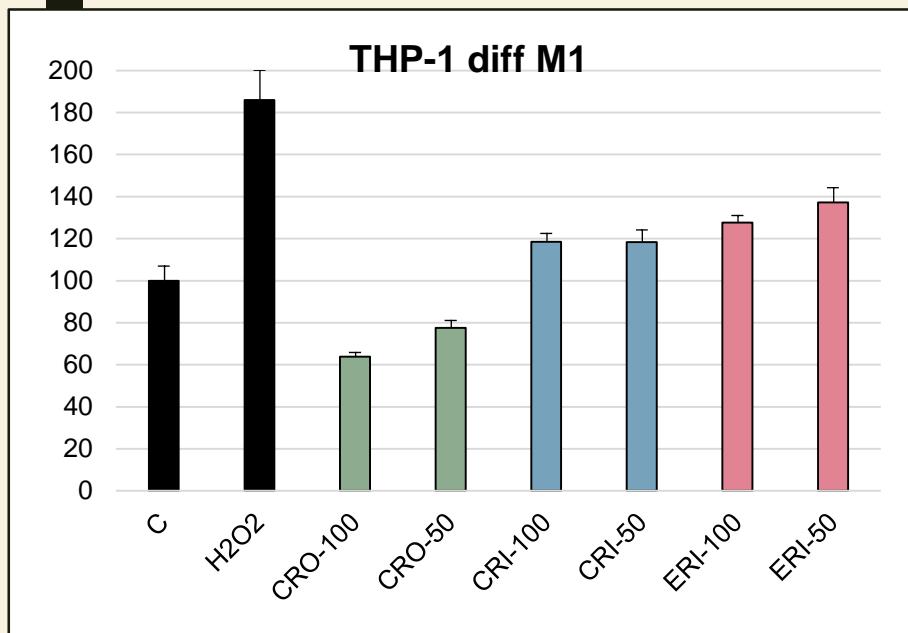
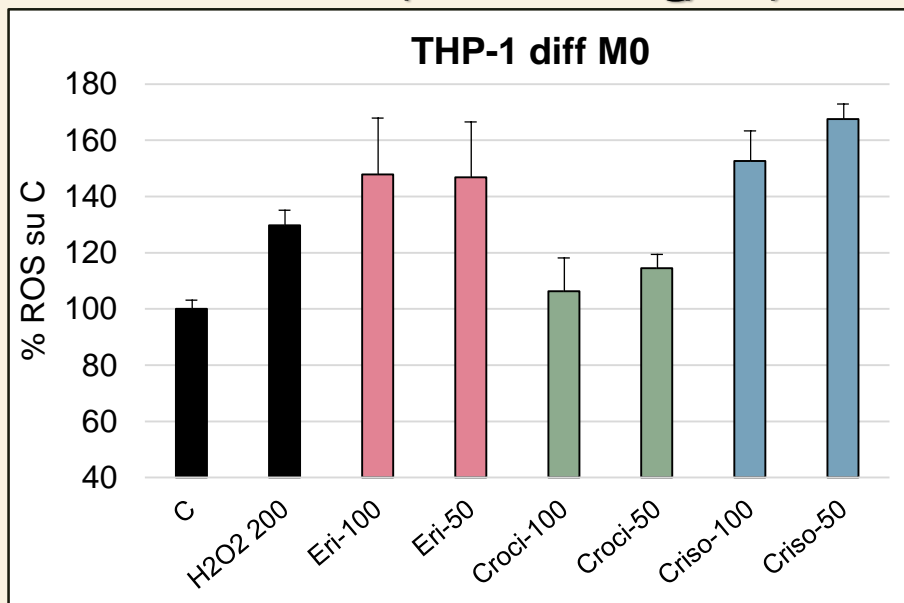
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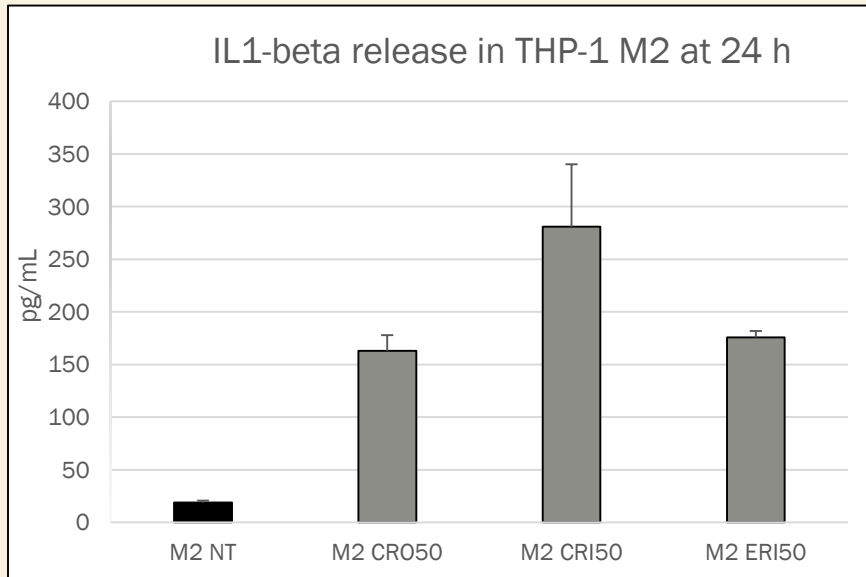
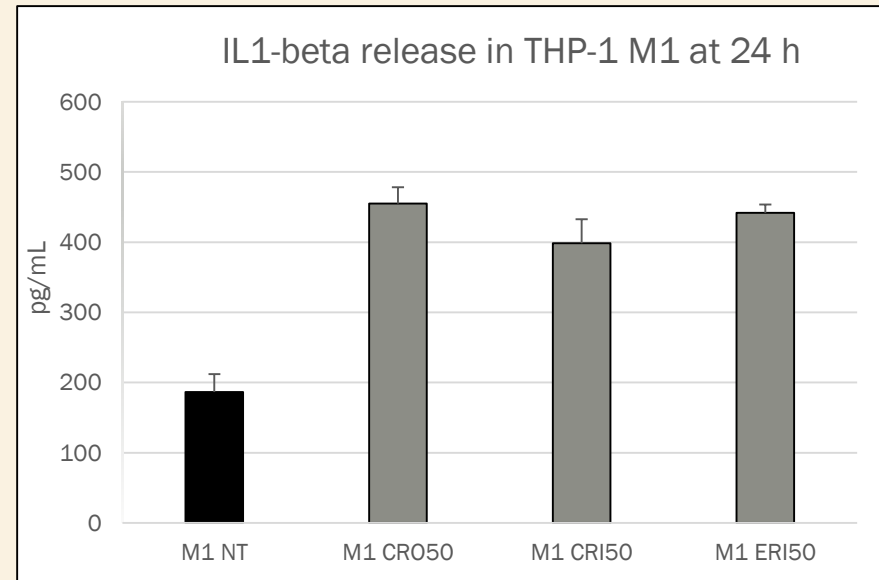
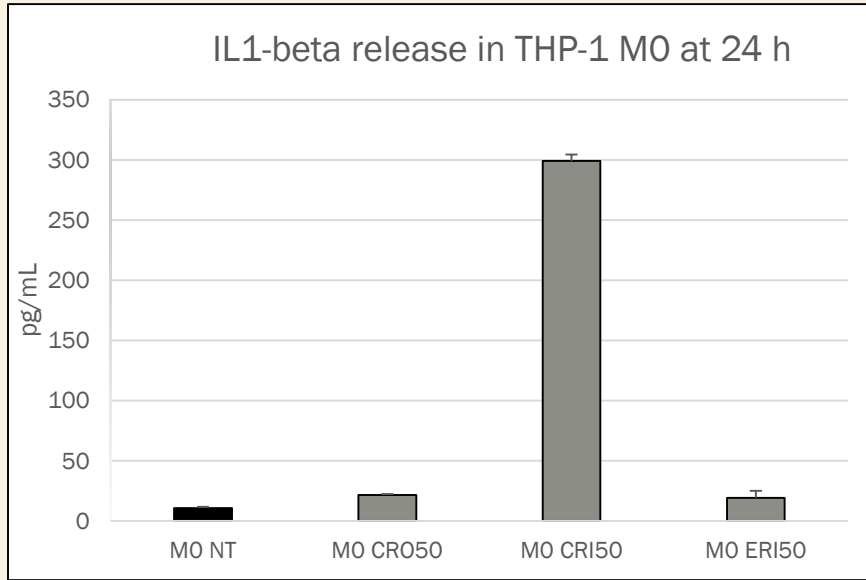
DOXORUBICINA



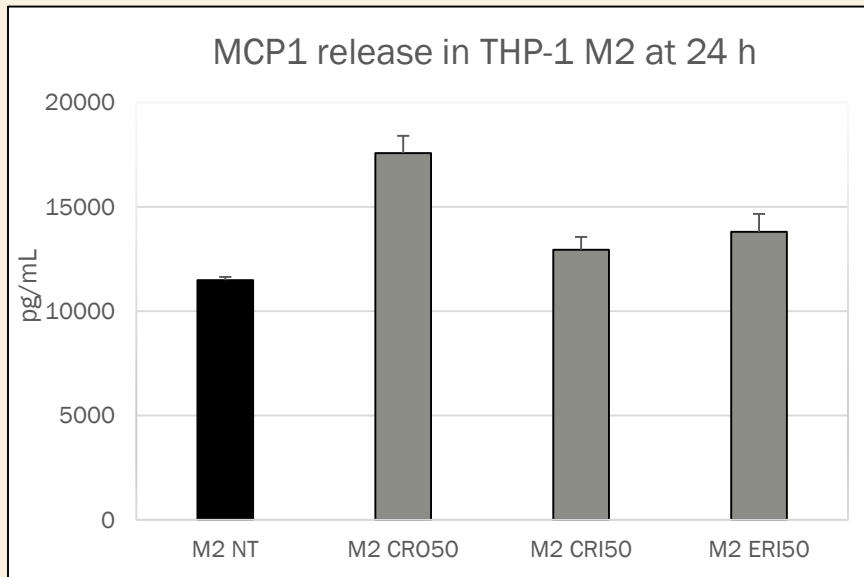
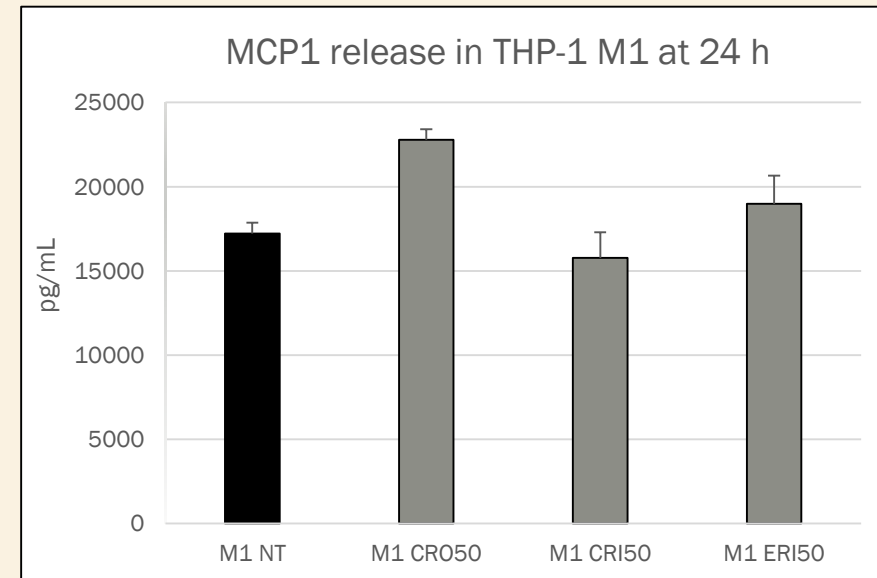
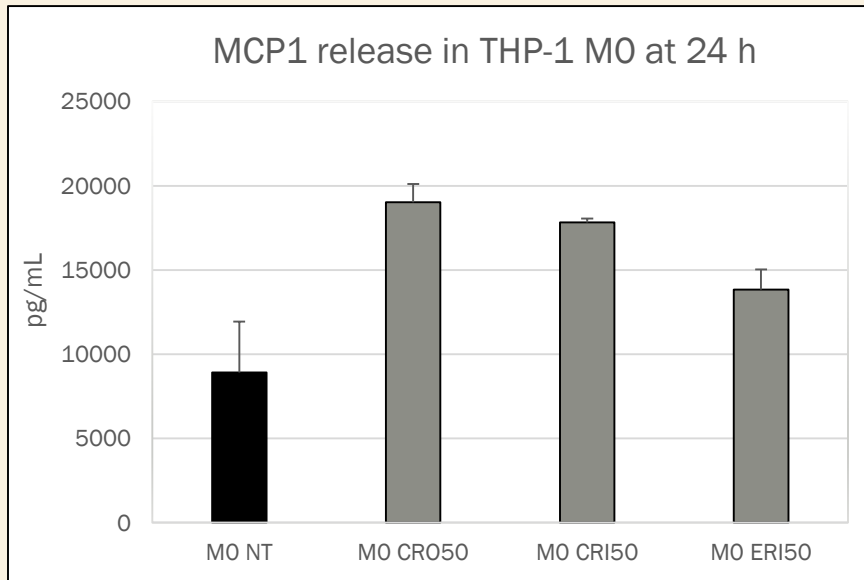
Valutazione della produzione intracellulare di ROS a 2h dalla somministrazione delle fibre (50 e 100 ug/ml) tramite la sonda DCF



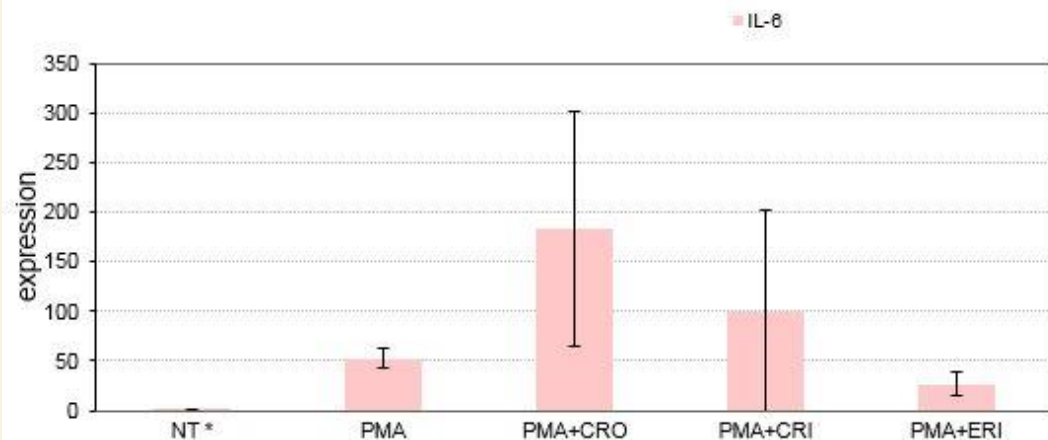
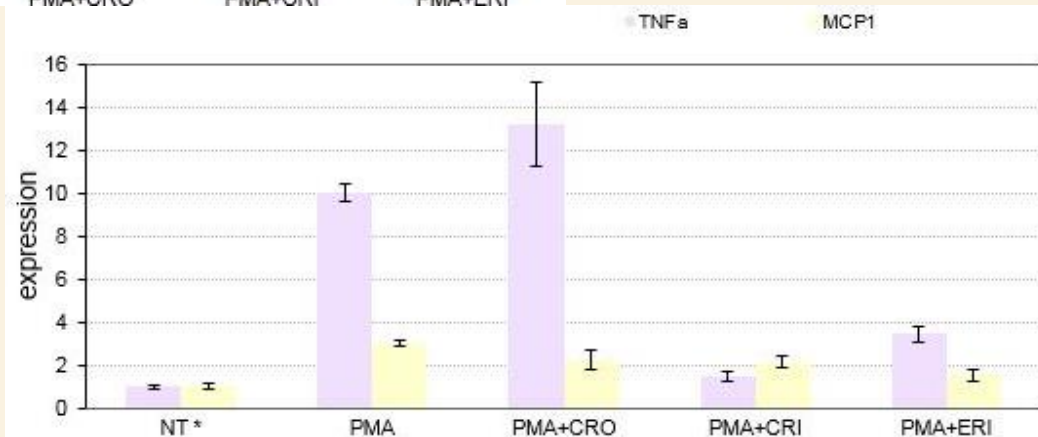
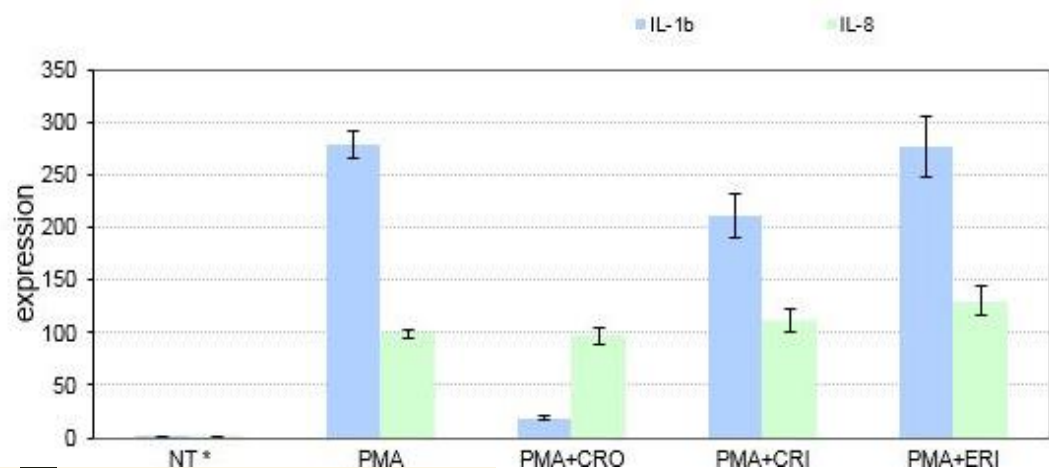
Valutazione della produzione extracellulare di IL-1 β con fibre a 50 ug/ml a 24 ore tramite kit ELISA



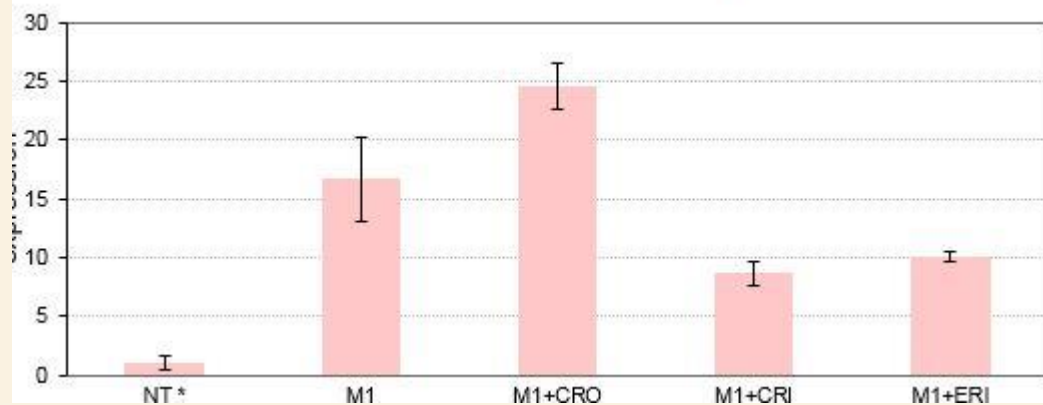
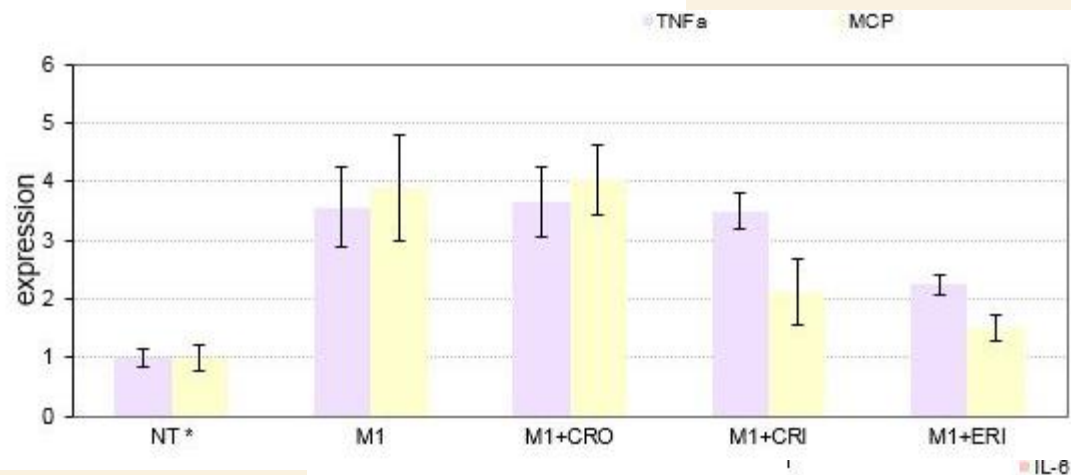
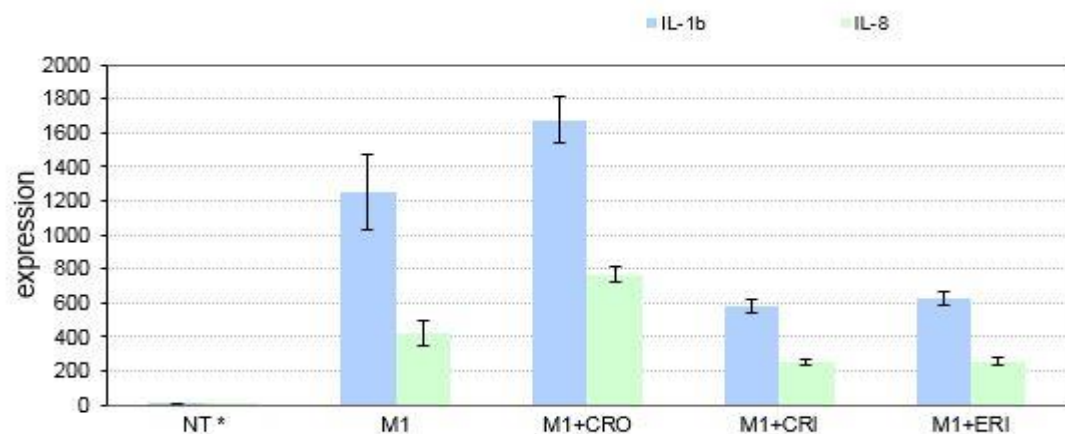
Valutazione della produzione extracellulare di MCP-1 con fibre a 50 ug/ml a 24 ore tramite kit ELISA



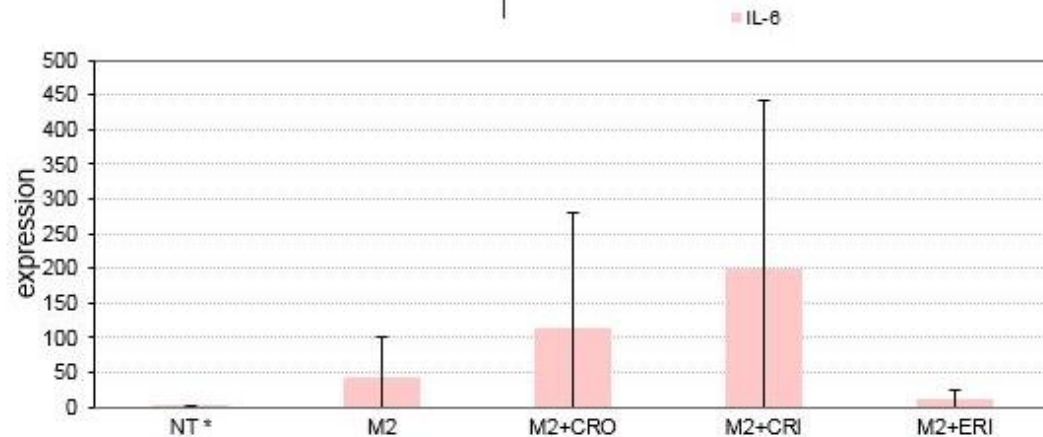
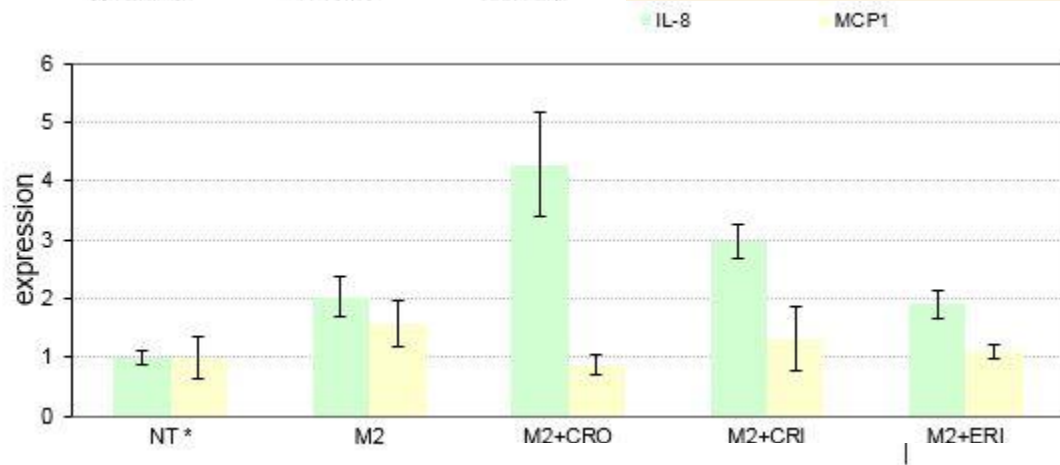
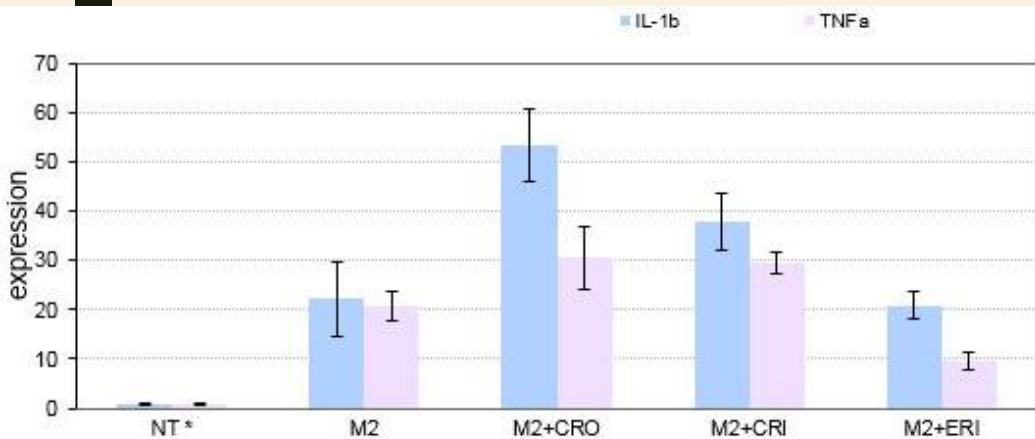
Espressione genica di geni pro-infiammatori di M0 a 6 h con fibre a 50 ug/ml



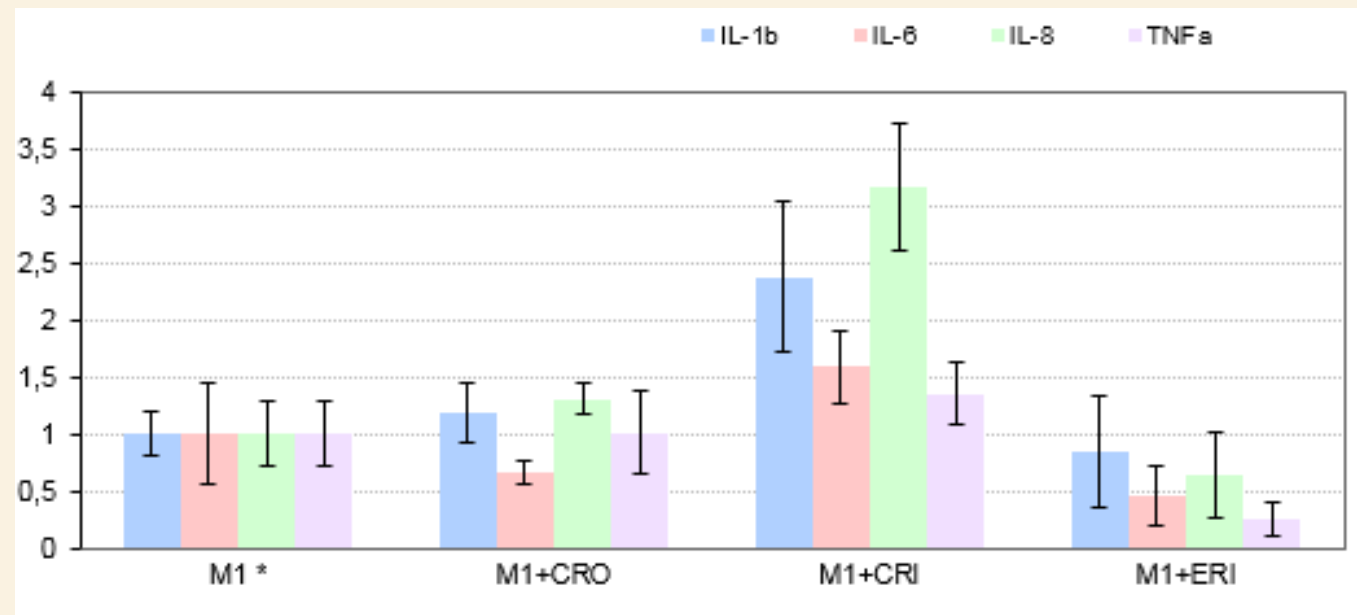
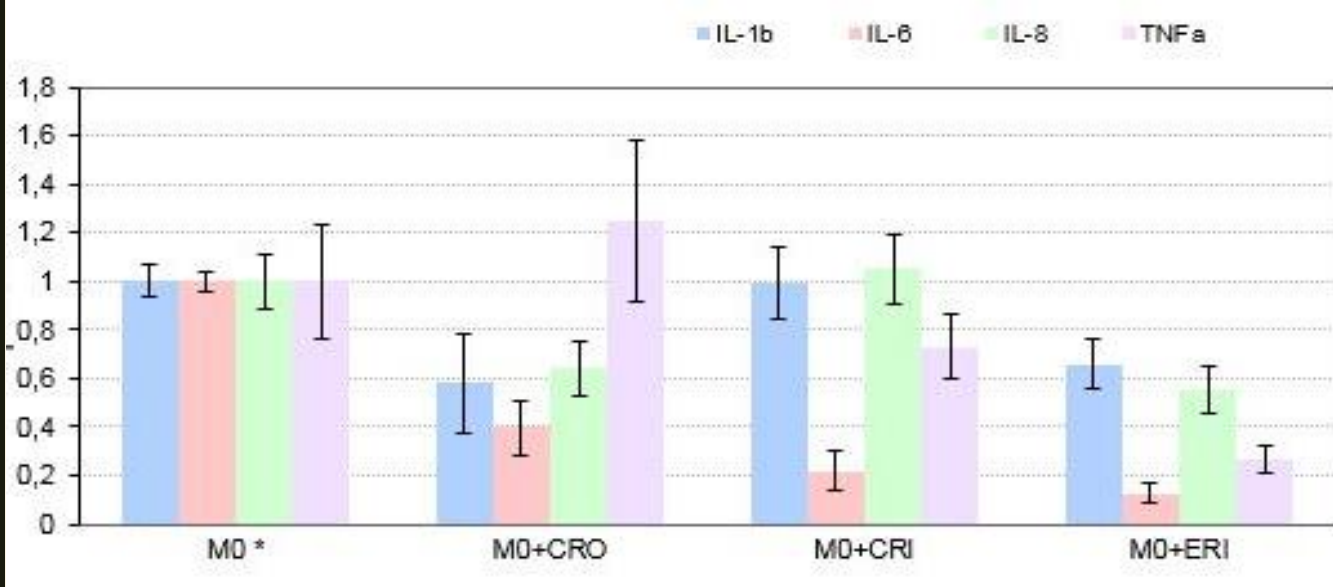
Espressione genica di geni pro-infiammatori di M1 a 6 h con fibre a 50 ug/ml



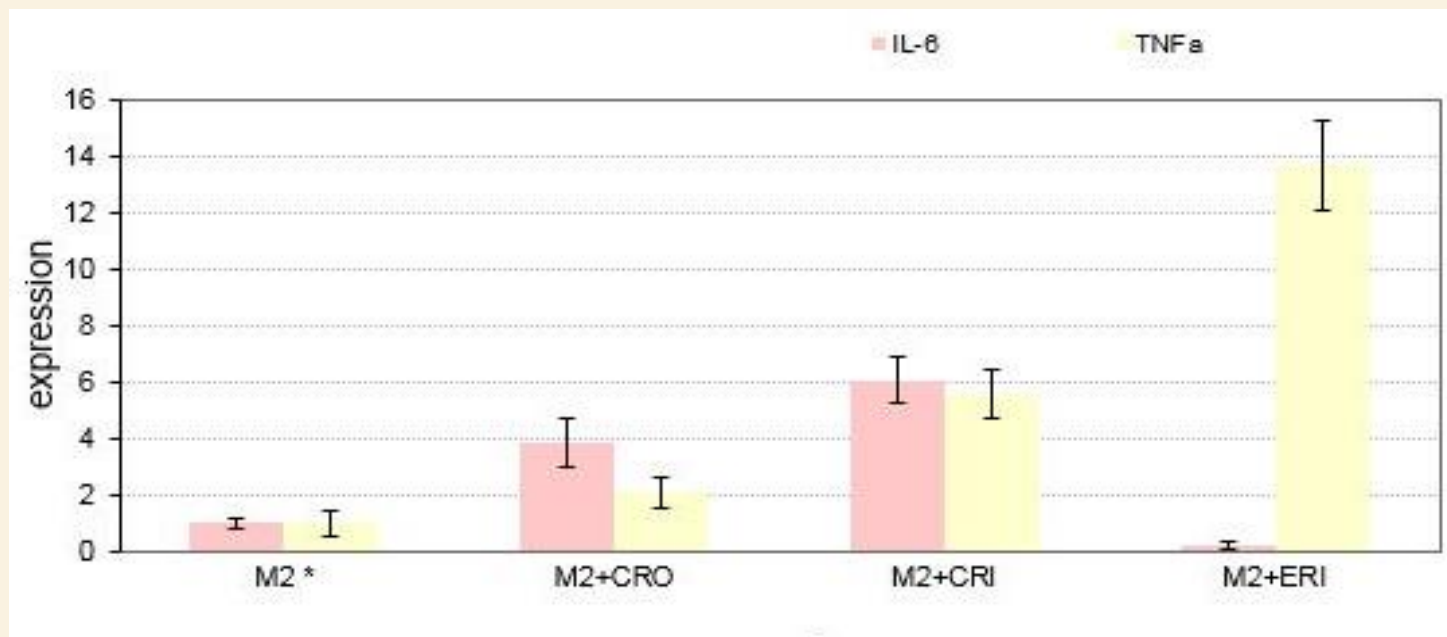
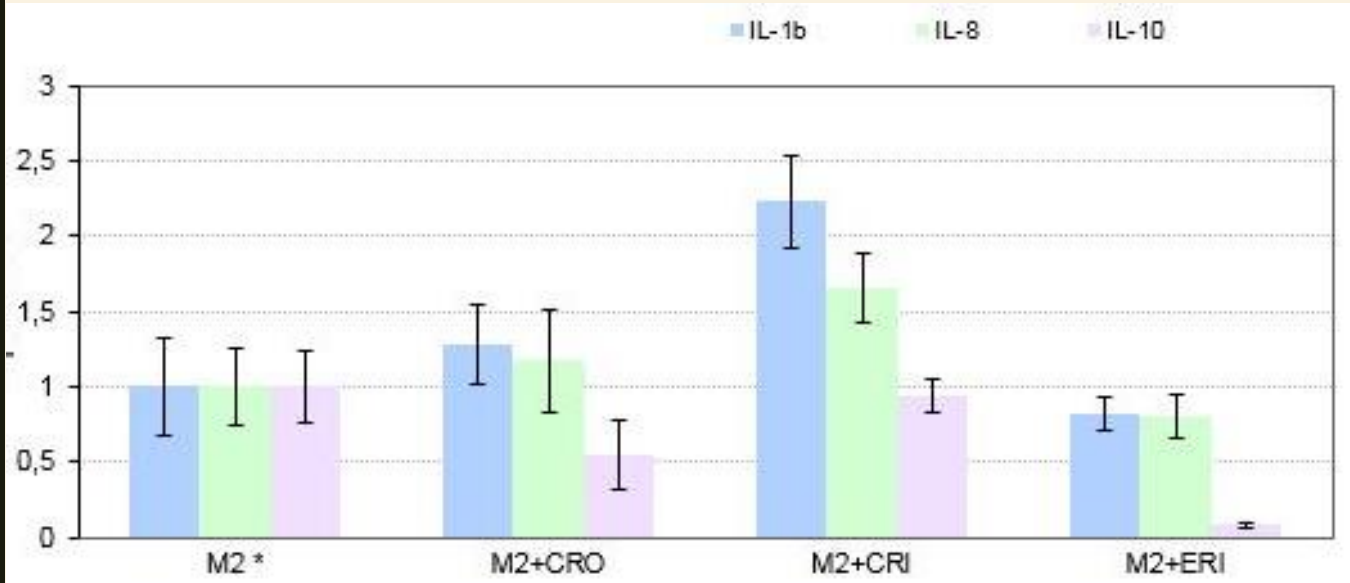
Espressione genica di geni pro-infiammatori di M2 a 6 h con fibre a 50 ug/ml



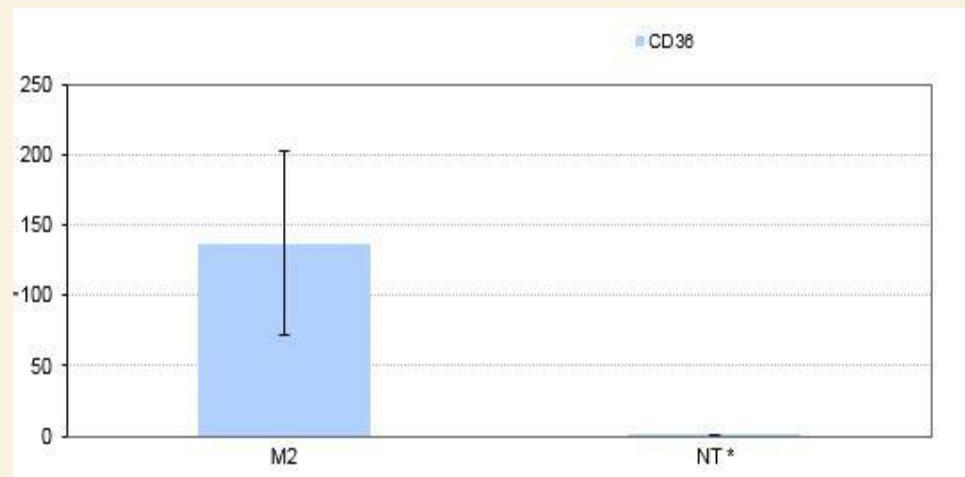
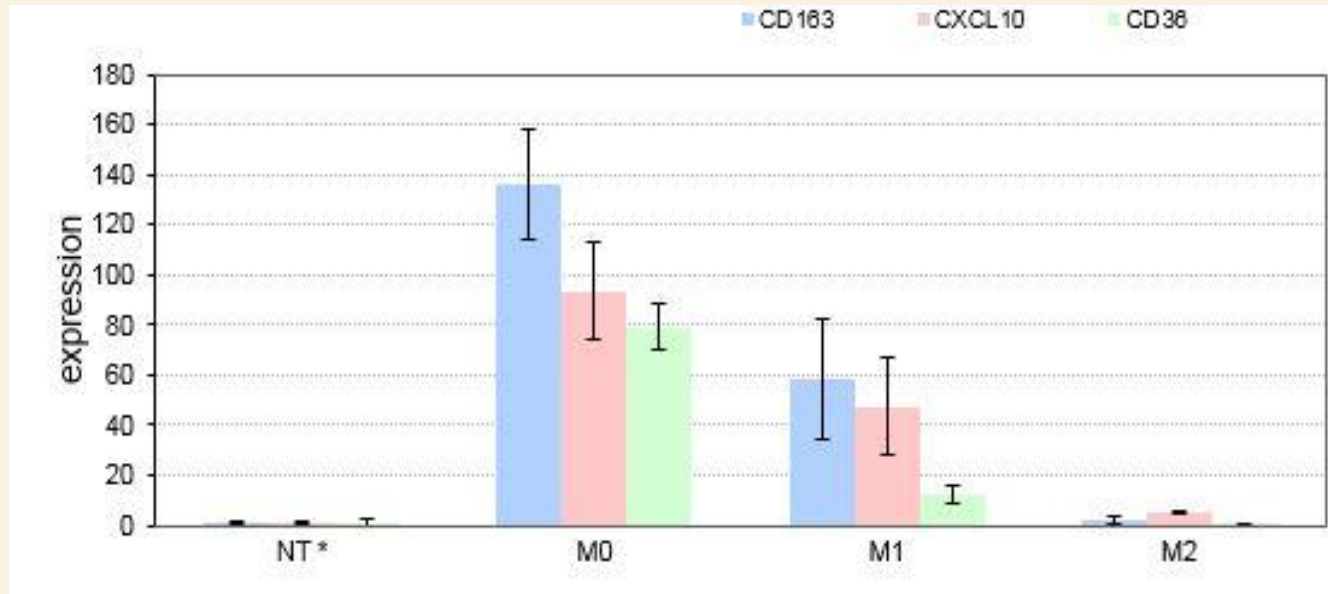
Espressione genica di geni pro-infiammatori con THP-1 diff M0 e M1 a 24 h con fibre a 50 ug/ml



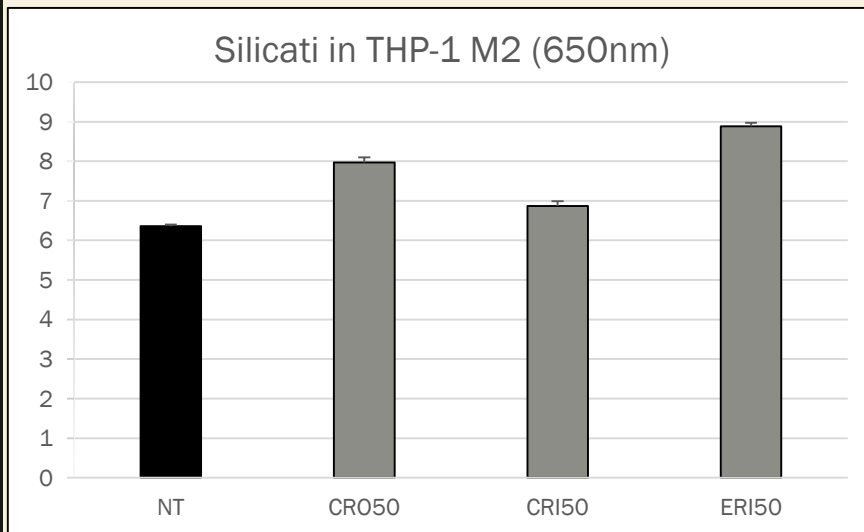
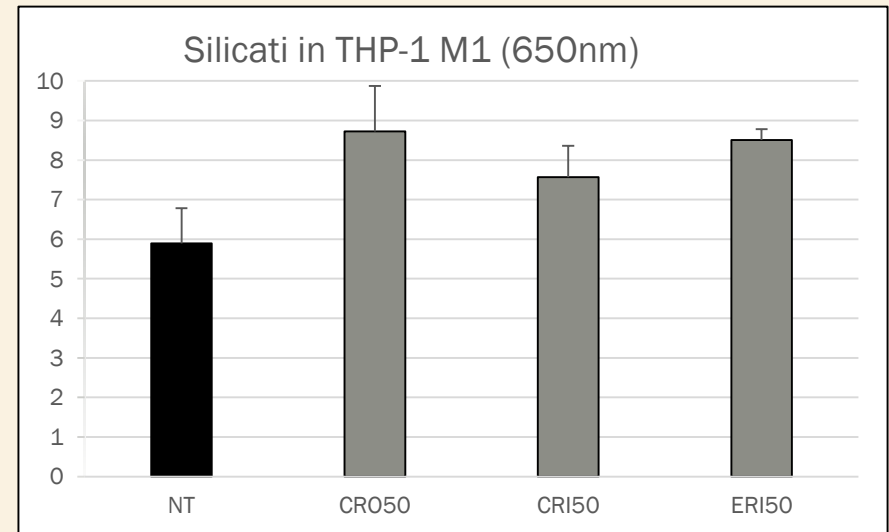
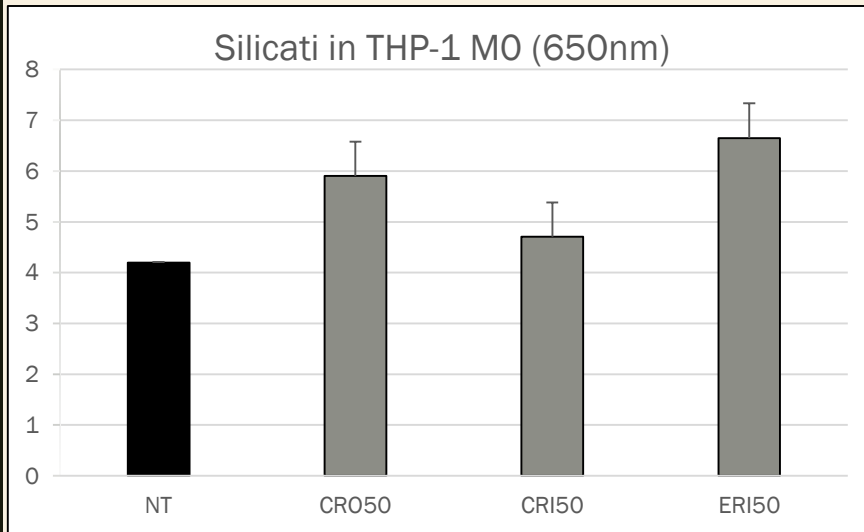
Espressione genica di geni pro-infiammatori di M2 a 24 h con fibre a 50 ug/ml



Espressione genica dei geni coinvolti nel differenziamento delle THP-1 nei diversi tipi di macrofagi M0, M1 e M2



Quantificazione dei SILICATI intracellulari con fibre a 50 ug/ml a 24 ore mediante kit spettrofotometrico



CONGRESSO CENTRO 3R

IN VITRO EFFECTS OF DIRECT OR INDIRECT EXPOSURE TO MINERAL FIBERS ON HUMAN ENDOTHELIAL CELLS AND MONOCYTES

Vanessa Almonti 1,4 , Serena Mirata 2,4 , Barbara Marengo 1,4 , Dario Di Giuseppe 3 , Alessandro F. Gualtieri 3 , Stefania Vernazza 1,4 , Sara Tirendi 1,4 , Susanna Penco 1,4 , Mario Passalacqua 1,4 , Sonia Scarfi 2,4 , Bassi Anna M. 1,4

11TH WORLD CONGRESS ON ALTERNATIVES AND ANIMAL USE IN THE LIFE SCIENCES

EFFECT OF MINERAL FIBRES ON ACUTE TOXICITY AND INFLAMMATION IN AN IN VITRO MODEL OF HUMAN M0-M1-M2 MACROPHAGES

Serena Mirata 1,4 , Vanessa Almonti 2,4 , Barbara Marengo 2 , Dario Di Giuseppe 3 , Alessandro Gualtieri 3 , Stefania Vernazza 2,4 , Sara Tirendi 2,4 , Anna Maria Bassi 2,4 , Sonia Scarfi 1,4

AN ALTERNATIVE IN VITRO APPROACH TO EVALUATE IN HUMAN ENDOTHELIAL CELLS AND MONOCYTES BOTH DIRECT AND INDIRECT CARCINOGENIC EFFECT OF ASBESTOS FIBRES

Vanessa Almonti 1,4 , Serena Mirata 2,4 , Barbara Marengo 1,4 , Dario Di Giuseppe 3 , Alessandro F. Gualtieri 3 , Stefania Vernazza 1,4 , Sara Tirendi 1,4 , Susanna Penco 1,4 , Mario Passalacqua 1,4 , Sonia Scarfi 2,4 , Bassi Anna M. 1,4