Lessons learned so far – *in vivo* analysis of Hazard Mechanism for Carbon Nanotubes vs. Asbestos

Marion MacFarlane

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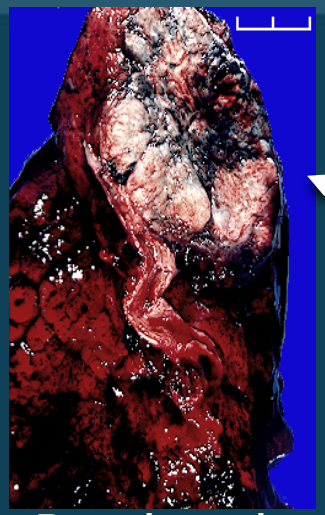
UK

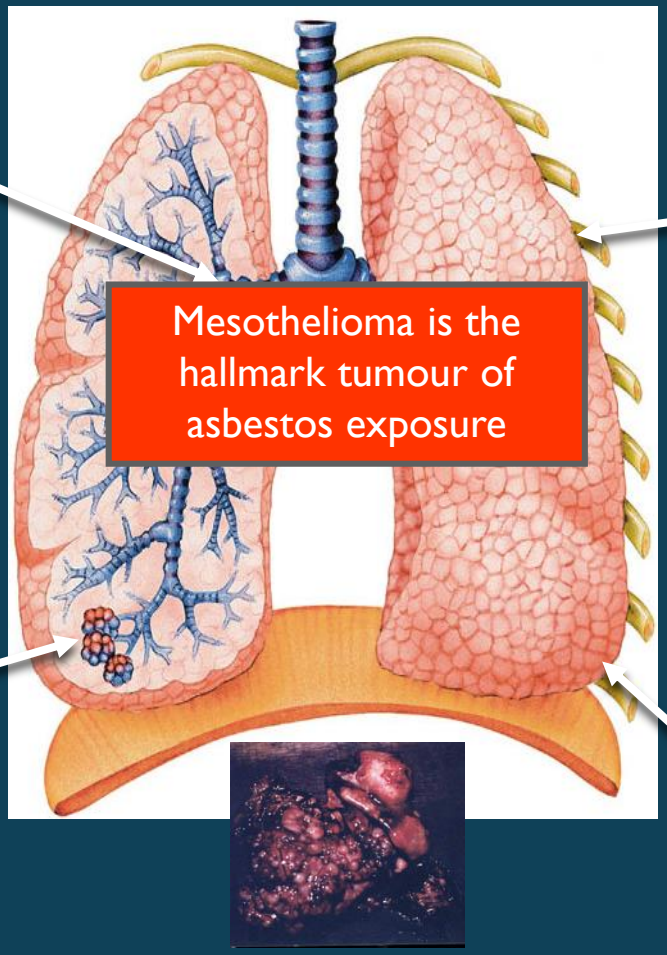
NO DISCLOSURES

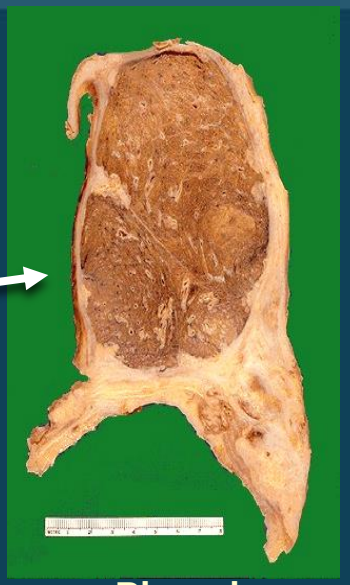
Asbestos-related Lung Disease



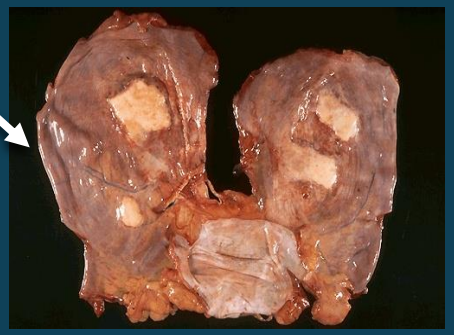
Bronchogenic carcinoma

Asbestosis
Honeycomb lung





Pleural mesothelioma



Pleural plaque

The Fibre Pathogenicity Paradigm

1) The fibre pathogenicity paradigm is the most robust SAR for any particle
2) Derived from human, animal and *in vitro* studies over 25 years
3) Holds true for asbestos, glass fibre, ceramic fibres – no fibre so far studied has violated the paradigm
4) So is regardless of chemistry, but is based on shape and persistence in the lungs
5) Paradigm states that only **long** (> 5μm), **thin** (< 3μm) and **biopersistent** fibres are pathogenic

Government Report: 'UK Nanotechnologies Strategy' (2010);

Poland et al (2008) 'Carbon nanotubes introduced into abdominal cavity display asbestos-like Pathogenicity'

Warnings About Carbon Nanotubes

Potential for Harm

'…Given previous experience with asbestos, we believe that nanotubes deserve special toxicological attention…' 2004

Vol 444|16 November 2006 nature

COMMENTARY

Safe handling of nanotechnology

The pursuit of responsible nanotechnologies can be tackled through a series of grand challenges, argue **Andrew D. Maynard** and his co-authors.

When the physicist and Nobel laureate Richard Feynman challenged the science community to think small in his 1959 lecture 'There's Plenty of Room at the Bottom', he planted the seeds of a new era in science and technology. Nanotechnology, which is about controlling matter at near-atomic scales to produce unique or enhanced materials, products and devices, is now maturing rapidly with more than 300 claimed nanotechnology products already on the market[1]. Yet concerns have been raised that the very properties of nanostructured materials that make them so attractive could potentially lead to unforeseen health or environmental hazards[2].

The spectre of possible harm — whether real or imagined — is threatening to slow the development of nanotechnology unless sound, independent and authoritative information is developed on what the risks are, and how to avoid them[3]. In what may be unprecedented pre-emptive action in the face of a new technology, governments, industries and research organizations around the world are beginning to address how the benefits of emerging nanotechnologies can be realized while minimizing potential risks[4]. Yet despite a clear commitment to support risk-focused research, opportunities to establish collaborative, integrated and targeted research programmes are being missed[5]. In September, Sherwood Boehlert, chair of the US House Science Committee, commented in a hearing that "we're on the right path to dealing with the problem, but we're sauntering down it when a sense of urgency is required". And in October, Britain's Royal Society raised concerns that the UK government had not made enough progress on reducing the uncertainties surrounding the health and

Potential health risks from exposure to engineered nanomaterials must be understood and minimized.

grand challenges to stimulate research that is imaginative, innovative and above all relevant to the safety of nanotechnology.

Fears over the possible dangers of some nanotechnologies may be exaggerated, but they are not necessarily unfounded. Recent studies examining the toxicity of engineered nanomaterials in cell cultures and animals have shown that size, surface area, surface chemistry, solubility and possibly shape all play a role in determining the potential for engineered nanomaterials to cause harm[6]. This is not surprising: we have known for many years that inhaled dusts cause disease, and that their harmfulness depends on both what they are made of and their physical nature. For instance, small particles of inhaled

"Understanding and preventing risk often has a low priority in the competitive world of research funding."

cause harm to people and the environment. But the way science is done is often ill-equipped to address novel risks associated with emerging technologies. Research into understanding and preventing risk often has a low priority in the competitive worlds of intellectual property, research funding and technology development. And yet there is much at stake in how potential nano-specific risks are understood and managed. Without strategic and targeted risk research, people producing and using nanomaterials could develop unanticipated illness arising from their exposure; public confidence in nanotechnologies could be reduced through real or perceived dangers; and fears of litigation may make nanotechnologies less attractive to investors and the insurance industry.

The science community needs to act now if strategic research is to support sustainable nano-

'….Fibre-shaped nanomaterials possibly represent a unique inhalation hazard, and their pulmonary toxicity should be evaluated as a matter of urgency….. failure to pick up asbestos-like behaviour as early as possible would be potentially devastating to the health of exposed people and to the future of the nanotechnology industry….' 2006

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Potential Carcinogenicity of Carbon Nanotubes – In Vivo Analysis

2014 – IARC: only one Carbon Nanotube - MWCNT-7 - classified in Group 2B

MWCNT-7

Long, large-diameter, rigid MW tubes - when delivered either IP or IS induced Mesothelioma (Tagaki, 2008; Nagai, 2011; Sakamoto, 2009)

Short, thin, tangled MWCNT delivered intra-peritoneally did NOT induce mesothelioma (Muller, 2009)

Long, rigid MWNT – more potent than thin, flexible or curved CNT in inducing Mesothelioma (Rittinghaussen, 2014)

Above studies, using bolus delivery to peritoneum, confirmed by trans-tracheal intrapulmonary spraying:

Longer, rigid MWCNT (~150 nm D/~8 µm L) translocate to the pleura & induce ↑Inflammation/Fibrosis than shorter/thinner CNT (Xu, 2014);
>100 weeks – induced Mesothelioma, plus Lung Ademoma and Carcinomas (Suzui 2016)

Chronic Inhalation – MWCNT-7 at 0.2 or 2mg/m^3 induced Lung Adenoma & Carcinoma but no Mesothelioma (Kasai, 2016)

CNT may act as Tumor Promoters in development of Lung Cancer – 3MC followed by inhaled MWCNT-7 (Sargent, 2013)

GAPS in our understanding of Mechanisms of Carcinogenicity of Asbestos & HARNs (Kuempel, 2017):

End-stage & Pre-neoplastic Endpoints in animal studies - defined in comparison with Human Pathology

Dissecting the Molecular Changes in MPM – a Disease linked with direct Fibre Exposure

Malignant Mesothelioma

The Worldwide Pandemic of Asbestos-Related Diseases

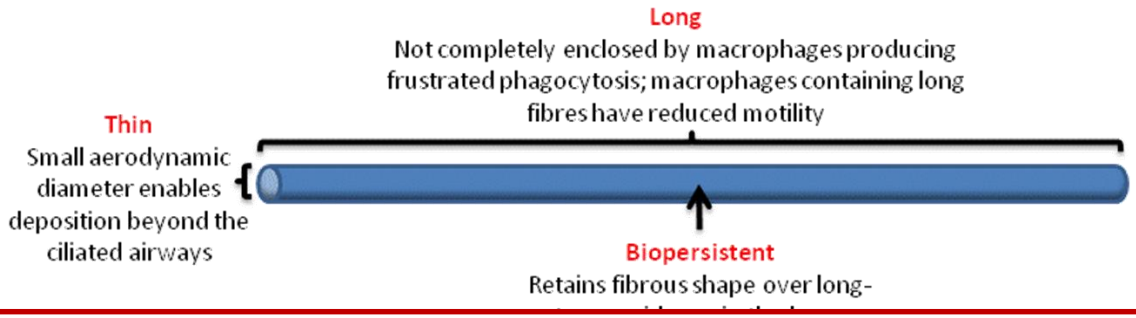
Leslie Stayner,[1] Laura S. Welch,[2] and Richard Lemen[3]

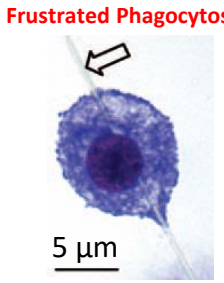
Annu. Rev. Public Health 2013. 34:1–4.12

Pathogenicity of Fibres in the Pleural Cavity

MRC Toxicology Unit

Pathogenic characteristics of fibres





Depos **nges**

- Meso
- Inflar
- Prolif
- Granuloma formation
- Fibrosis

Aim

To investigate the molecular changes that occur at the mesothelium as a consequence of direct exposure to fibres

Underlying molecular mechanisms are not fully understood

Exposure → Chronic Inflammation → Mesothelioma

Fibre Panel

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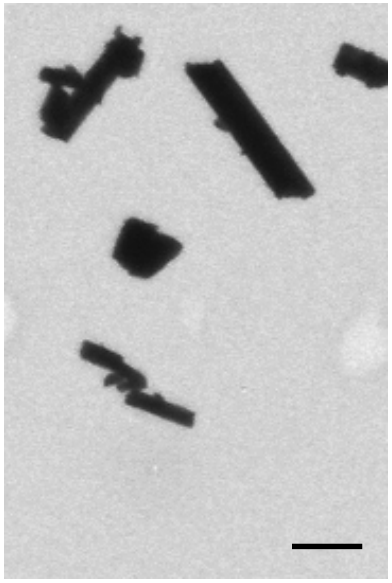
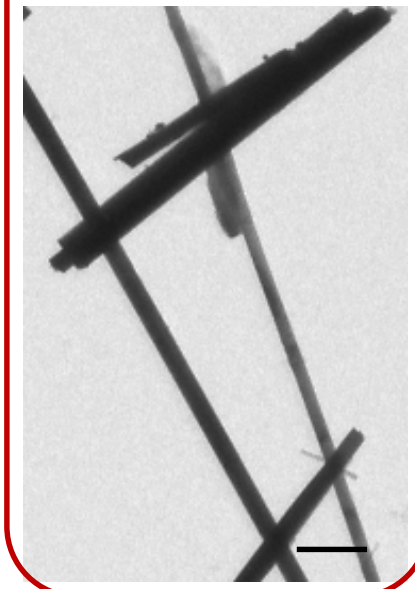
Short Fibre Asbestos **(SFA)**

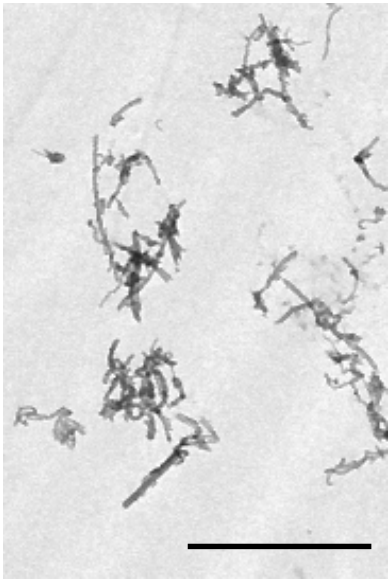


Long Fibre Asbestos **(LFA)**

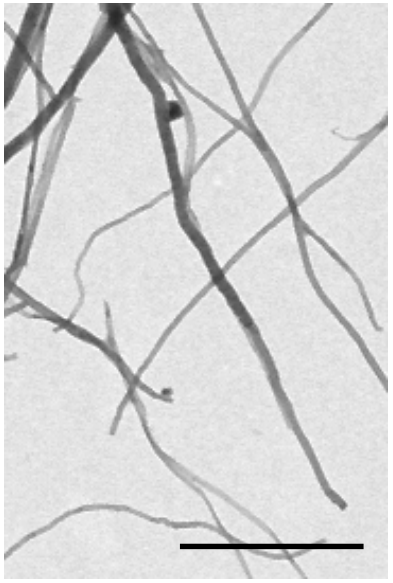


Induced Lung Tumours and Mesothelioma in previous *in vivo* studies

Short Carbon Nanotubes **(SNT)**



Long Carbon Nanotubes **(LNT)**



Scale bar = 1 µm

Fibre Panel

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	LFA	SFA	LNT	SNT
Sample	Long fibre amosite asbestos	Short fibre amosite asbestos	Long straight carbon nanotubes	Short straight carbon nanotubes
Source	Manville Corporation, South Africa	Manville Corporation, South Africa	University of Manchester, Dr. Ian Kinloch	Nanostructured and Amorphous Material Inc.
Diameter (nm)	1000	700	165	125
% fibres > than 15µm	50	4	85	0

Soluble Aqueous Extract of Metal Contaminants

Sample	Cd	Co	Cr	Cu	Fe	Mn	Ni	Ti	V	Zn
SFA	<0.1	2.1	<0.1	3.1	547	36.3	18.4	31.5	3.1	10.5
LFA	<0.1	1.4	3.4	5.2	853	104.8	5.1	2.0	<0.1	27.3
SNT	<0.1	<0.1	<0.1	<0.1	24.2	50.3	21.6	0.4	<0.1	5.3
LNT	<0.1	3.4	<0.1	1.2	37.3	3.6	6.2	0.3	<0.1	<0.1

Metal concentration expressed as µg/g. The limit of detection by this method is 0.1 µg/g.

Animal Model to explore Fibre-induced changes in the Pleura

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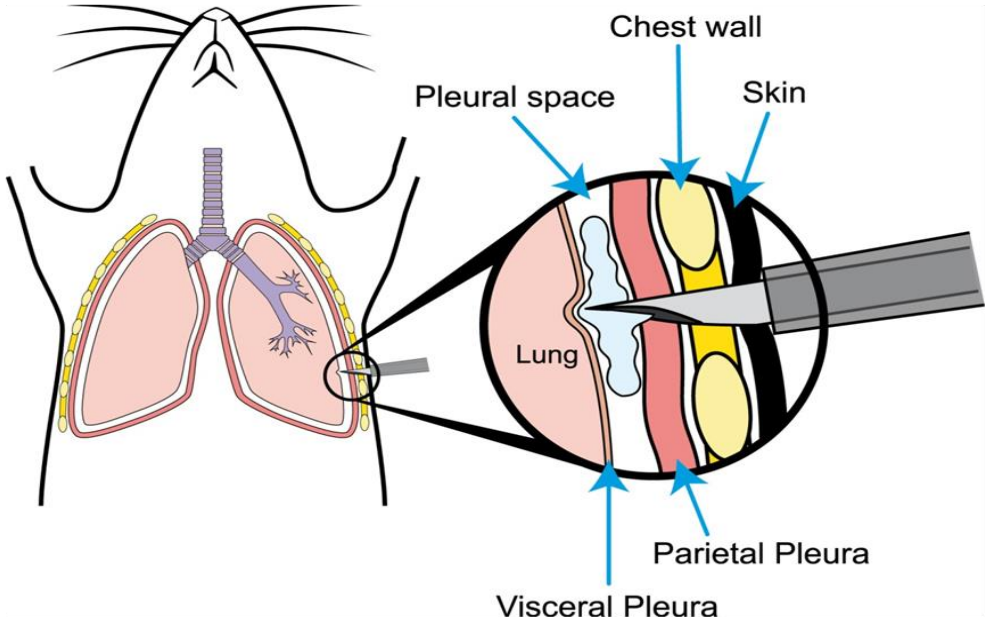
Aim

To investigate the molecular changes that occur at the mesothelium as a consequence of direct exposure to fibres

Mouse model



Pleural Instillation

SFA/LFA 25 µg/mouse

SNT/LNT 0.5, 1.0, 2.5 or 5 µg/mouse

Exposure

- 1 week
- 12 weeks
- 6 months
- Up to 20 months

Wild type C57/Bl6 mice
Single injection

Murphy et al 2011 Am J Path

Experimental Design

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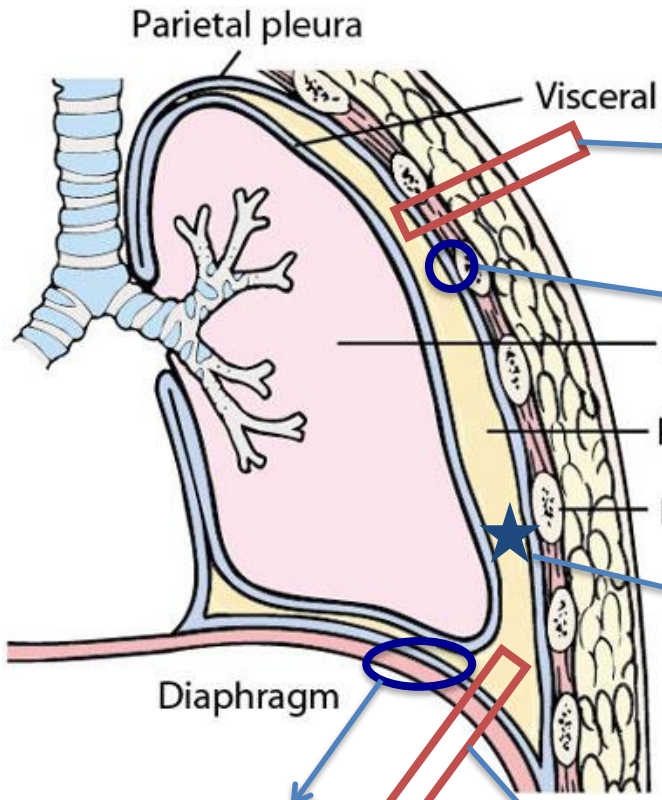
End points

1 and 12 weeks, 6 months after single injection

Too early for mesothelioma development (1-2 yrs in wild type mice) – extended to 20 months



Histology
Immunostaining

Kinome profiling

Activation of signalling pathways

Pleural Lavage
Cell counts

mRNA array

Laser Microdissection
q-PCR
BiSulfite Sequencing/Me-DIP Chip Array

fm124@le.ac.uk

Length-dependent Pleural Lesion Development

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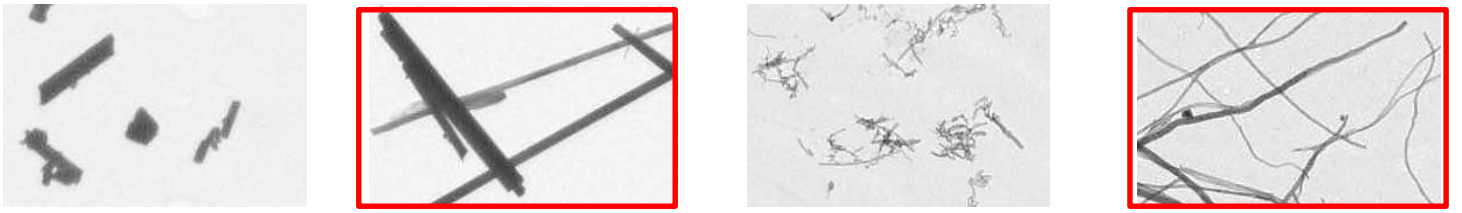
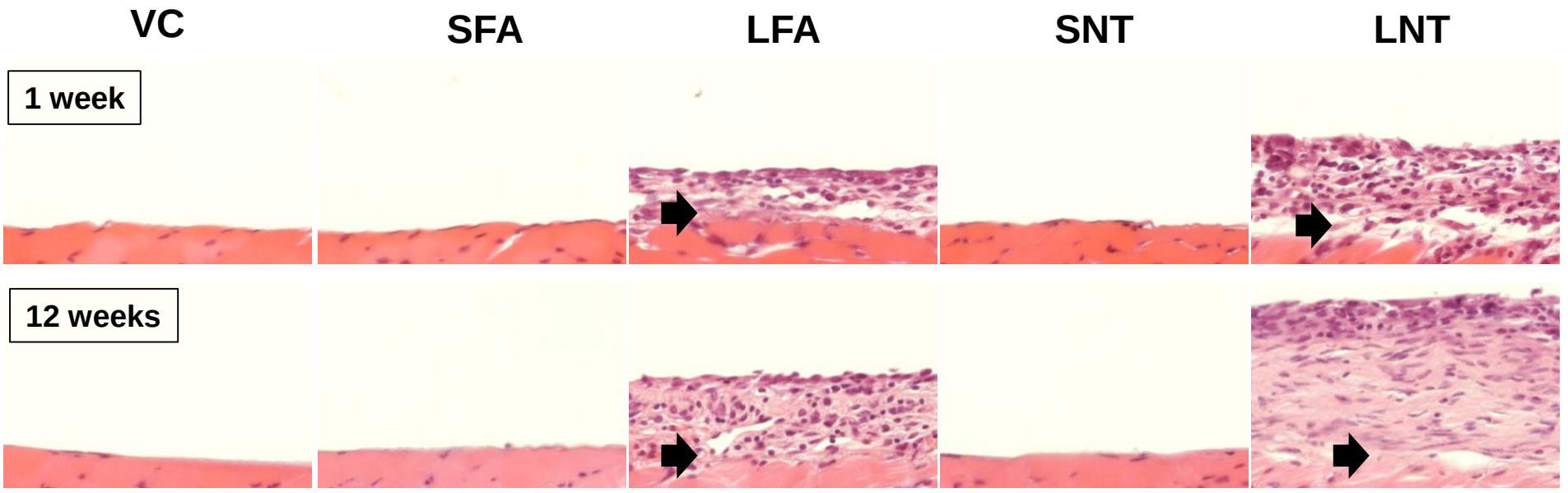
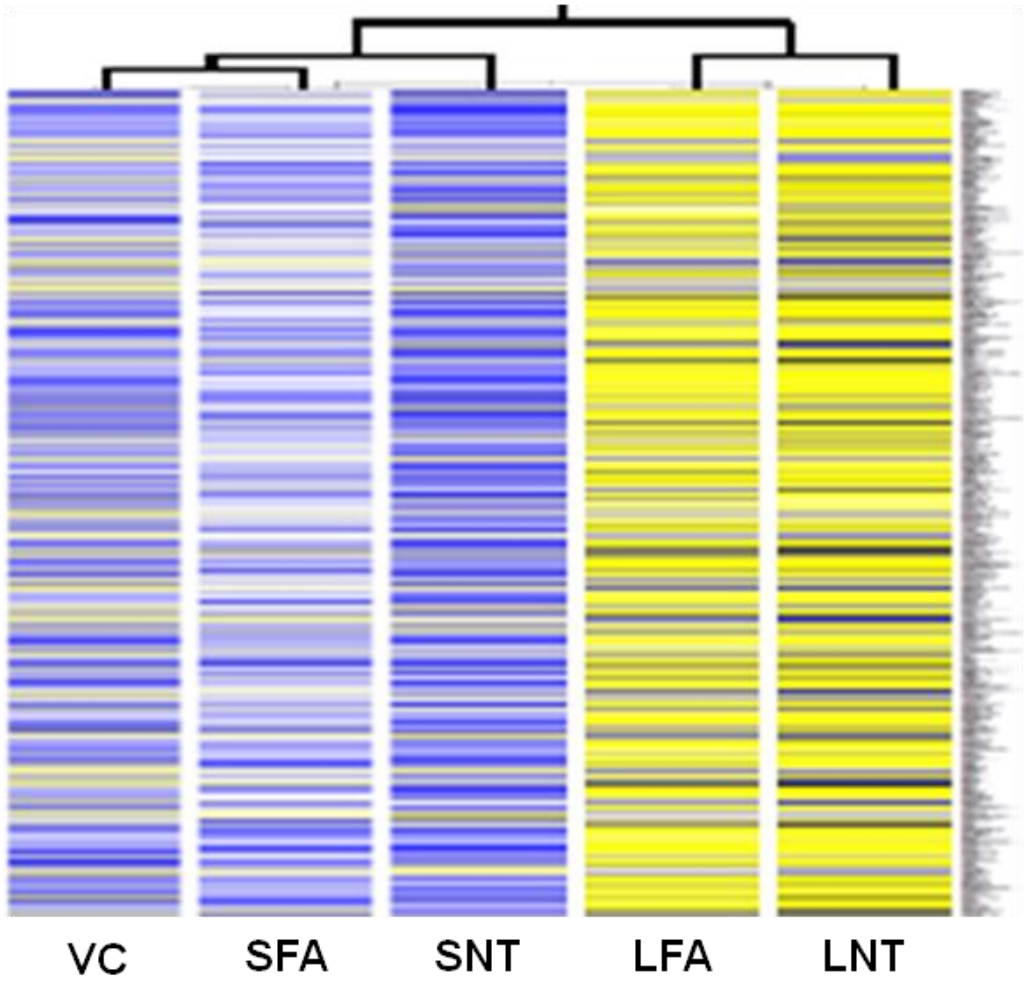




Common Molecular Signature in Long Fibre-induced Lesions



Changes in mRNA levels in whole diaphragm of animals exposed to SFA, SNT, LFA and LNT compared to VC.

'Cluster Analysis' reveals common gene expression signature between LFA & LNT - induced lesions.

Pathways involved:

- Inflammatory processes,
- Macrophage recruitment,
- Cytokine production, etc

Common Pattern of Signaling Pathway Activation in Long Fibre-induced Lesions & Mesothelioma Tissue from Patients

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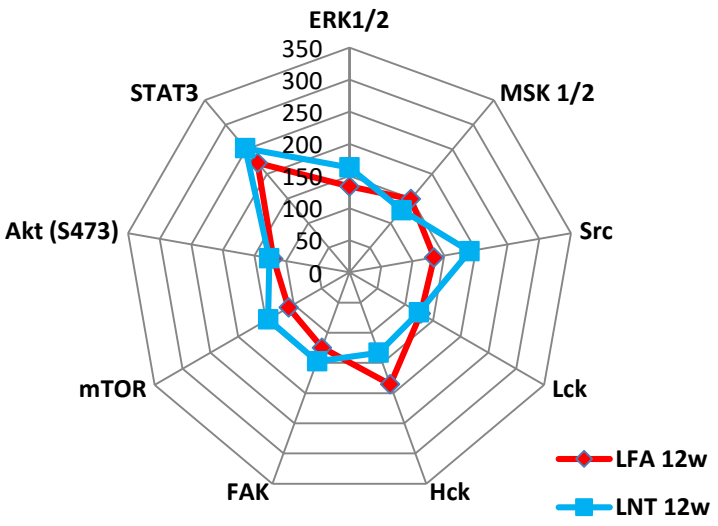

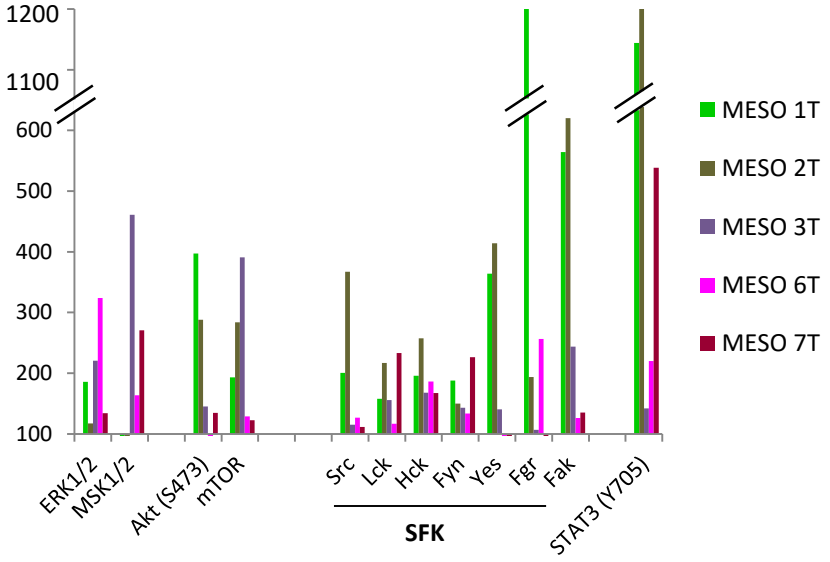

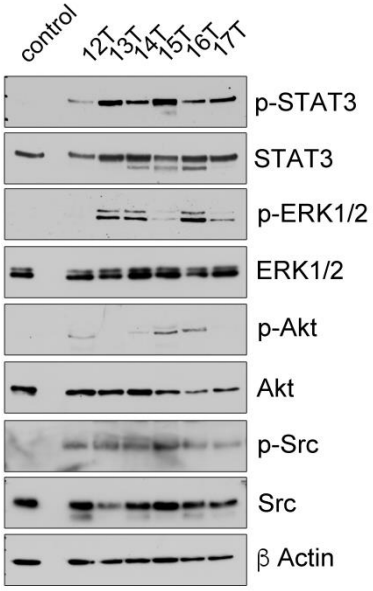

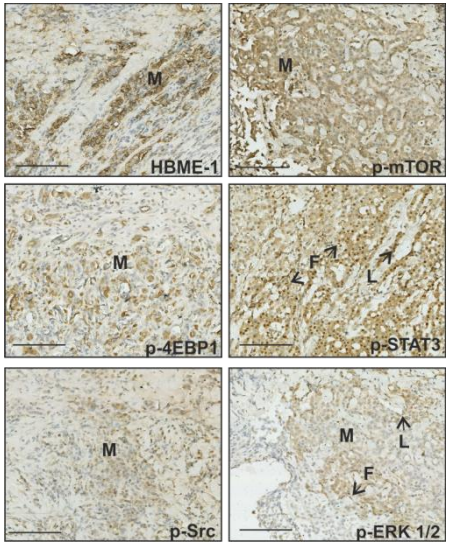

Do Long Fibre-Induced Inflammatory Lesions Progress to Malignant Mesothelioma?

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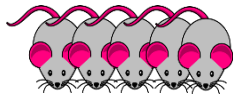
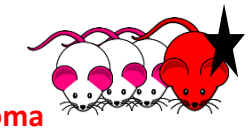
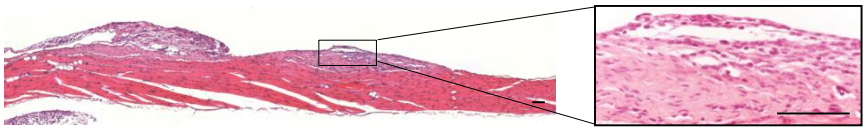
6 months exposure
Inflammatory Lesions



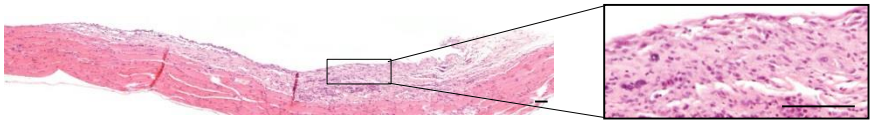
1 year exposure
LNT-induced mesothelioma

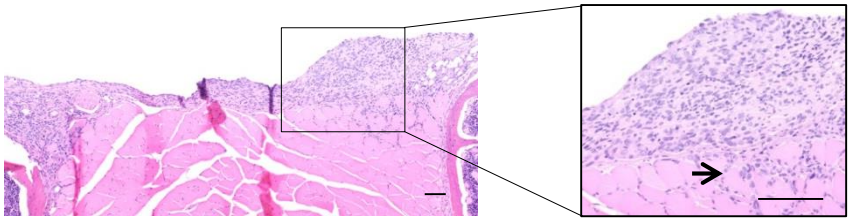


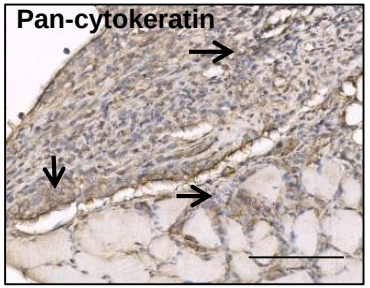
LFA

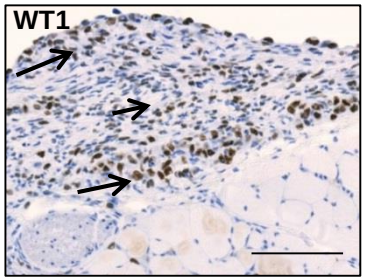


LNT

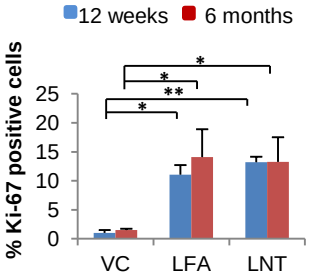


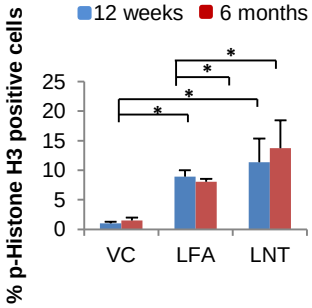




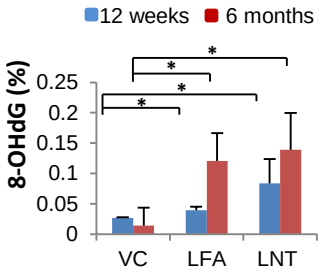


Proliferation



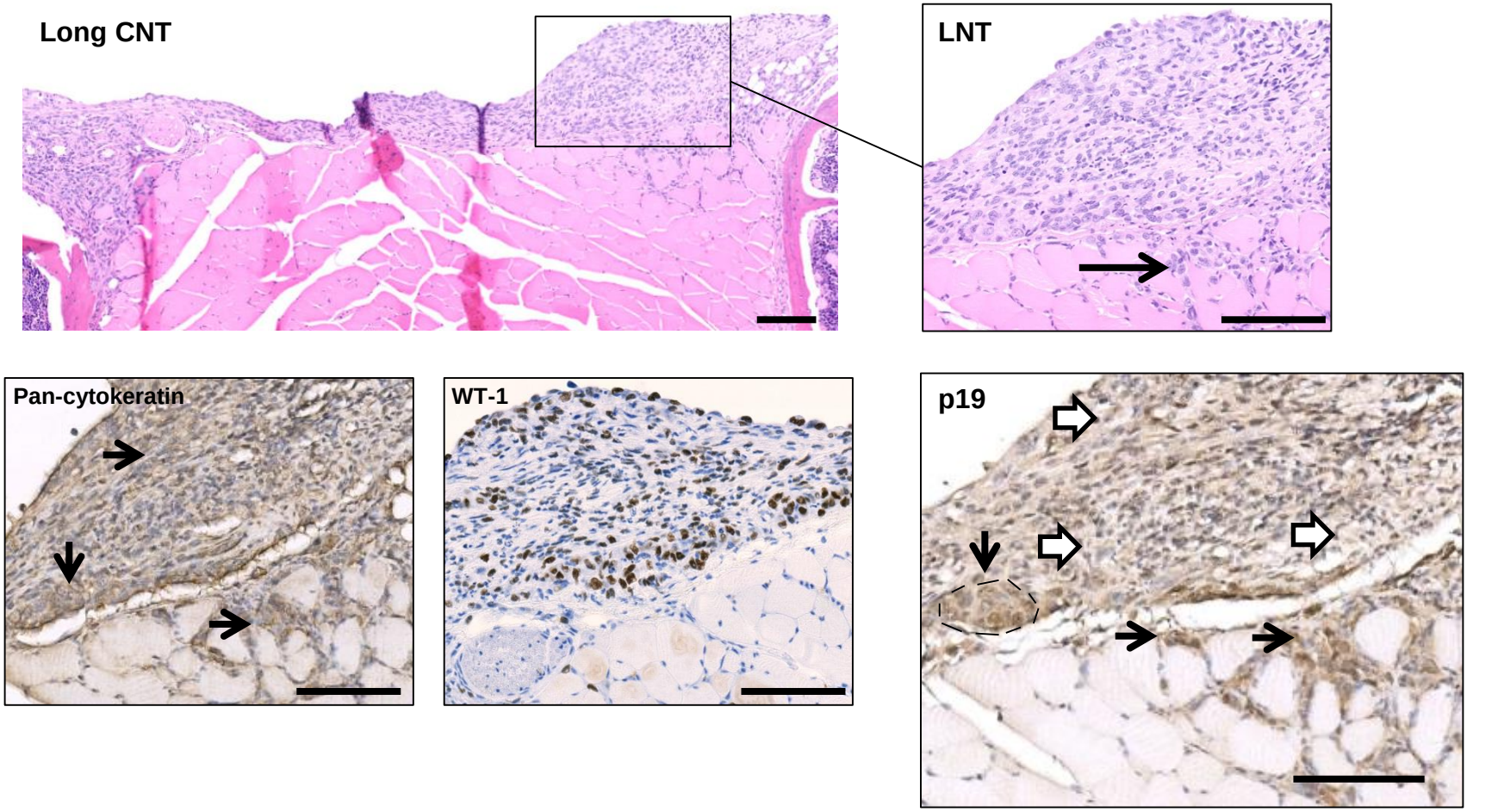


Oxidative DNA damage



Progression of Long-Fibre Pleural Lesions to Malignant Mesothelioma

MRC Toxicology Unit

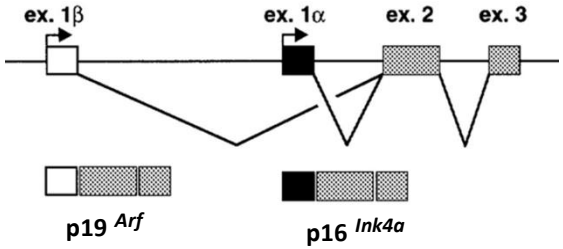


Long Fibre-induced Pleural Lesions progress to Mesothelioma with loss of p19

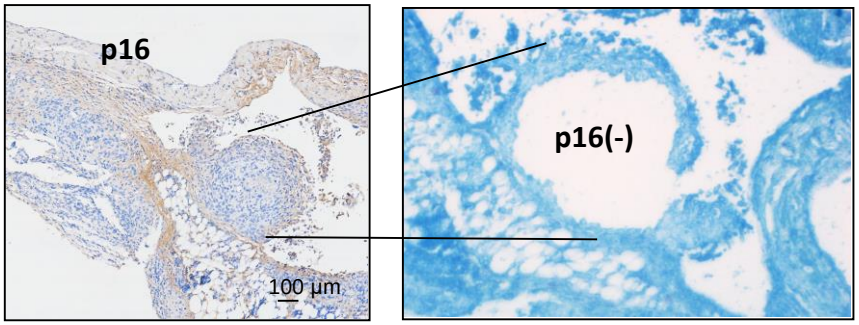
LNT-induced Tumour Displays Loss of the Tumour Suppressor Gene *Cdkn2a*

MRC Toxicology Unit

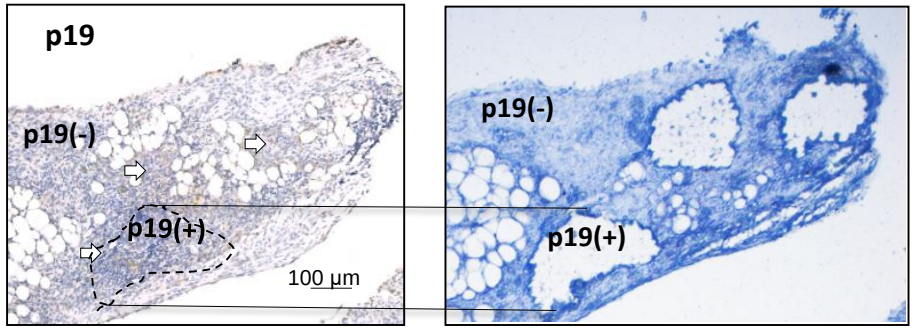
Cdkn2a ($p16^{Ink4a}$/$p19^{Arf}$)

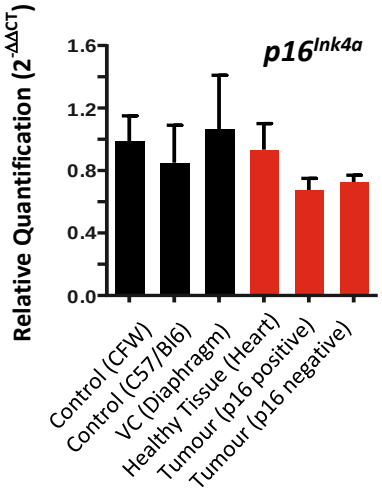


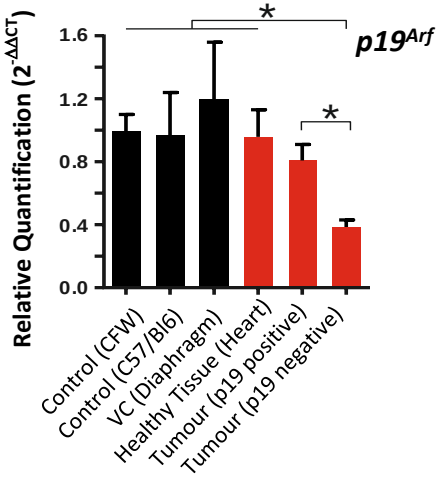
Long CNT-induced Tumour



Long CNT-induced Tumour

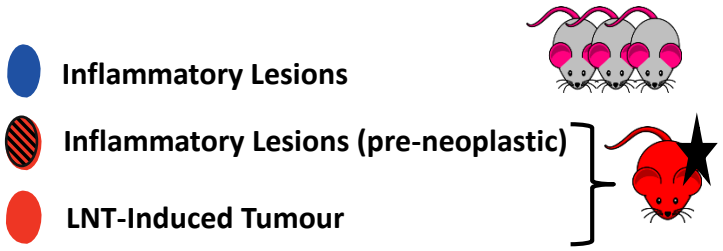




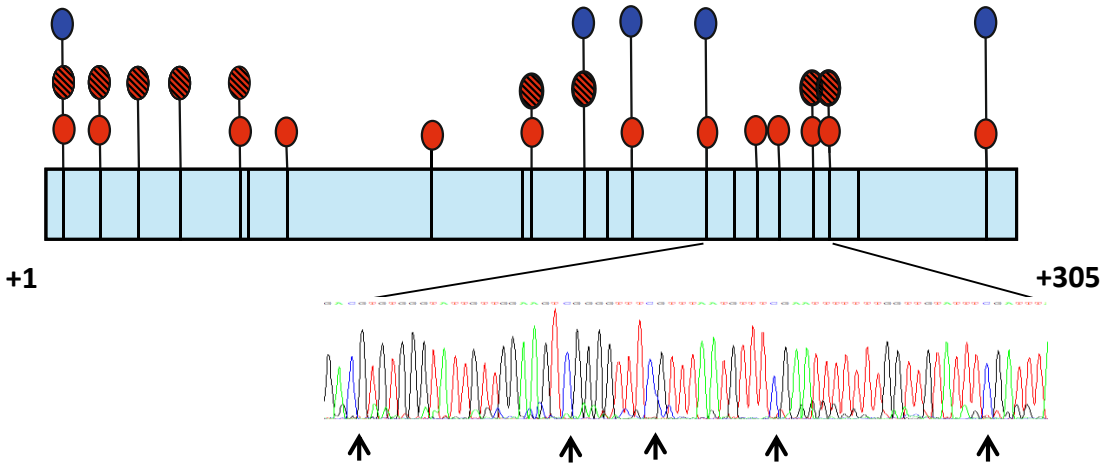


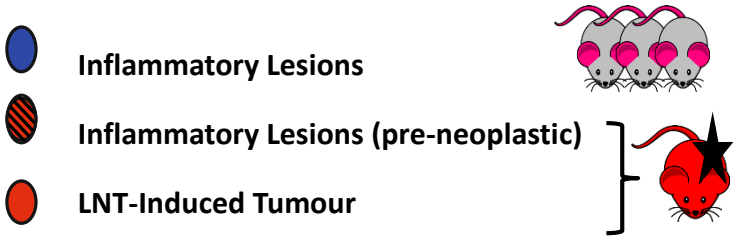
Hypermethylation of the *Cdkn2a* (*p16^{Ink4a}/p19^{Arf}*) Locus in LNT-induced Mesothelioma & LNT-induced Inflammatory Lesions

MRC Toxicology Unit

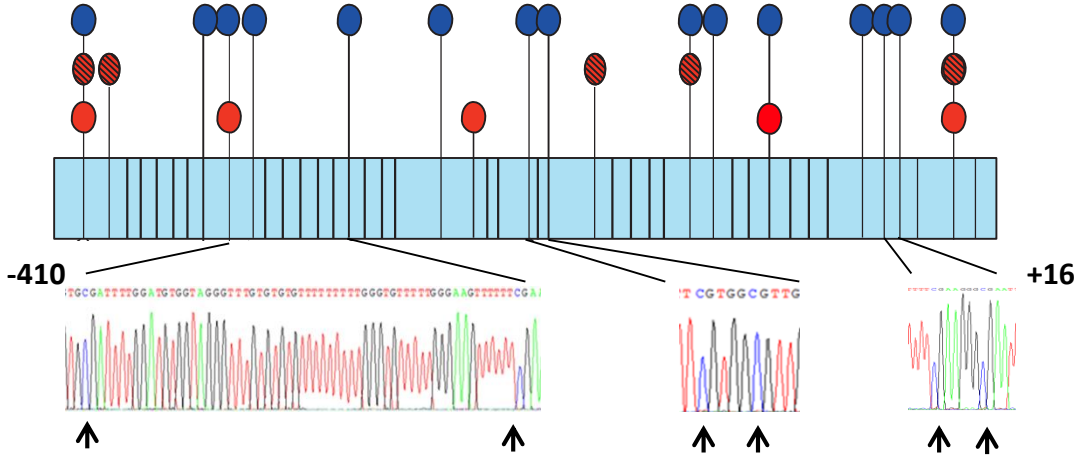


***Cdkn2a p16^{Ink4a}* exon 1α CpG island**





***Cdkn2a p19^{Arf}* CpG island (5'region flanking exon 1β)**



Mouse Model Identifies Epigenetic Signatures during Disease Progression

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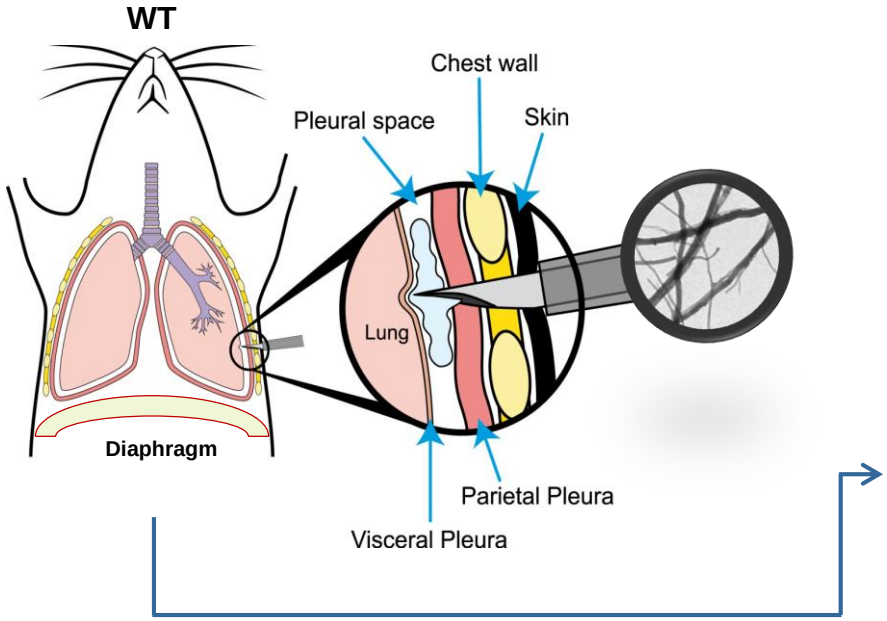
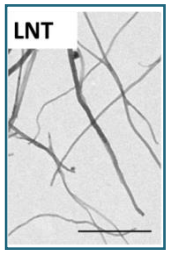


Occupationally-relevant dose

25 μg
(**LFA**)

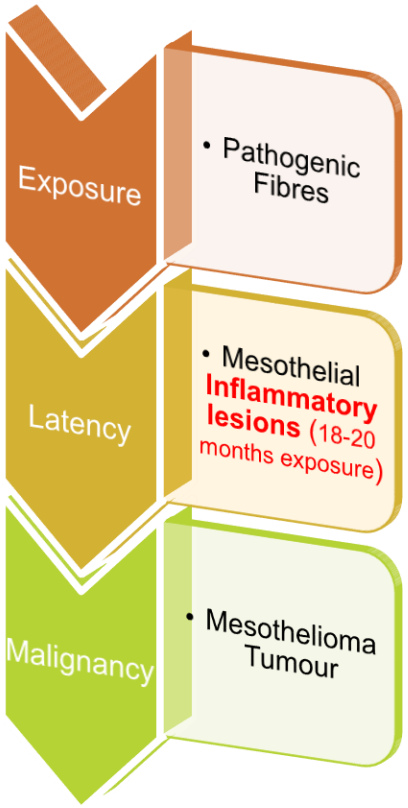


1.0 μg
(**LNT1.0**)

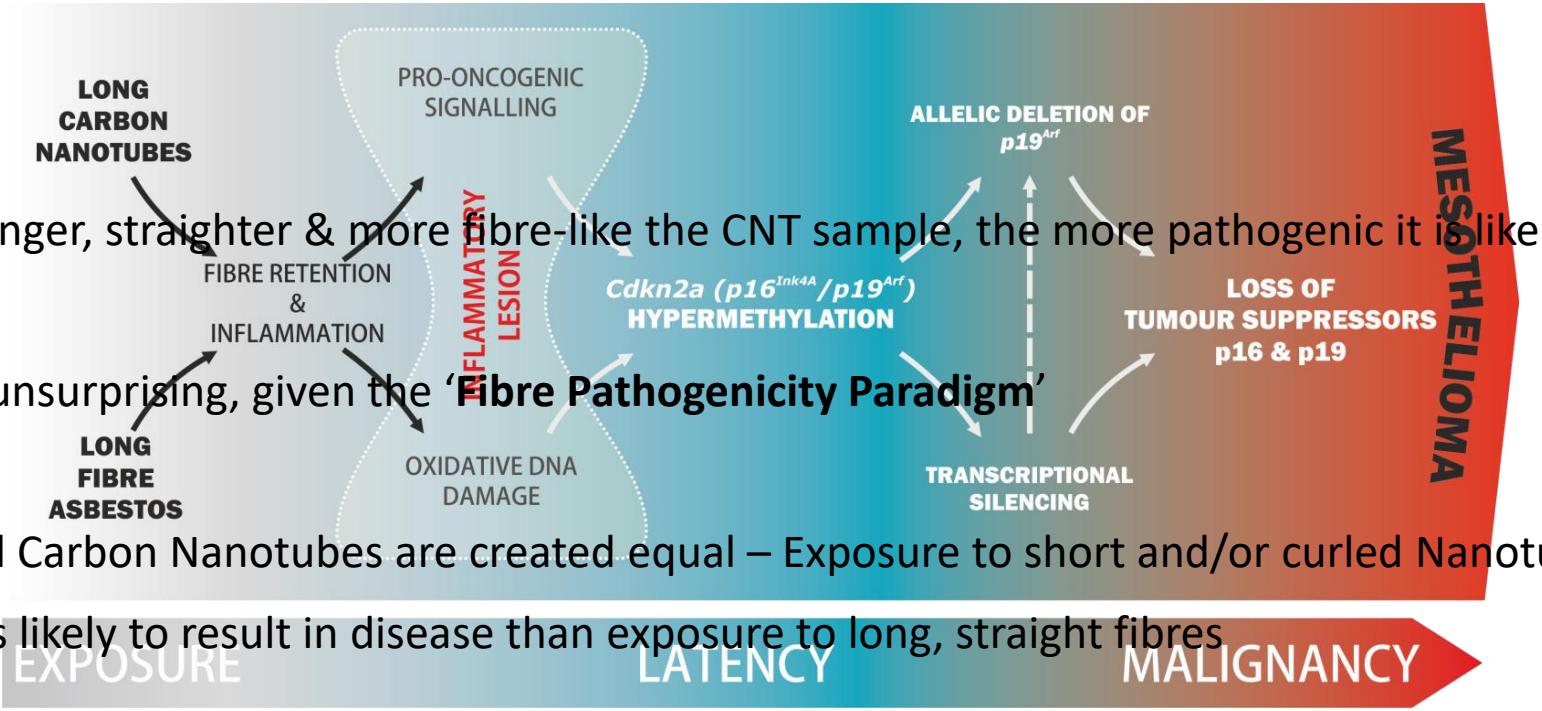
DNA methylation
Microarray

VC **LFA** **LNT**

mRNA expression
Microarray



Conclusions



- The longer, straighter & more fibre-like the CNT sample, the more pathogenic it is likely to be
  - unsurprising, given the '**Fibre Pathogenicity Paradigm**'
- Not all Carbon Nanotubes are created equal – Exposure to short and/or curled Nanotubes is less likely to result in disease than exposure to long, straight fibres
  - Common molecular changes occur in LFA- and LNT-induced pleural lesions that progress to malignant mesothelioma

'Fibre Pathogenicity Paradigm' Update:

Width, Length, Biopersistence & a 4th factor 'mechanical bending stiffness' (Kane et al, *TAP* 2018)

- Aberrant signalling pathway activation, hypermethylation of *Cdkn2a,* and deletion of *p19^{Arf}* in LNT-induced tumours recapitulates common features of human mesothelioma

- The common molecular signature of LFA- and LNT-induced pathology demonstrates a similar hazard mechanism leading to pleural disease, including malignant mesothelioma

Chernova et al, Current Biology. 2017 & Unpublished

Acknowledgements

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University of Edinburgh Biological Services

NIOSH
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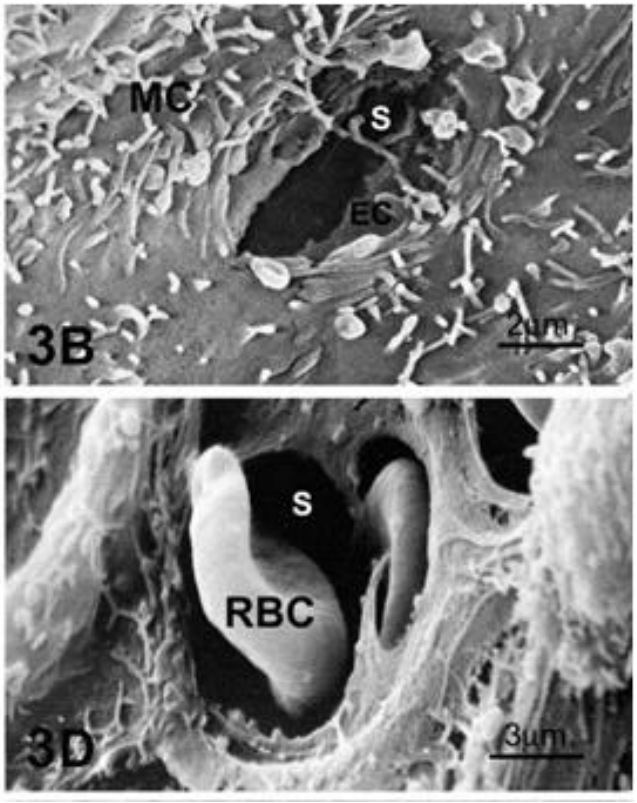
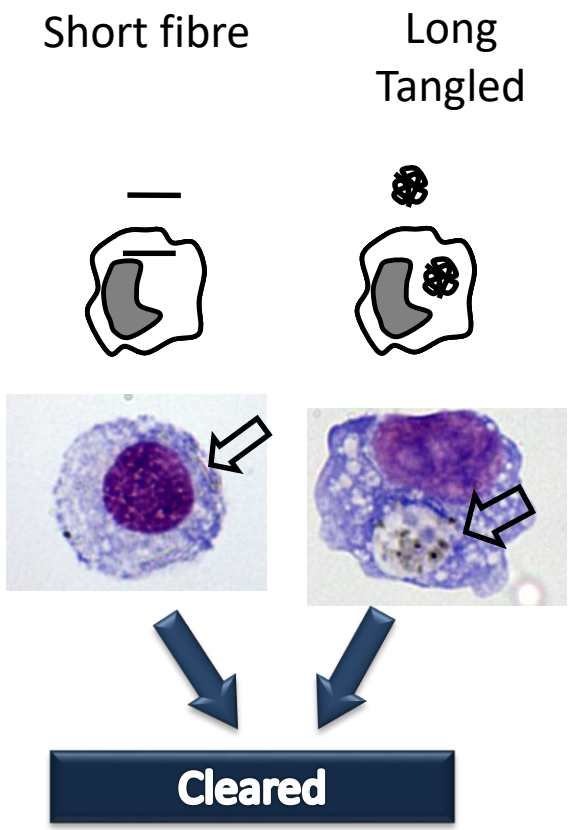
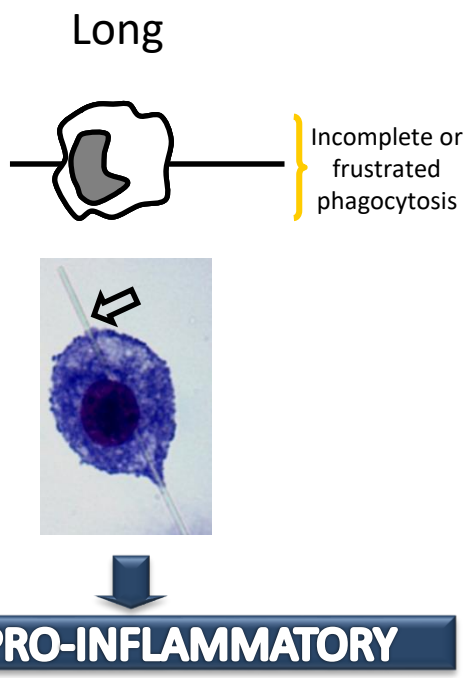
Frustrated Phagocytosis







Ken Donaldson et al 2010

Experimental Dose and Relevance to Human Exposure

Mice were exposed to 5 μg of CNT per animal

The ratio of human to mouse alveolar surface is 1255

The equivalent exposure of 5 μg CNT in mouse is 6.275 mg for a human

The 2013 exposure limit for carbon nanotubes recommended by NIOSH is 1 μg/m3

A volume of 10 m^3 of inspired air per 8-hr shift

48 weeks per year

40 year working life-time

A worker exposed to 1 μg/m3 would inhale 96 mg of carbon nanotubes

Loss of the Tumour Suppressor Gene *Cdkn2a in* LNT-induced Tumours

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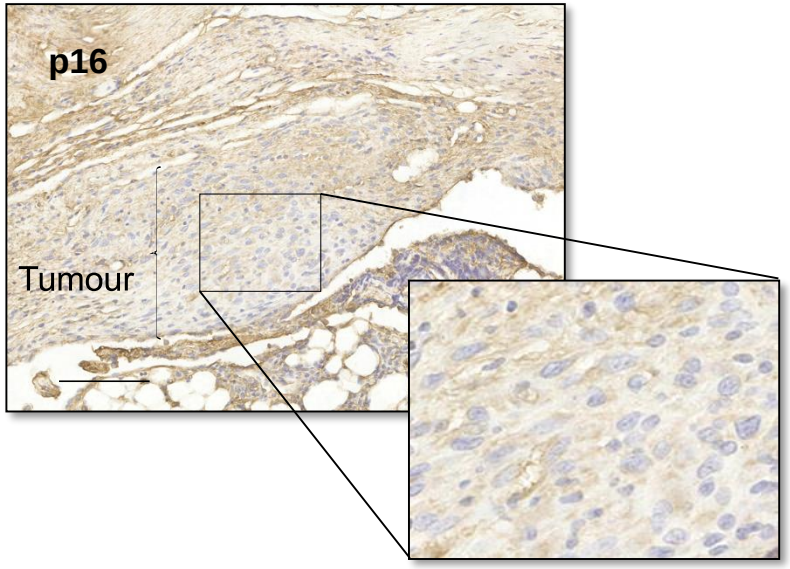
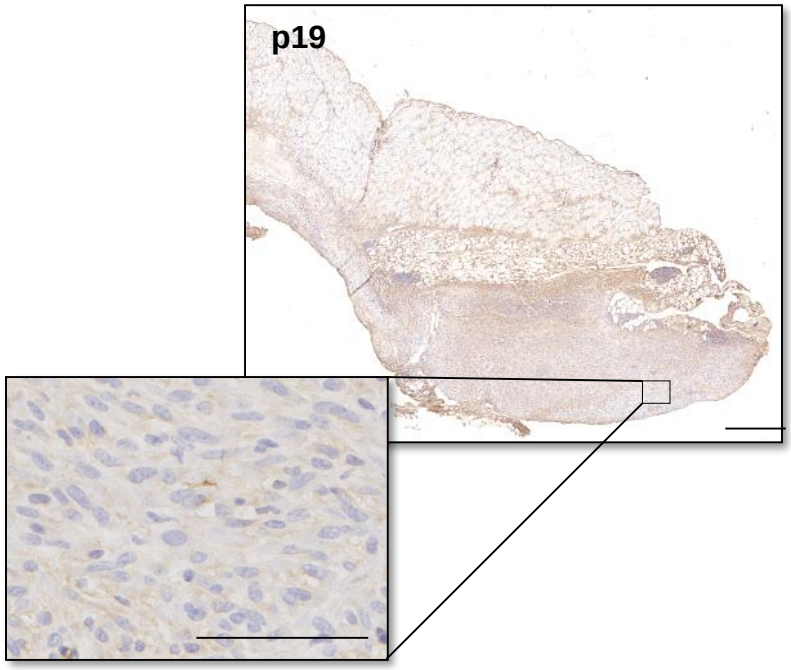
In 3 independent studies 20-25% of animals exposed to LNT developed pleural mesothelioma

All mesotheliomas displayed loss of p16 and p19 protein expression



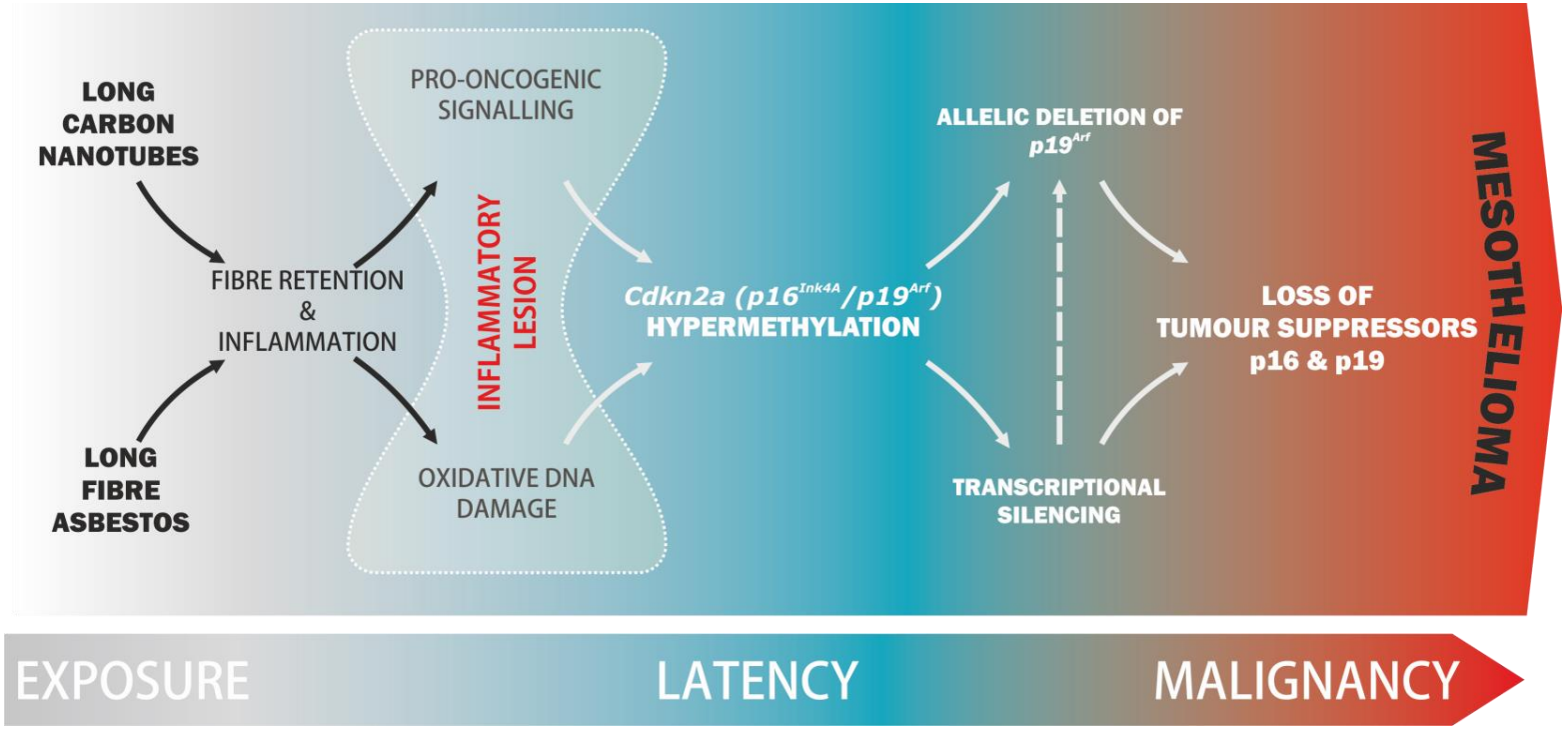


Summary & Open Questions

MRC Toxicology Unit

- Aberrant signalling pathway activation, hypermethylation of *Cdkn2a,* and deletion of *p19^{Arf}* in Long Fibre-induced tumour recapitulates common features of human mesothelioma

- The common molecular signature of LFA- and LNT- induced pathology demonstrates a similar mechanism leading to pleural disease, including malignant mesothelioma



- Longitudinal Study of molecular determinants of Fibre-induced malignant transformation

- WT vs. GEMMs; Cre-targeted deletion of key tumour suppressor genes in target tissues

Chernova et al, Current Biol. 2017; Unpublished

LNT-induced Inflammatory Lesions Display Loss of the Tumour Suppressors p16 and p19

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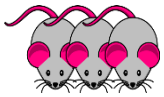
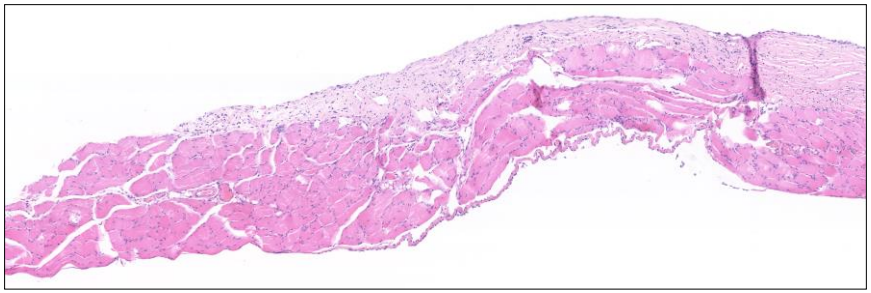
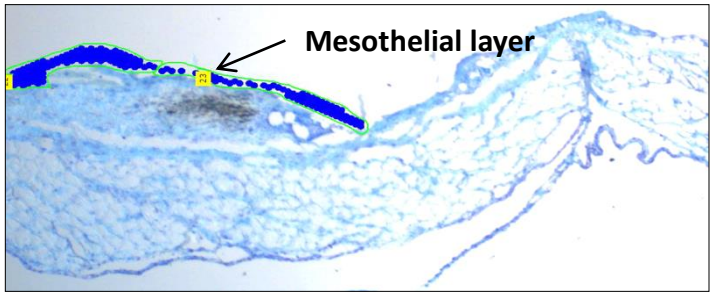
LNT-induced Inflammatory Lesions at 1 year

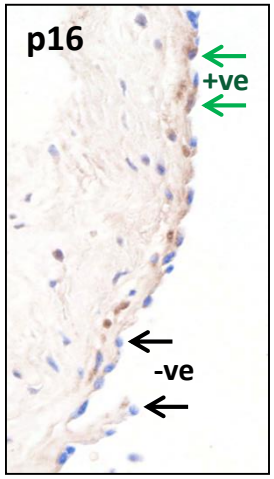


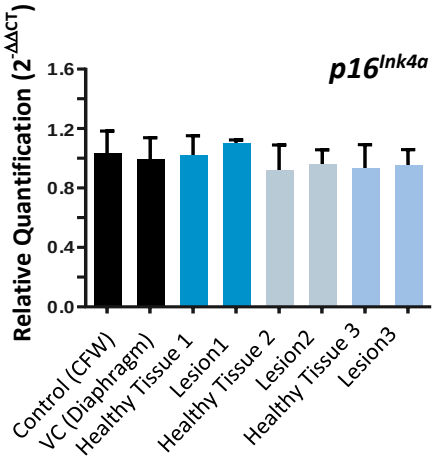


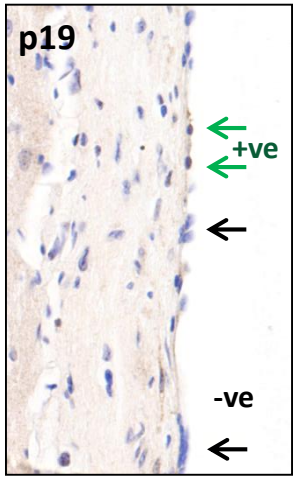
LNT-induced Lesion

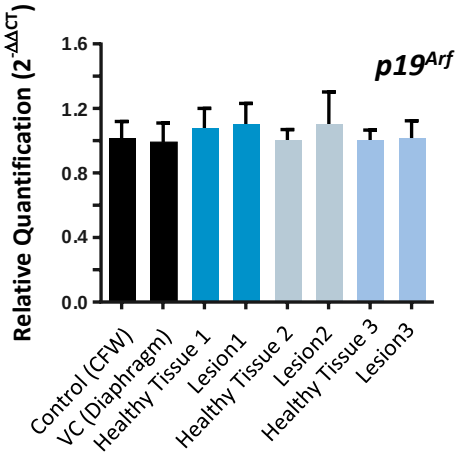


LNT-induced Inflammatory Lesions: loss of p16 and p19 protein in mesothelial cells



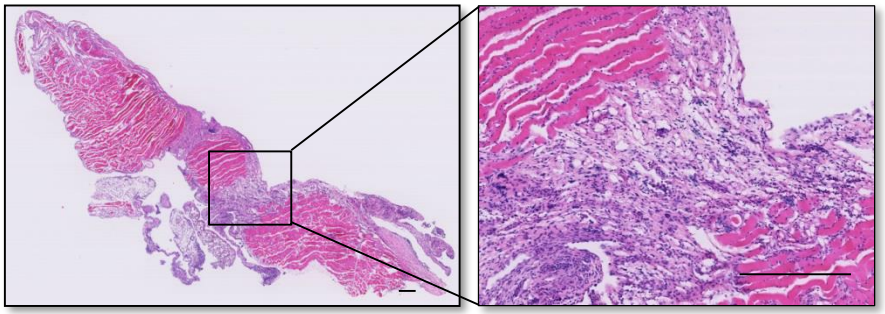


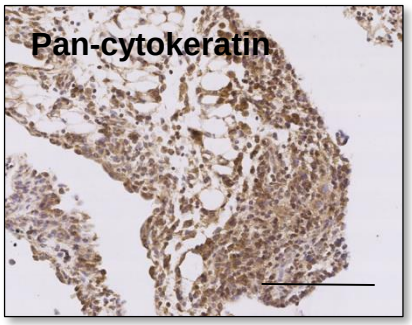


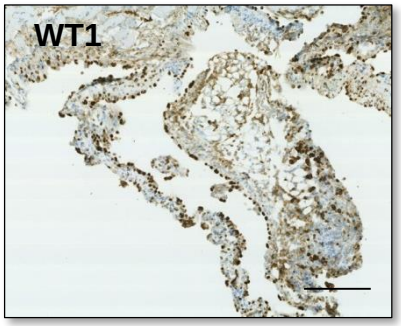


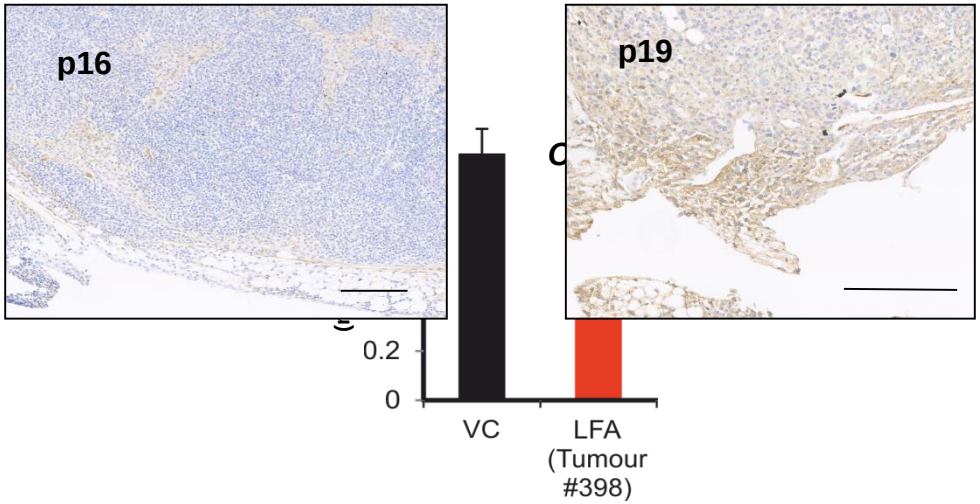
LFA-induced Mesothelioma Displays Loss of the Tumour Suppressor Proteins p16 and p19

MRC Toxicology Unit









Hypermethylation of the *Cdkn2a* (*p16^{Ink4a}/p19^{Arf}*) Locus in LFA-induced Mesothelioma and LFA-induced Inflammatory Lesions

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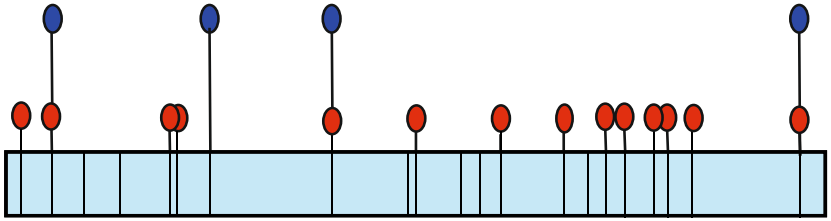
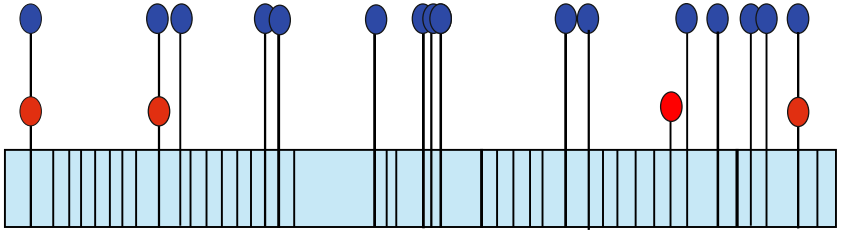
***Cdkn2a p16^{Ink4a}* exon 1α CpG island**



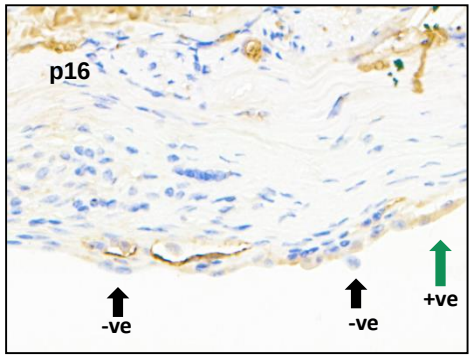
***Cdkn2a p19^{Arf}* CpG island (5'region flanking exon 1β)**

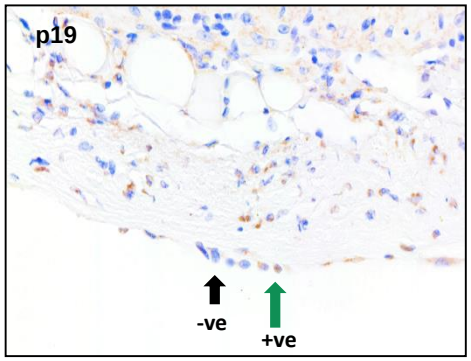


LFA-induced Inflammatory Lesions

LFA-Induced Tumour

LFA-induced Lesions





Hypermethylation is a Common feature of Long-Fibre-induced Chronic Inflammatory Lesions

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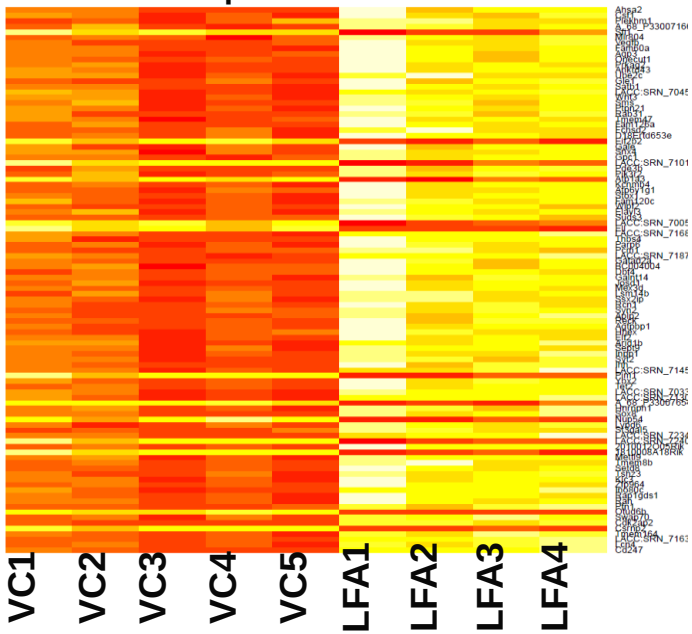
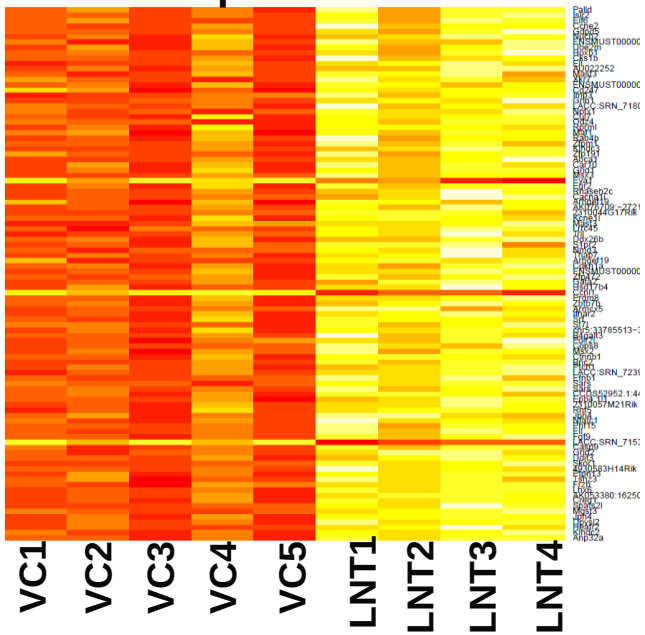
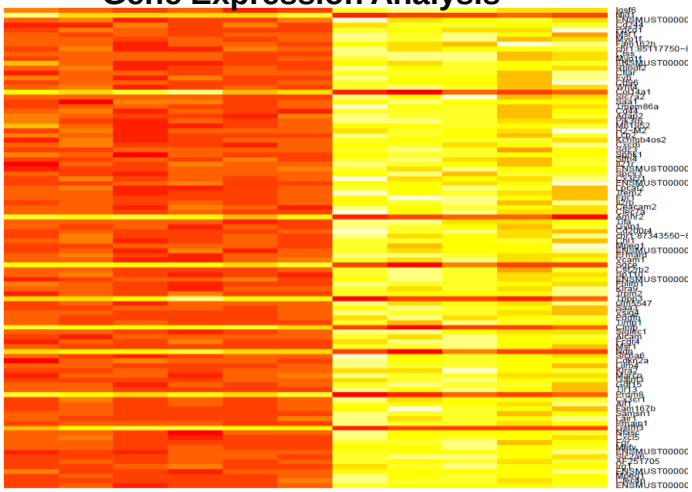
Heat Maps - VC vs LFA



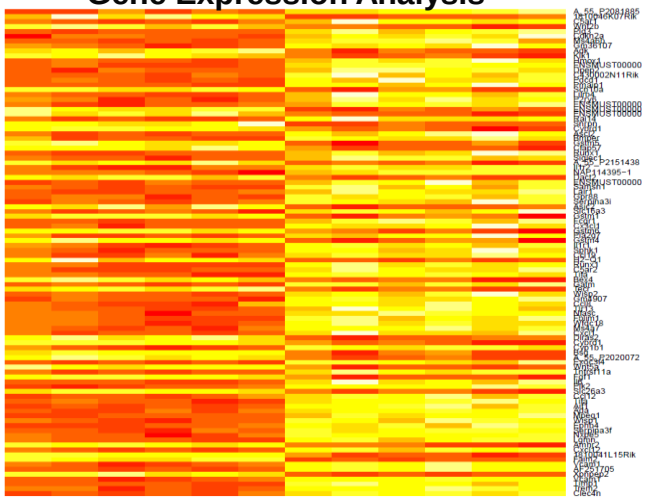
Heat Maps - VC vs LNT



Gene Expression Analysis



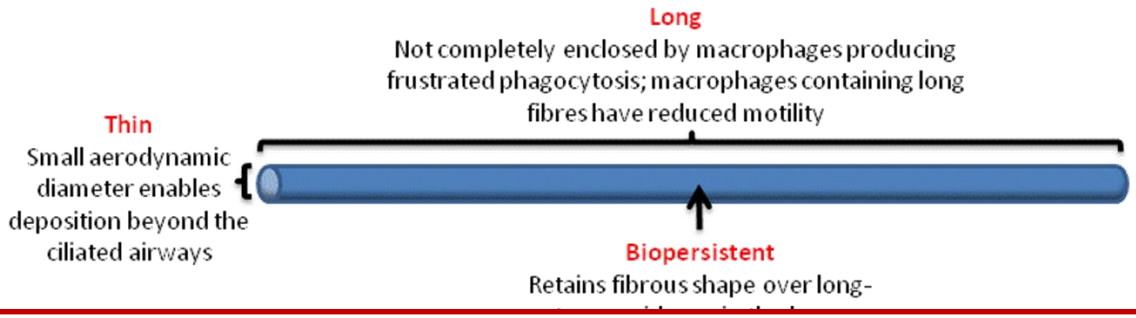
Gene Expression Analysis

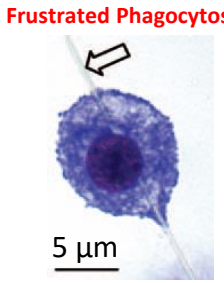


Pathogenicity of Fibres in the Pleural Cavity

MRC Toxicology Unit

Pathogenic characteristics of fibres





Depos **nges**

- Meso
- Inflar
- Prolif
- Granuloma formation
- Fibrosis

Aim

To investigate the molecular changes that occur at the mesothelium as a consequence of direct exposure to fibres

Underlying molecular mechanisms are not fully understood

Exposure → Chronic Inflammation → Mesothelioma

Dissecting the Molecular Changes in MPM – a Disease linked with direct Fibre Exposure

Malignant Mesothelioma

Leslie Stayner,[1] Laura S. Welch,[2] and Richard Lemen[3]
Annu. Rev. Public Health 2013. 34:4.1–4.12

MRC Team – Collaborators & Partners

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Dr. Xiao-Ming Sun
Dr Joaquin Zacarias-Cabeza

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Institute of Occupational Medicine

NIOSH
Dr. Dale Porter
Dr. Linda Sargent

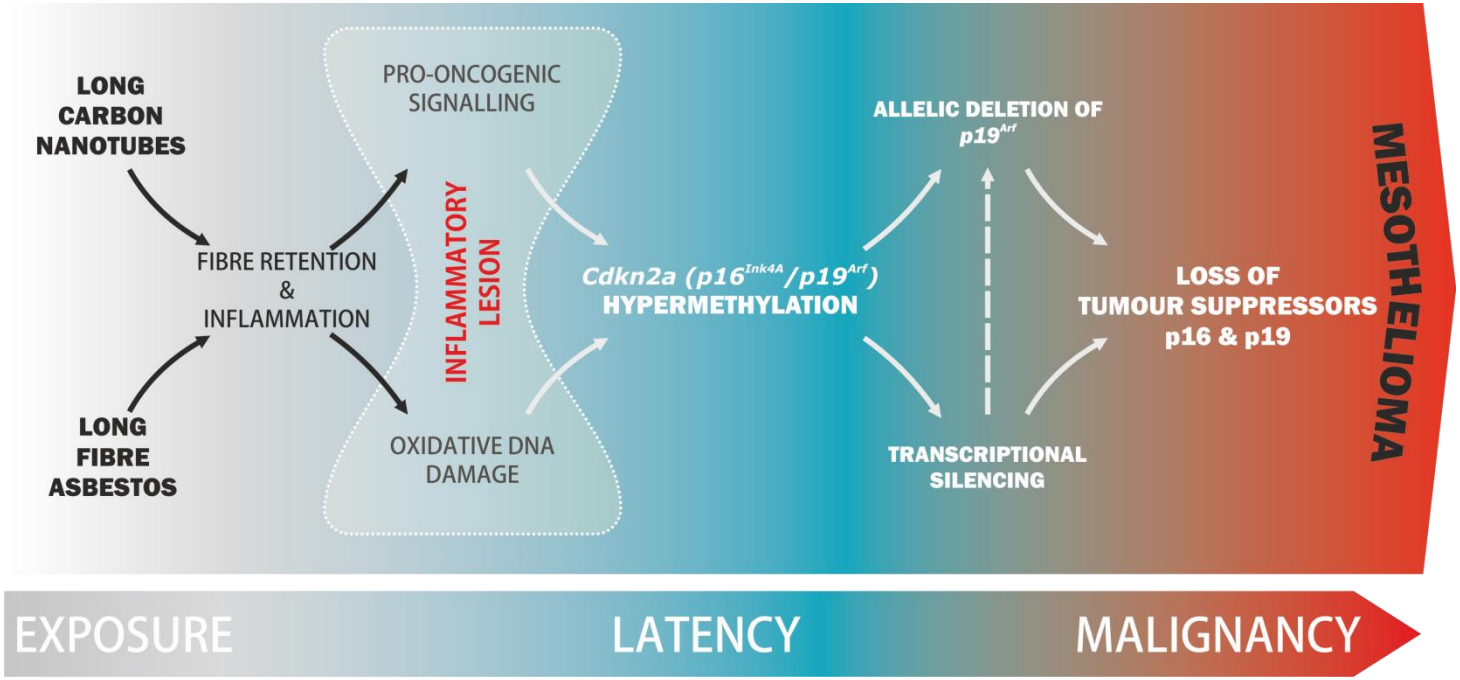
University Hospitals of Leicester NHS
NHS Trust

Mr. Apostolos Nakas
Dr. Jonathan Bennett
Prof. Mick Peake

Conclusions

MRC Toxicology Unit

- Common molecular changes occur in LFA- and LNT-induced pleural lesions that progress to mesothelioma
- Aberrant signalling pathway activation, hypermethylation of *Cdkn2a,* and deletion of *p19^{Arf}* in LNT-induced tumour recapitulates common features of human mesothelioma
- The common molecular signature of LFA- and LNT- induced pathology demonstrates a similar mechanism leading to pleural disease, including malignant mesothelioma

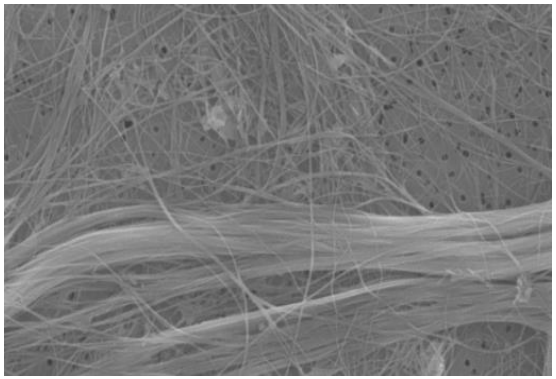


Chernova et al, Current Biol. 2017; Unpublished

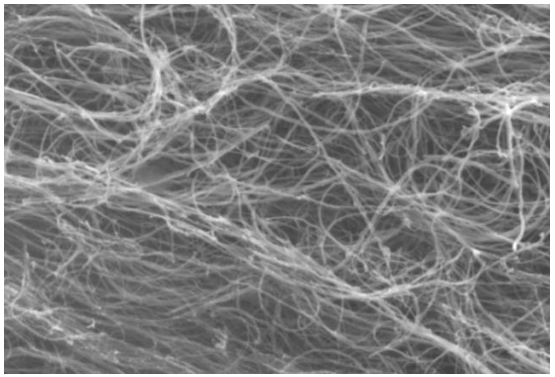
Carbon Nanotubes

MRC Toxicology Unit

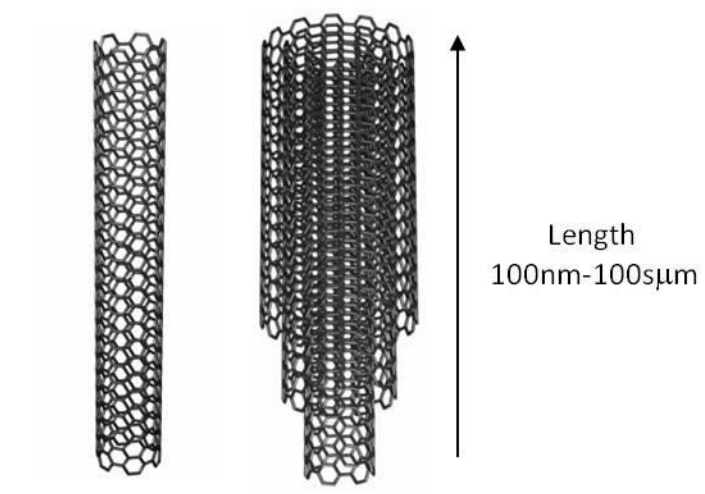
- Hexagonal arrangements of carbon atoms built up to form a fibre
- Exceptional properties including strength & conductivity
- Capacity for production estimated >2 Kilotonnes/year…. rapidly increasing
- Global market for carbon nanotubes is estimated to be worth over $1 billion (2014)
- Similar structure to asbestos



Asbestos (x4000)



Carbon Nanotubes (x6000)



The Fibre Pathogenicity Paradigm

1) The fibre pathogenicity paradigm is the most robust SAR for any particle
2) Derived from human, animal and *in vitro* studies over 25 years
3) Holds true for asbestos, glass fibre, ceramic fibres and the only organic fibre so far studied in this context (p-aramid) – no fibre so far studied has violated the paradigm
4) So is regardless of chemistry but is based on shape and persistence in the lungs
5) Paradigm states that only **long** (>20μm), **thin** (<3μm) and **biopersistent** fibres are pathogenic

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Conclusions

- Long MWCNT behave like long asbestos in showing rapid inflammatory and fibrogenic effects in a model of direct mesothelial exposure
- The longer, straighter and more fibre-like the CNT sample, the more pathogenic it is likely to be
  - unsurprising given the Fibre Pathogenicity Paradigm
- Not all nanotubes are created equal – Exposure to short and/ or curled nanotubes is less likely to result in disease than exposure to long, straight fibres

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Future Research

- Are long CNT released into the occupational environment in a respirable form in significant amounts?
- This model bypassed the lungs and delivered the CNT straight onto the mesothelium
- Would inhaled CNT reach the pleural mesothelium in sufficient amounts to cause disease?
- This study only addresses the fibre effect and the mesothelioma risk
- Research should address a long CNT effect in the lung (? fibrosis/lung cancer) and a compact particulate CNT effect in the lungs (?fibrosis)

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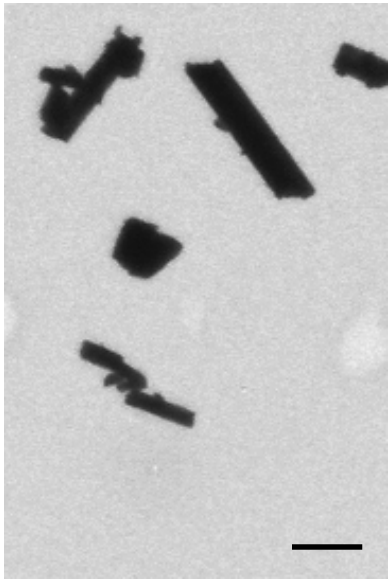
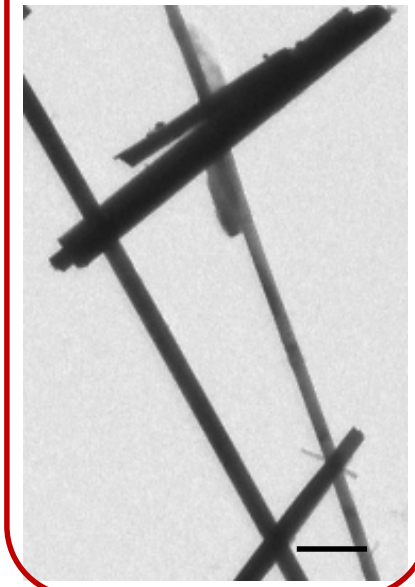
Fibre Panel

MRC Toxicology Unit

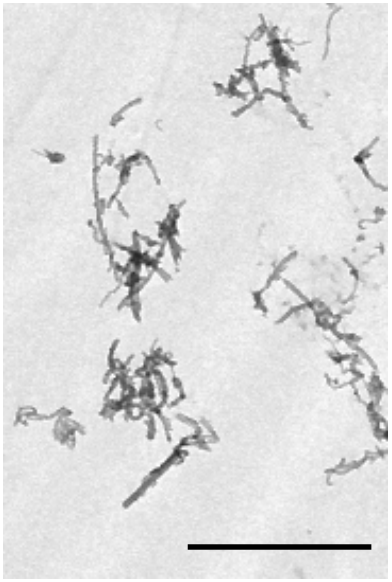
Short Fibre Asbestos **(SFA)**



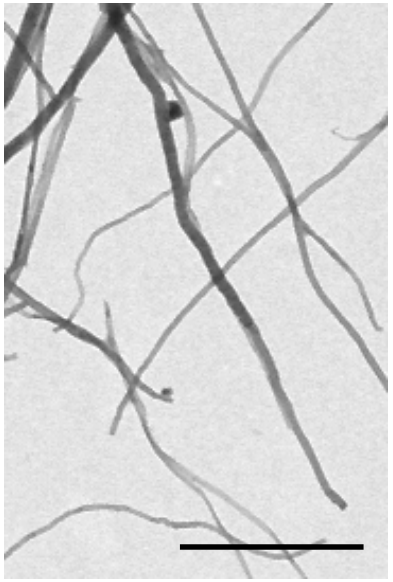
Long Fibre Asbestos **(LFA)**



Short Carbon Nanotubes **(SNT)**



Long Carbon Nanotubes **(LNT)**



Mice were exposed to 0.5, 1.0, 2.5 or 5 **µg CNT per animal**

Induced Lung Tumours and Mesothelioma in previous *in vivo* studies

Equivalent exposure to 2.5 µg CNT in mouse is 3.137 mg for a human

2013 exposure limit to carbon nanotubes recommended by NIOSH is 1 µg/m^3

A worker exposed to 1µg/m^3 would inhale ~96 mg of carbon nanotubes

Scale bar = 1 µm

Experimental Dose and Relevance to Human Exposure

Mice were exposed to 0.5, 1.0, 2.5 or 5 **μg of CNT per animal**

Equivalent exposure to 2.5 μg CNT in mouse is 3.137 mg for a human

2013 exposure limit to carbon nanotubes recommended by NIOSH is 1 μg/m^3

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Mercer et al. Particle and Fibre Toxicology 2010, 7:28
http://www.particleandfibretoxicology.com/content/7/1/28

PARTICLE AND FIBRE TOXICOLOGY

RESEARCH Open Access

Distribution and persistence of pleural penetrations by multi-walled carbon nanotubes

Robert R Mercer[1,2*], Ann F Hubbs[1], James F Scabilloni[1], Liying Wang[1], Lori A Battelli[1], Diane Schwegler-Berry[1], Vincent Castranova[1], Dale W Porter[1,2]

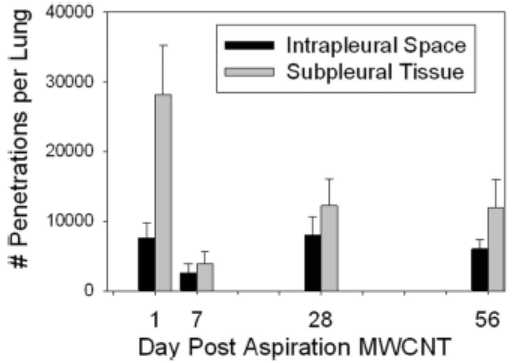
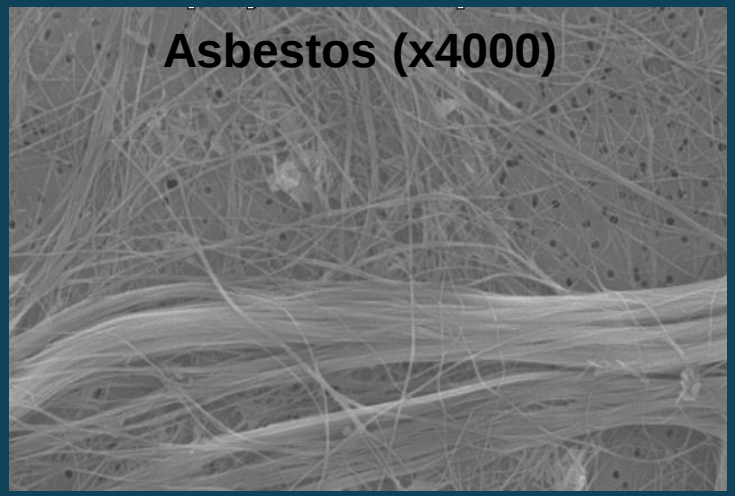


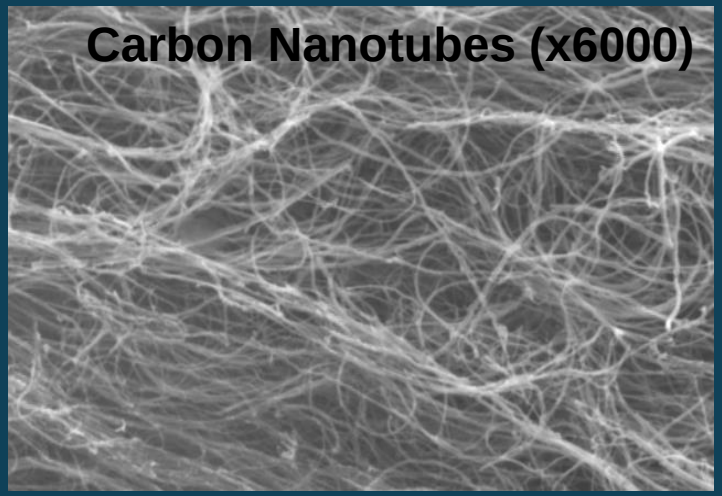
Figure 9 Time course of MWCNT penetrations into the subpleural tissue and intrapleural space. There was a significant number of penetrations into the subpleural tissue and intrapleural space with an initial spike at one day after aspiration with some initial clearance evident by the decline at 7 days. However the number of penetrations in both spaces significantly increased after day 7 and was still elevated 56 days after the initial aspiration. Data from animals given a single aspiration dose of 80 μg. As indicated by the asterisk, the number of subpleural tissue penetrations at 1 day was significantly different from the number of penetrations at 7, 28 and 56 days. (Mean ± SE, N = 8).

Carbon Nanotubes

Advantages and Applications

- New form of manufactured carbon fibre
- Hexagonal arrangement of carbon atoms built up to form a fibre with diameter in the nano range
- Extraordinary physicochemical characteristics
  - Exceptional strength, electrical and thermal conductance
- Generally assumed that carbon nanotubes are no more harmful than graphite





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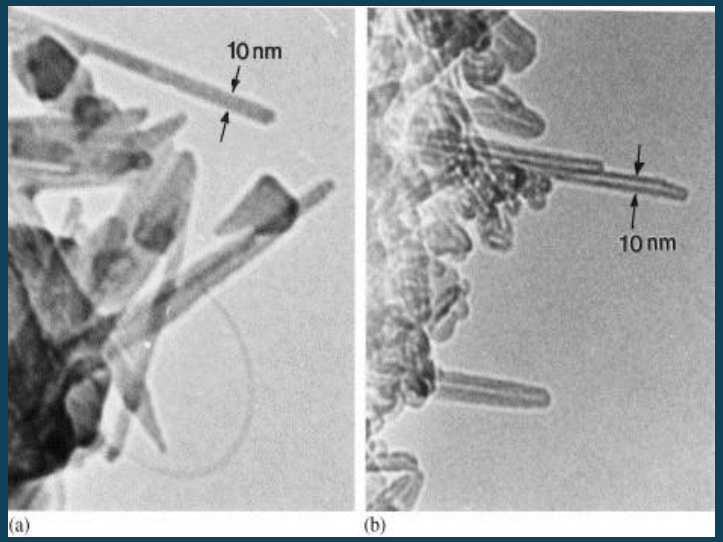
Nanotubes have always been around, produced by combustion

CNT: accidental production



From 10,000 year – old ice melt water

From lean burning flame (methane plus air).

'….Particulates extracted from a single section of a 10,000 year-old ice core melt ….. Particularly significant were the presence of carbon nanotubes and fullerene nanocrystals composing aggregated particulates reflecting global combustion products similar to contemporary, airborne carbon nanocrystal aggregates..'.[1]

CNT – industrial production

Global market for carbon nanotubes is predicted to grow to over $1 billion by 2014[2]

1) Murr, L. E., Esquivel, E. V., Bang, J. J., de la Rosa, G., and Gardea-Torresdey, J. L. (2004). Chemistry and nanoparticulate compositions of a 10,000 year-old ice core melt water. Water

2) Thayer, A. M. Carbon nanotubes by the metric ton: Anticipating new commercial applications, producers increase capacity. Chem. Eng. News 85, 29-38 (2007)

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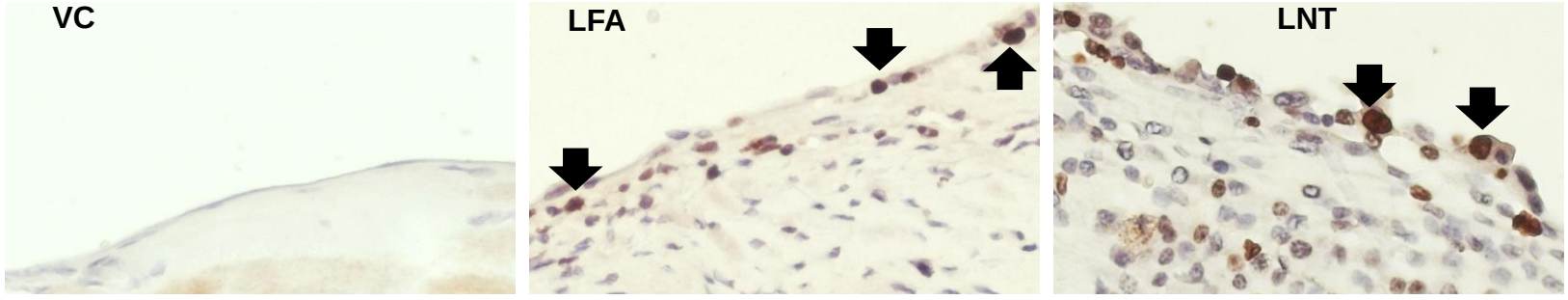
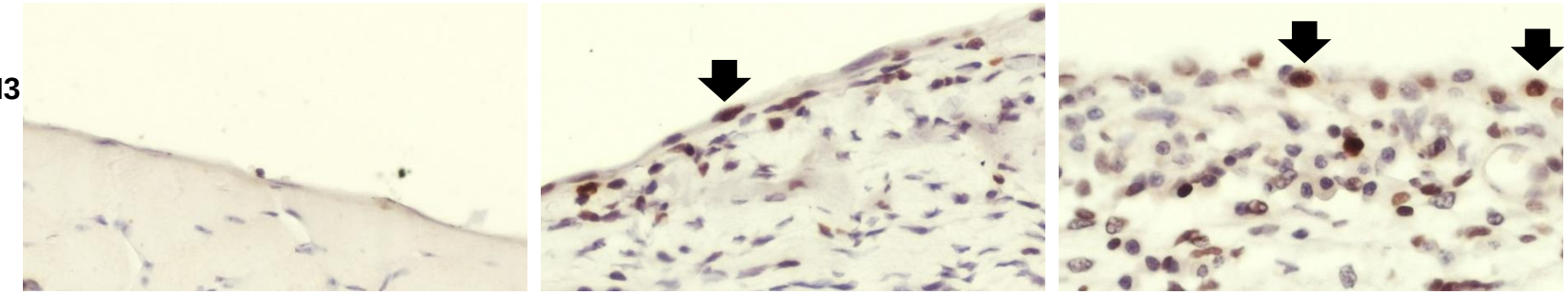
Progression of Lesions at 6 Months Post-Injection: LNT-induced Mesothelioma at 1 Year Post-Injection?

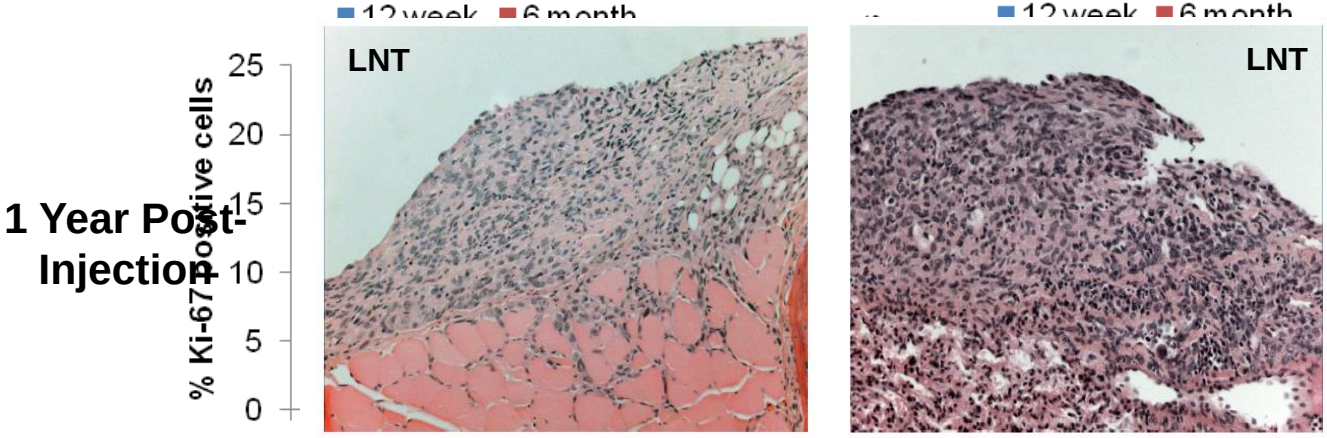
MRC Toxicology Unit



Ki-67



p-Histone H3



1 Year Post-Injection

610/2014: Parietal pleura with spindle cell proliferation x 20

610/2014: Visceral pleural tumour predominantly composed of spindle cells. x 20

Dissecting the Molecular Changes in MPM - a Disease linked with direct Fibre Exposure

MPM Patient Samples
(late stage disease)

Freshly-derived MPM cell lines

CAFs; Tumour-associated Macrophages

3D Organotypic Model - MPM Explants

Mouse Models
(early and late stage disease)

<2 Year Study –
- Fibrotic/Inflammatory Lesions
- Mesothelioma?

Modulation of Lesion Development

- **Mechanism of Fibre-induced Carcinogenesis**
- **Carbon Nanotubes Hazard Mechanism Study**