Anna Maria Bassi
Vanessa Almonti
Stefania Vernazza
Sara Tirendi

DEPARTMENT OF EXPERIMENTAL MEDICINE
and
DEPARTMENT OF EARTH ENVIRONMENT AND LIFE SCIENCES

Sonia Scarfì
Serena Mirata

PRIN project n° 20173X8WA4

IN VITRO 2D AND 3D HUMAN LUNG MICROENVIRONMENT MODELS TO EVALUATE THE BIOLOGICAL IMPACT OF MINERAL FIBRES

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

PRIN 2017

Progetti di ricerca di Rilevante Interesse Nazionale

Università di Genova

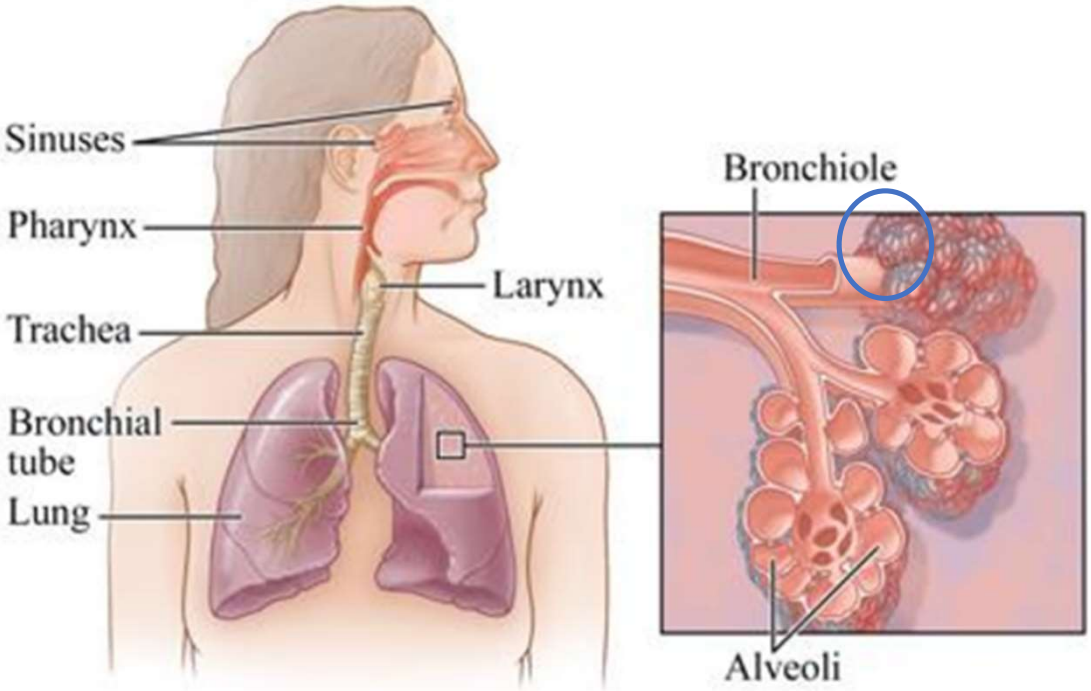
PRIN project n° 20173X8WA4

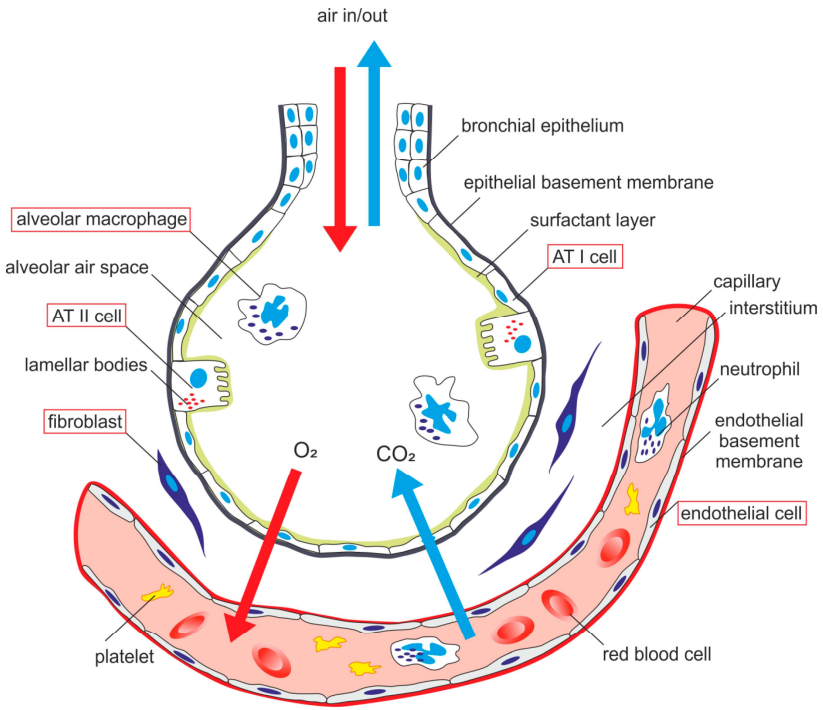
✓Genoa Unit

To study fibre effects on:

- 2D models of human cell cultures, as part of the lung environment
- 3D tissue of the human airway to investigate the biological potential impact of exposure in a more physiologically relevant environment.

Lung environment: what we are trying to reproduce





Alveolar-Capillary Membrane-Related Pulmonary Cells as a Target in Endotoxin-Induced Acute Lung Injury; Nova et al., 2019; IJMS 20(4), 831

Biological impact: what we are trying to investigate

Table 1. Key characteristics of carcinogens.

Characteristic	Examples of relevant evidence
1. Is electrophilic or can be metabolically activated	Parent compound or metabolite with an electrophilic structure (e.g., epoxide, quinone), formation of DNA and protein adducts
2. Is genotoxic	DNA damage (DNA strand breaks, DNA–protein cross-links, unscheduled DNA synthesis), intercalation, gene mutations, cytogenetic changes (e.g., chromosome aberrations, micronuclei)
3. Alters DNA repair or causes genomic instability	Alterations of DNA replication or repair (e.g., topoisomerase II, base-excision or double-strand break repair)
4. Induces epigenetic alterations	DNA methylation, histone modification, microRNA expression
5. Induces oxidative stress	Oxygen radicals, oxidative stress, oxidative damage to macromolecules (e.g., DNA, lipids)
6. Induces chronic inflammation	Elevated white blood cells, myeloperoxidase activity, altered cytokine and/or chemokine production
7. Is immunosuppressive	Decreased immunosurveillance, immune system dysfunction
8. Modulates receptor-mediated effects	Receptor in/activation (e.g., ER, PPAR, AhR) or modulation of endogenous ligands (including hormones)
9. Causes immortalization	Inhibition of senescence, cell transformation
10. Alters cell proliferation, cell death or nutrient supply	Increased proliferation, decreased apoptosis, changes in growth factors, energetics and signaling pathways related to cellular replication or cell cycle control, angiogenesis

First part: evaluate how direct or indirect exposure to mineral fibres (Crocidolite, Chrysotile and Erionite) affects human cells belonging to the lung environment.



❑ 2D cell culture models:

1. THP1 human monocyte cell line → Indirect and direct fibre exposure
2. THP1-derived macrophages → direct exposure
3. HECV human endothelial vein cell line → direct fibre exposure
4. A549 human alveolar-like cells → direct fibre exposure

❑ Endpoints investigated:

Metal ion release in a biological environment (intra and extracellular) → related to IARC point 2, 4 and 5
Cytotoxicity (apoptosis, cell membrane direct damage) → related to IARC point 6
Oxidative stress (ROS production) → related to IARC point 5
Genotoxicity (DNA damage) → related to IARC point 2
Inflammation (macrophage spontaneous differentiation, cytokine overexpression and release) → related to IARC point 6

Second part: evaluate how direct exposure to a Russian Chrysotile, divided in two different fractions (< and > 5μm), affects human cells belonging to the lung environment. Compare the results to carcinogenic crocidolite and non-carcinogenic wollastonite

- ❑ 2D cell culture models:

1. THP1-derived macrophages → direct exposure
2. HECV human endothelial vein cell line → Indirect and direct fibre exposure
3. MET5A human mesothelial cells →direct exposure

- ❑ 3D co-culture set up:

THP1 derived macrophages exposed to fibres with HECV endothelial cells not-exposed to fibres

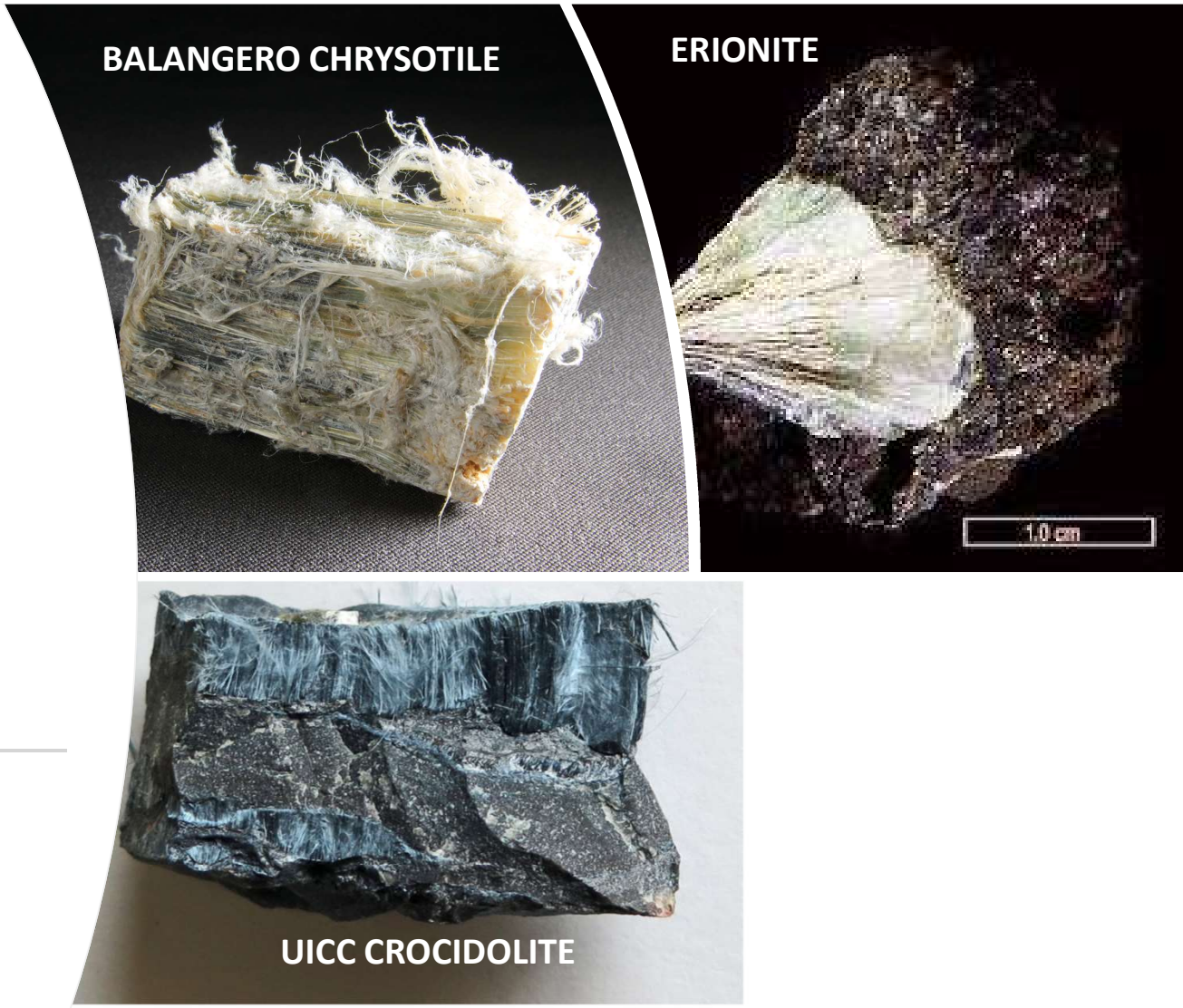
- ❑ 3D tracheal/bronchial epithelial tissue Epiairway™ → Reconstructed human tissue direct exposure to fibres

Versus:

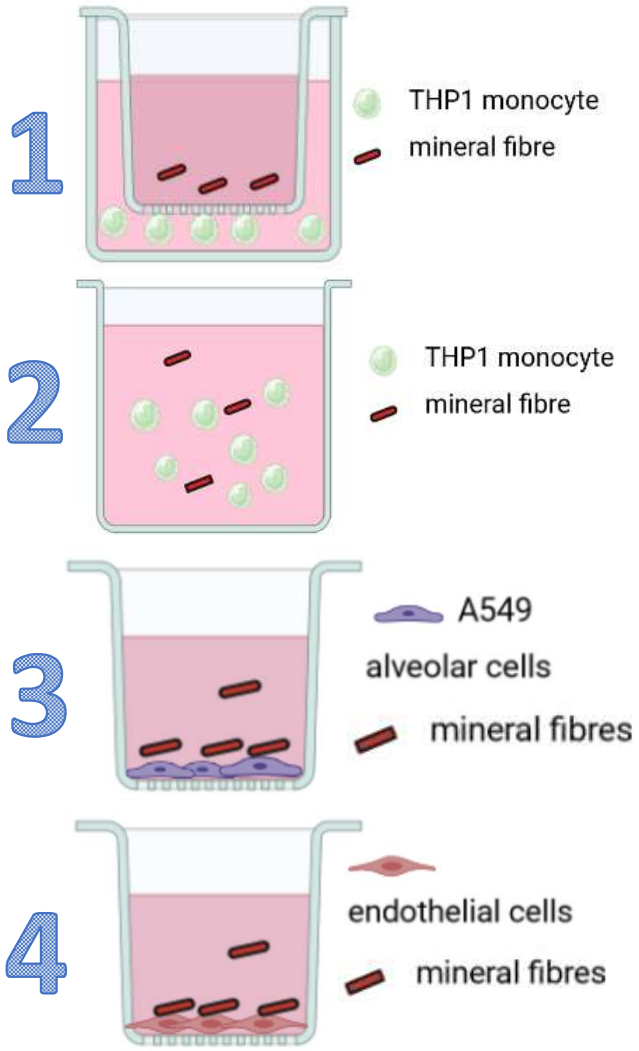
- ❑ Endpoints investigated:

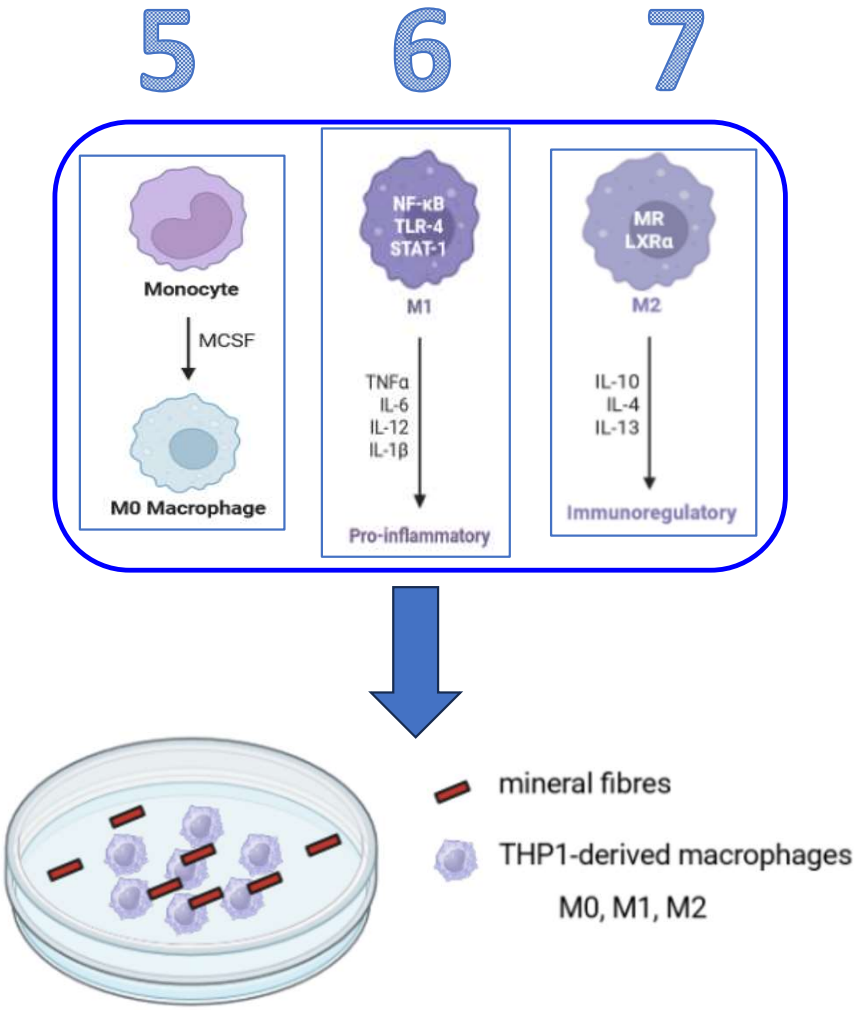
Cytotoxicity (apoptosis, cell membrane direct damage) → related to IARC point 6
Oxidative stress (ROS production) → related to IARC point 5
Genotoxicity (DNA damage) → related to IARC point 2
Inflammation → related to IARC point 6
Cell immortalization and Epithelial to Mesenchymal transition (EMT) → related to IARC point 9
Changes in signal transduction → related to IARC point 8
Changes in growth factors and in cell proliferation → related to IARC point 10

FIRST PART OF THE STUDY



Experimental design: 7 CELL MODELS!

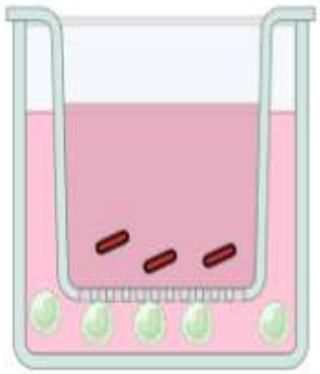




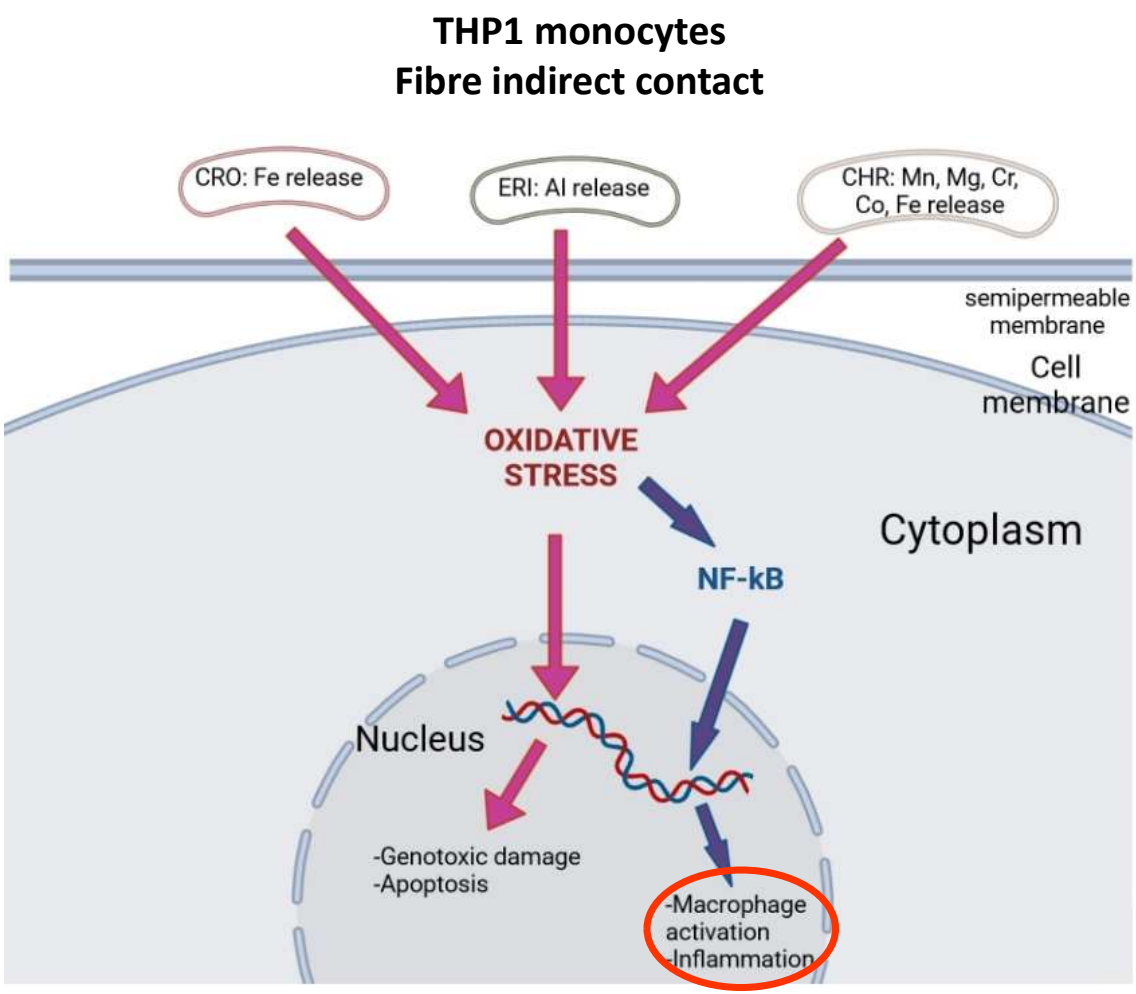
24-48 hrs
acute effects!

Endpoints
Proliferation
Apoptosis
Macrophage activation
Oxidative stress
Inflammation
Genotoxic damage

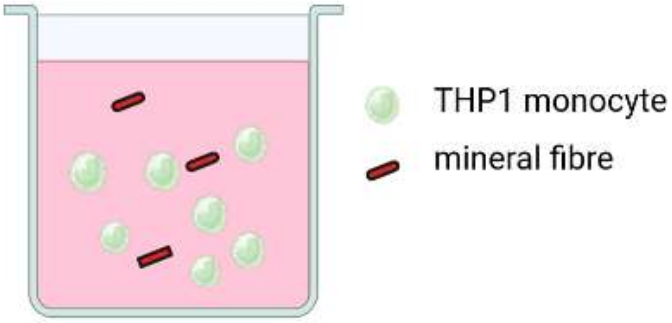
Main results: Model 1 with CRO, CHR-B, ERI



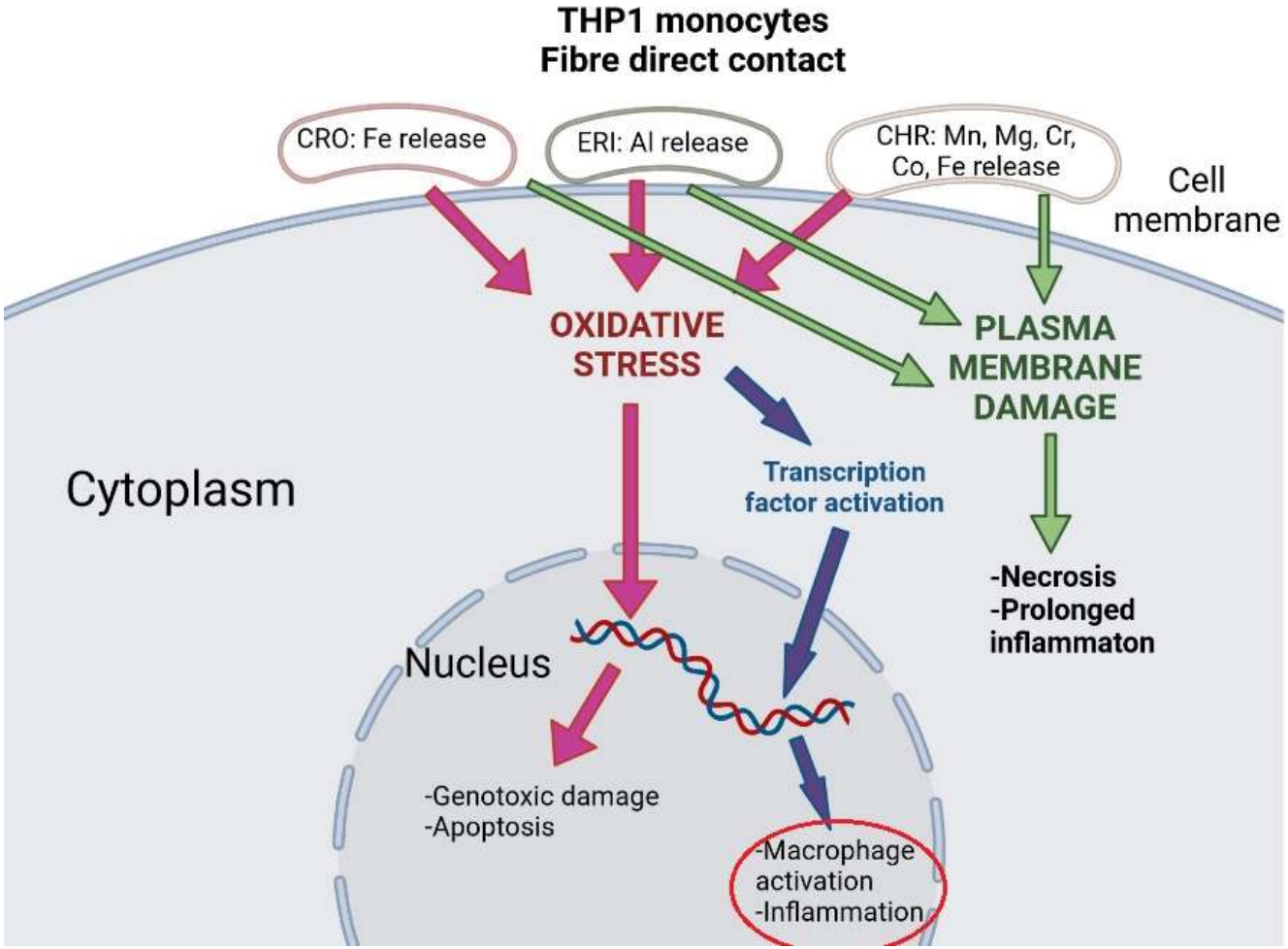
	CRO	CHR-B	ERI
Cell proliferation		- -	
Apoptosis: **-Early** **-Late**	++ ++++	++++ +++	
M0 **-CD163** **-CXCL10**	+	+	
ROS - **4hrs** - **24hrs**	+++ +	+++ ++	+++ +++
NF-kB		++	
Genotoxicity	++++	++++	++++



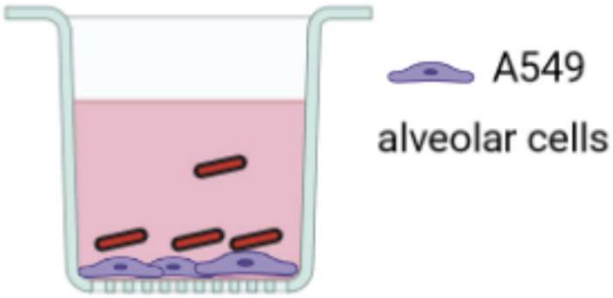
Main results: Model 2 with CRO, CHR-B, ERI



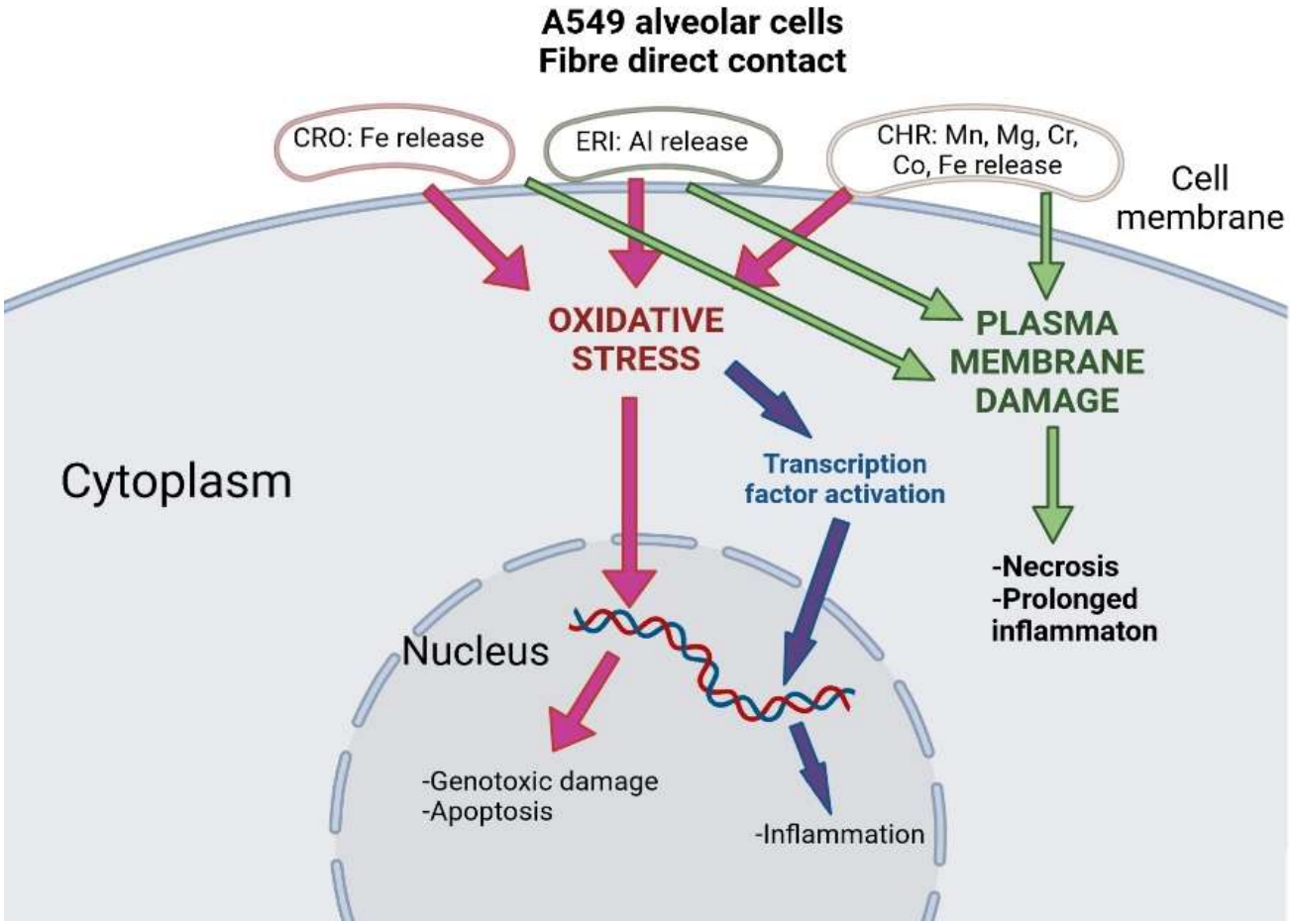
	CRO	CHR-B	ERI
Cell proliferation	---	--	---
M0 differentiation	++	++	+
ROS	+++	+++	+
Inflammatory genes	+	++	+



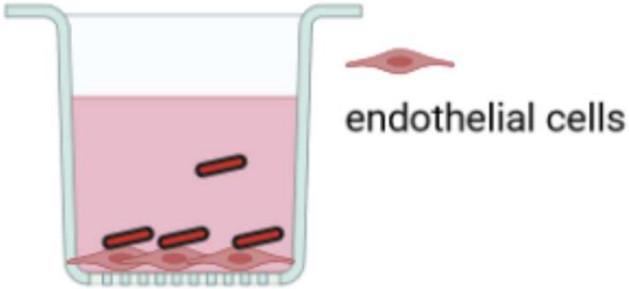
Main results: Model 3 with CRO, CHR-B, ERI



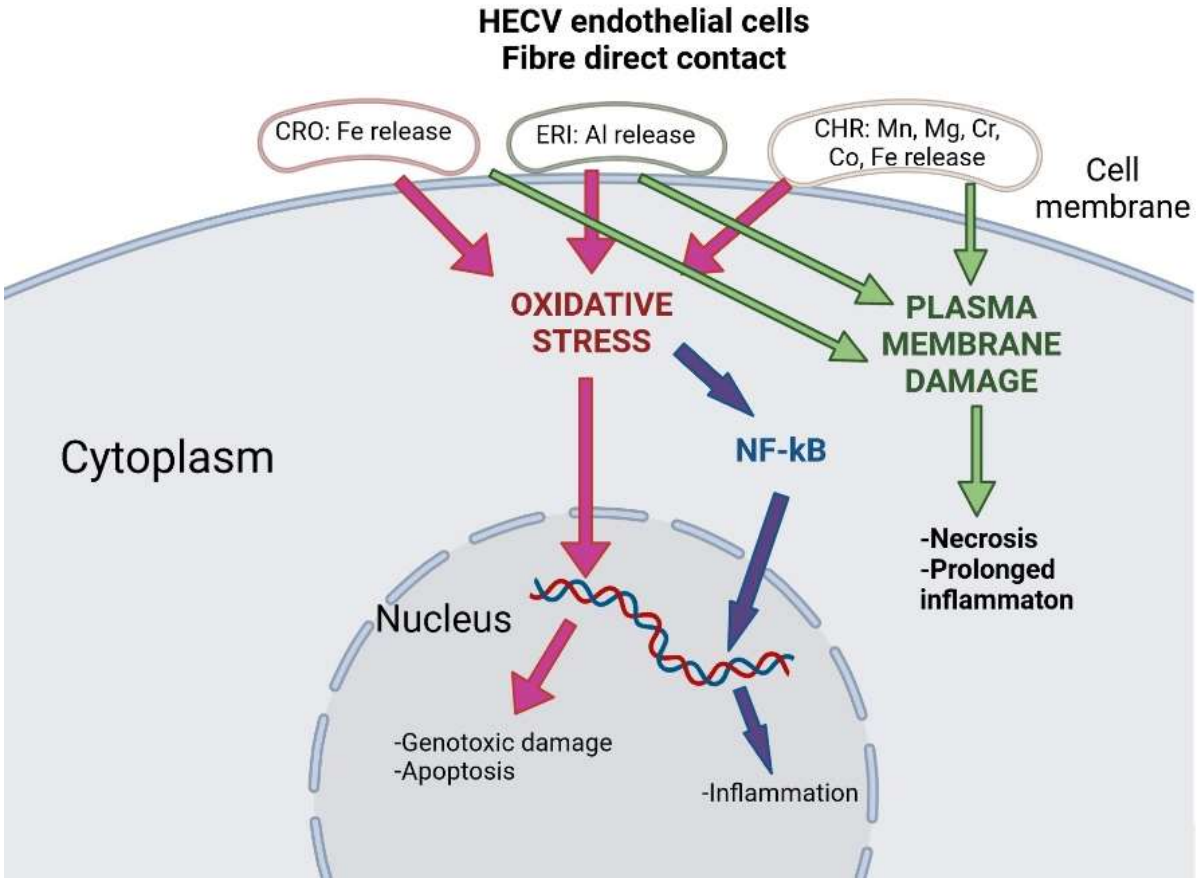
	CRO	CHR-B	ERI
Cell proliferation	---	---	---
Induction of M0 differentiation	++	+	++
ROS	++	++	+
Inflammatory genes	+	++	+



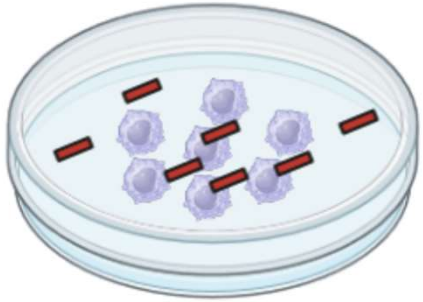
Main results: Model 4 with CRO, CHR-B, ERI



	CRO	CHR-B	ERI
Cell proliferation	--	--	
Apoptosis: -Early -Late	 ++	 ++ ++++	 ++
ROS		++++	++
NF-kB	++		
Genotoxicity	+	+	



Main results: Model 5, 6, 7 with CRO, CHR-B, ERI

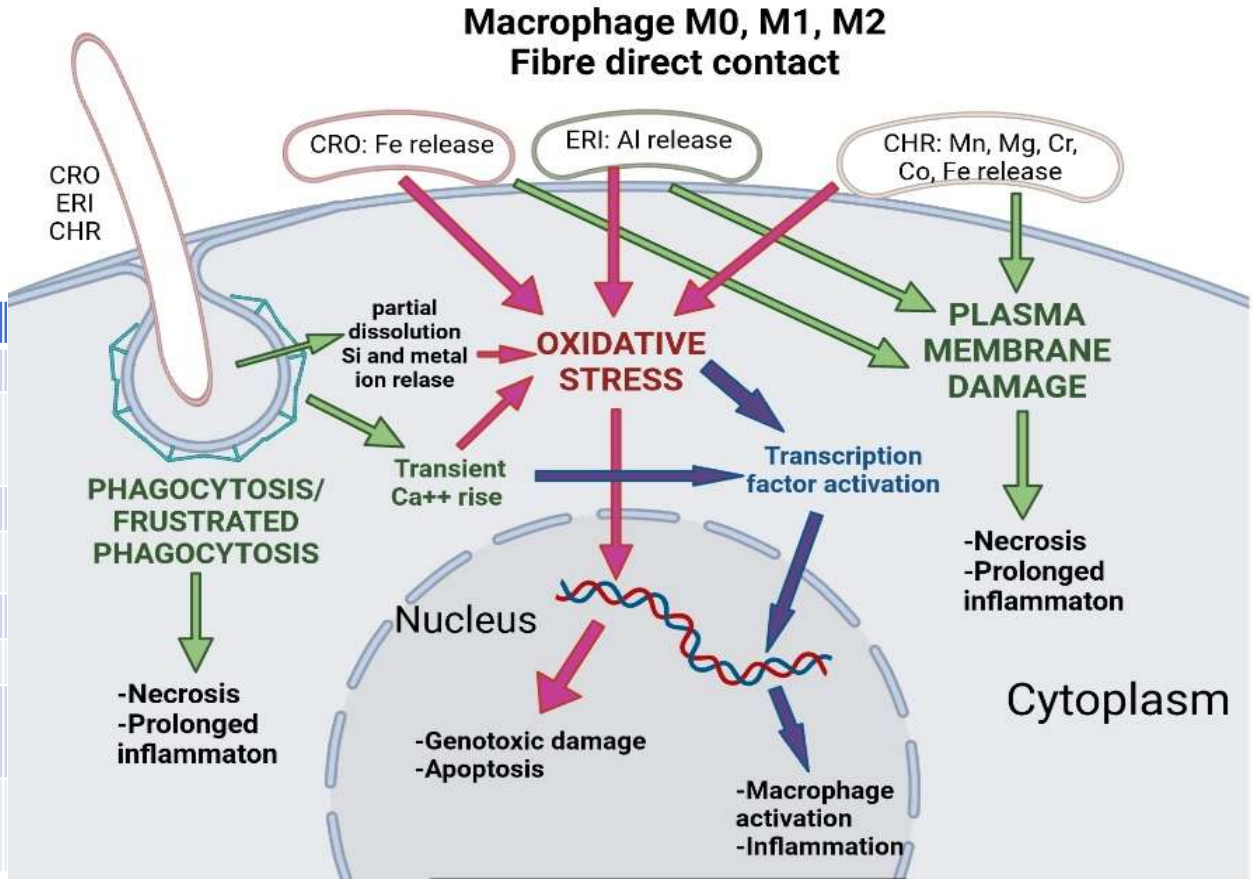


mineral fibres

THP1-derived macrophages

M0, M1, M2

	CRO	CHR-B	ERI
Biodurability	+++ (66 yrs)	+ (0.3 yrs)	+++ (181 yrs)
Cell death	Mainly apoptosis	Apoptosis and cell lysis	Apoptosis and cell lysis
ROS production	+++	++	++
Redox-active metal release	Fe	Mg, Fe, Ni, Cr, Co	Al
Genotoxicity	+++	+++	+++
Cytokine release	+++	+++	+++
Inflammatory gene upregulation	+++	++	
Cation-exchange capacity (CEC)			+++ (Ca++ depletion, Na+ increase)



CONCLUSIONS I

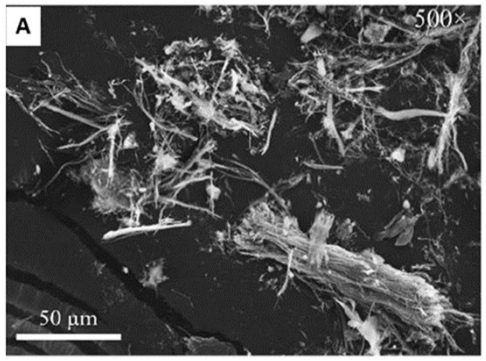
First conclusion…

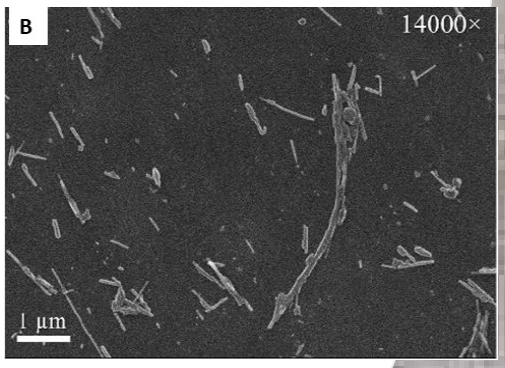
- **Crocidolite** seems to exert its toxic effects mostly as a result of its **high biodurability** and **iron content**, with significant levels of oxidative stress, DNA damage and the transcriptional upregulation of pro-inflammatory mediators.

- Despite its low biodurability, the adverse effects of **chrysotile** seem to be due to the dissolution process itself, with the **rapid release of several toxic metals** (i.e., Mg, Fe, Cr, Ni and Co), enhancing oxidative stress, DNA damage and the upregulation of pro-inflammatory mediators.

- **Erionite** releases minimal amounts of toxic metals and produces low levels of ROS and a lower inflammatory response, but allegedly exhibits a **cation exchange capacity** which alters the intracellular homeostasis of important cations (Ca^{2+} depletion, Na^{+} increase).

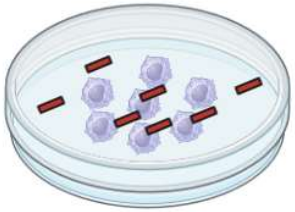
SECOND PART OF THE STUDY





Experimental design: 5 CELL MODELS!

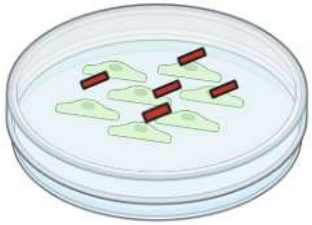
1



Russian Chrysotile

THP1-M0 macrophages

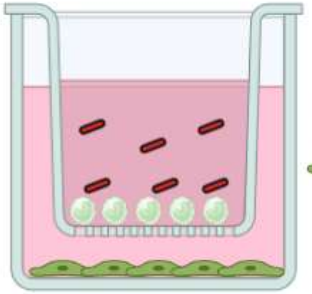
2



Russian Chrysotile

HECV endothelial cells

3

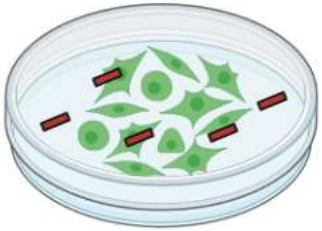


Russian Chrysotile

THP1-M0 macrophages

HECV endothelial cells

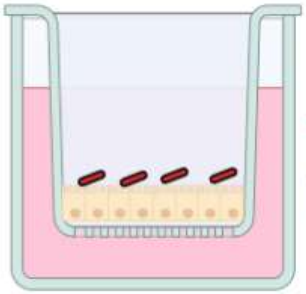
4



Russian Chrysotile

MET5a mesothelial cells

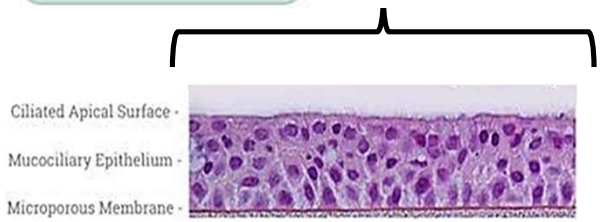
5



EpiAirway™

Russian Chrysotile

mature bronchial stratified epithelium



MATTEK
A BICO COMPANY

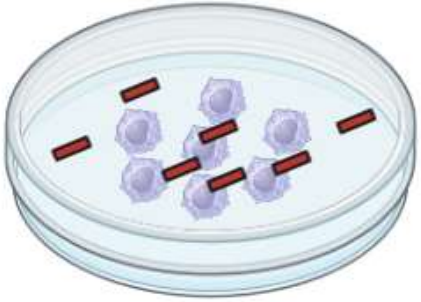
24-48-72 hrs acute

7-14-21 d chronic

Endpoints:

- Proliferation
- Apoptosis
- Oxidative stress
- Inflammation
- Genotoxic damage
- Immortalization
  EMT
- Receptor activation
  Growth factor production

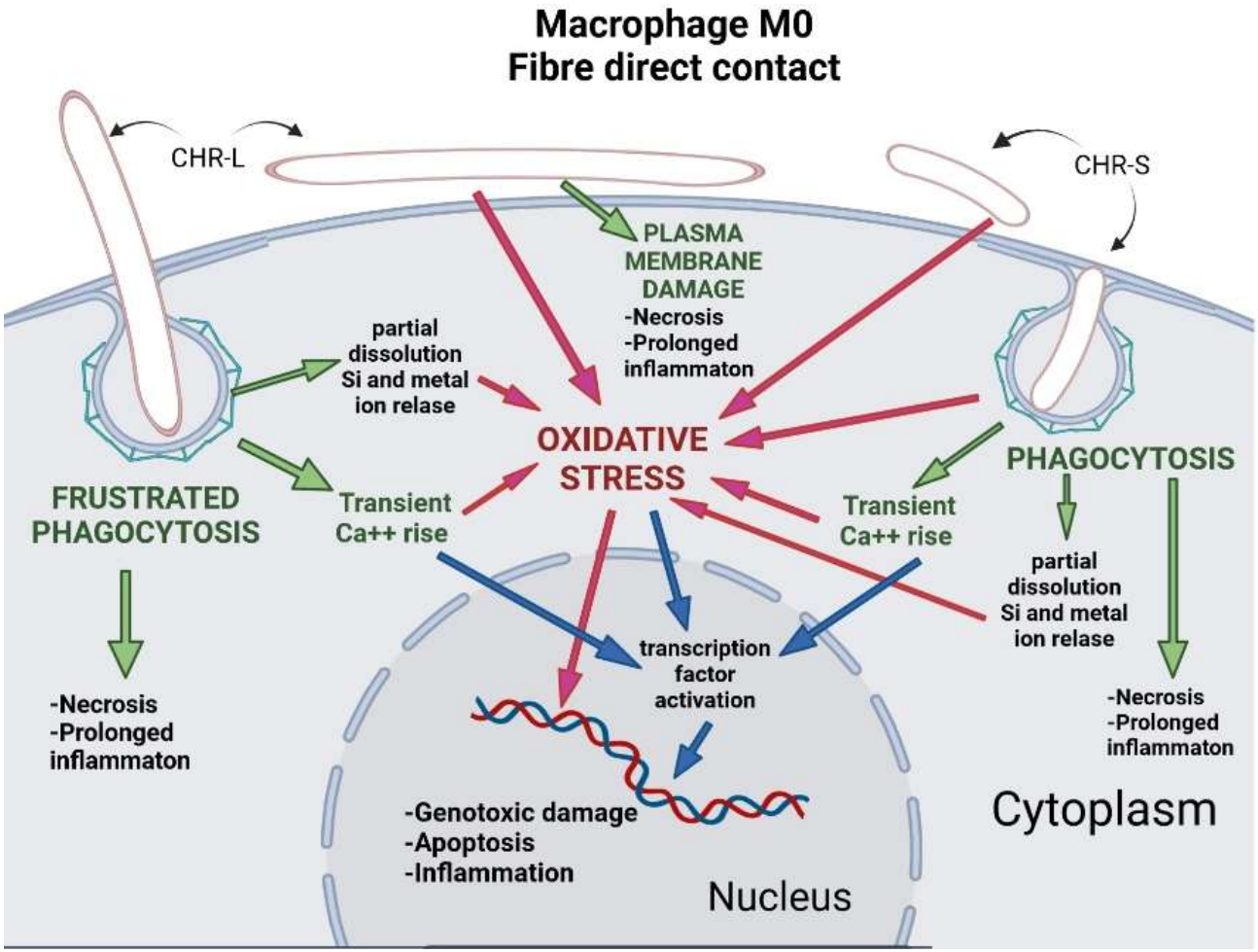
Main results: Model 1 with CHR-S, CHR-L, CRO, WOLL



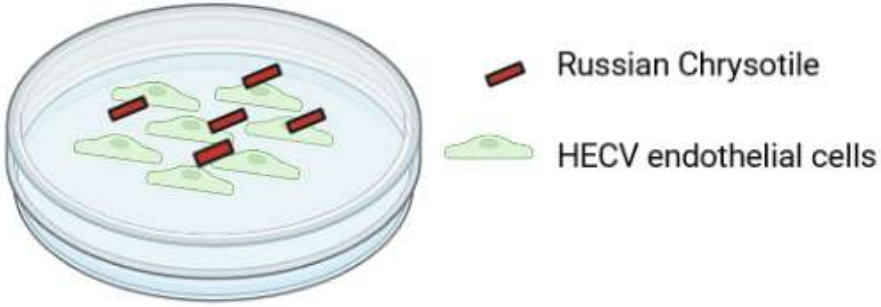
Russian Chrysotile

THP1-M0 macrophages

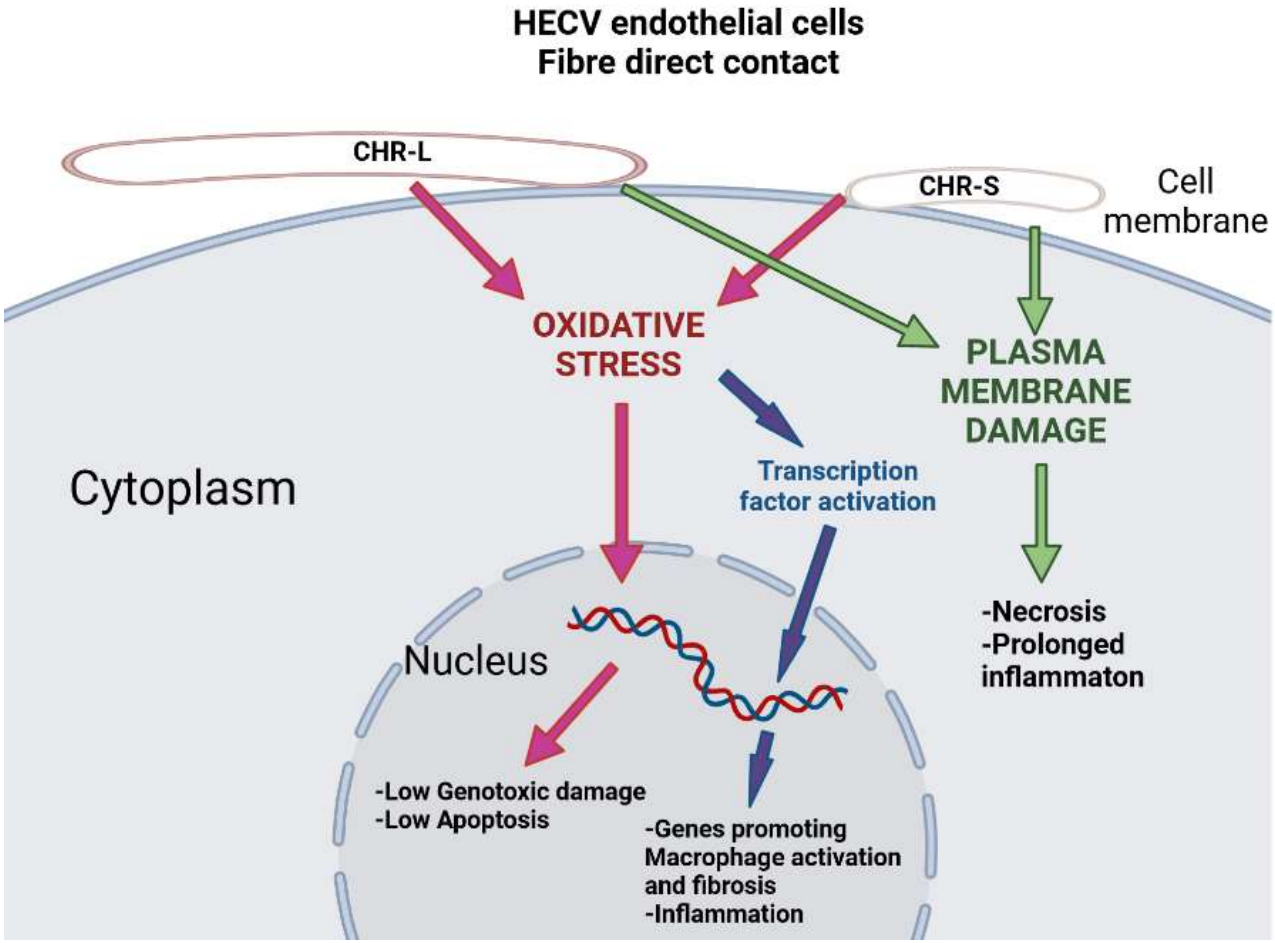
	CRO	CHR-L	CHR-S	WOLL
Biodurability	+++ (66 yrs)	+ (0.3 yrs)	+(0.3 yrs)	+(0.1 yrs)
Cell death	Apoptosis	Apoptosis and cell lysis	Apoptosis	Low effect
ROS production	+++	++++	++++	++++
Genotoxicity	+++	+++	++	no
Acute Inflammatory gene upregulation	+	+++	+	+
Chronic Inflammatory gene upregulation	no	++	no	no



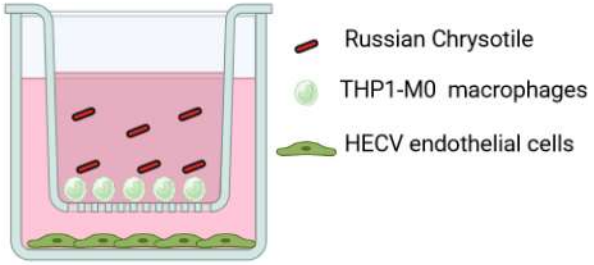
Main results: Model 2 with CHR-S, CHR-L, CRO, WOLL

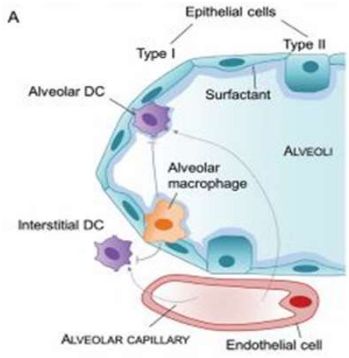


	CRO	CHR-L	CHR-S	WOLL
Biodurability	+++ (66 yrs)	+ (0.3 yrs)	+(0.3 yrs)	+(0.1 yrs)
Cell death	Mainly cell lysis	Mainly cell lysis	Mainly cell lysis	Low effect
ROS production	+++	++++	++++	++++
Acute Inflammatory gene upregulation	++	++	+++	++
Chronic Inflammatory gene upregulation	no	no	no	no
Fibrosis gene upregulation	+	no	+	+

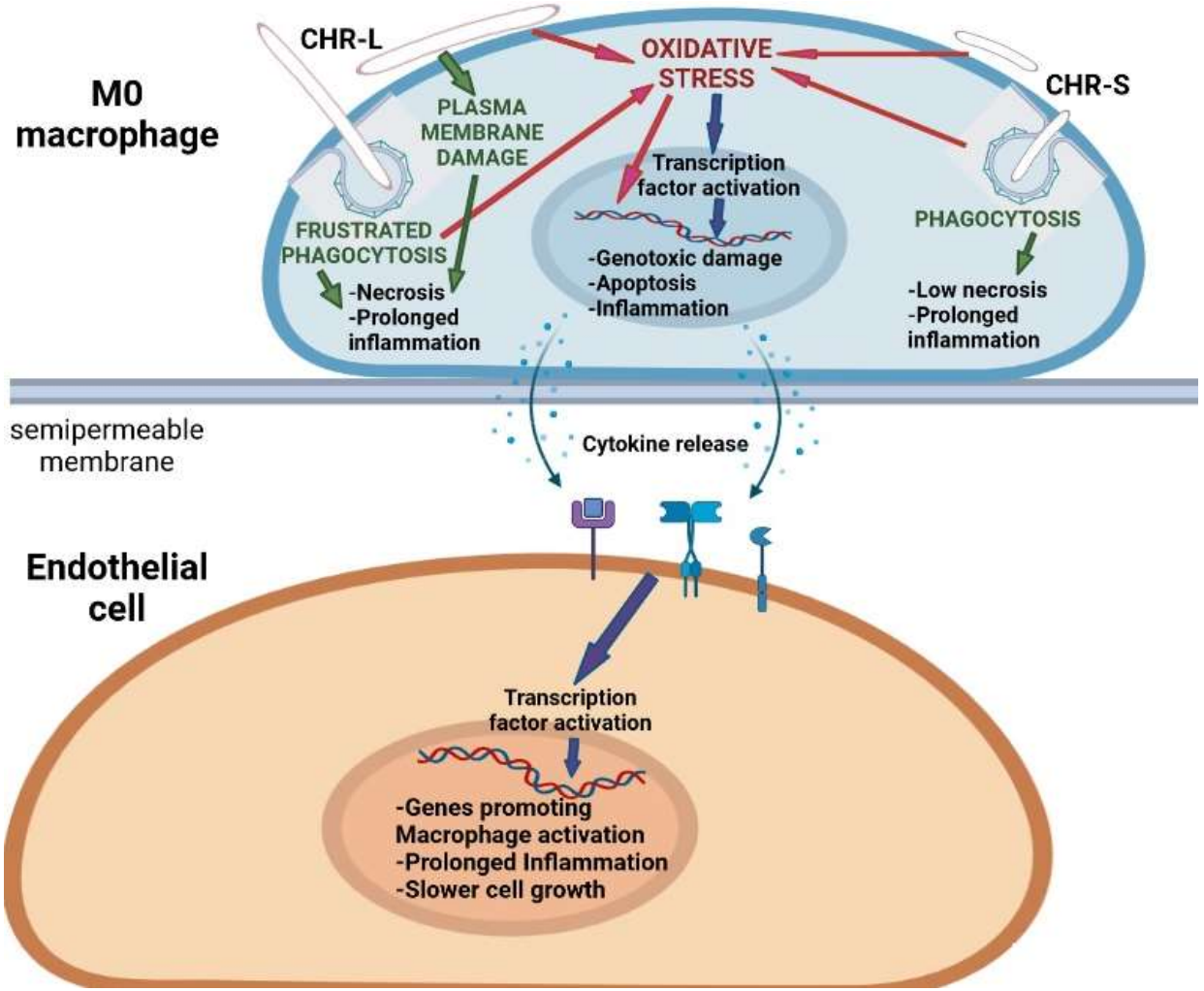


Main results: Model 3 with CHR-S, CHR-L, CRO, WOLL

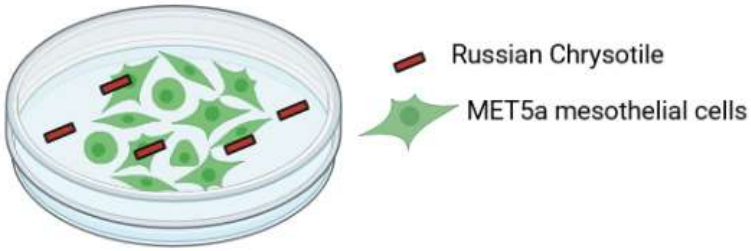




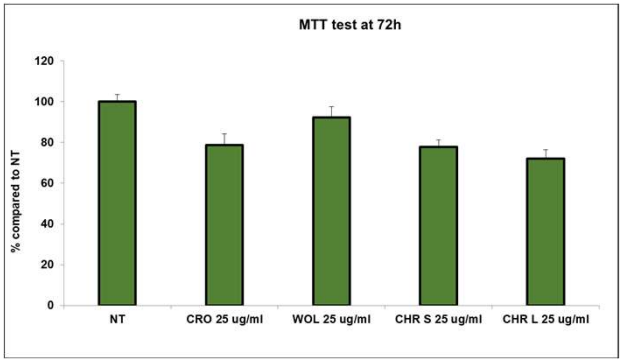
Indirect effects on HECV cells	CRO	CHR-L	CHR-S	WOLL
Acute Inflammatory gene upregulation	+++	+++	+++	+++
Chronic Inflammatory gene upregulation	no	no	no	no
Fibrosis gene upregulation	no	no	+	+
Cell growth inhibition	+	+	+	+

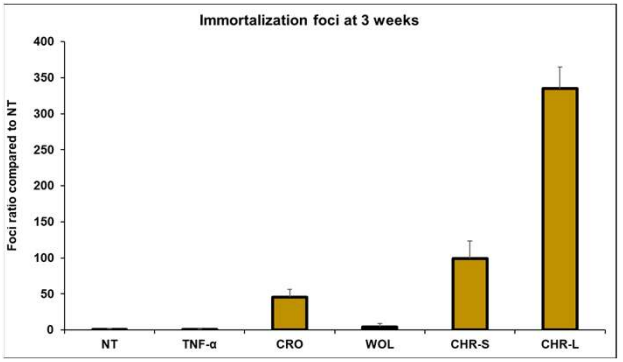


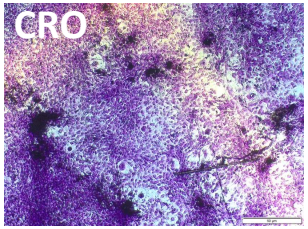
Main results: Model 4 with CHR-S, CHR-L, CRO, WOLL

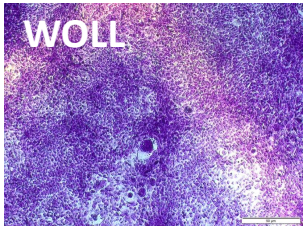


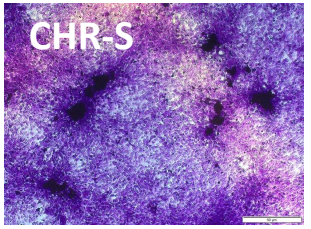
	CRO	CHR-L	CHR-S	WOLL
Cytotoxicity	++	++	++	+
ROS production	+	++	++	++
Immortalization foci	+	++++	++	
EMT	+	++	++	+
Acute inflammation	+++	+++	+	++
Chronic Inflammation	+	+		
Mesothelin overexpression			+	
Growth factor overexpression	+	+	+	+

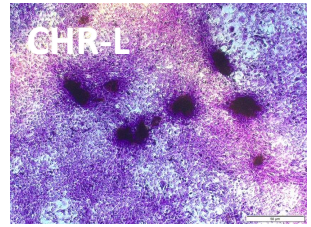


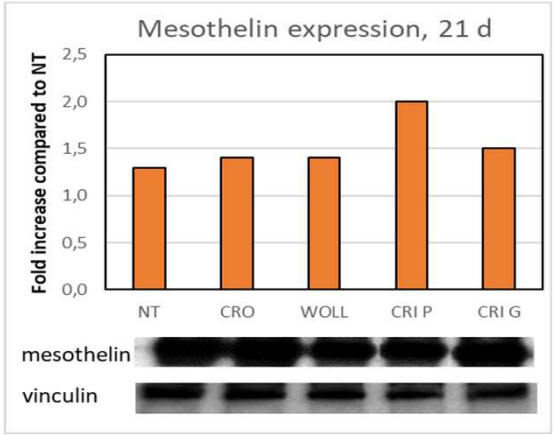


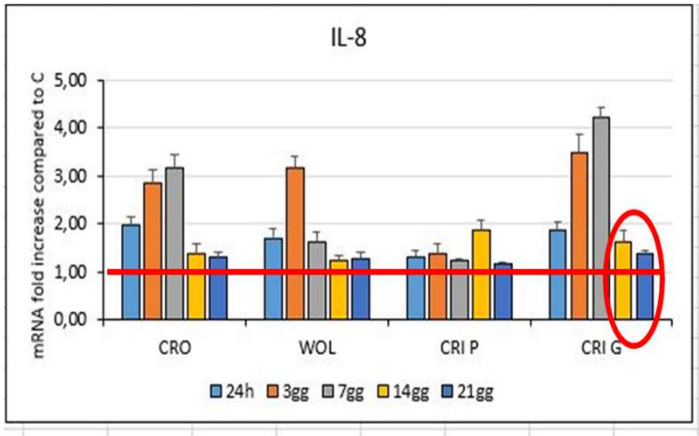






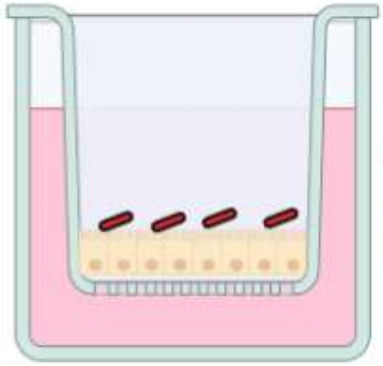






Experimental design: Model 5 with CHR-S, CHR-L, CRO

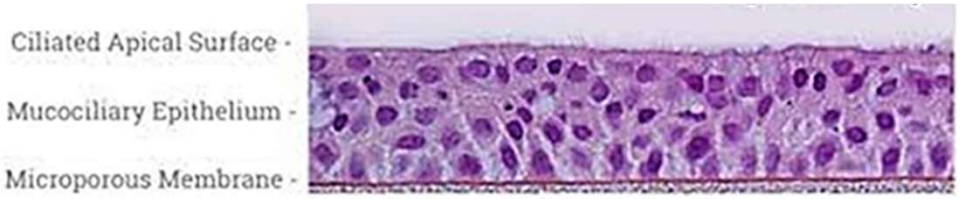
EpiAirway™



Russian Chrysotile

mature bronchial stratified epithelium

Tissue Culture Insert



MATTEK
A BICO COMPANY

24-48 hrs,12 days

EpiAirway™
MTT cytotoxicity index
Trans-Epithelial Electrical Resistance (TEER)
Histology
Acute and chronic inflammation
Genotoxic damage

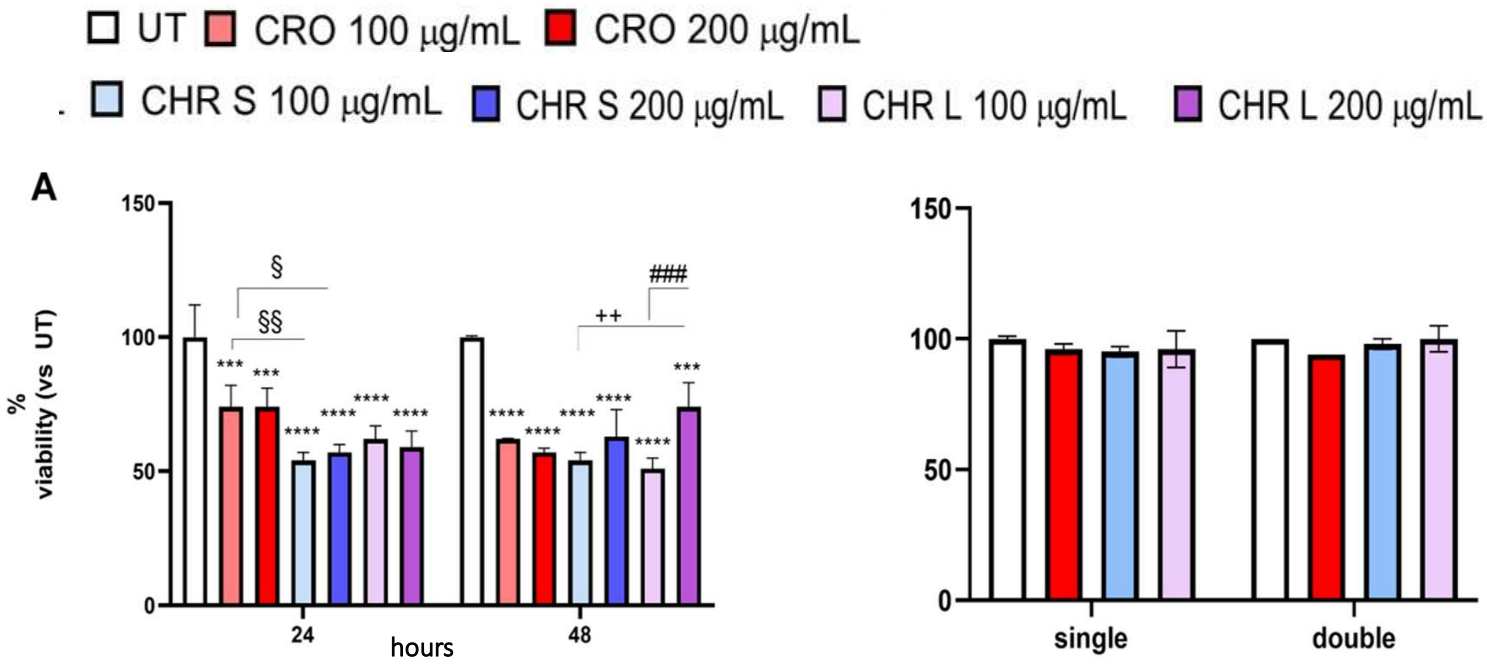
EPIAIRWAY™ RESULTS

MTT index: acute and chronic toxicity

Fibre concentration: 100-200 µg/ml
Time: 24-48hrs (A), 12days (B)
CRO positive control

EpiAirway™

MTT index

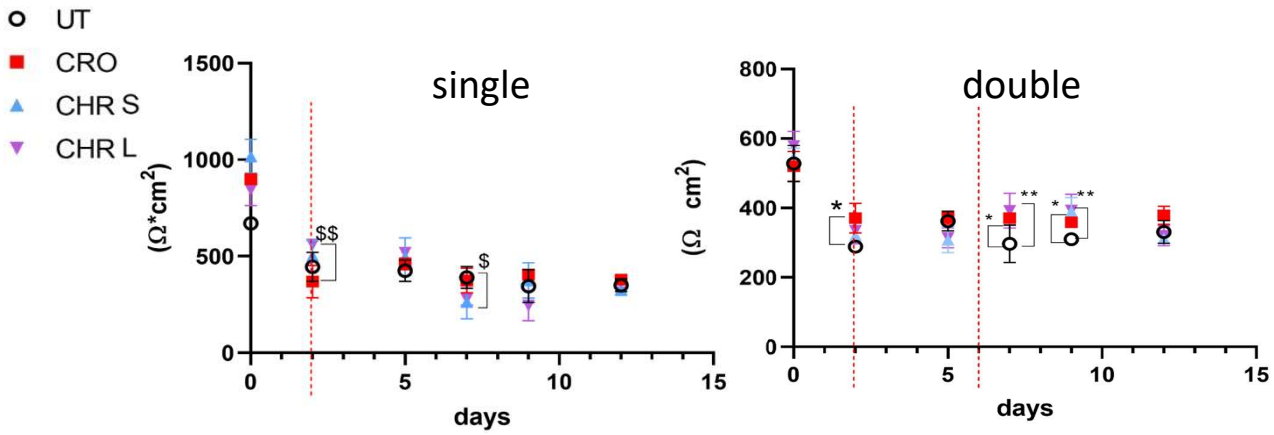


TEER and Histology: tissue integrity

Fibre concentration: 100 µg/ml
Time: 48 hrs, 12 days
CRO positive control

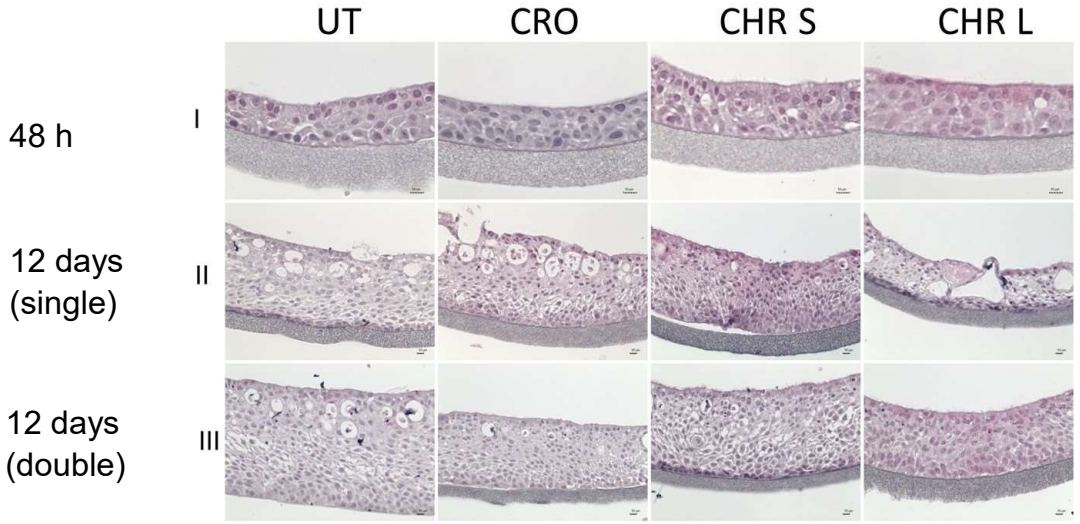
EpiAirway™

Trans-Epithelial Electrical Resistance (TEER)



EpiAirway™

Haematoxylin/Eosin staining

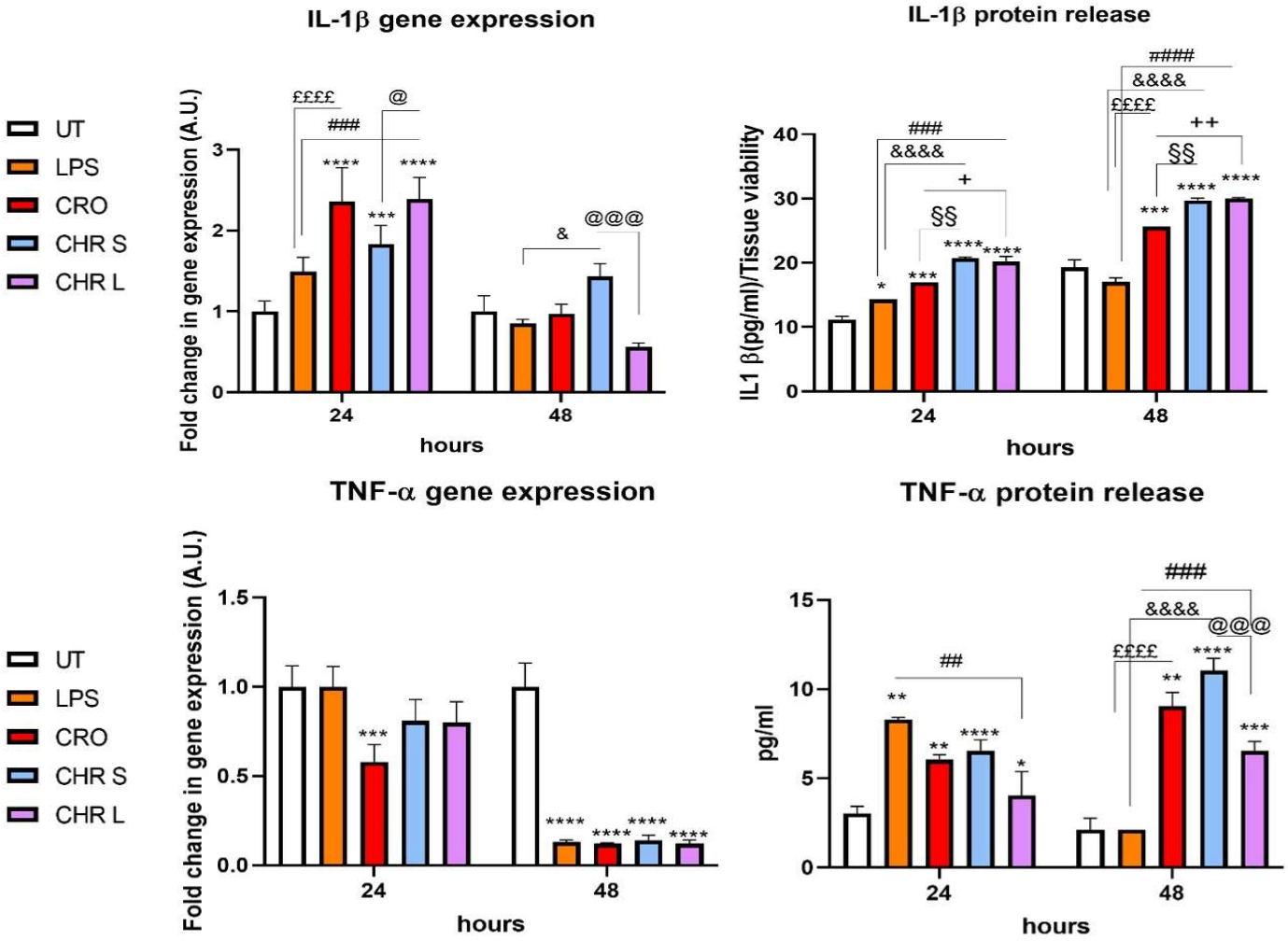


IL-1β/TNFα gene and protein: inflammatory acute response

Fibre concentration: 100 µg/ml
Time: 24-48 hrs
CRO positive control;
LPS pro-inflammatory stimulus

EpiAirway™

Pro-inflammatory genes and proteins

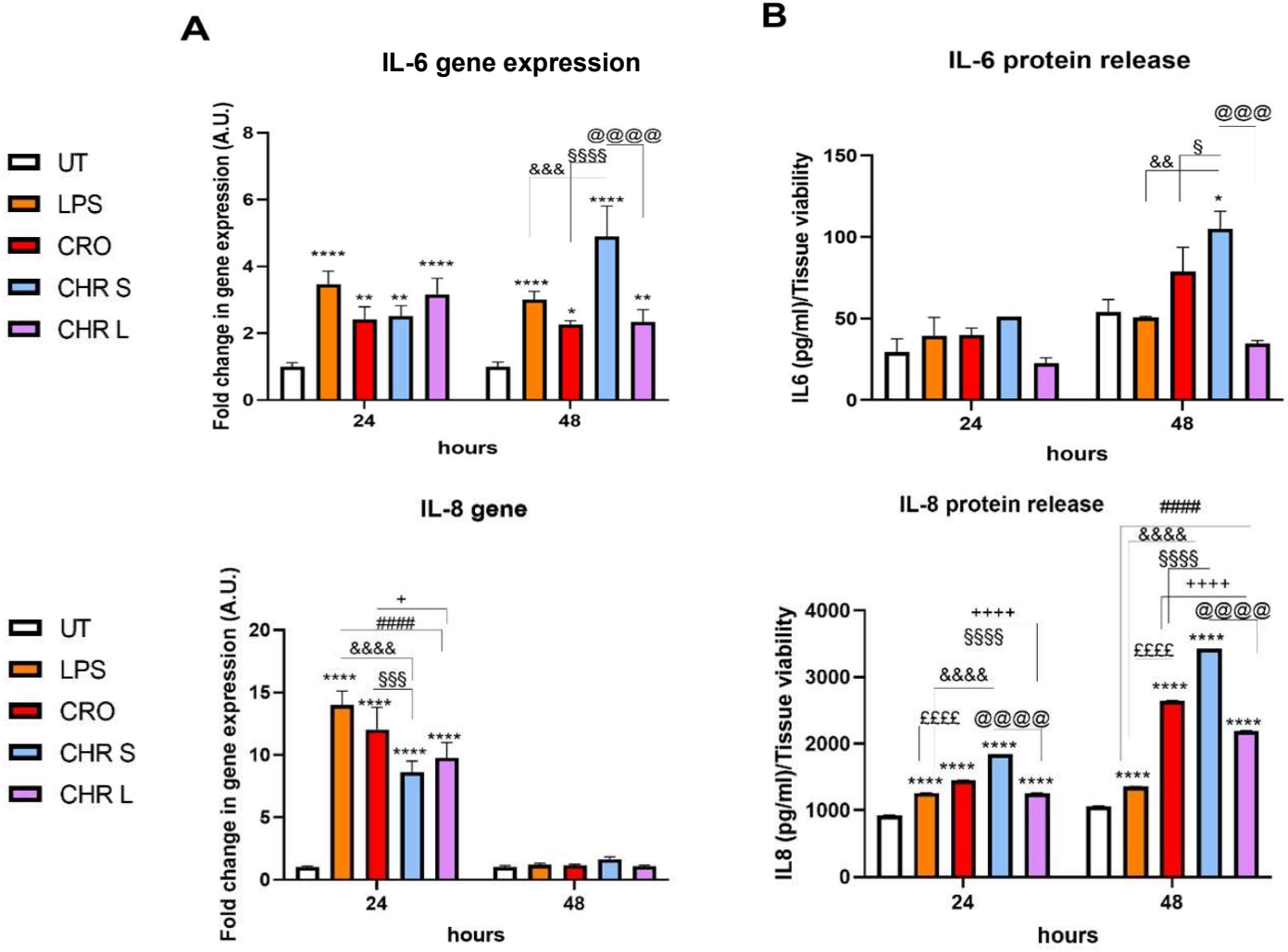


IL-6/IL-8 gene and protein: inflammatory acute response

Fibre concentration: 100 µg/ml
Time: 24-48 hrs
CRO positive control;
LPS pro-inflammatory stimulus

EpiAirway™

Pro-inflammatory genes and proteins

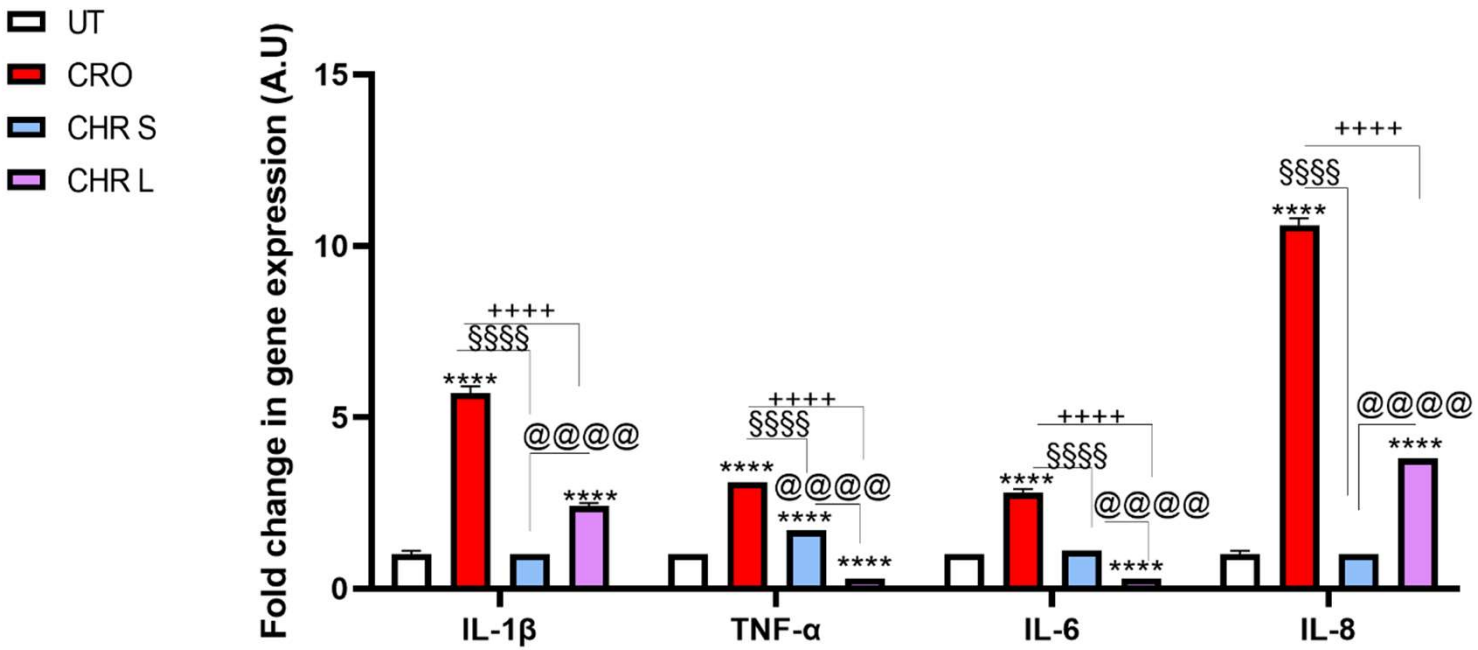


Marker genes: chronic inflammatory response

Fibre concentration: 100 µg/ml
Time: 12 days
CRO positive control

EpiAirway™

Pro-inflammatory genes

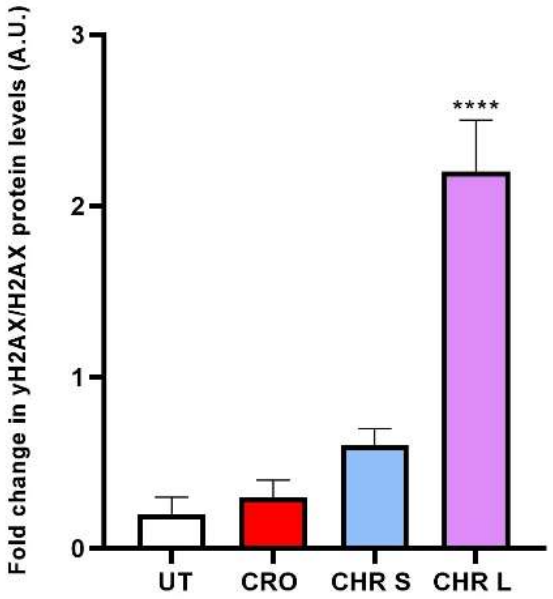


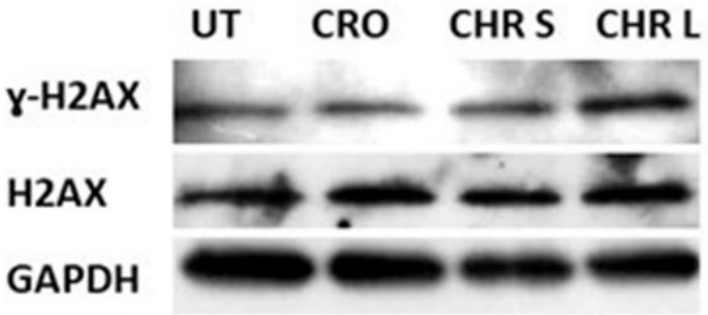
Acute genotoxic damage via H2AX signal

Fibre concentration: 100 µg/ml
Time: 24 hrs
CRO positive control;

EpiAirway™

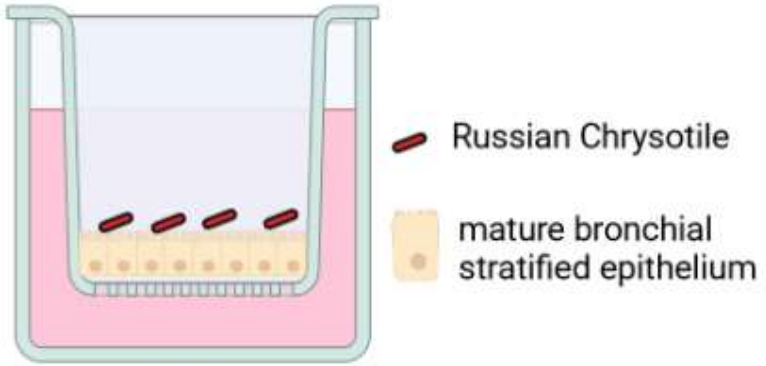
Genotoxic damage





Summary Model 5

EpiAirway™



24-48 hrs,12 days

Acute and chronic effects	CRO	CHR-L	CHR-S
Acute cytotoxicity	++++	++++	++++
Chronic cytotoxicity	no	no	no
TEER (tissue integrity)	stable	stable	stable
Histology (tissue integrity)	stable	stable	Stable
Acute Genotoxic damage		++++	+
Acute cytokine release	++++	++++	++++
Acute gene expression Inflammation	++++	++++	+++
Chronic gene expression Inflammation	++++	+++	++

CONCLUSIONS II

Second conclusion….

- Both **chrysotile fractions** display significant acute cytotoxic effects, with results that are comparable to the well-known damaging effects of crocidolite.

- The **long fraction** (> 5 μm) shows a higher cytotoxic potential, with high levels of oxidative stress, inflammatory response (acute and chronic), genotoxic damage and induction of cell death, both by plasma membrane damage and by apoptosis.

- The **short fraction** (< 5 μm) of chrysotile displays significant genotoxicity, inflammatory response and cytotoxicity, mainly through an apoptotic mechanism.

- **Wollastonite** shows minimal acute toxicity and no significant genotoxic effect, while the transcriptional upregulation of inflammatory mediators returns to basal levels within a short amount of time (i.e., 7 days).

Conclusions: 3D lung tissue model EpiAirway™

- The 3D lung tissue model is able to highlight the cyto/genotoxic and inflammatory effects of asbestos mineral fibres at early and, especially at late time-points.
- EpiAirway™ tissue proves to be a valuable tool for investigating short and long-term fibre effects resulting in a sensitive and reliable *in vitro* model.
- More sophisticated alveolar tissue models are still needed to mimic the fundamental crosstalk between the alveolar tissue and the immune component.

New funded project!

Best team ever!

DIMES:
Anna Maria Bassi
Stefania Vernazza
Sara Tirendi
Serena Mirata

DISTAV:
Sonia Scarfì
Vanessa Almonti

Anna Maria

Sonia

Serena

Vanessa

Stefania

Sara

DISTAV
Università degli Studi di Genova

Centro 3R

DIMES

UNIVERSITÀ DEGLI STUDI DI GENOVA