UNIVERSITÀ
POLITECNICA
DELLE MARCHE

FACOLTÀ
DI MEDICINA
E CHIRURGIA

Unità operativa di Ancona

- DIP. SCIENZE CLINICHE E MOLECOLARI – DISCLIMO
  Sezione di Patologia Sperimentale

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- DIP. MEDICINA SPERIMENTALE E CLINICA - DISMC
  Sezione Anatomia

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METHODS

Human mesothelial (MeT-5A) and alveolar endothelial (A549) cells were exposed to asbestos fibres:

- Chrysotile UICC, widely used in the production of asbestos containing material;
- Chrysotile Valmalenco (Central Alps, Sondrio, Italy),
- Erionite from Jersy Nevada (USA) both naturally occurring and airborn asbestos fibres.
- Crocidolite UICC, well known for its carcinogenic power;

- Morphological and microanalitical investigations performed by SEM-EDX.

- Citotoxic effects evaluated by MTT test and intracellular glutathione content assay.

- DNA Double-Strand Breaks by immunofluorescence analysis of gamma H2AX phosphorylation (Kinner, 2008) and Comet assay (Cardile 2004).

For in vitro cytotoxicity assays

the fibers were administred at 6, 24, 48h at 50μg/ml

in human

- mesothelial MeT-5A cells
- alveolar endothelial A549 cells,

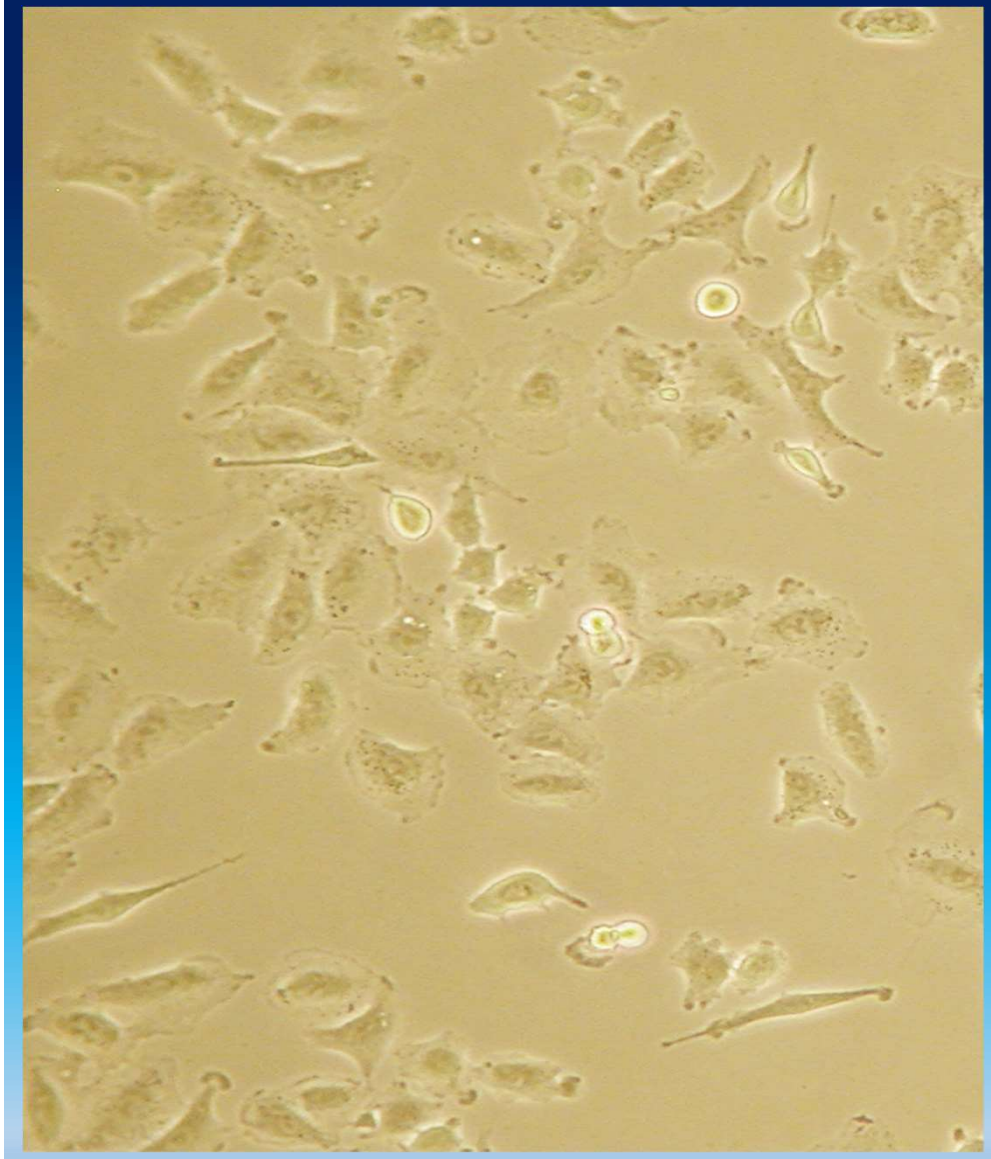
mimicking human respiratory microenvironment

representing the first target of respired fibers.

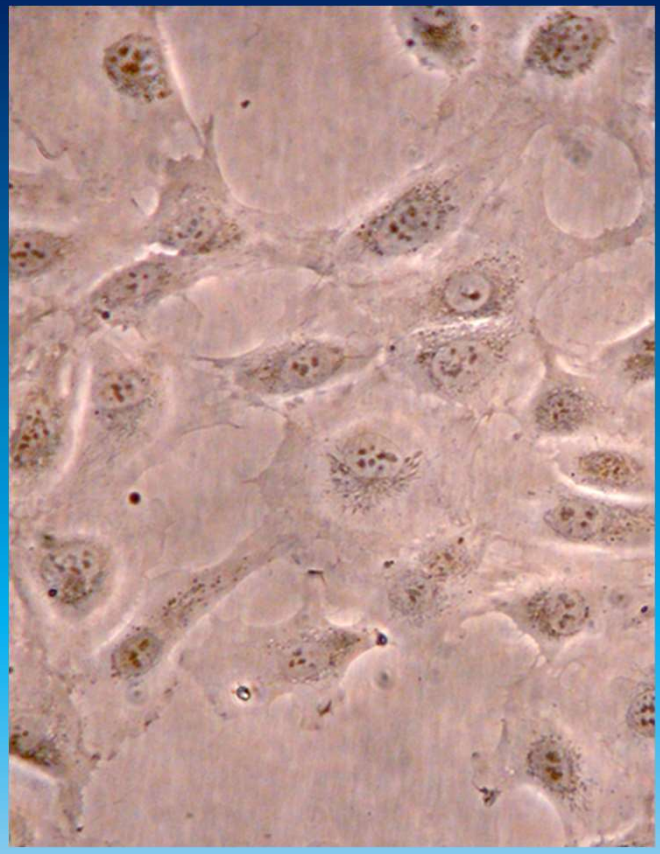
Widely used in asbestos exposure and pulmonary research

MeT5A

CTRL 6h



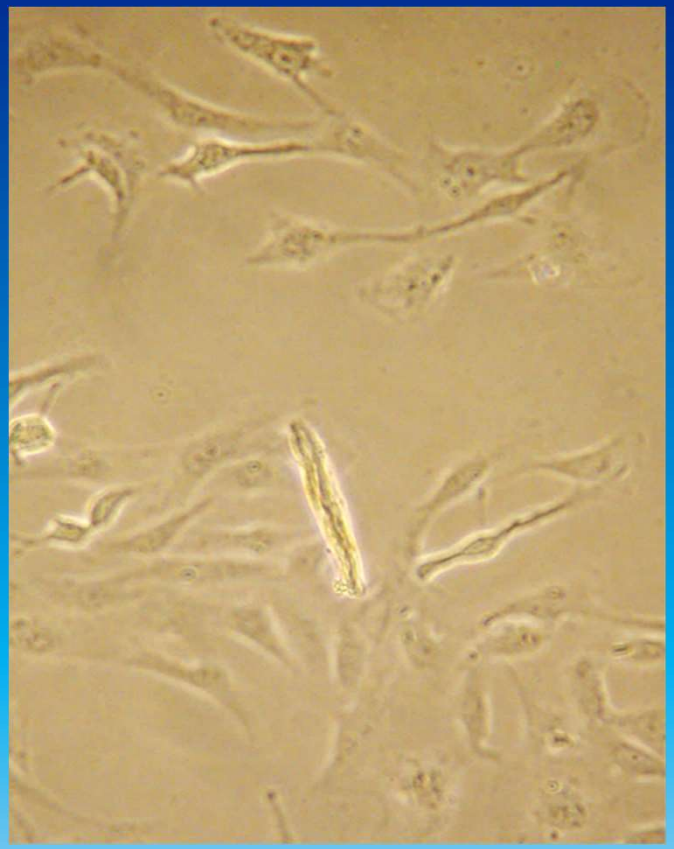
24h



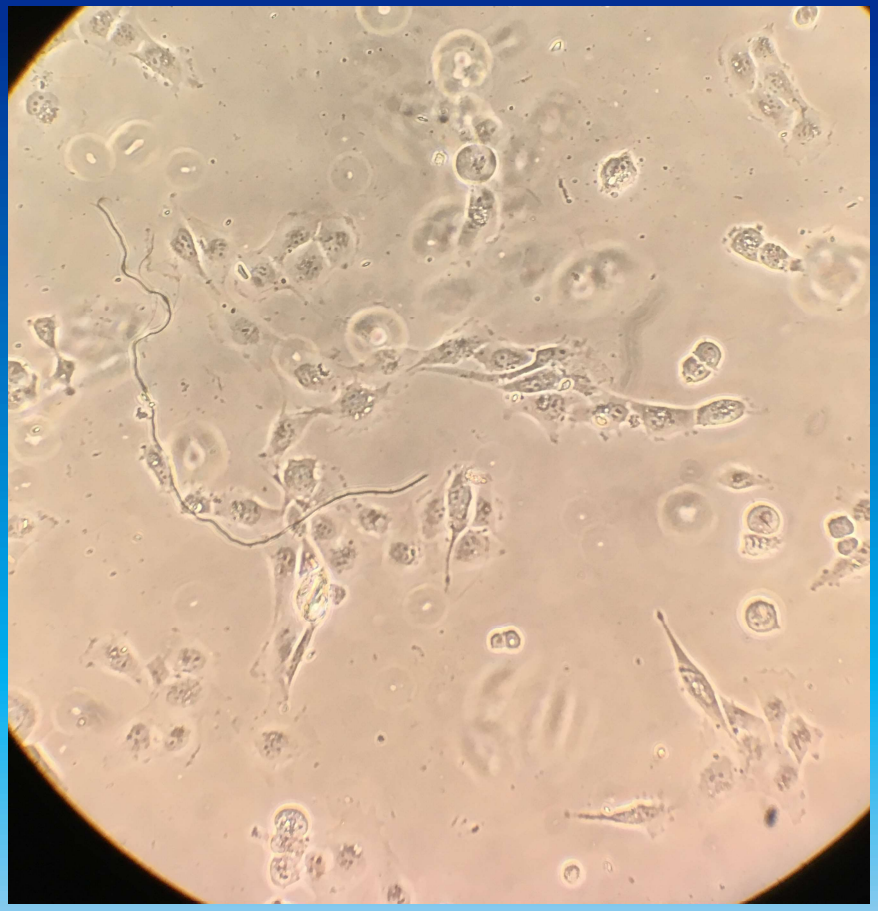
MeT5A

CRY VM

6h

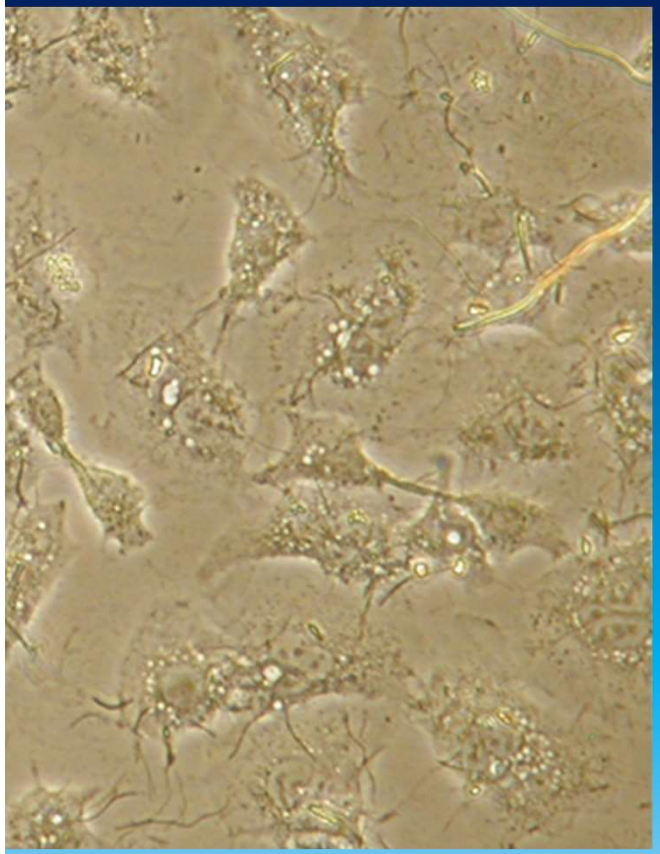


24h

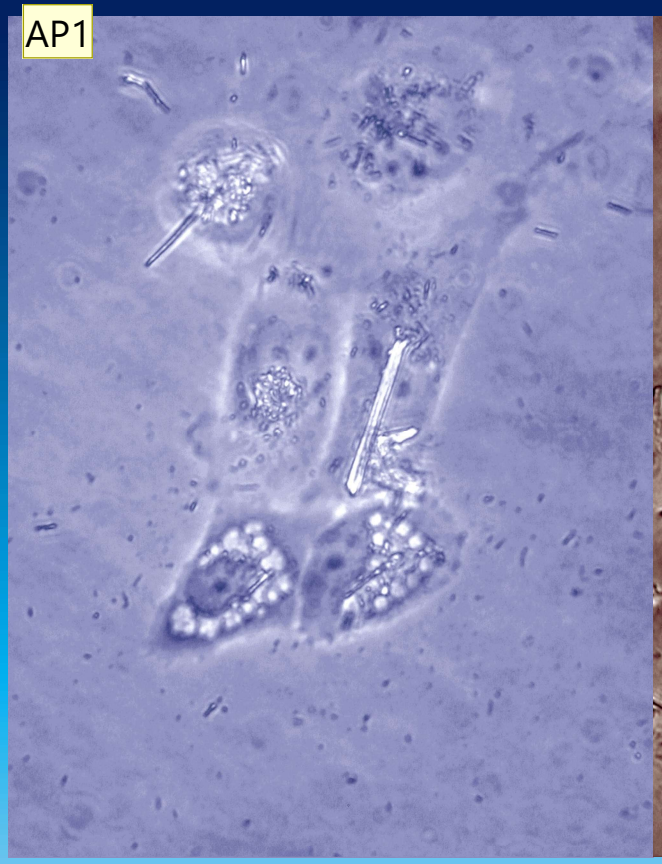


MeT5A

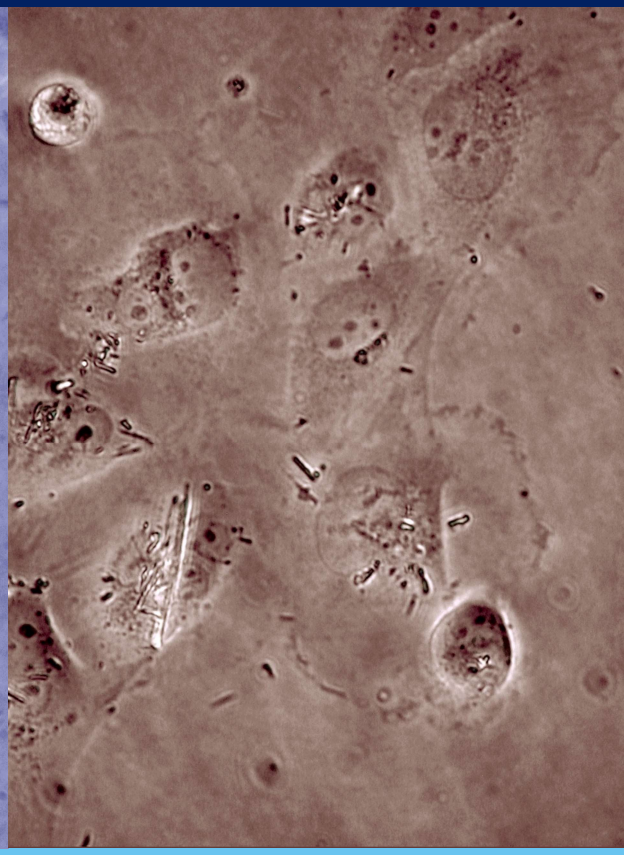
CRY UICC



Crocidolite UICC



Erionite



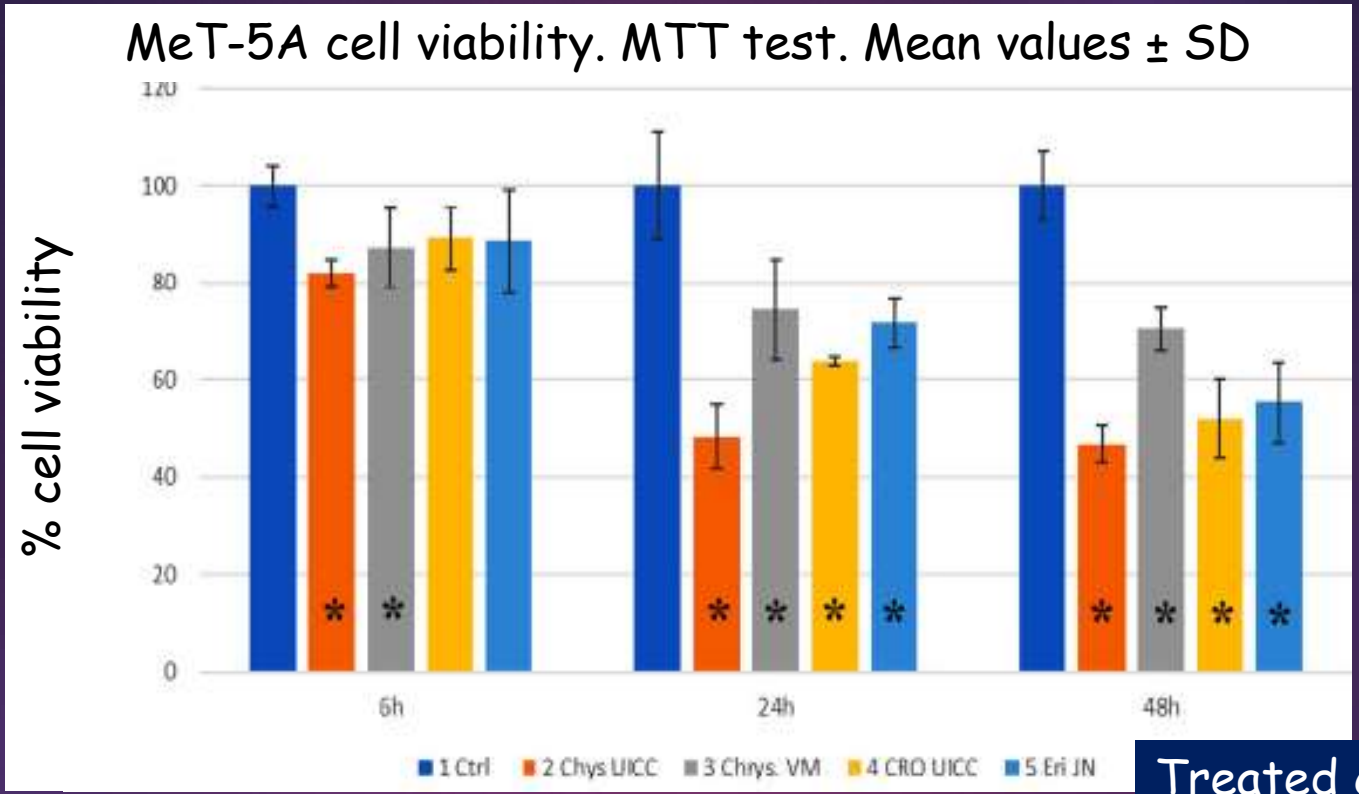
Diapositiva 6

AP1 ARMANDA PUGNALONI; 08/10/2020

Cell viability

Effects of fibres treatment on MeT-5A and A549 cells viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] colorimetric assay testing mitochondrial succinate dehydrogenase activity.

Absorbance values of control and treated cell lines at 6, 24 and 48 h are means ± SD of at least three different experiments. Control cell absorbance values were taken as 100% of cell viability.

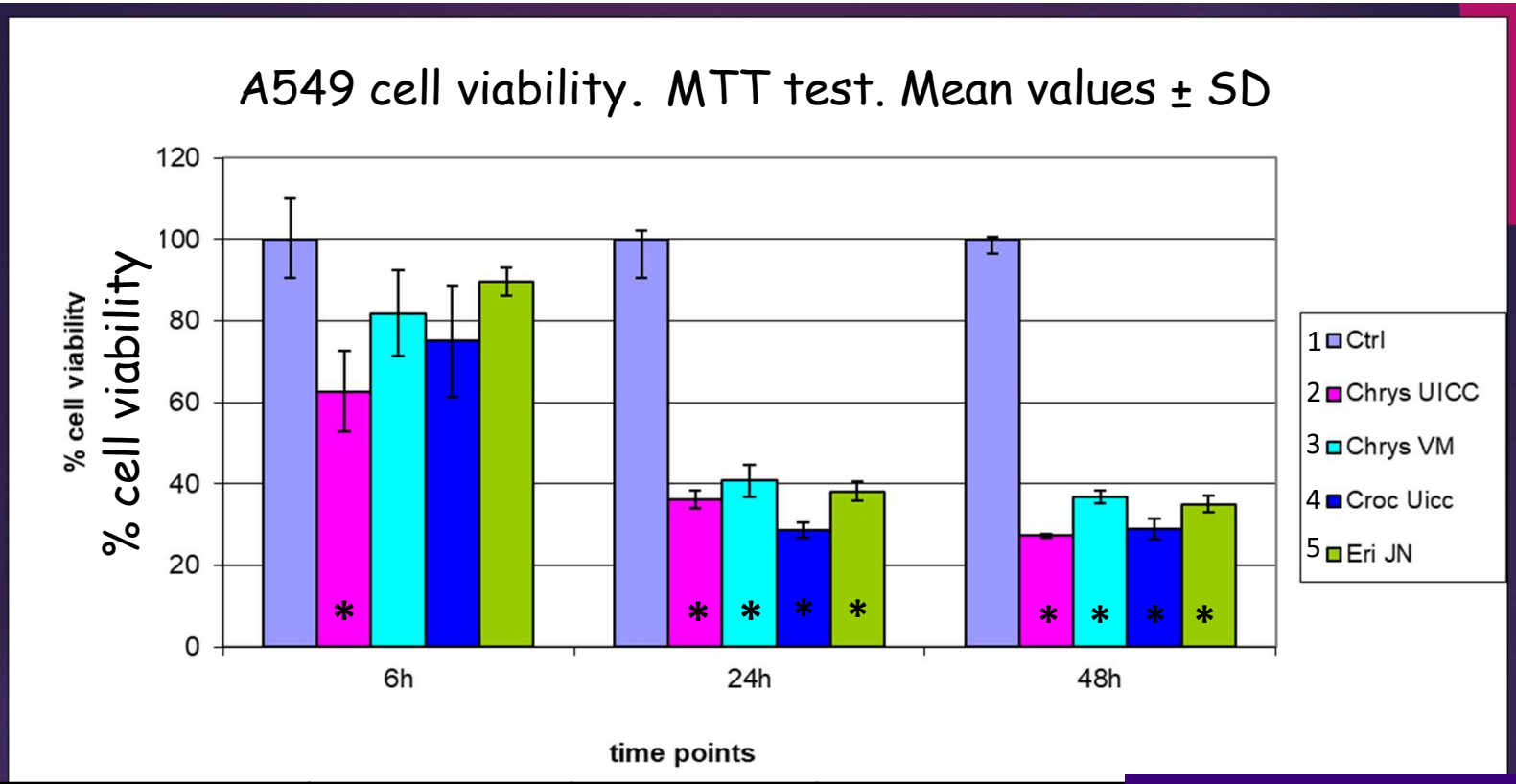


MTTtest	6h	24h	48h
% values X ± SD			
1 Ctrl	100 ± 4	100 ± 11	100 ± 7.10
2 Chrys. UICC	82.10 ± 2.72	48.30 ± 6.55	46.80 ± 4
3 Chrys. VM	87.20 ± 8.20	74.40 ± 10.20	70.50 ± 4.45
4 Crocidolite UICC	89.10 ± 6.59	63.90 ± 0.93	52 ± 8.20
5 Erionite JN	88.50 ± 10.70	71.70 ± 5.03	55.50 ± 8.24
t test Bonferroni's correction p<0.05	1-3, 1-2, 4-3, 5-3.	3-1, 3-2, 5-1, 5-2, 4-2, 4-1, 2-1.	1-2, 1-4, 4-5, 1-3, 3-2, 3-4, 3-5

Chrys. UICC exerts higher cytotoxic effects since 6h

Crocidolite UICC and Erionite JN shows higher effects at 24 and 48h

Treated cultures show lower though still moderate absorbance reductions in all the treated cultures at 6h compared with the control ones. Higher reductions of cell viability were found at 24 and 48h.



MTT test % values X ± SD	6h	24h	48h
1 Ctrl	100 ± 9.5	100 ± 9.3	100 ± 3.5
2 Chrys. UICC	62.6 ± 9.9	36.2 ± 2.1	27.3 ± 0.5
3 Chrys. VM	81.9 ± 10.4	40.8 ± 4.0	36.8 ± 1.6
4 Crocidolite UICC	75 ± 13.8	28.7 ± 1.9	28.9 ± 2.4
5 Erionite JN	89.5 ± 3.5	38.1 ± 2.4	35 ± 2.0
T test Bonferroni's corrections	1-2 ; 2-5; 4-5	1-2; 1-3; 1-4; 1-5	1-2; 1-3; 1-4; 1-5; 2-3; 2-5; 3-4

Treated cultures show lower though still moderate absorbance reductions at 6h compared with control cultures. Higher reductions of cell viability were found at 24 and 48h.

Chrys. UICC exerts higher cytotoxic effects since 6h
Crocidolite UICC and Erionite JN shows higher effects at 24 and 48h

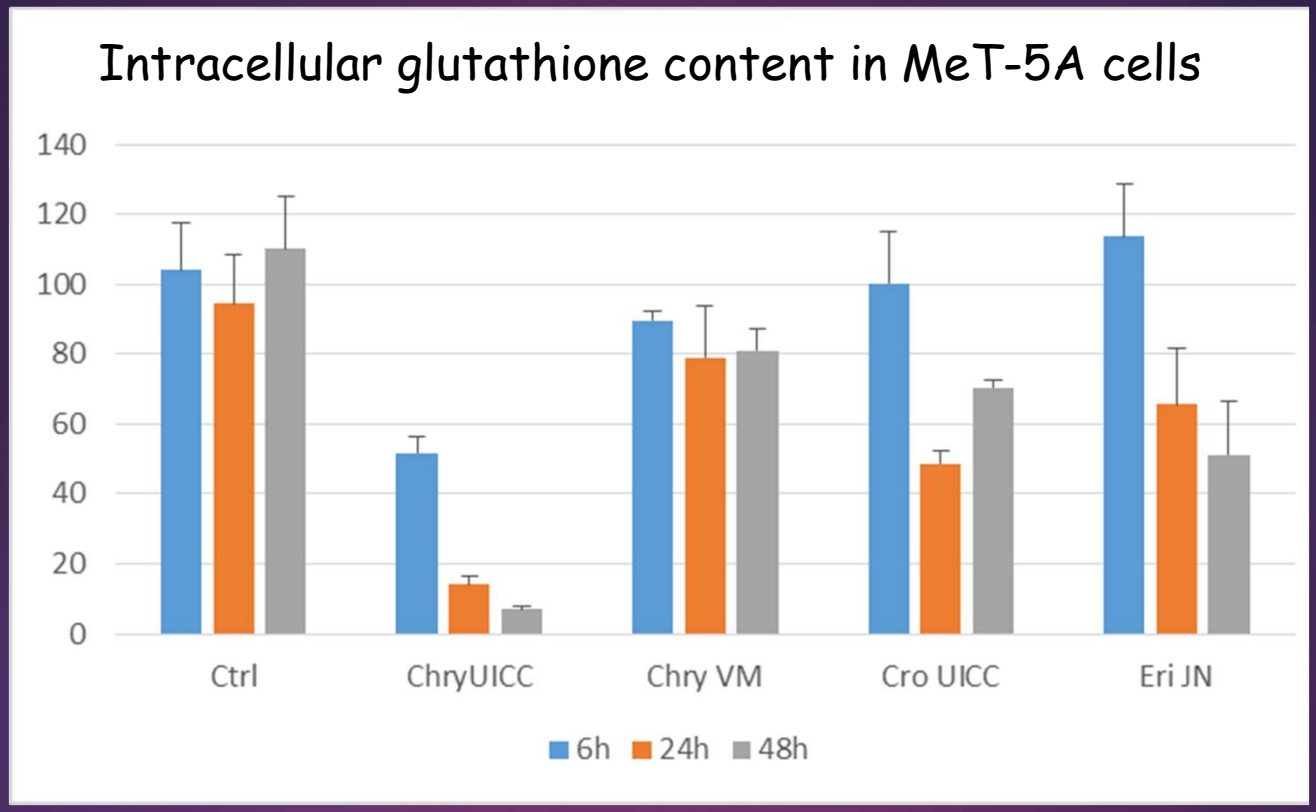
Intracellular glutathione (GSH) content

Measuring glutathione can help assess oxidative stress status of an organism and the potential for downstream oxidative damage

Glutathione is a structural mitochondria defense against excessive ROS production.

Cells produce glutathione as an antioxidant to help resist oxidative stresst to maintain homeostasis.

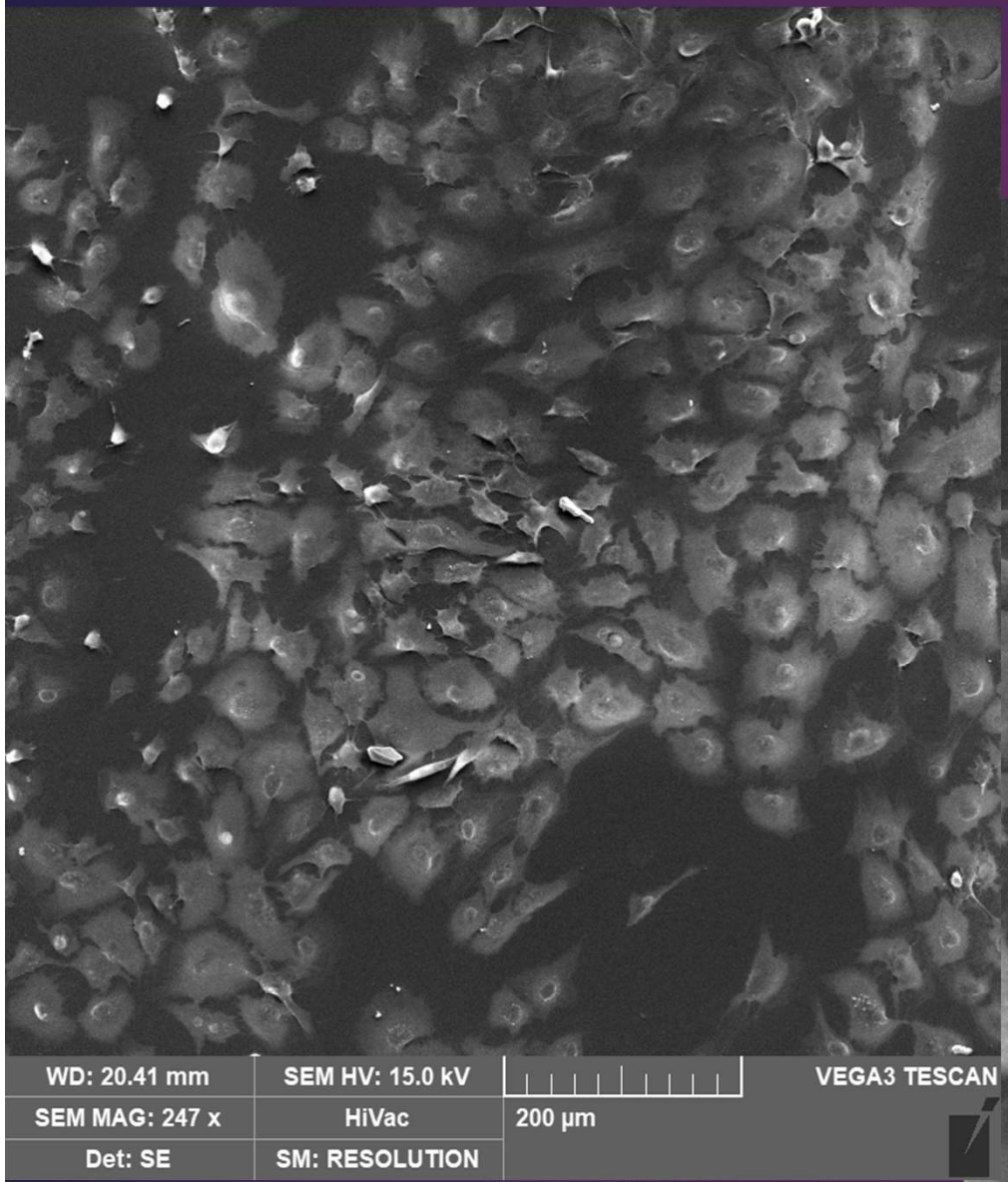
Total glutathione (GSH+GSSG) was measured spectrophotometrically by the Glutathione Reductase (GR) recycling assay at 412 nm in presence of 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) (Brigelius et al., 1983).

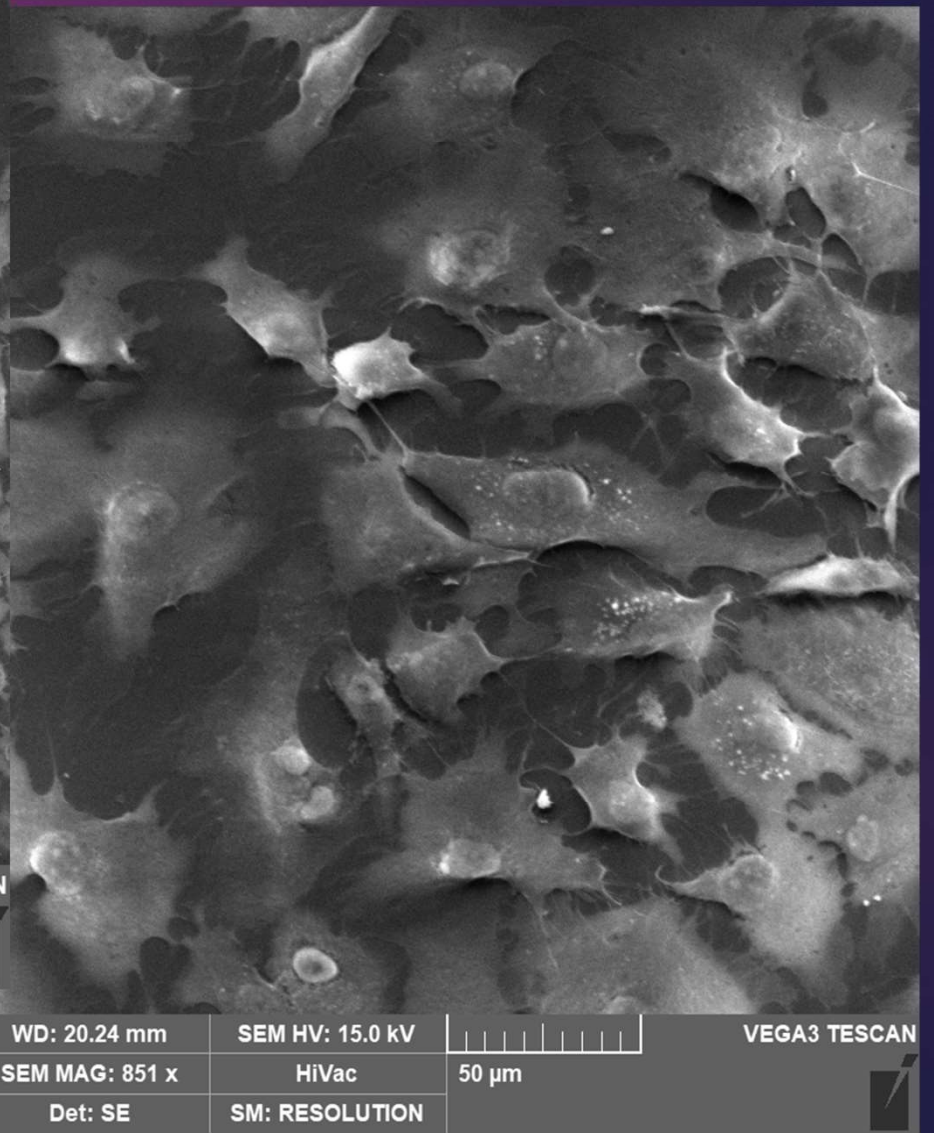


Intracellular glutathione content in MeT-5A cells at 48h resulted globally decreased in all treated cultures, with decrements after fibres administration.

Chrisotile UICC treatments exerted most drastic effects since 6 h.

Human mesothelial MeT-5A cells



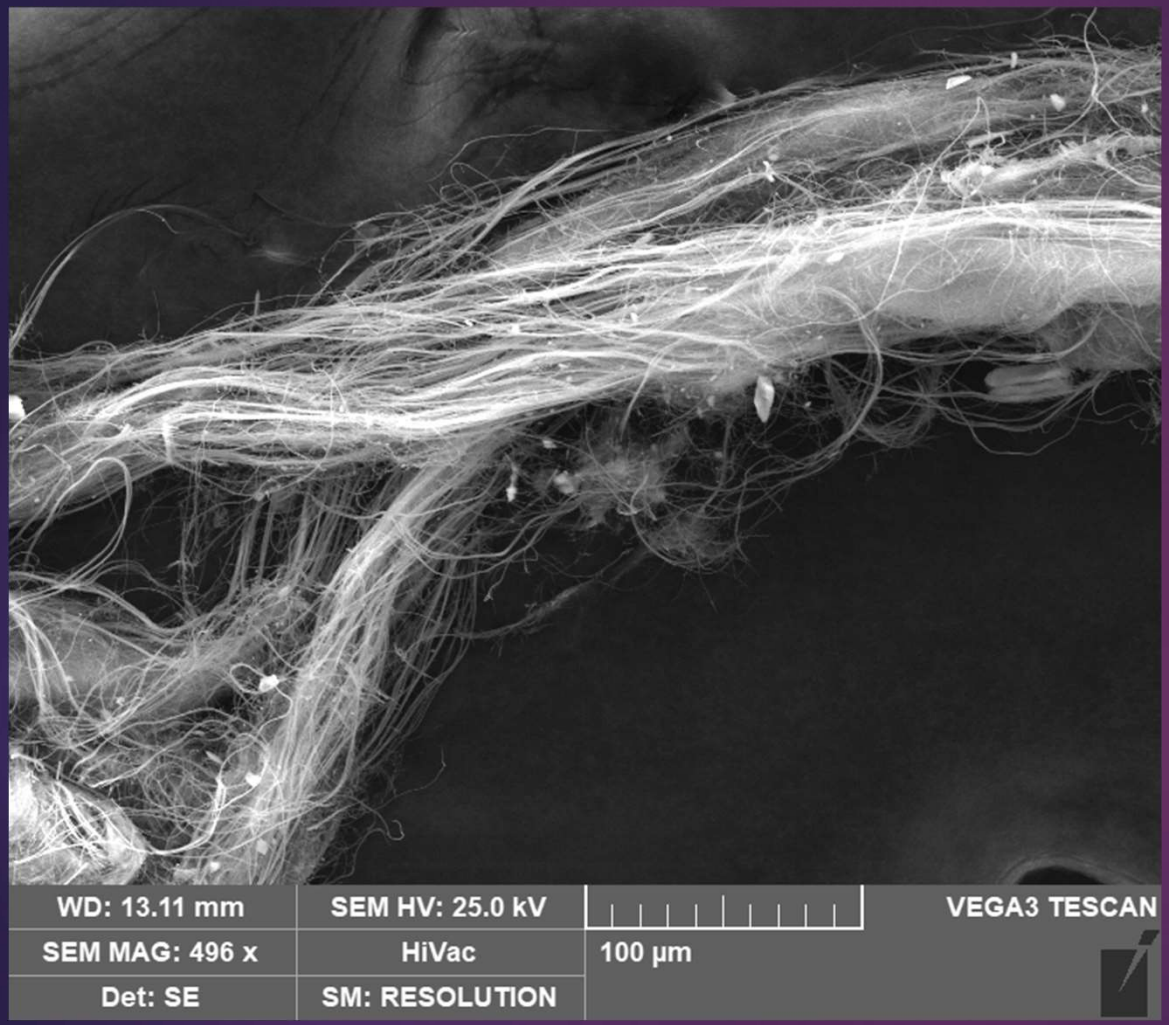


High resolution SEM image and EDS investigations.

- Secondary electron SEM image of fibres and cultured cell exposed to fibres at 48 h.

- Dispersive energetic spectrum EDS-SEM of fibres performed in selected area of original fibres (not in culture) and fibres in contact with cells at 48h.

Differences were found in element content expressed as weight % among fibres before and after cell culture contact.



Secondary electron SEM image of

Chrysotile UICC fibres

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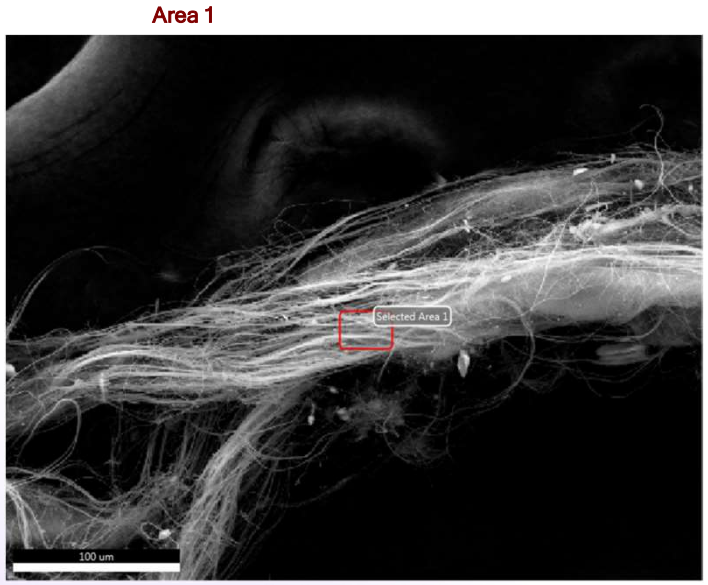
EDAX APEX

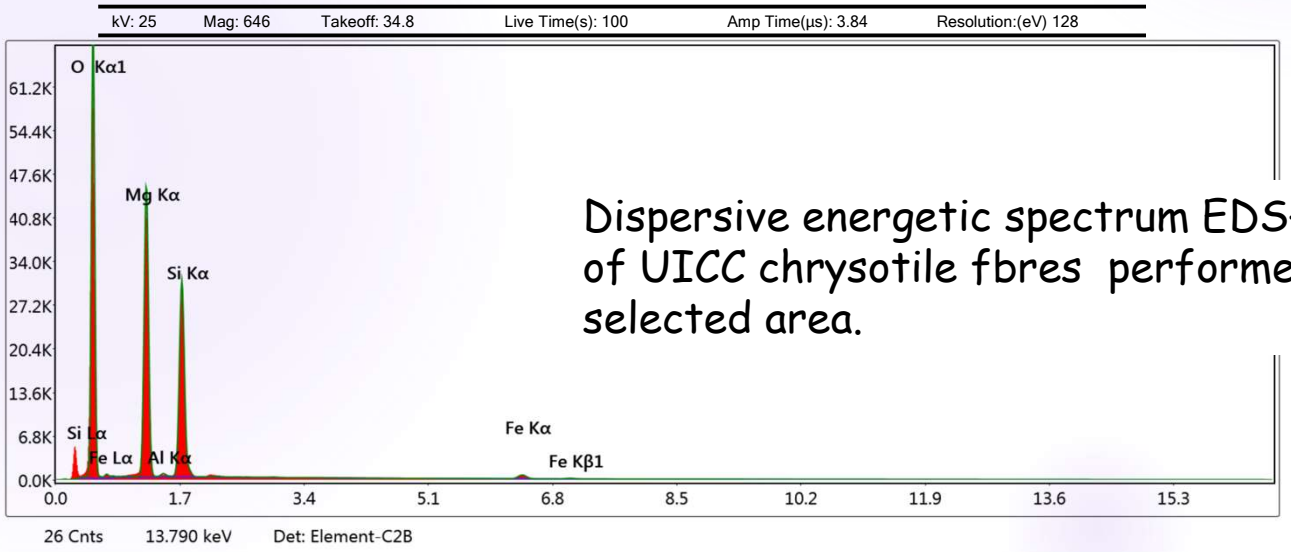
UICC chrysotile fbres



Smart Quant Results

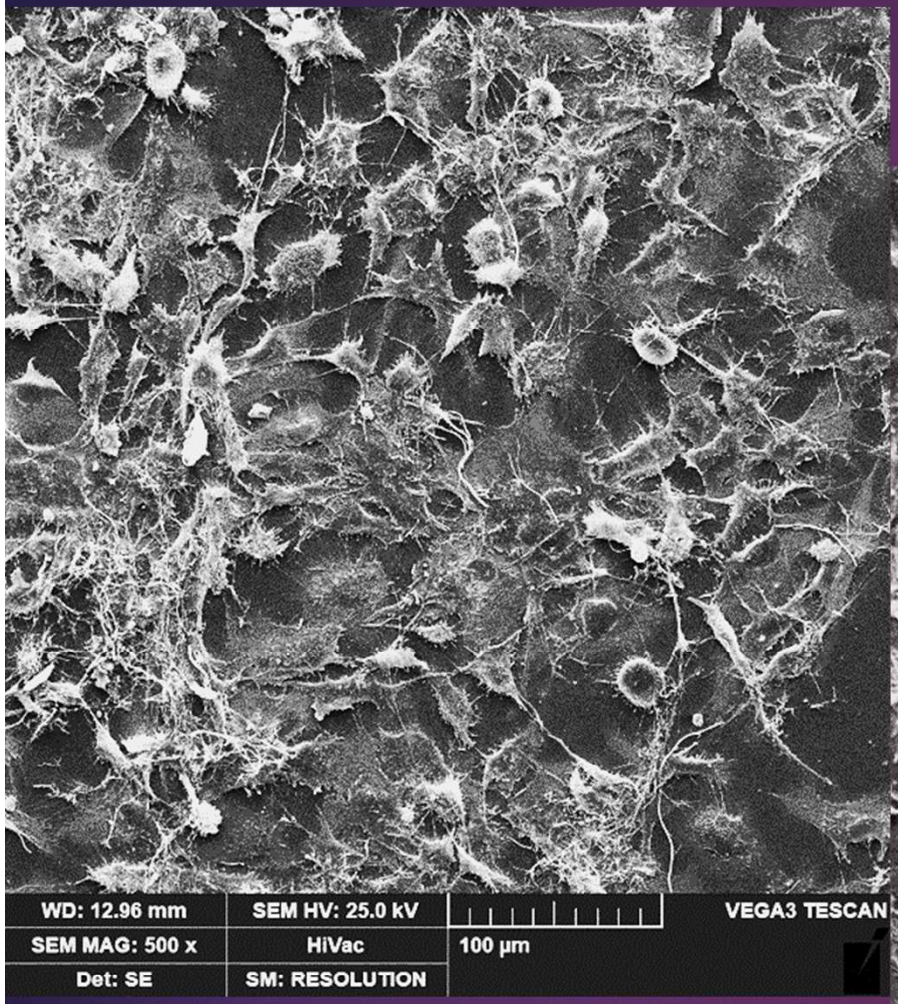
Element	Weight %	Atomic %
O K	54.51	66.03
MgK	26.68	21.27
AlK	0.36	0.26
SiK	17.62	12.16
FeK	0.84	0.29

Chemical components characteristic of Chrysotile UICC represented by Si, Al, Fe, Mg.

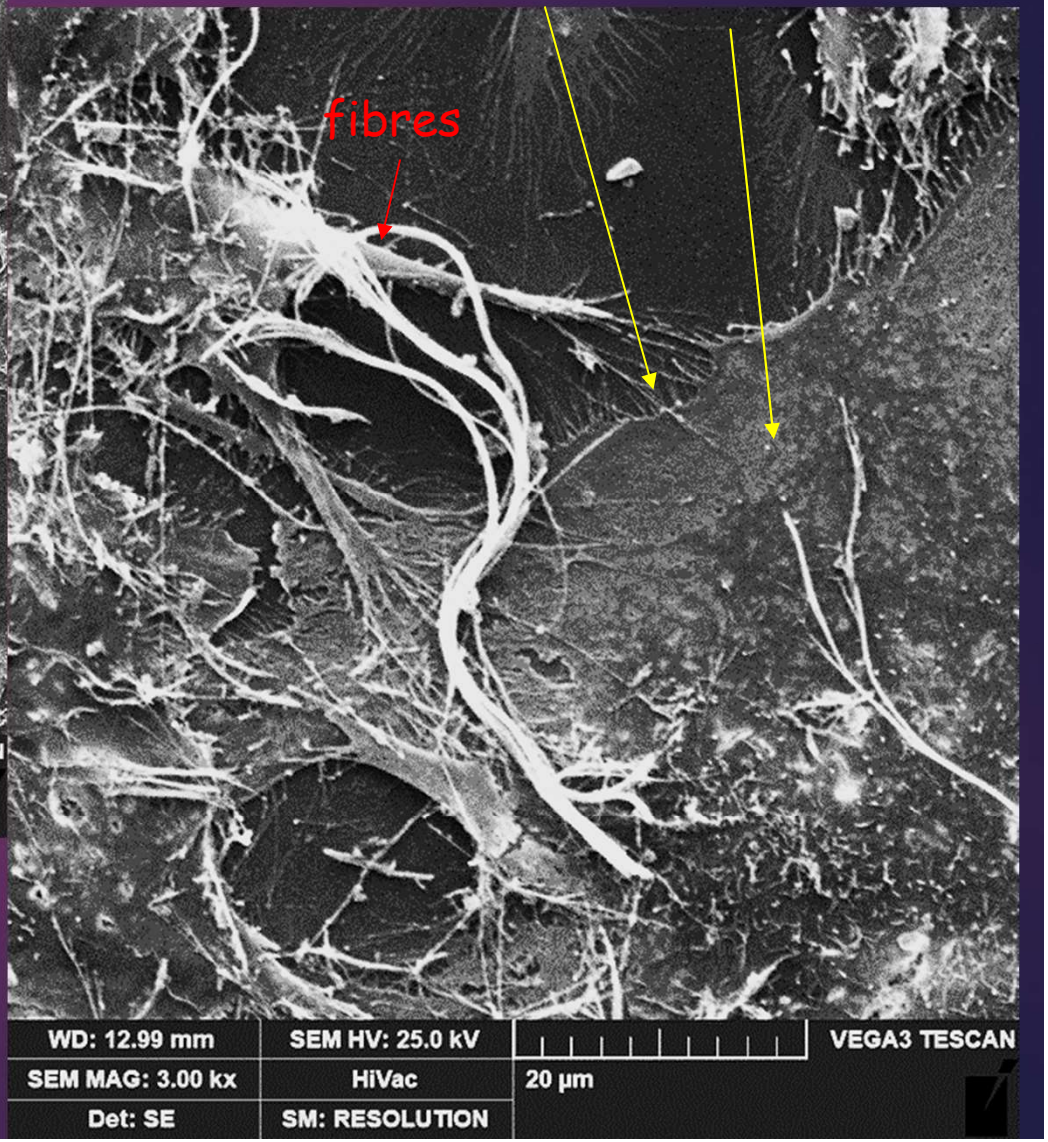


Dispersive energetic spectrum EDS-SEM of UICC chrysotile fbres performed in selected area.

Chrysotile UICC fibres in MeT-5A culture



cell sprouts and cytoplasm



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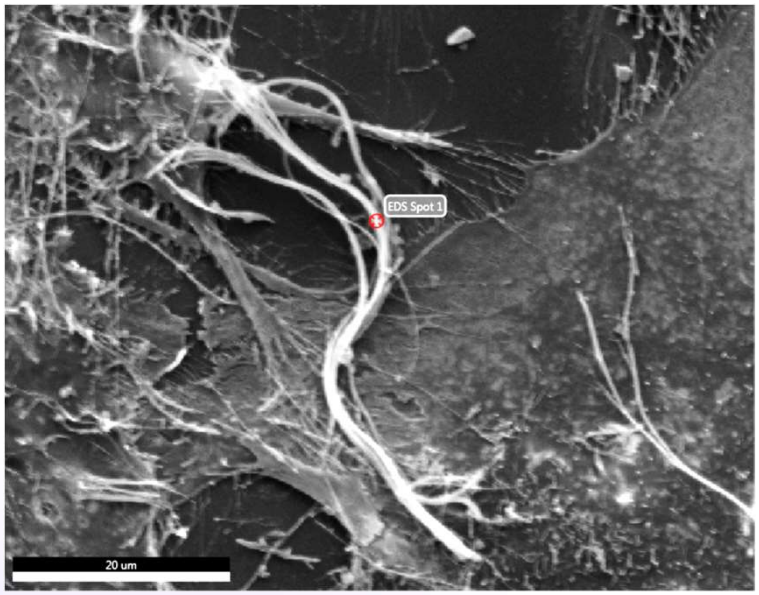
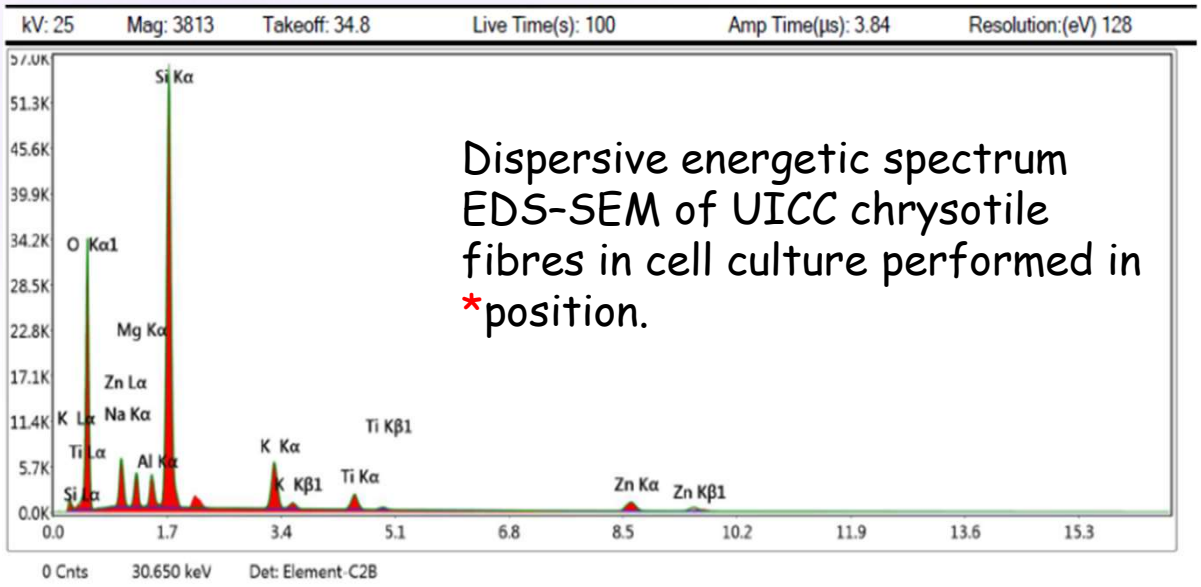
Chrysotile UICC fibres in MeT-5A culture

Area 1



Smart Quant Results

Element	Weight %	Atomic %
O K	47.67	62.27
NaK	4.64	4.22
MgK	6.95	5.97
AlK	2.4	1.86
SiK	28.92	21.52
K K	4.32	2.31
TiK	1.78	0.77
FeK	0.26	0.1
ZnK	3.06	0.98

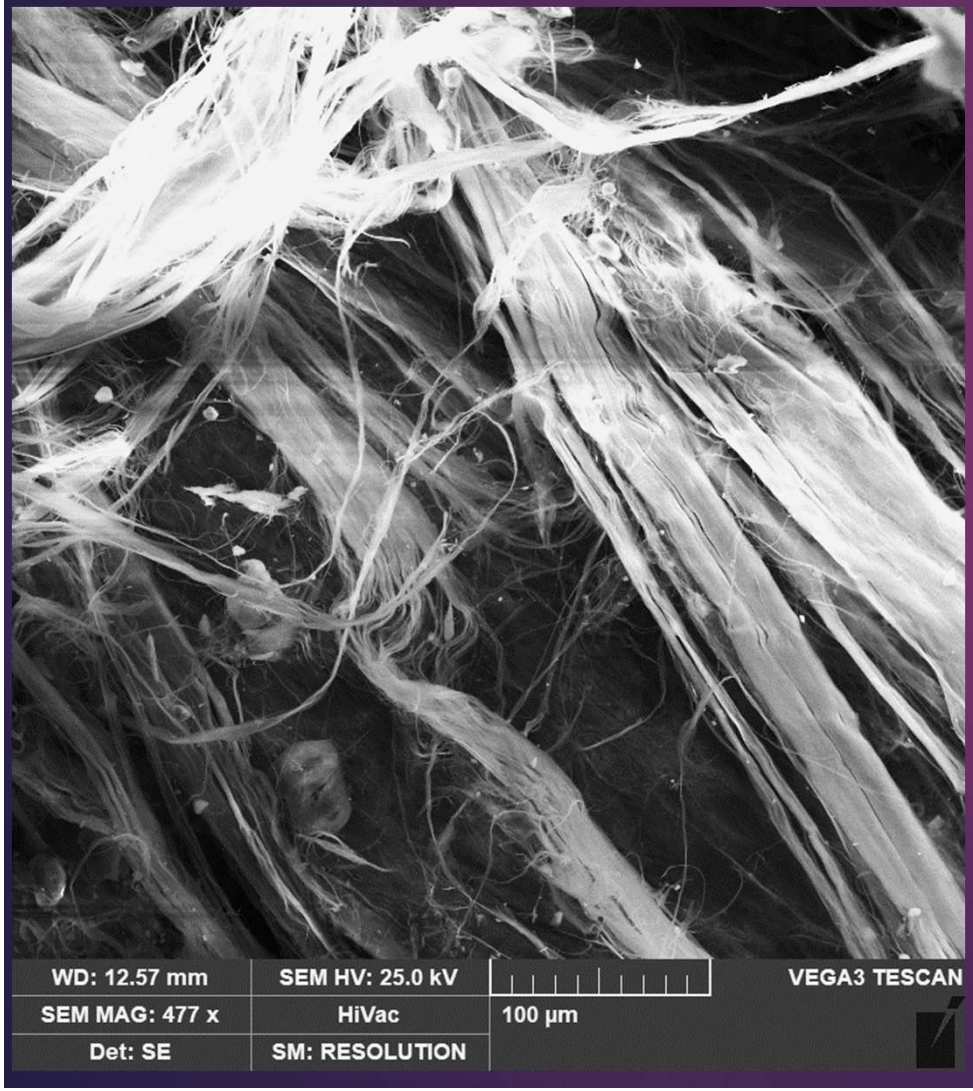


Dispersive energetic spectrum EDS-SEM of UICC chrysotile fibres in cell culture performed in *position.

Differences were found with respect to the original fibres in element content expressed as weight %:

- decrements of Mg and Fe content released in the colture environment.
- Other elements are released from culture medium and cell metabolism. Ti: from champion mounting glass.

Secondary electron SEM image of Chrysotile asbestos from Valmalenco (Sondrio, Italy)



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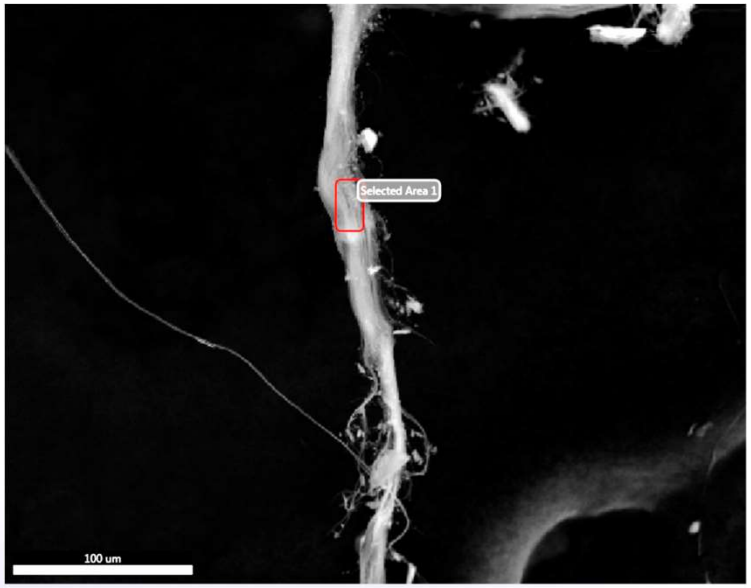
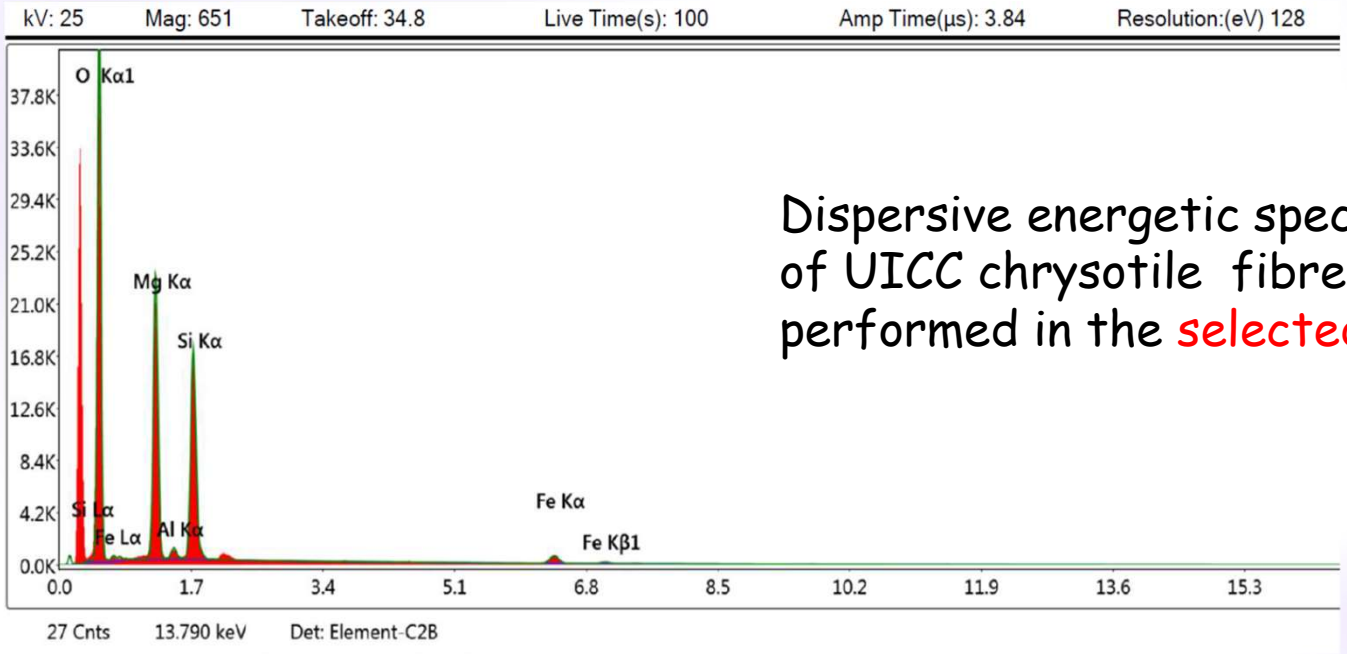
Chrysotile Valmalenco

Area 1



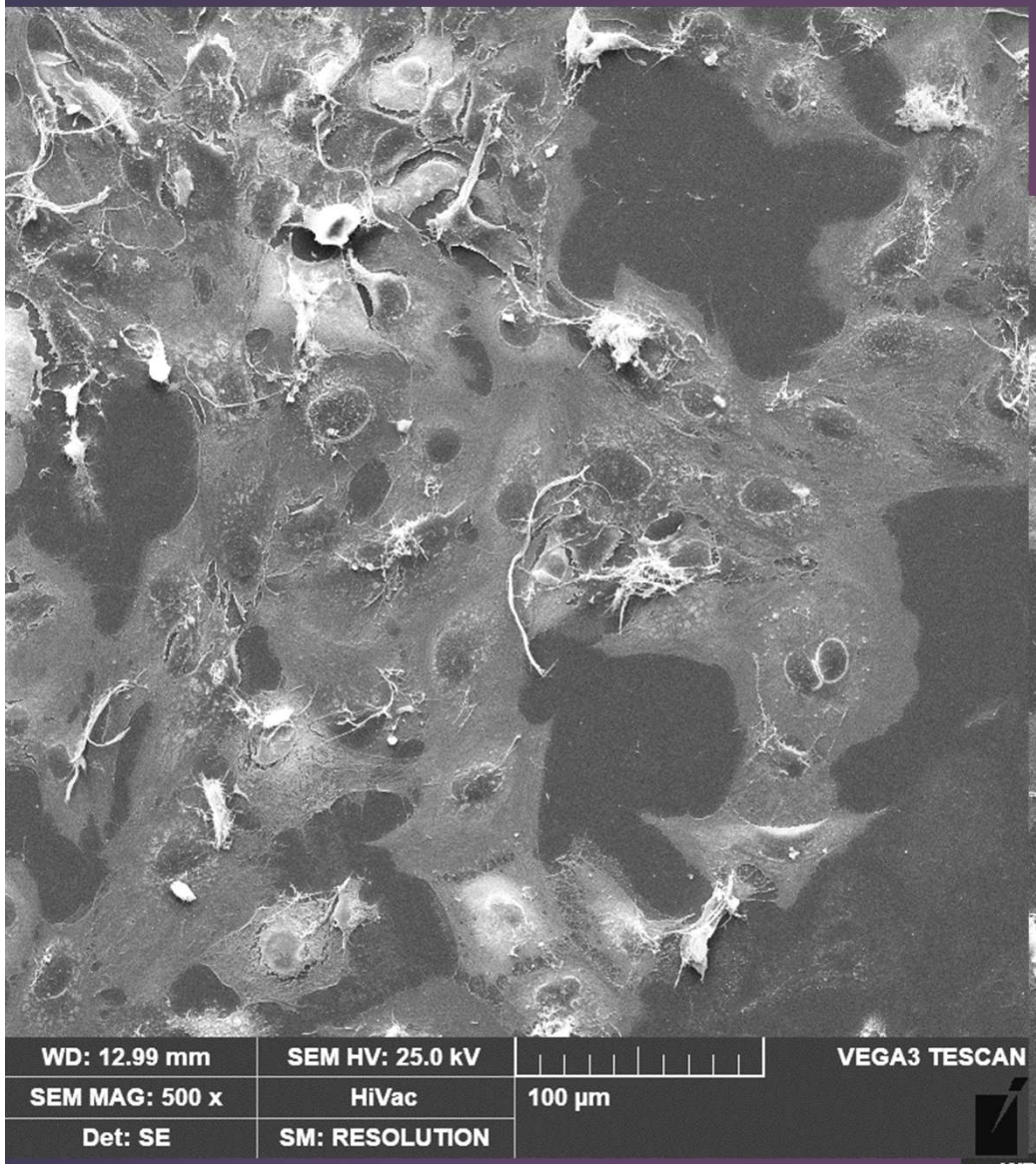
Smart Quant Results

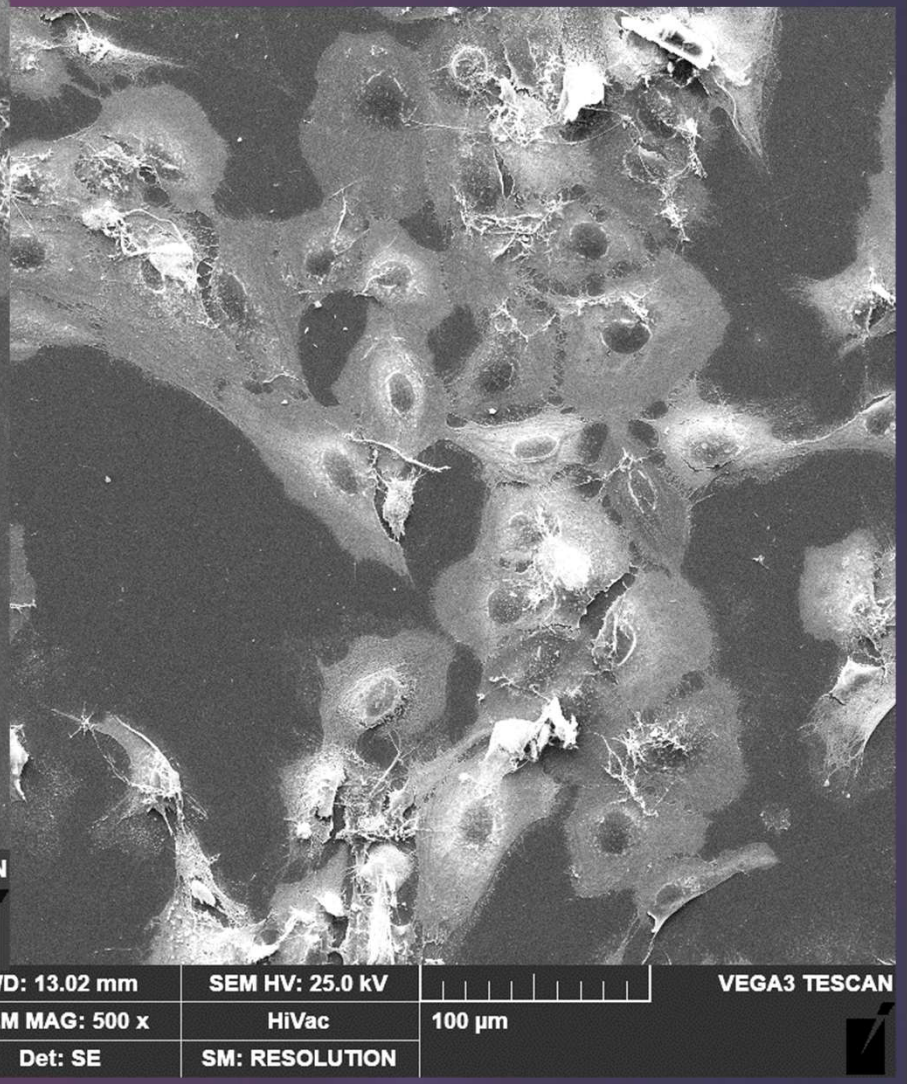
Element	Weight %	Atomic %
O K	56.42	67.97
MgK	24.23	19.21
AlK	1.14	0.81
SiK	16.77	11.51
FeK	1.44	0.5



Dispersive energetic spectrum EDS-SEM of UICC chrysotile fibres in cell culture performed in the selected area.

Chrysotile Valmalenco in MeT5A culture





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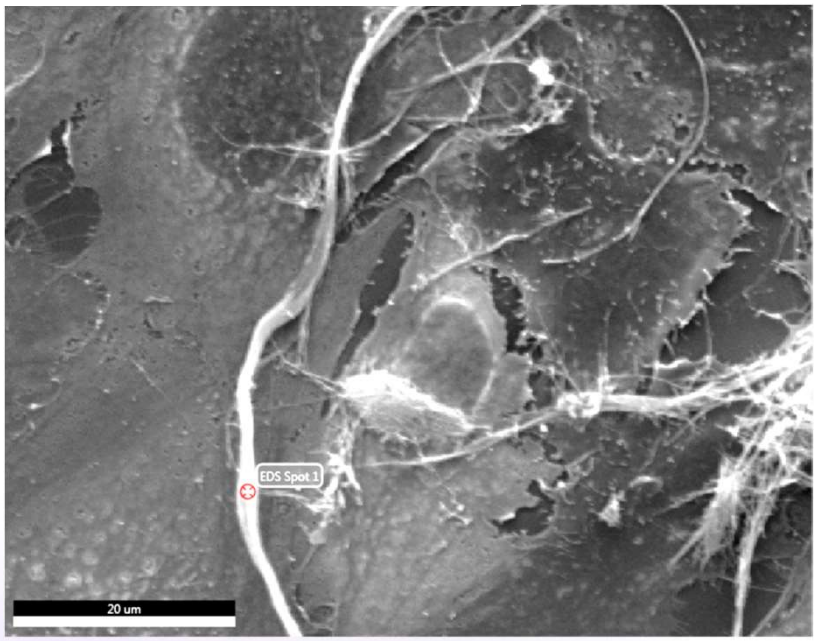
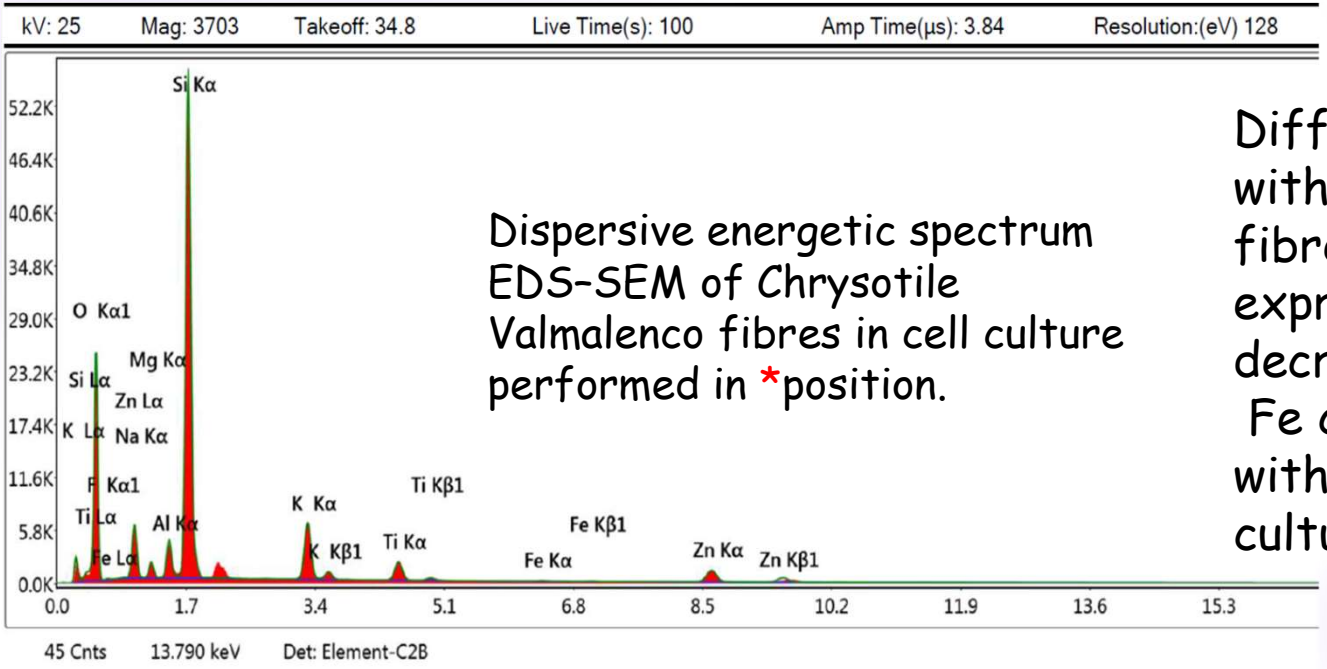
Chrysotile Valmalenco in MeT5A culture



Element	Weight %	Atomic %
O K	43.95	59.42
F K	0.3	0.35
NaK	5.6	5.27
MgK	1.64	1.46
AlK	3.02	2.42
SiK	32.9	25.34
K K	5.75	3.18
TiK	2.42	1.09
FeK	0.14	0.06
ZnK	4.27	1.41



Dispersive energetic spectrum EDS-SEM of Chrysotile Valmalenco fibres in cell culture performed in *position.

Differences were found with respect to the original fibres in element content expressed as weight% : decrements of Mg and Fe content are compatible with their release in the culture microenvironment.

Crocidolite UICC

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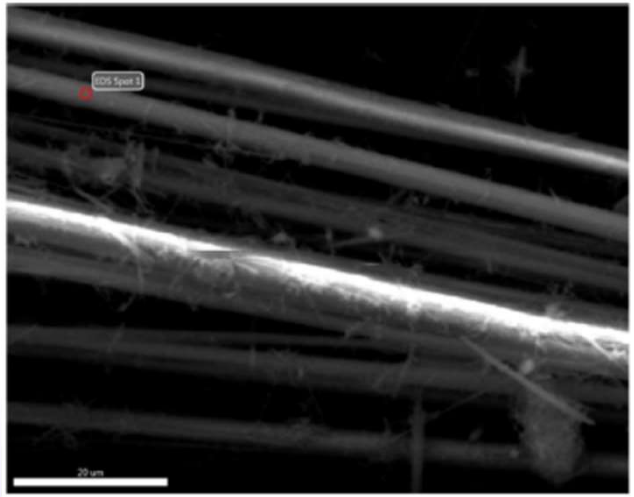
EDAX APEX

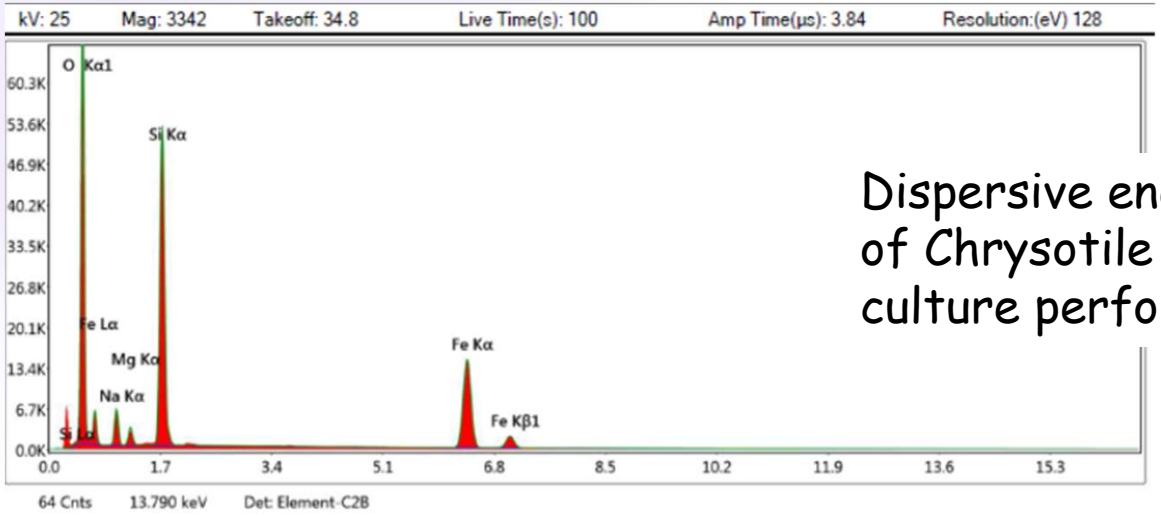
Crocidolitec UICC

Area 2



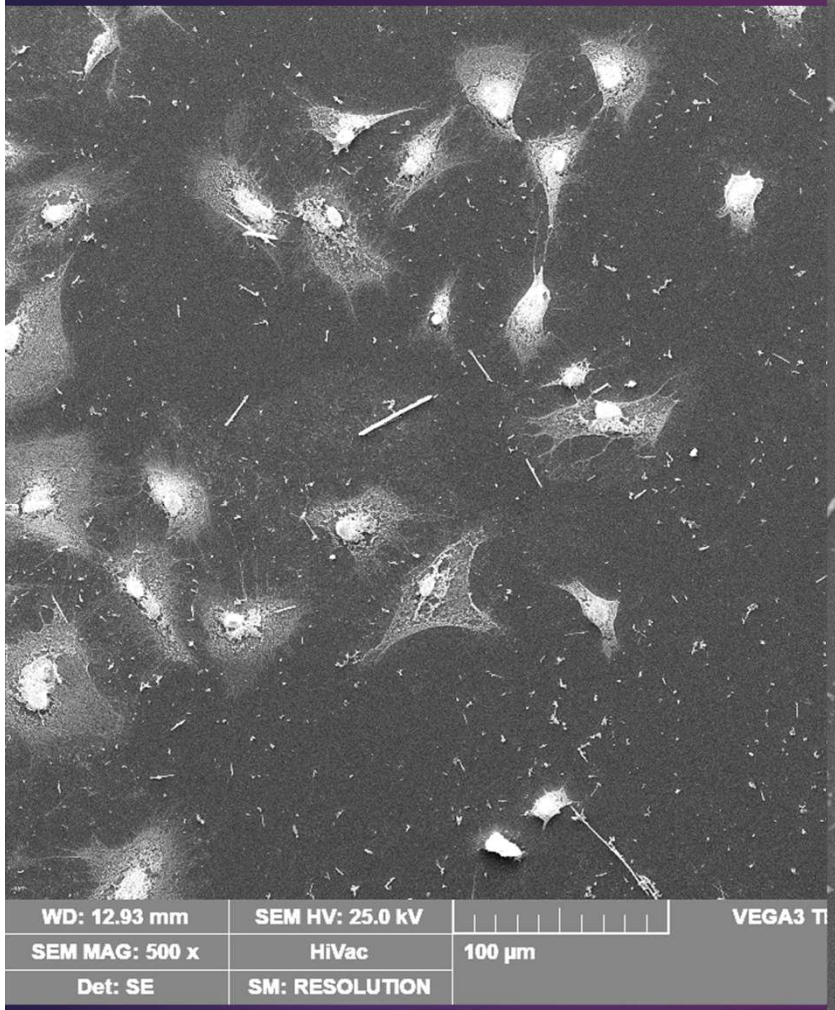
Smart Quant Results

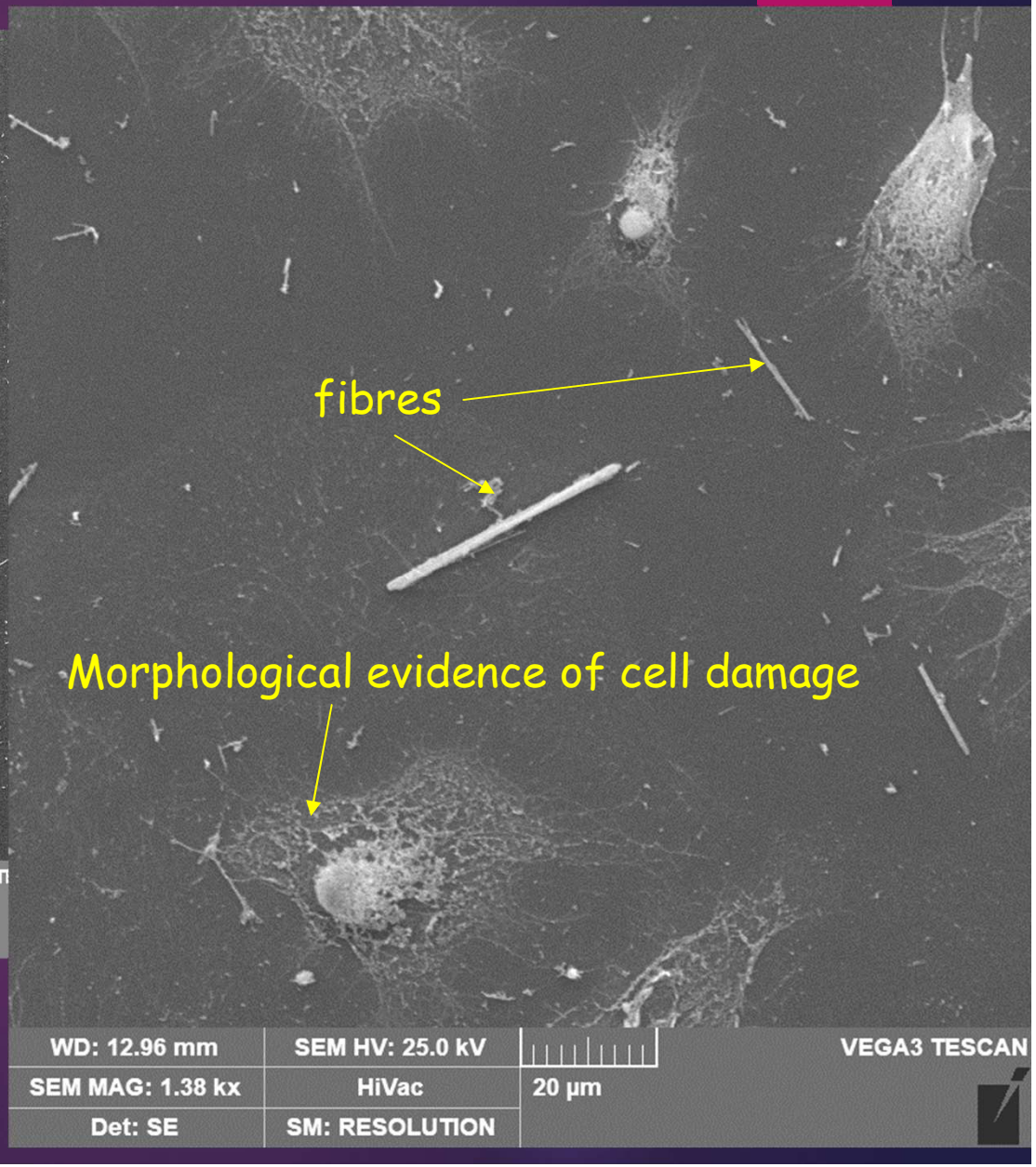
Element	Weight %	Atomic %
O K	46.99	64.39
NaK	7.99	7.62
MgK	2.34	2.11
SiK	23.52	18.36
FeK	19.15	7.52



Dispersive energetic spectrum EDS-SEM of Chrysotile Valmalenco fibres in cell culture performed in *position.

Crocidolite UICC fibres in MeT5A culture





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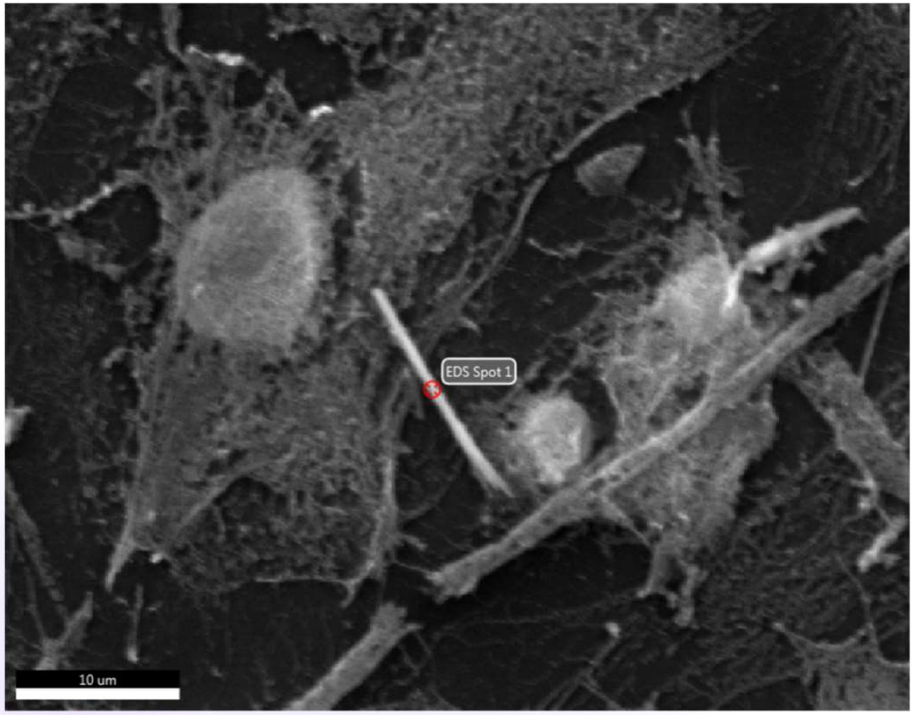
EDAX APEX

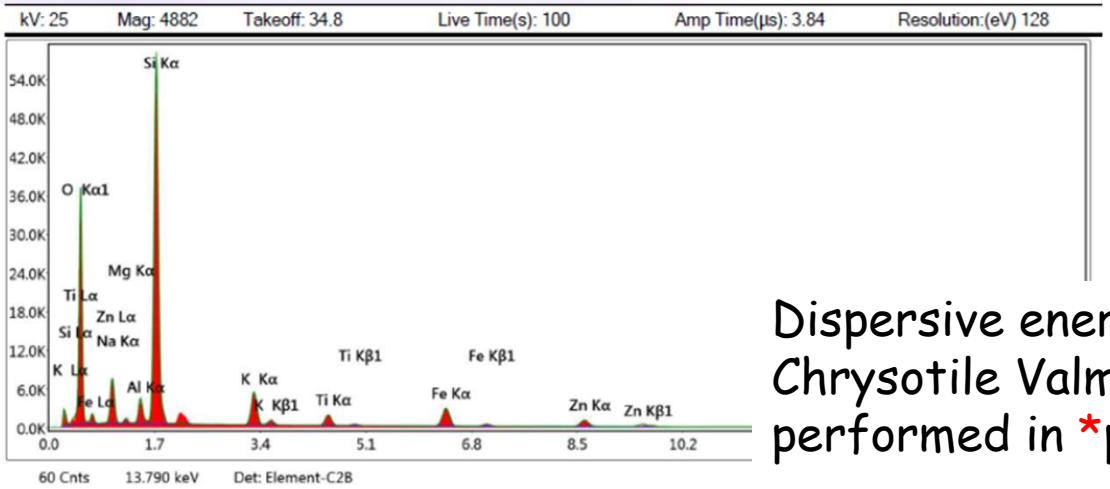
Crocidolite UICC fibres in MeT5A culture



Smart Quant Results

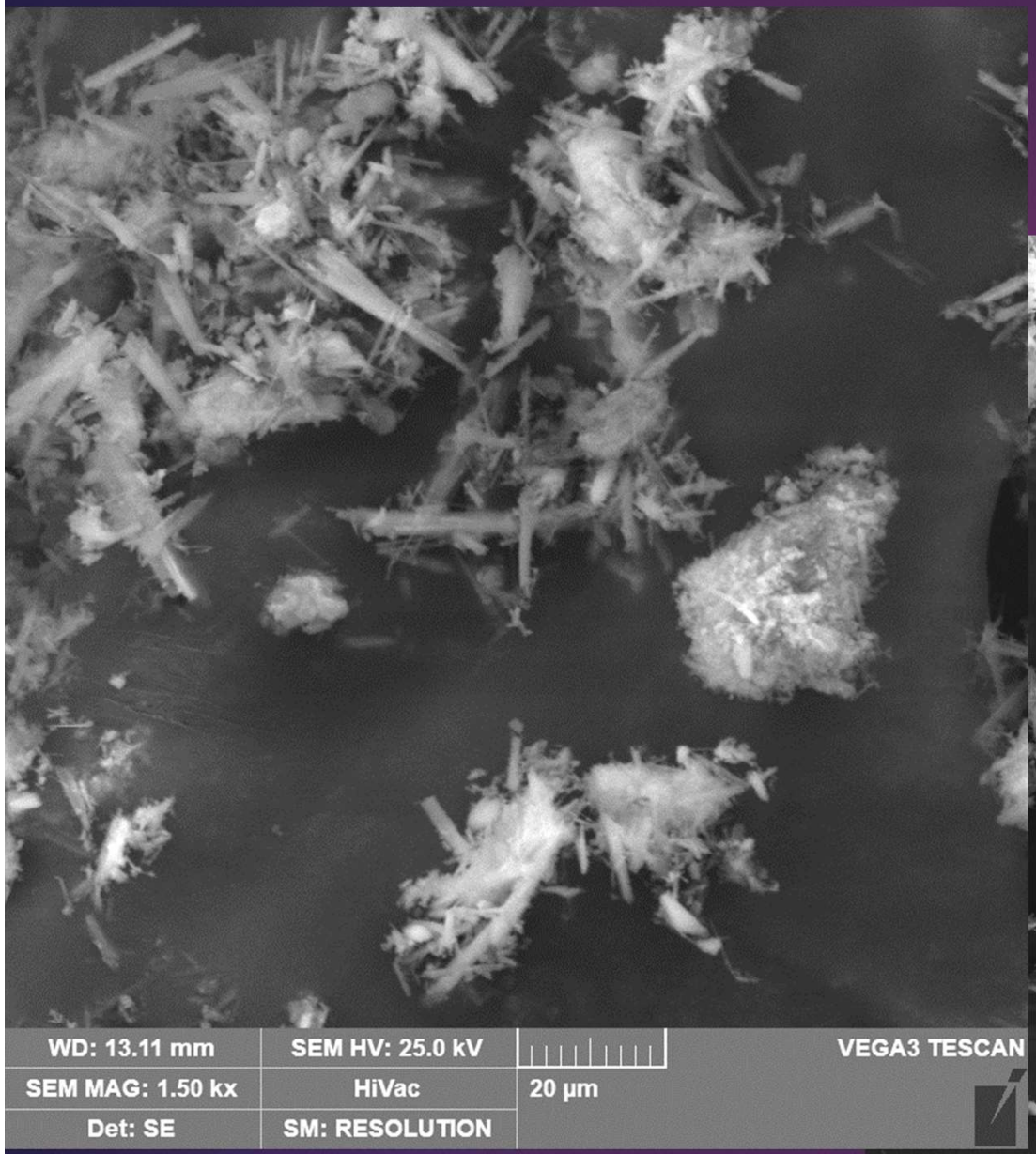
Element	Weight %	Atomic %
O K	47.33	63.04
NaK	6.91	6.4
MgK	0.6	0.52
AlK	2.46	1.94
SiK	29.72	22.55
K K	3.98	2.17
TiK	1.65	0.74
FeK	4.42	1.69
ZnK	2.93	0.96

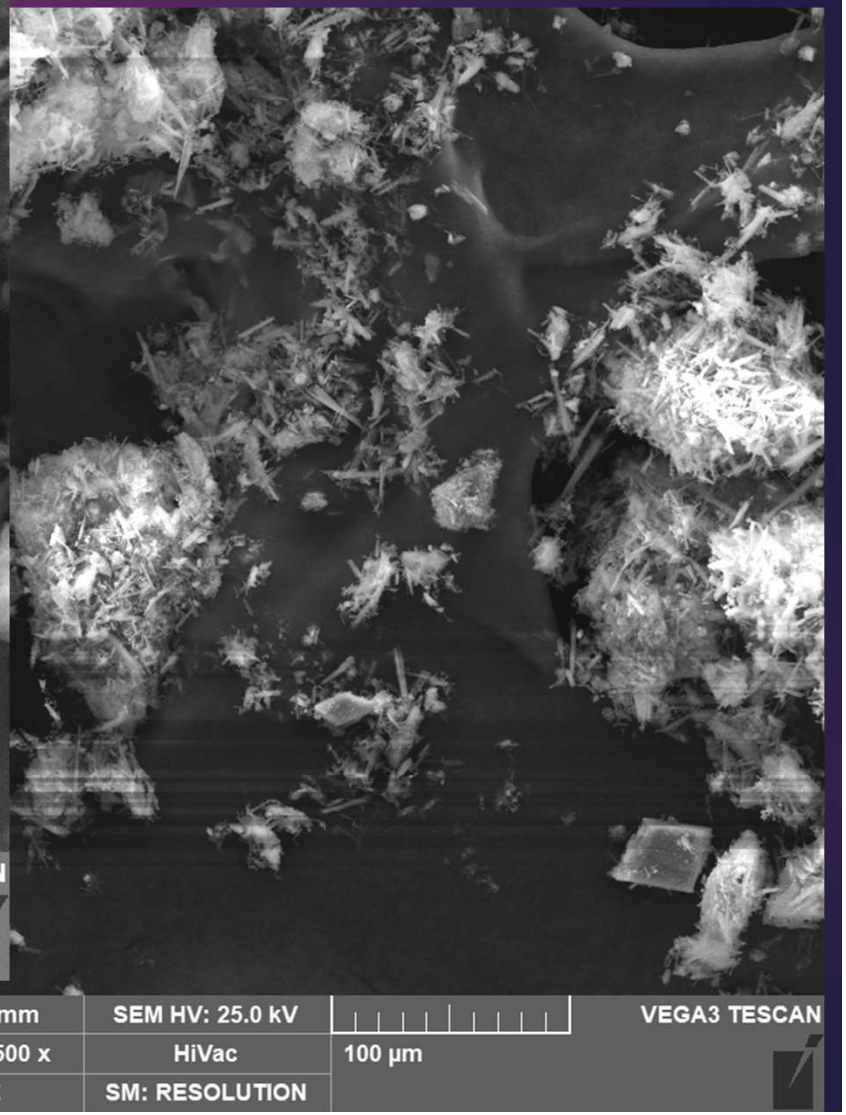
Differences were found with respect to the original fibres in element content expressed as weight %: decrements of Mg and Fe content are compatible with their release in the culture microenvironment



Dispersive energetic spectrum EDS-SEM of Chrysotile Valmalenco fibres in cell culture performed in *position.

Erionite Jersy Nevada





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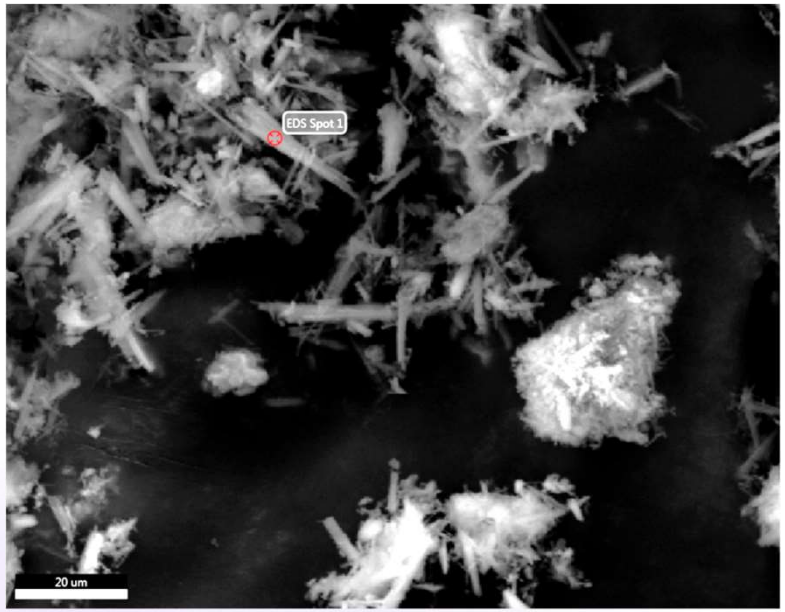
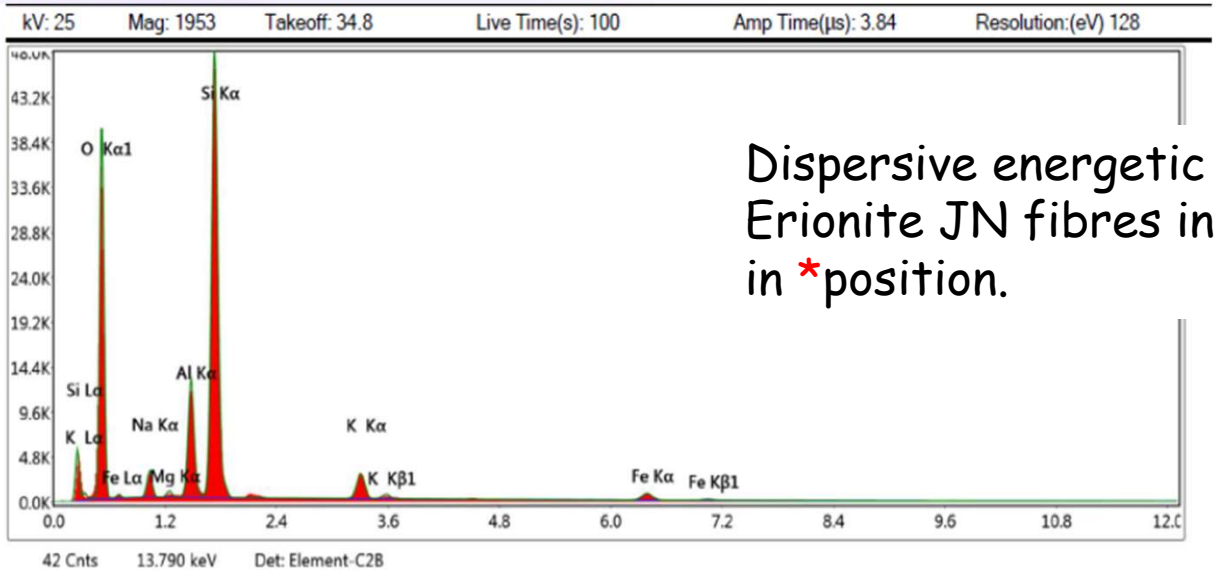
Erionite JN

Area 1



Smart Quant Results

Element	Weight %	Atomic %
O K	53.1	66.55
NaK	4.2	3.66
MgK	0.55	0.45
AlK	8.46	6.28
SiK	29.73	21.23
K K	2.56	1.31
FeK	1.41	0.5

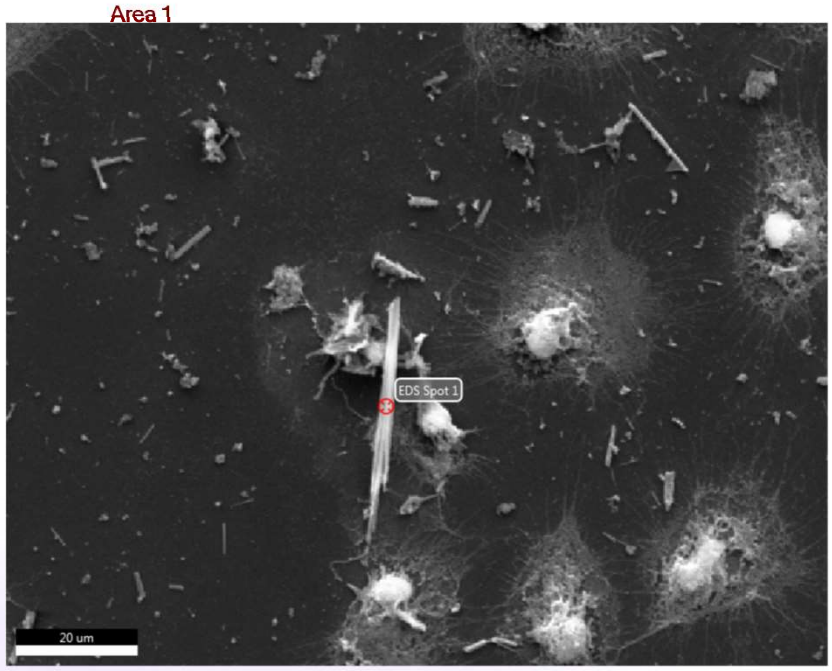


Dispersive energetic spectrum EDS-SEM of Erionite JN fibres in cell culture performed in *position.

Erionite JN in MeT5A colture

TESCAN VEGA3 LMU
EDAX APEX

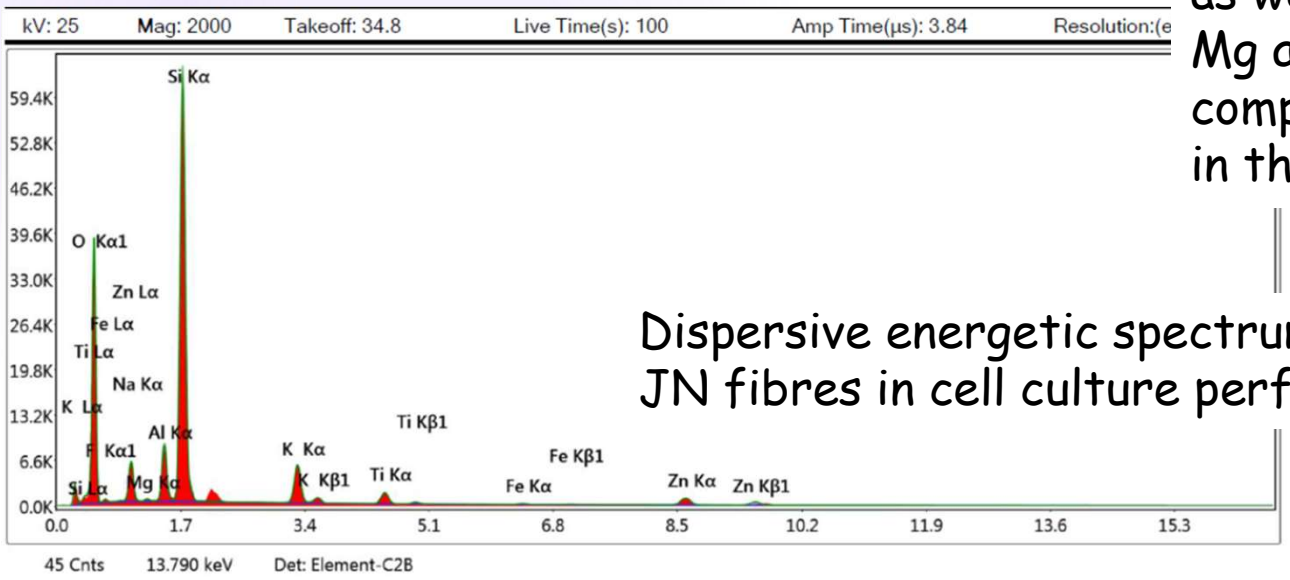
Erionite J N in MeT5A colture



Smart Quant Results

Element	Weight %	Atomic %
O K	49.74	64.27
F K	1.01	1.1
NaK	4.67	4.2
MgK	0.24	0.2
AlK	4.77	3.66
SiK	30.89	22.74
K K	4.22	2.23
TiK	1.56	0.67
FeK	0.24	0.09
ZnK	2.65	0.84

Differences were found with respect to the original fibres in element content expressed as weight% : decrements of Mg and Fe content are compatible with their release in the culture environment.



Dispersive energetic spectrum EDS-SEM of Erionite JN fibres in cell culture performed in *position.

Genotoxicity

DNA damage investigation

Nuclear H2AX phosphorylation related to DNA damage

As a result of the DNA double-strand breaks, the histone H2AX protein can be distinguished from other histones by a unique carboxy-terminal sequence that is rapidly phosphorylated at the serine-139 position (γH2AX) in response to DNA damage.

H2AX phosphorylation is a very rapid and sensitive response to DNA damage and occurs within a short time after exposure to ionizing radiation and environmental stress
(Redon et al. 2002).

Immunofluorescence analysis of gamma H2AX phosphorylation were performed in MeT-5A and A549 cells at 24 and 48h

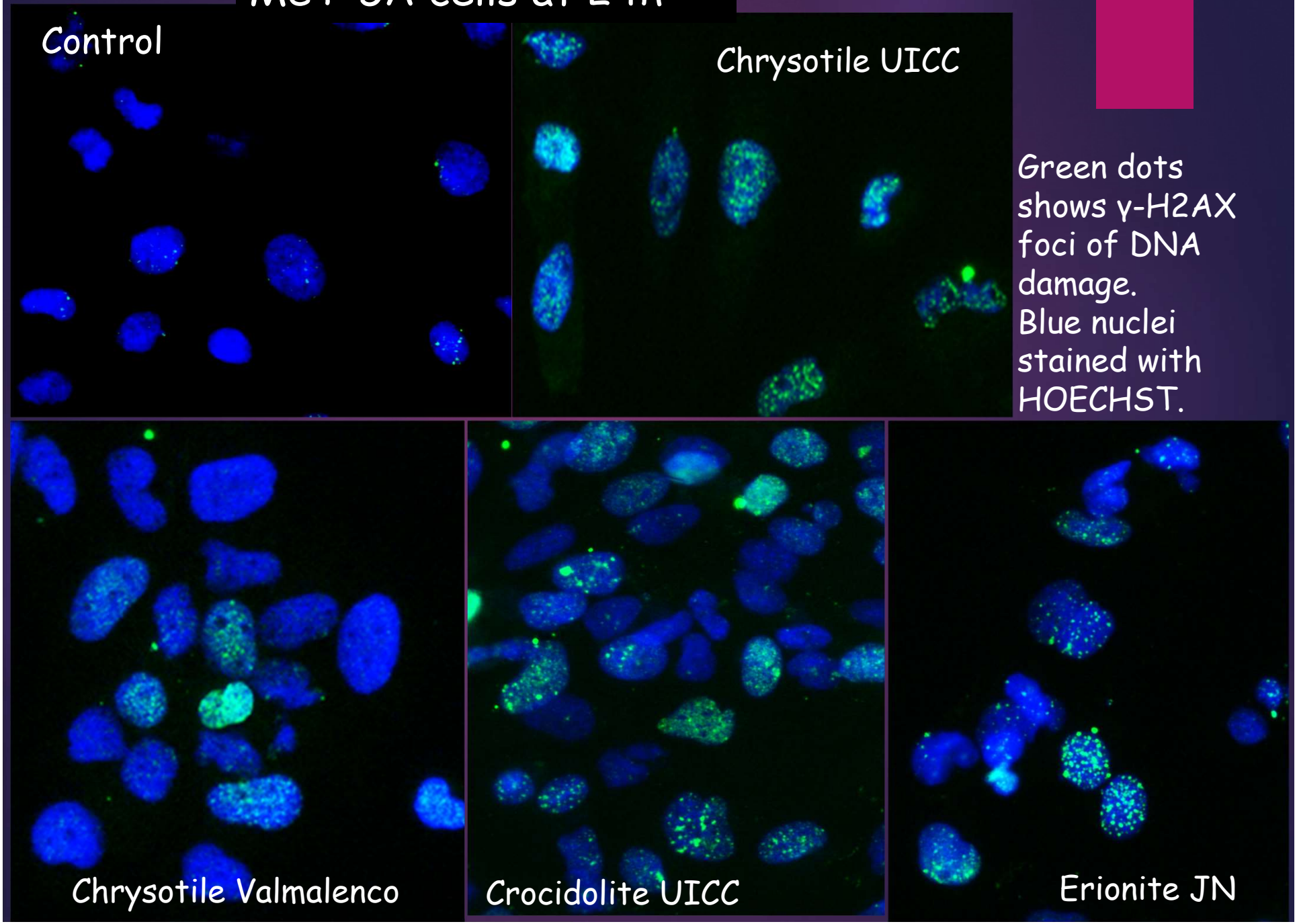
Immunofluorescence analysis of gamma H2AX phosphorylation assay

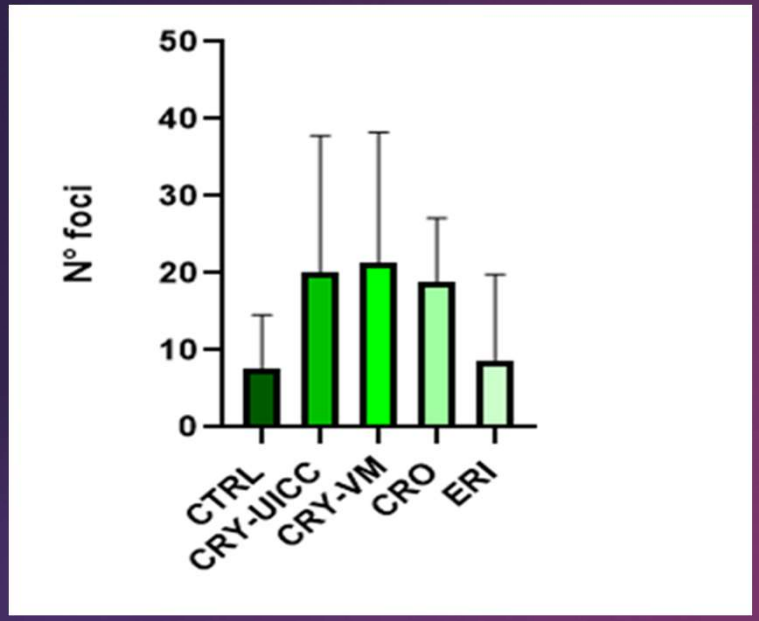
MeT-5A and A549 cells seeded in RPMI media.

- Phospho-Histone H2A.X (Ser139) antibody (Cell Signaling Technology, Danvers, MA, USA.
- secondary anti-rabbit Alexa Fluor 488 antibody (Jackson Laboratories, Baltimore Pike, West Grove, PA,USA);
- nuclear staining with HOECHST 33342 (Molecular Probes, Oregon, USA)
- Observation with fluorescence microscopy.

- Omission of the primary antibody resulted in lack of labeling, confirming the specificity of the antibody.

MeT-5A cells at 24h





Immunofluorescence analysis of gamma H2AX phosphorylation assay

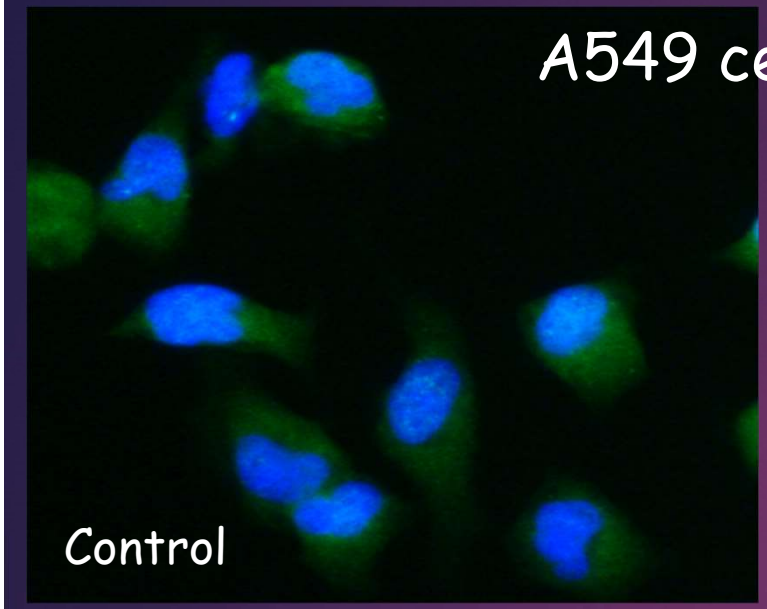
Quantification of DNA damage induced γH2AX focus formation

1. CTRL
2. CRY-UICC
3. CRY-VM
4. CRO
5. ERI

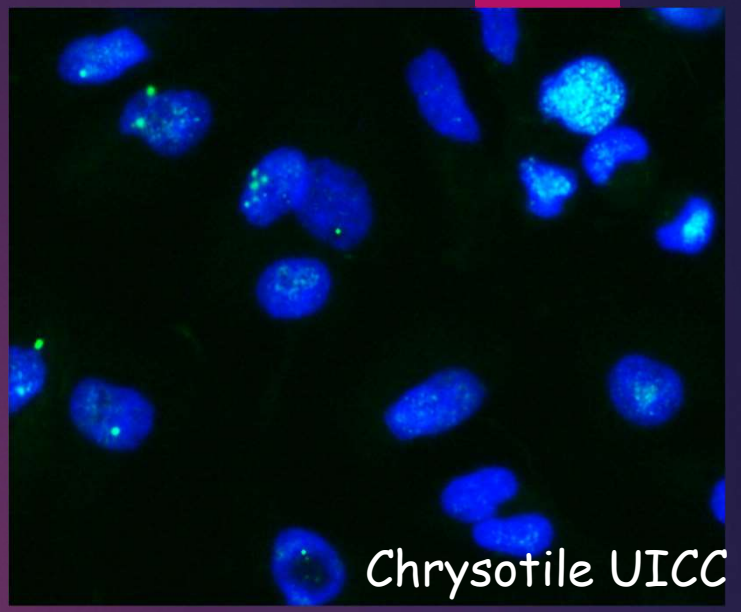
Cell line: **Met5a**
Fibers concentration: 50µg/ml
Exposure time: 24h
Multiple comparisons test: *Statistic P<0.05 *; P<0.01 **
1-2; 1-3; 1-4; 2-5; 3-5; 4-5

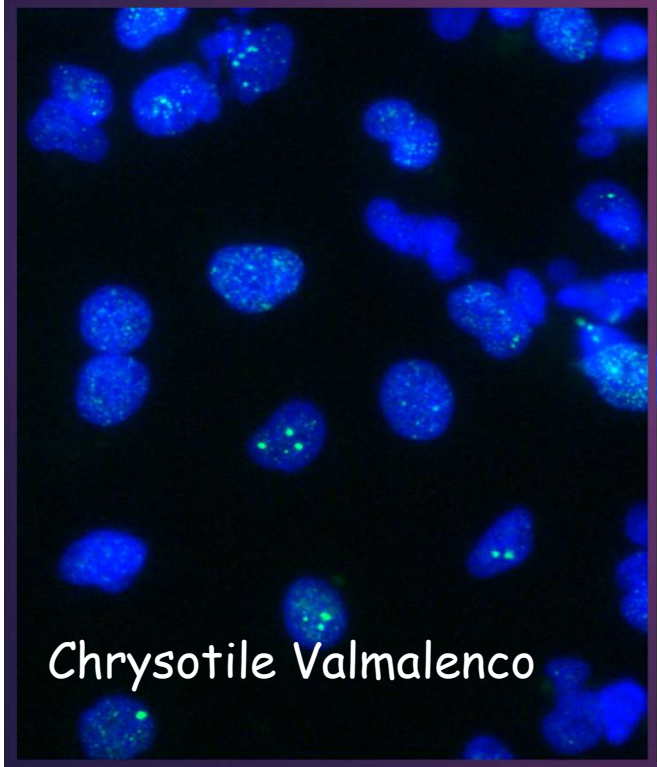
PRELIMINARY DATA

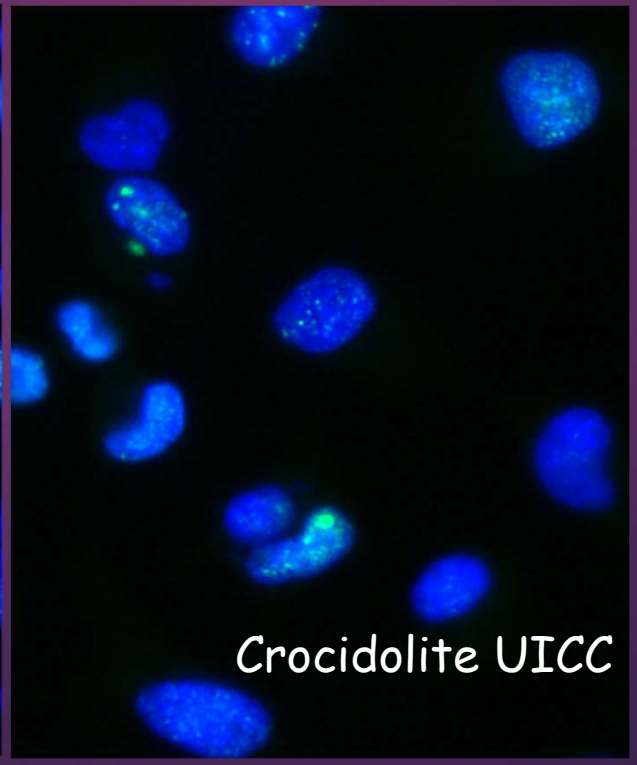
A549 cells at 24h

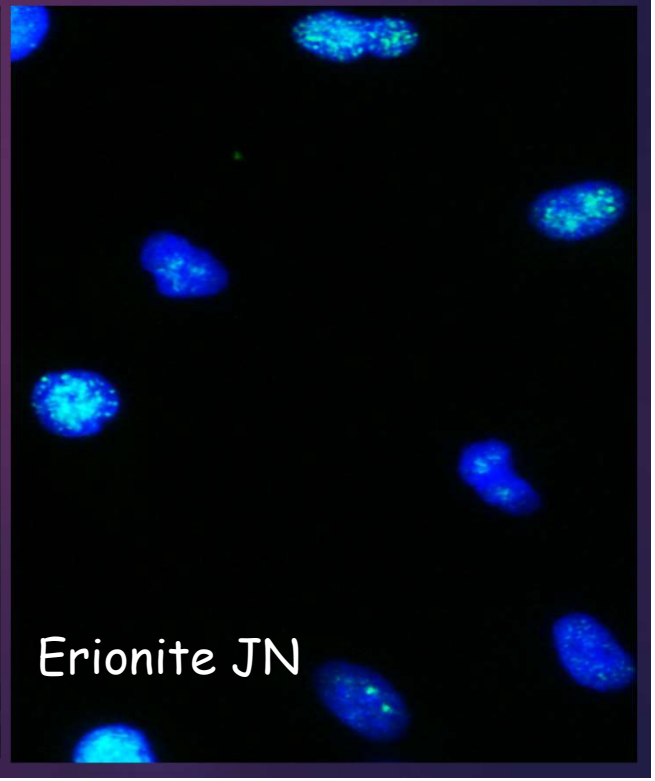


Green dots shows γ-H2AX foci of DNA damage.
Blue nuclei stained with HOECHST.

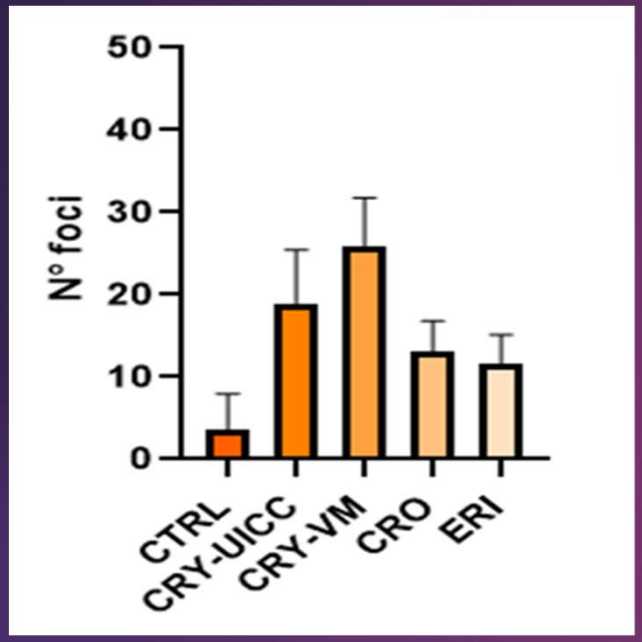








Immunofluorescence analysis of gamma H2AX phosphorylation assay



Quantification of DNA damage induced γH2AX focus formation

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Cell line: **A549**
Fibers concentration: 50µg/ml
Exposure time: 24h
Multiple comparisons test: *Statistic P<0.05 *; P<0.01 **
1-2; 1-3; 1-4; 1-5; 2-3; 2-4; 2-5; 3-4; 3-5

PRELIMINARY DATA

Our preliminry data suggest that all fibres shows nuclear H2AX phosphorylation related to DNA damage both in MeT-5A and A549 cells

Also after Chrysotile Valmalenco contacts fluorescent green spots related to foci of DNA double strands brake can be detectd.

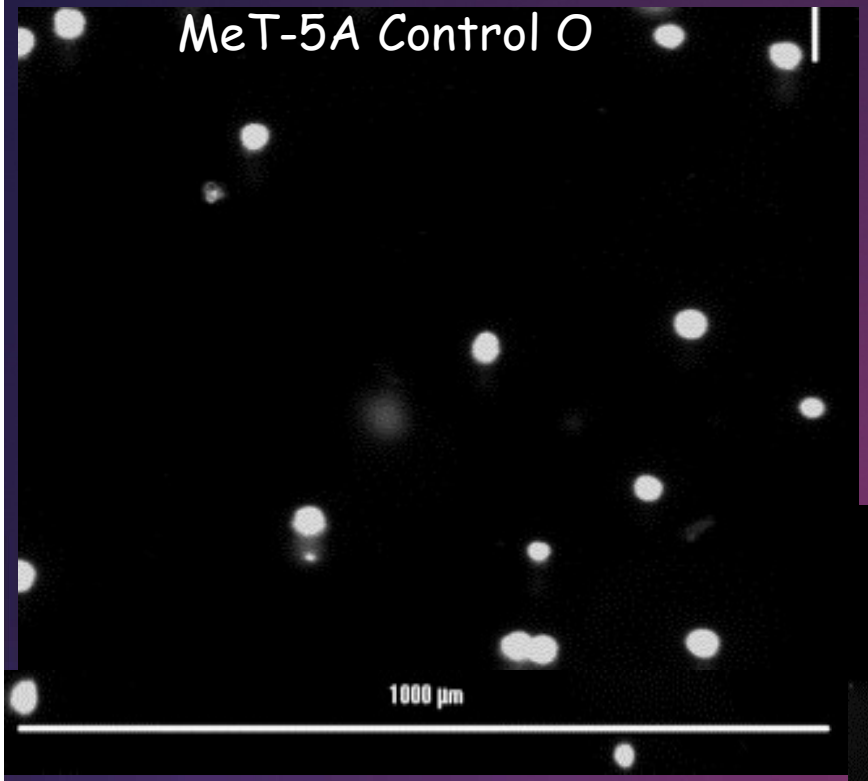
COMET assay

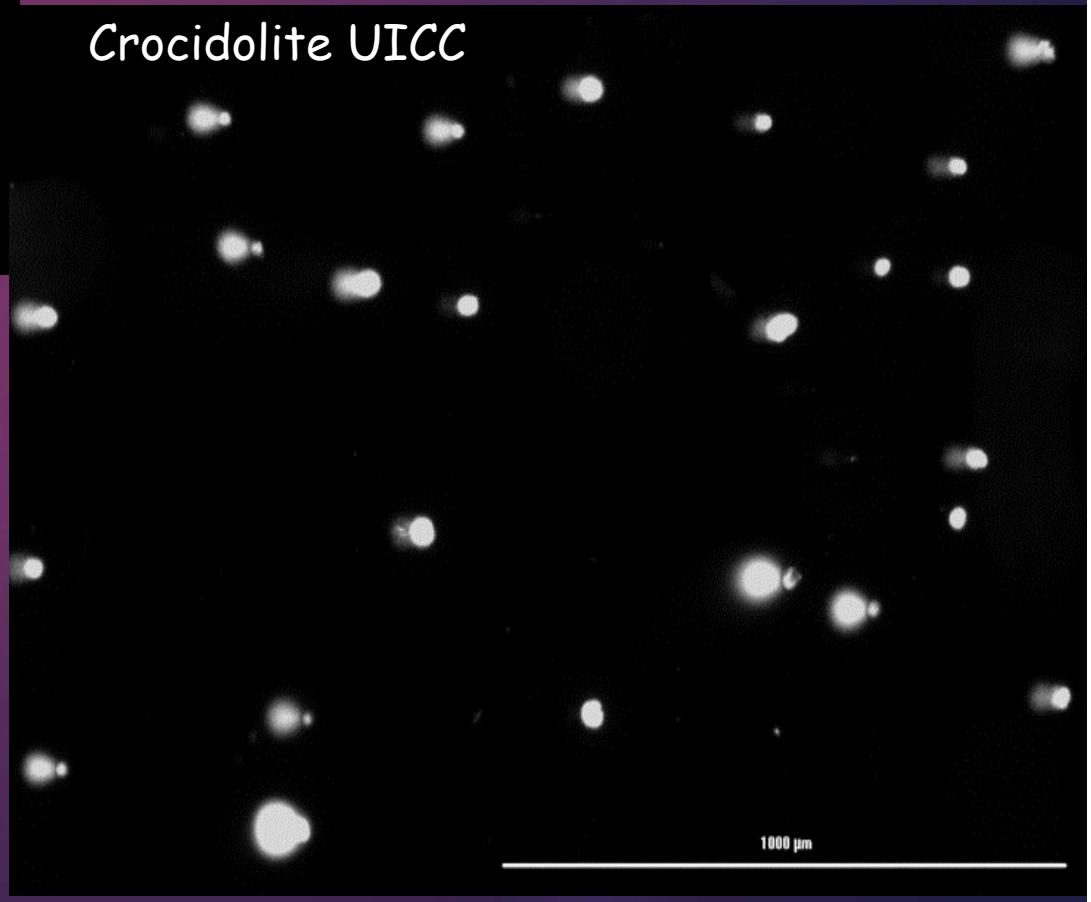
DNA strand breakage assay,
also known as single cell gel electrophoresis assay,
is a sensitive and rapid test for quantifying and analysing DNA damage in individual cells.

DNA damage can be detected and quantified at the level of each single cell by staining with ethidium bromide and measuring the displacement of genetic material between the cell nucleus (comet "head") (Brugè et al. 2014).

- Electrophoresis performed for 20 min at 1 V/ cm
- DNA on each slide stained with 0.015 ml ethidium bromide (20 mg/ml)
- comets are analyzed using fluorescence microscopy

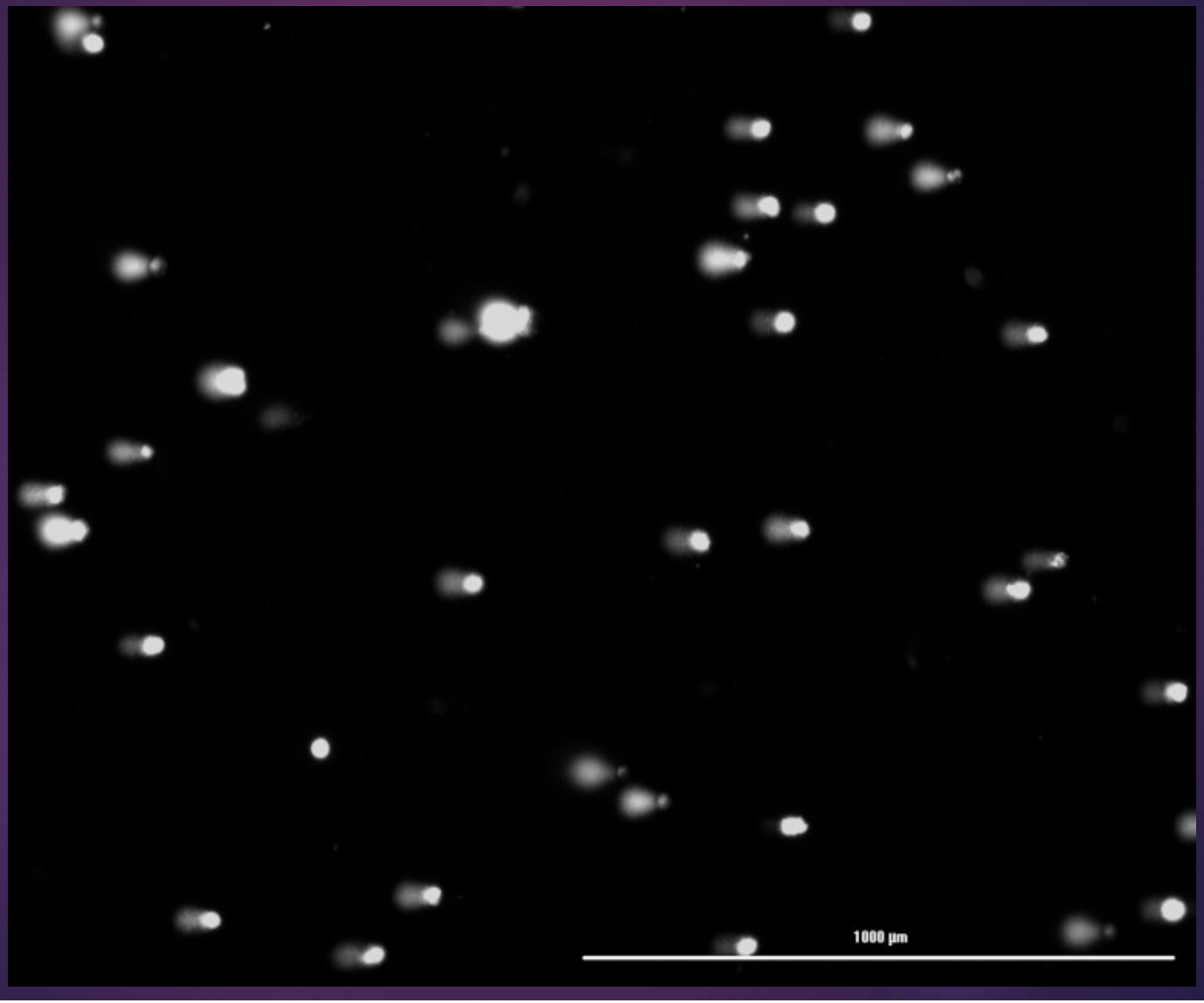
MeT- 5A COMET assay at 24h



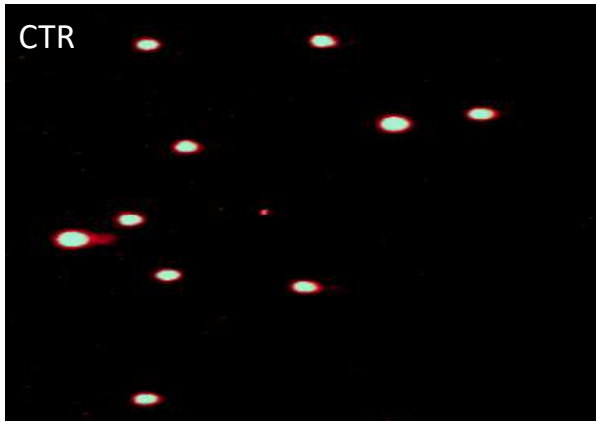


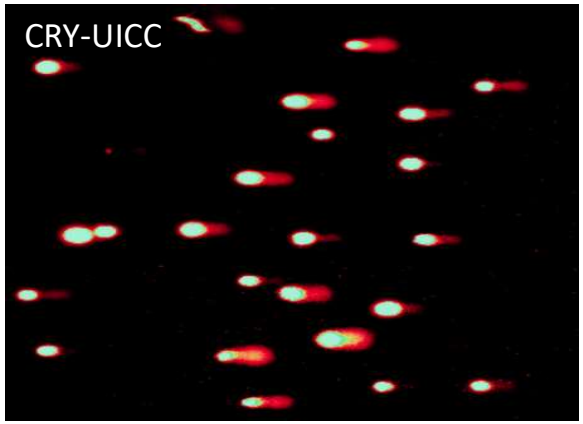
Undamaged DNA is visualized as a fluorescent core;
the presence of strand breaks (damaged DNA) induces DNA to migrate during the electrophoresis originating a tail, which is visualized as a "comet".
The bigger and the more fluorescent the tail is, the higher is the DNA damage induced.

COMET assay
MeT- 5A CROCIDOLITE at 48h



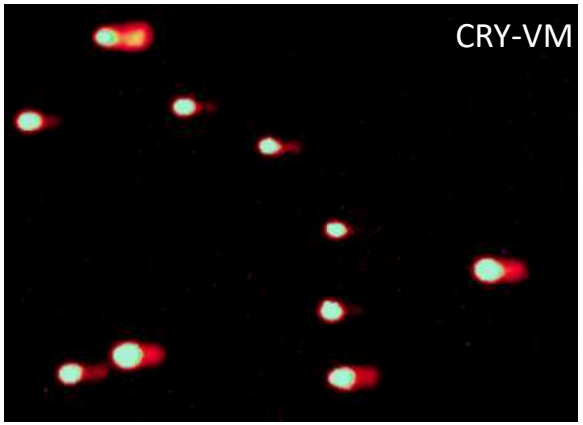
Immagini Comet assay Met5a 24h

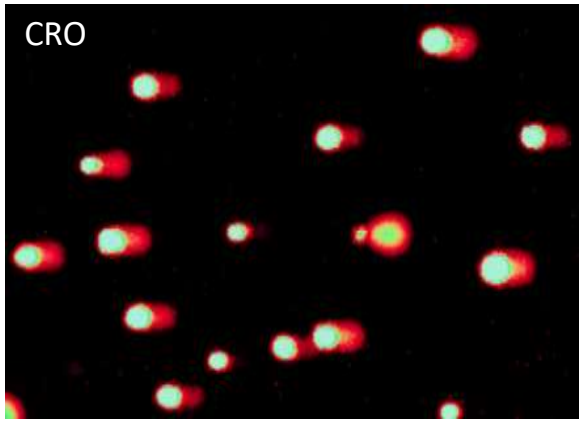


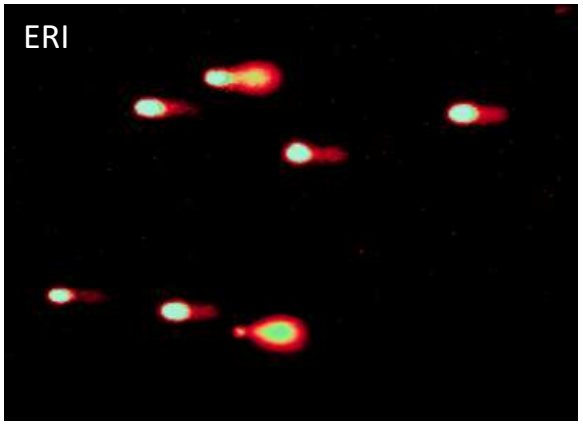


Parametri calcolati:
TL – tail Length (um) parametro misura la distanza di migrazione del DNA dal nucleo cellulare.

TMOM- Tail Moment:
TL rapportta a Tail intensity





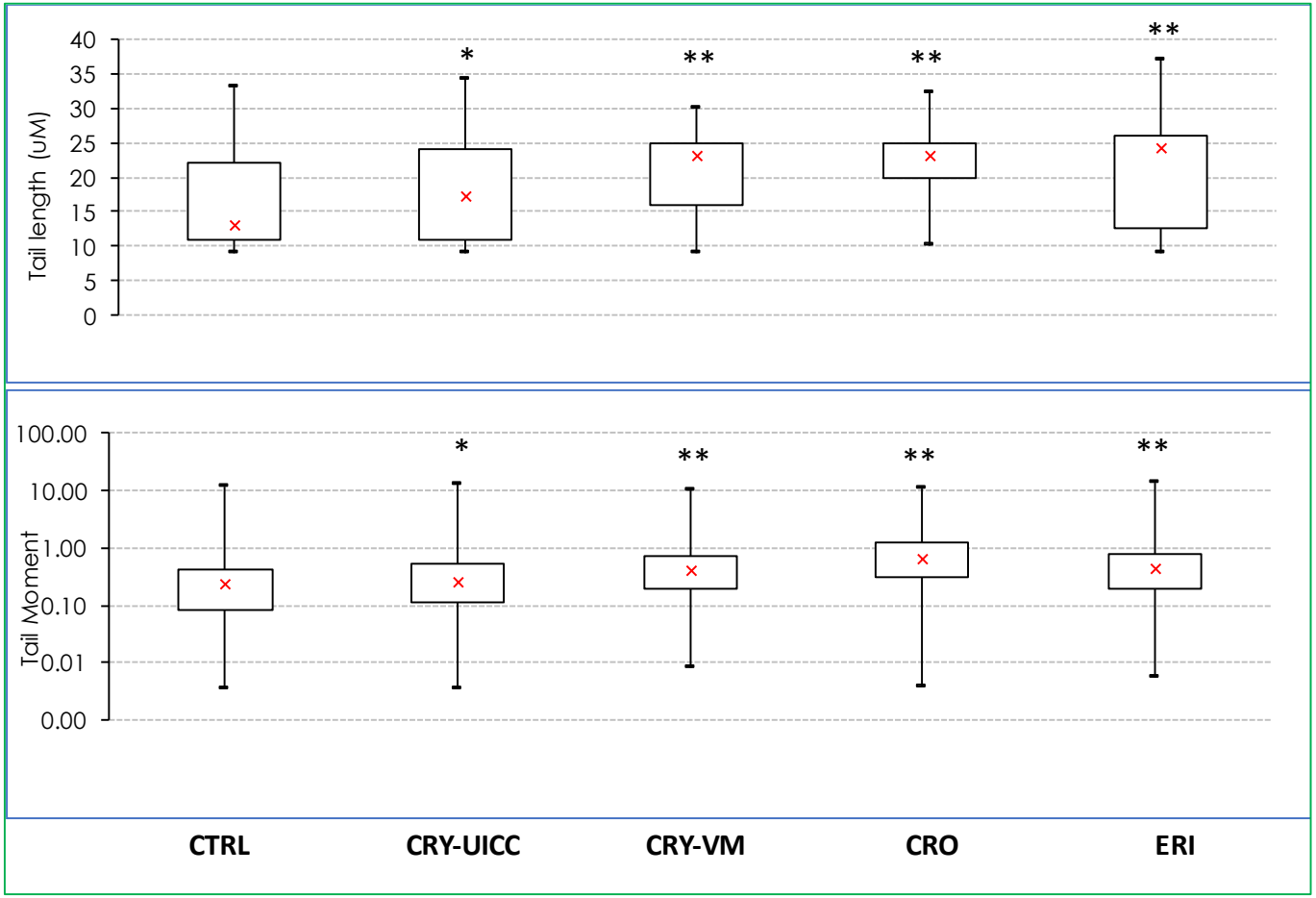


COMET assay Met5a at 24h

TL – tail Length (um) parametro misura la distanza di migrazione del DNA dal nucleo cellulare.

TMOM- Tail Moment: TL rapportata a Tail intensity

Size parameters distribution of the fibers used in the study at 24h



Legend
-minimum
-25th percentile
X=median
-75th percentile
-maximum

*Statistic P<0.05 *; P<0.01 **

24h

Preliminary data of DNA fragmentation suggest **increase in TL and TMOM**

Results and Discussion

All fibres induced

- Reduction of cell viability,
- Reduction glutatione content
- Increased presence of immunofuorescent γ-H2AX foci

Comets detectd after Crocidolite UICC contacts considerd as singns of DNA damage.

Cytotoxicity and genotoxic cancerogen potential of all fibres, including airborn fibres from natural expositions, related to their chemical and structural features and their dissolution capacity (Gualtieri, *Sci Rep*, 2018).

In conclusion

Morpho-functional perturbations evidenced in vitro
further highlight the risk of in vivo contact with natural fibres such as
Erionite from Jersy Nevada and
Chrysotile form Valmalenco that .

Our current results suffer from the freezing of our laboratories activities in the COVID period

For this reason our next intents will be:

- to better define the different grades of cyto-genotoxicity among the different fibres;
- to investigate the imbalance effects of the same fibres on DNA activity repair, by the repair factors Rad50 and Rad51 detection, an interest event that with Histone oxidation state should help to deeper genome damages exerted by asbestos.

Asbestos induced toxicity: in vitro different effects of different fibres.

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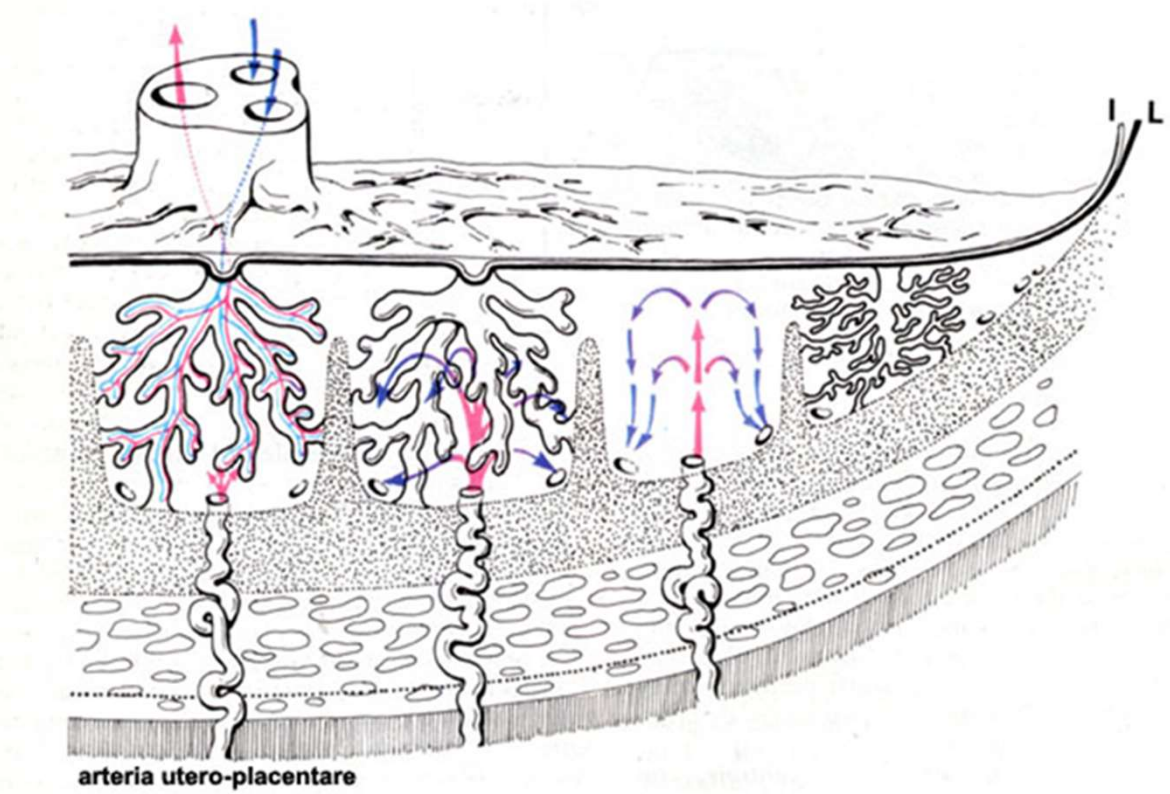
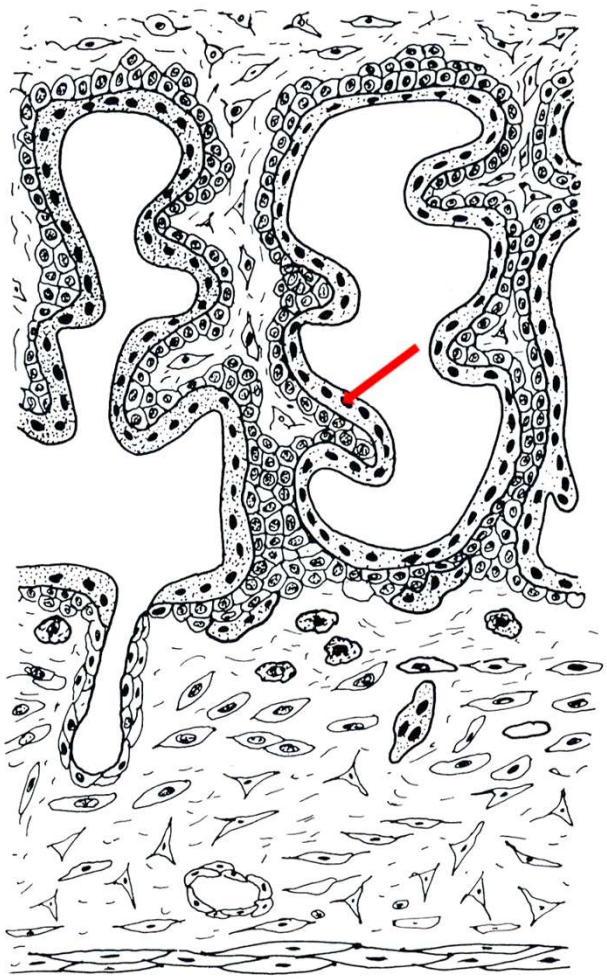
- DIP. MEDICINA SPERIMENTALE E CLINICA - DISMC
  Sezione Anatomia
  G.Tossetta, S. Fantone, D. Marzioni

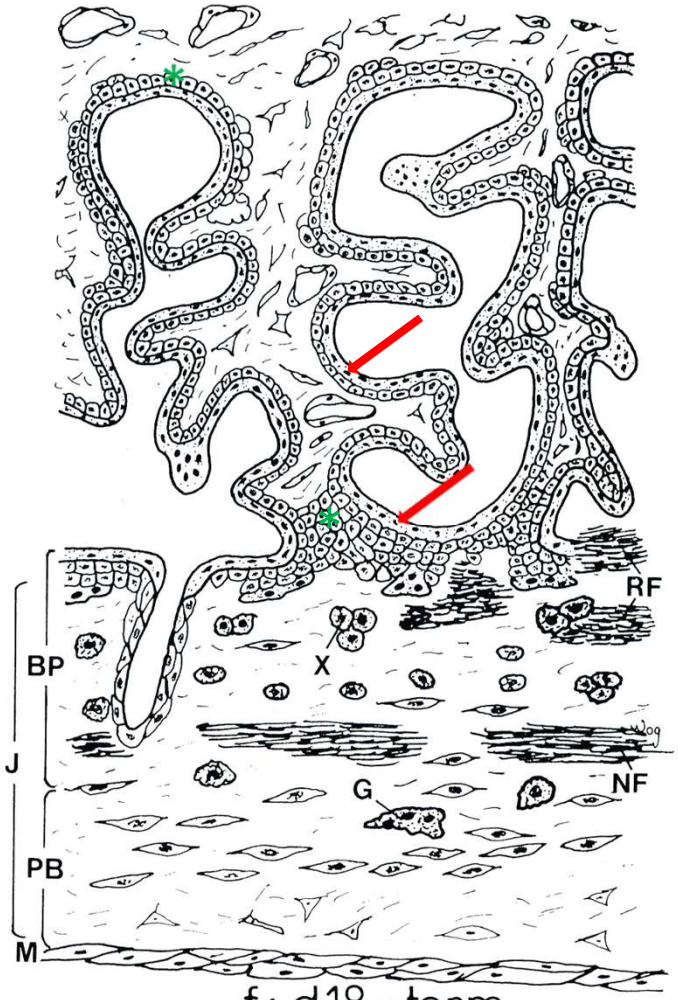
Versante fetale



Versante materno



e: d15-21



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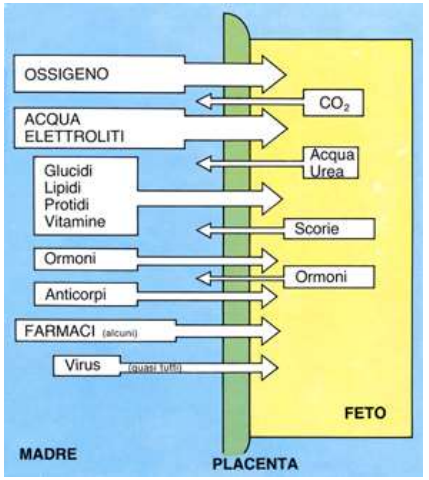
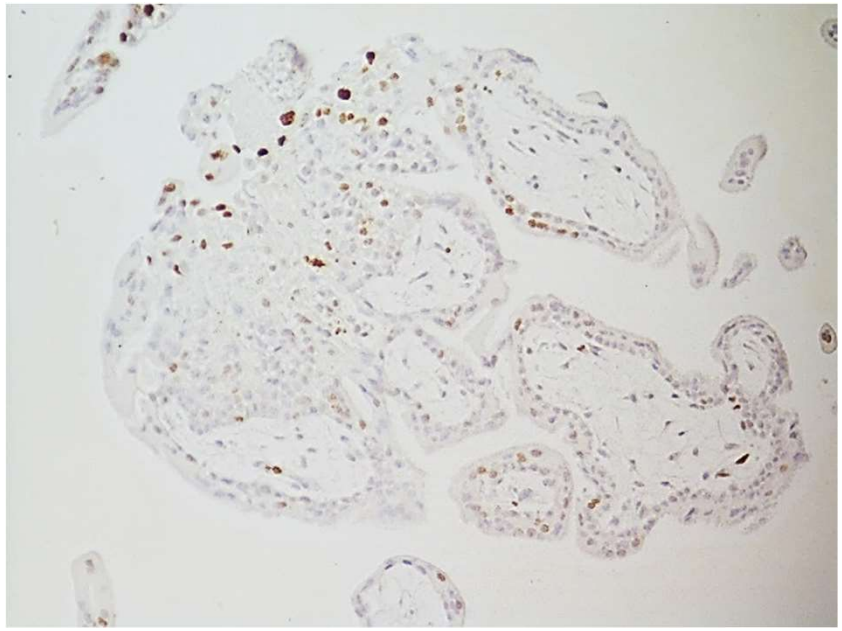
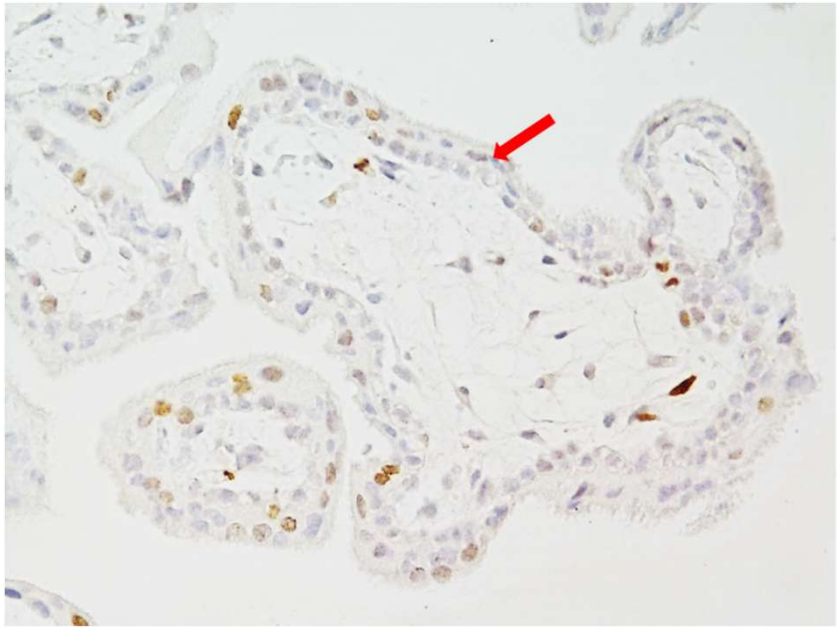
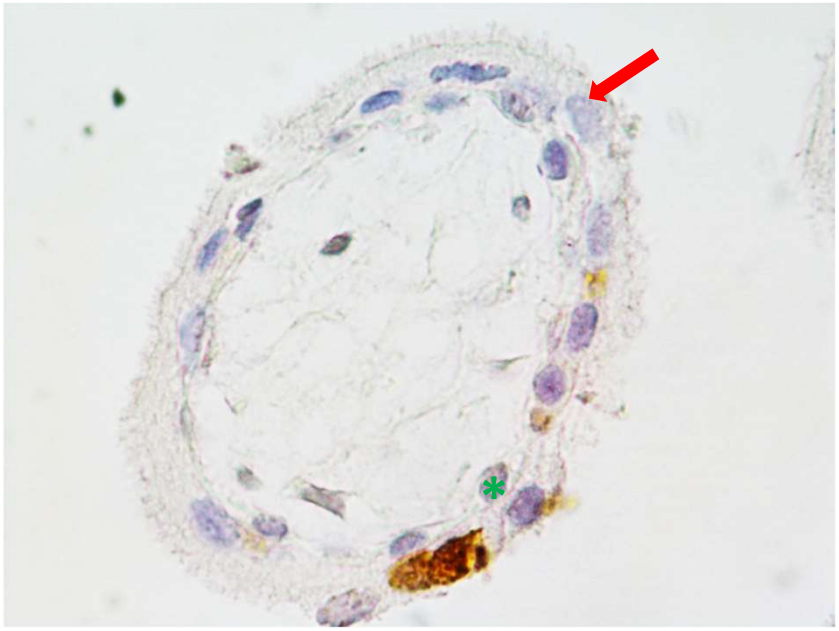


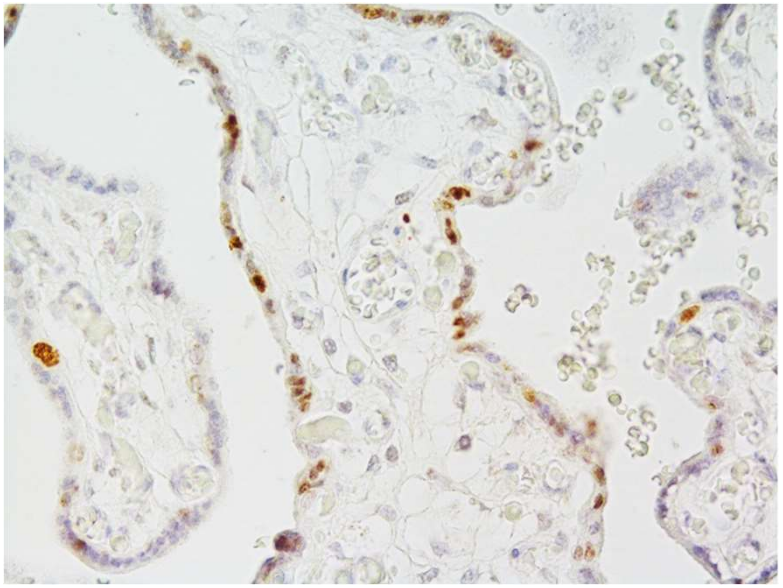


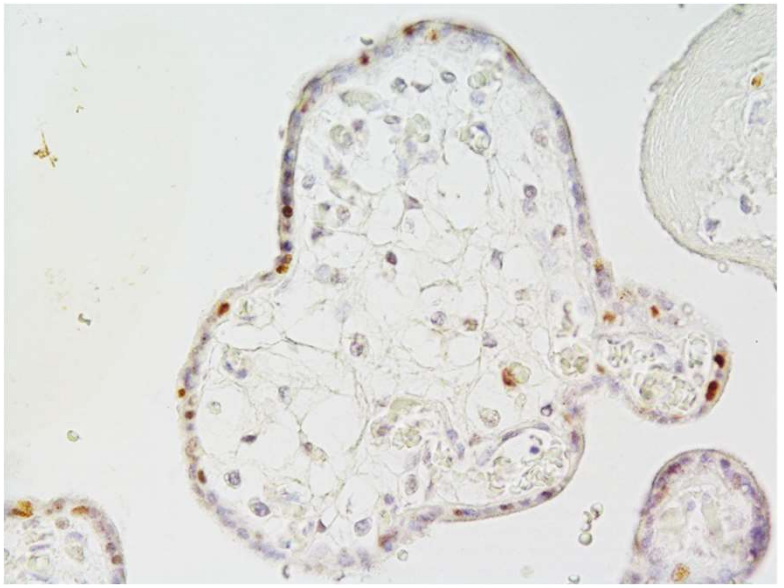


Staining per pH2A.X



Primo trimestre
9-12 settimane





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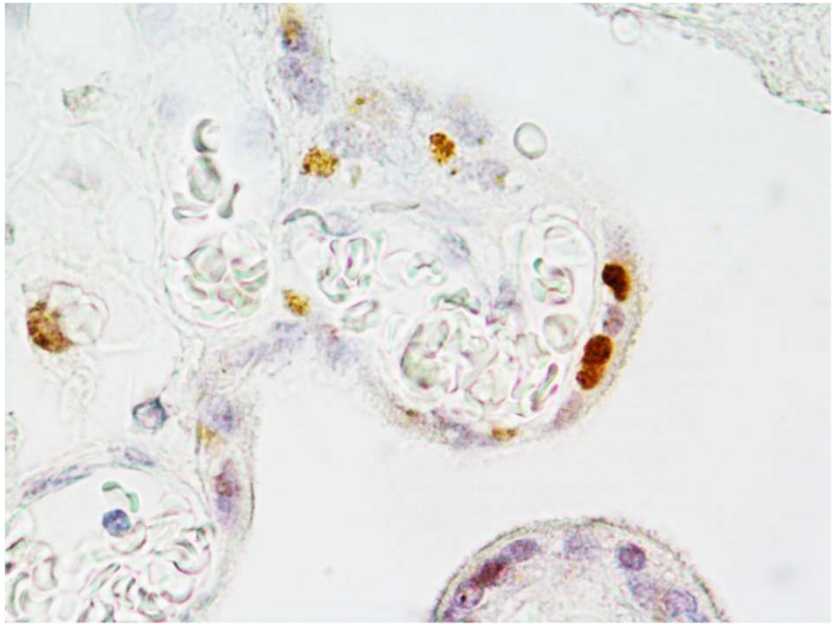
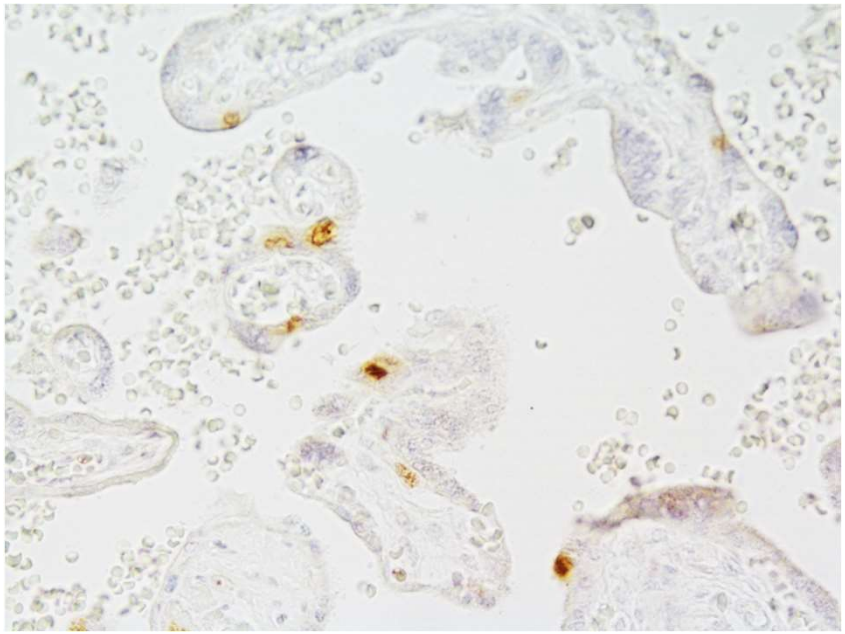
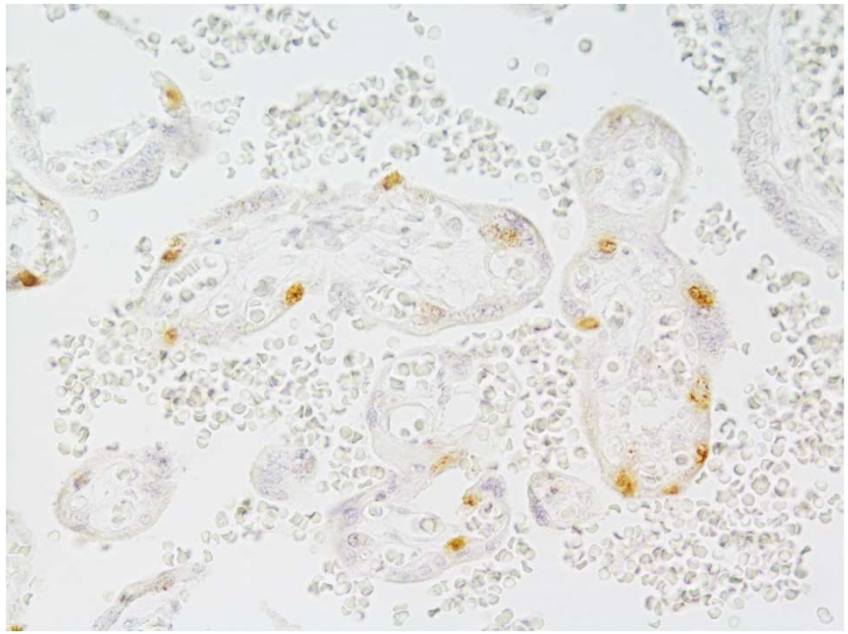
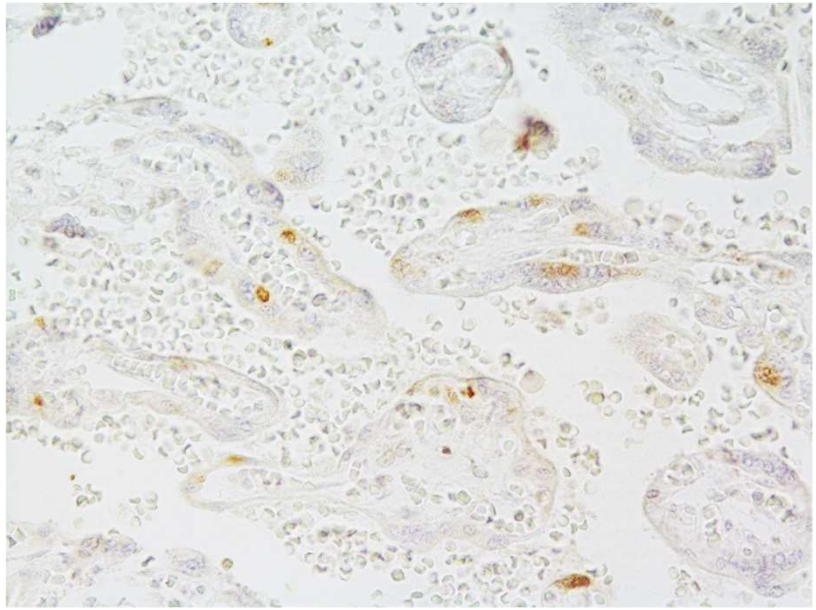


Terzo trimestre
35 settimane



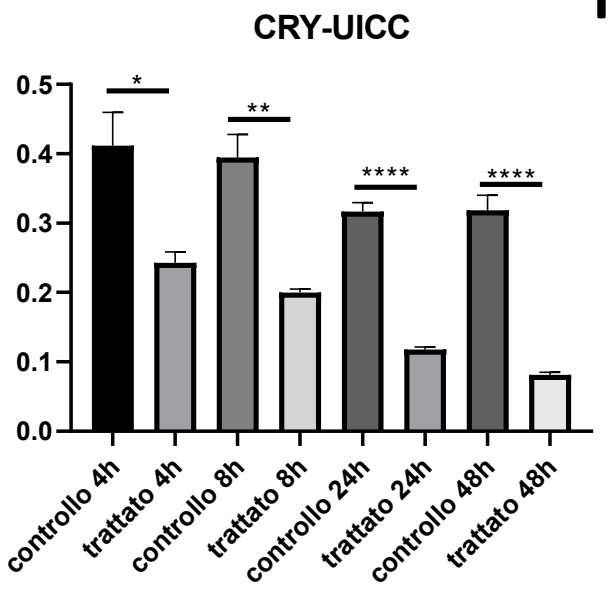


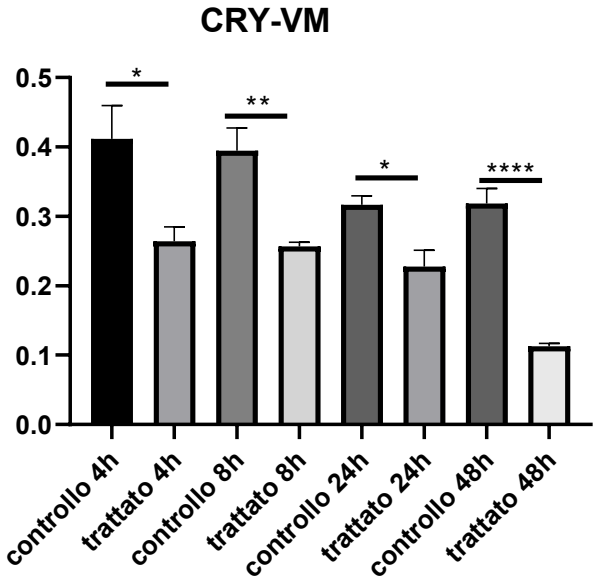
Staining per pH2A.X

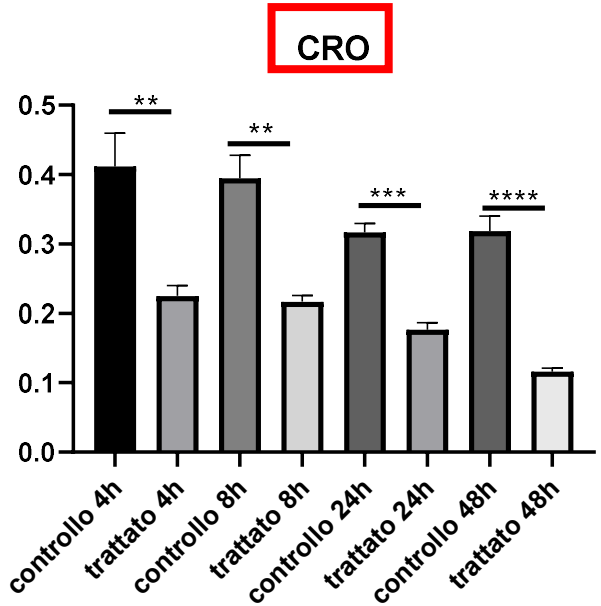


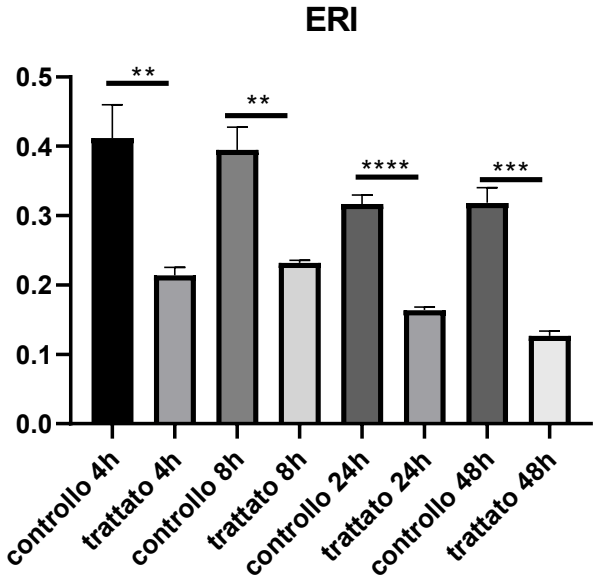
Preeclampsia
38 settimane

MTT BeWo


CRY-UICC
0.5
0.4
0.3
0.2
0.1
0.0
*
**
****
****
controllo 4h
trattato 4h
controllo 8h
trattato 8h
controllo 24h
trattato 24h
controllo 48h
trattato 48h


CRY-VM
0.5
0.4
0.3
0.2
0.1
0.0
*
**
*
****
controllo 4h
trattato 4h
controllo 8h
trattato 8h
controllo 24h
trattato 24h
controllo 48h
trattato 48h


CRO
0.5
0.4
0.3
0.2
0.1
0.0
**
**
***
****
controllo 4h
trattato 4h
controllo 8h
trattato 8h
controllo 24h
trattato 24h
controllo 48h
trattato 48h


ERI
0.5
0.4
0.3
0.2
0.1
0.0
**
**
****
***
controllo 4h
trattato 4h
controllo 8h
trattato 8h
controllo 24h
trattato 24h
controllo 48h
trattato 48h

BeWo
trattamenti 8/24 h CRO