GERMAN FEDERAL INSTITUTE FOR RISK ASSESSMENT

20 YEARS

BfR
German Federal Institute for Risk Assessment

"Establishing an *in vitro* fiber toxicity test strategy to support risk assessment and facilitate grouping"

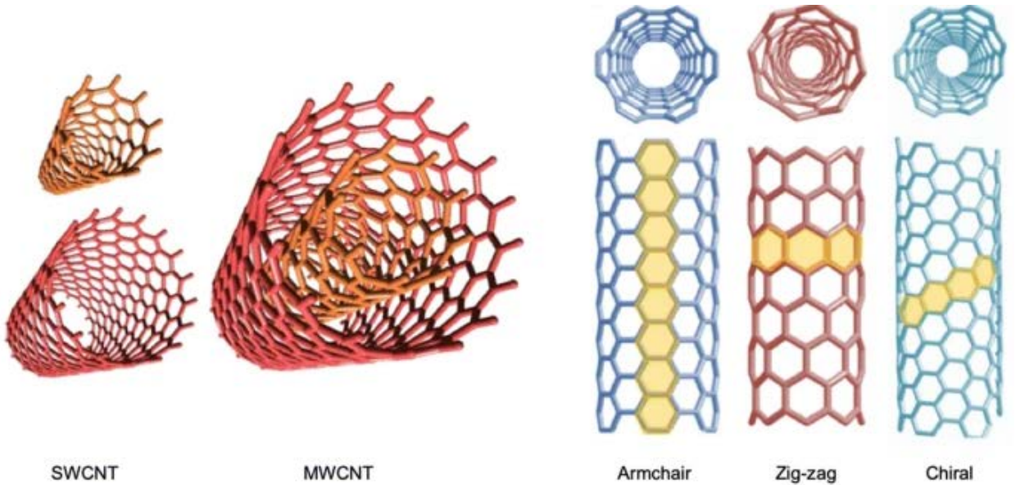
4th Joint Symposium on Nanotechnology

Rico Ledwith

Carbon Nanotubes (CNTs)

- **CNTs** are tubular structures made of layer(s) of graphene
- Single-walled CNTs **(SWCNTs):** Single graphene sheet rolled up
- Multi-walled CNTs **(MWCNTs):** Consisting of multiple sheets forming concentric cylinders

CNT classifications:



Negri, V et al., *Top Curr Chem (Z)* 378, 15 (2020). DOI: 10.1007/s41061-019-0278-8

- **Properties** that make **MWCNTs** useful for industrial applications
  - Tensile strength
  - Electrical and thermal conductivity

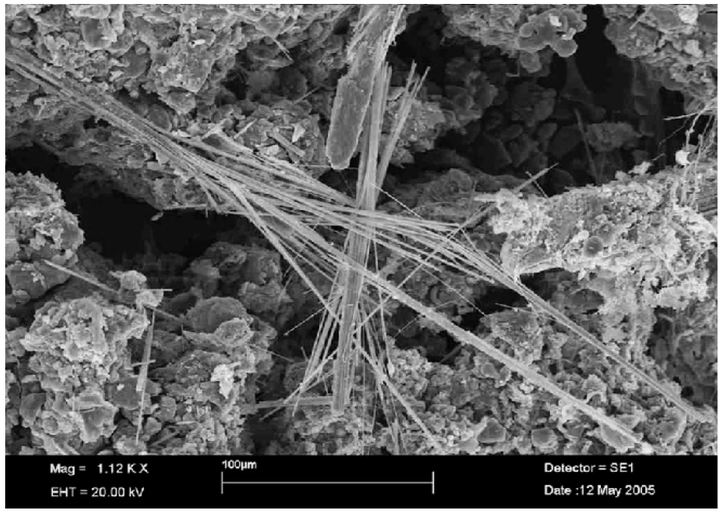
- Fiber - shaped nanomaterials are also referred to as High-Aspect Ratio Nanomaterials **(HARNs)**

- **Usages**
  - Antistatic and electro-paintable thermoplastics
  - Anti-fouling coatings
  - Batteries (Li-ion)
  - Structural composites (*e.g.*, for windmill blades and high performance sporting goods)
  - Possibly printed electronics (conductive inks) and conductive coatings for displays and touch screens

Concerns

- **MWCNT**s may elicit similar toxicity to highly pathogenic **asbestos** fibers due to their morphological similarity
- Inhalation is the main route of exposure

Amphibole class: Amosite (needle-like fibers)



SEM image of amosite fibers detached from an asbestos cement roof

Campopiano et al. *Annals of Occupational Hygiene*, Vol 53 (6) 2009, DOI: 10.1093/annhyg/mep036

- Asbestos is banned in the EU
  - Amphiboles - 1991
  - Serpentines - 2005

Asbestos associated pathologies:

- **Fibrosis:** Long term inflammation and scarring of the lungs
- **Lung Cancer:** Malignant neoplasm of the lung arising from the epithelium
- **Mesothelioma:** Cancer arising from the transformation of **mesothelial cells** lining the thoracic (pleura) or the abdominal (peritoneum) cavities
  - Aggressive, non-curable cancer
  - Uniquely associated with asbestos exposure
  - **Long latency period!**

What physiochemical features render a fiber pathogenic?

→ **Fiber pathogenicity paradigm**

What properties of fibers imbue them with pathogenicity?

❖ **Fiber Pathogenicity Paradigm (FPP):** Structure toxicity model that predicts whether a fiber is, or is not pathogenic

World Health Organization **(WHO)** Criteria:

1. Inhalable diameter (D_{ae} < 3 µm):

- Deposition in the distal lung beyond ciliated airways

2. Length (> 5 µm):

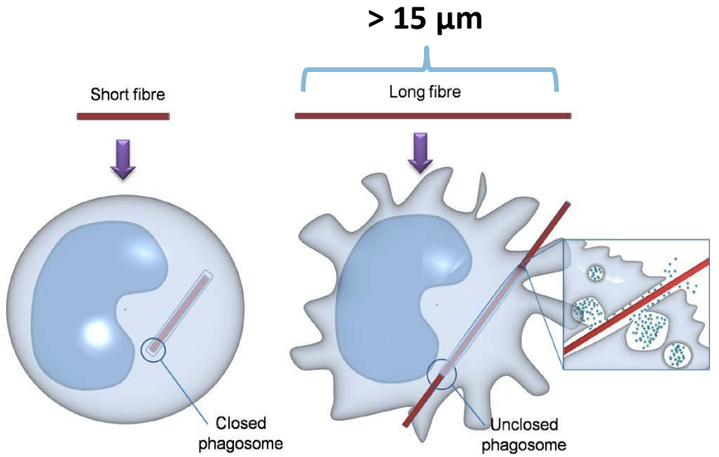
- Escape clearance mechanisms, e.g.:
  - **Alveolar Macrophages (> 15 µm):** Long fibers can not be engulfed

→ **Frustrated Phagocytosis!**

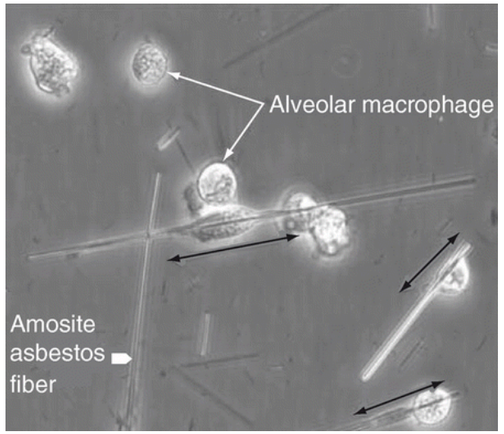
3. Aspect Ration: > 3:1

4. Biopersistance:

- Leads to prolonged retention in lung and accumulation over time
- Pathogenic fibers are typically not dissolvable



Schinwald, A.et al. *Part Fibre Toxicol* 9, 34 (2012). doi.org/10.1186/1743-8977-9-34



Frustrated phagocytosis observed for amosite asbestos by NR8383 rat alveolar macrophages

Donaldson et al., *Nanomedicine*, doi.org/10.2217/nnm.10.139

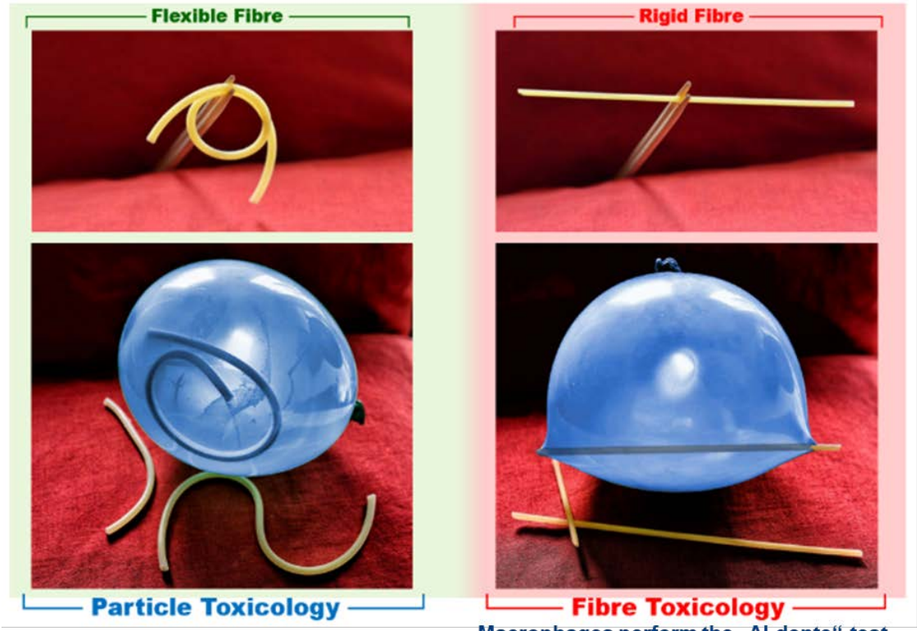
Very long fibers cannot be fully engulfed by macrophages, causing frustrated phagocytosis → **Inflammation** → **Cancer development**

Challenges of nanofibers

❖ Nanofibers pose a challenge to the WHO fiber criteria

Classical FPP: Respirable, long and biopersistent fibers are carcinogenic

Rigidity hypothesis: Only long, rigid nanofibers act as fibers while flexible (i.e. thin) nanofibers curl up



Macrophages perform the „Al-dente"-test
(Images by Asmus Meyer-Plath, BAuA)

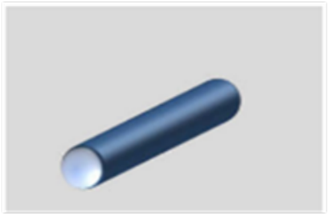
- *In vivo* studies on MWCNTs indicated a diameter threshold for carcinogenicity **> 30 nm**

❖ Further implications of secondary structures

- Some types of non-rigid MWCNTs can form highly ordered **secondary structures**
- **Aligned bundles** comply with the WHO criteria

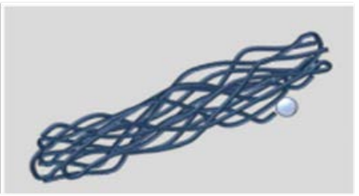
  → **Fiber paradigm might apply**

Primary structure:



Singlet tube

Possible secondary structure:



Aligned bundle

Simonow, B.K. et al. *J Nanopart Res* 20, 154 (2018). DOI: 10.1007/s11051-018-4262-y

Regulatory activities on MWCNT by Germany (BfR)

Rigid MWC(N)T (diameter > 30 nm)

- Harmonised classification (2018-21)

Classification and labelling should be harmonised throughout the EU to ensure a high level of protection of human health.

→ Fiber-like pathomechanism

Carc. 1B, "*Presumed human carcinogen*"

STOT-RE 1, Specific Target Organ Toxicity – Repeated Exposure, lung by inhalation
(„*Substance produces significant toxicty*")

Enables authorities to conduct further regulatory risk management.
E.g. substitution of CMR substances

Tangled MWCNT (diameter < 30 nm)

- Substance Evaluation (SEv, 2019)

For clarifying whether their use poses a risk to human health.

During the SEv additional information can be requested from the registrants of the substance to verify the suspected concern, if necessary.

Registrants applied **grouping and read across approaches** to avoid additional testing.

What is Grouping?

- ❖ Testing of each nanomaterial for their potential adverse effects is virtually impossible
- ❖ Grouping is the most commonly used alternative approach for filling data
- ➢ Structural similarity allows to predict properties of substances without having to test them all for each endpoint (‘read-across’)

Endpoint-specific grouping

- For toxicity evaluation (endpoint-specific category building)

Endpoint	
Acute toxicity	
Sub-chronic toxicity	
Mutagenicity	
Skin sensitization	
….	

- o A high aspect ratio and potential biopersistence are key factors for the toxicity and pathogenicity of fibres, these properties can be used for grouping
- o Frameworks have been developed in the form of integrated approach to testing and assessment **(IATA)** to support this grouping

GRACIOUS grouping framework

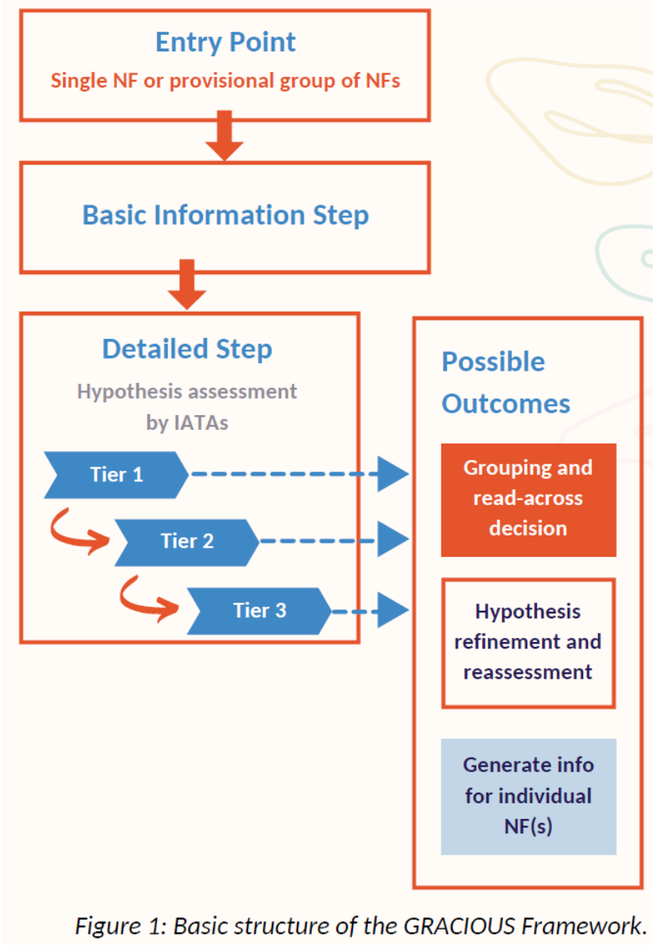
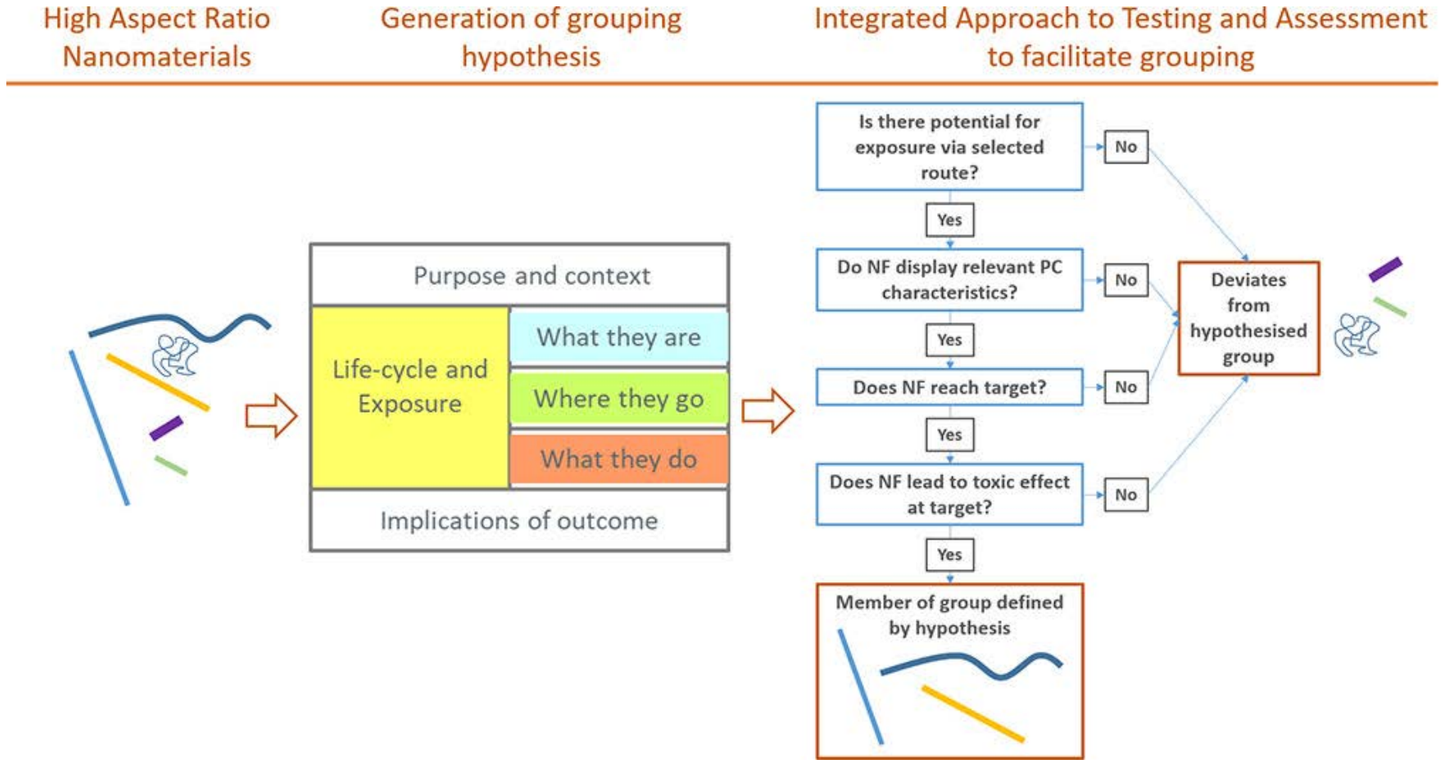


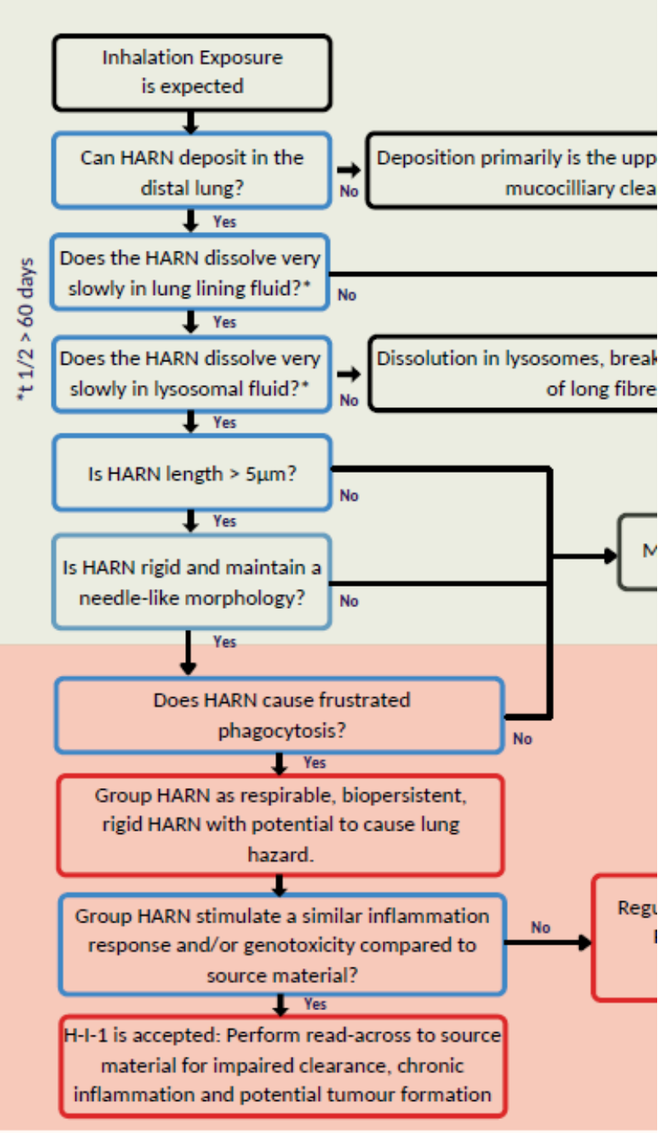
Figure 1: Basic structure of the GRACIOUS Framework.

GRACIOUS Framework Guidance Document



IATA consisting of multiple **decision nodes** (blue boxes) are used to collate the required information to test a grouping hypothesis

Testing strategies and uncertainties



GRACIOUS Framework Guidance Document

Pre-defined HARN hypothesis:

"Respirable, biopersistent, rigid HARN: Following inhalation exposure, long-term pulmonary retention of HARNs can occur, resulting in lung toxicity"

Uncertainty associated with HARN IATA

- How to measure rigidity?
  - No standardized or validated method available
- How to measure inflammation that is fiber specific?
- How to measure frustrated phagocytosis?
  → Biomarker detection by means of omics

EU HARMLESS project

HARMLESS

Grant Agreement : 953183

Advanced **H**igh **A**spect **R**atio and **M**ulticomponent Materials: Towards comprehensive intel**L**igent t**E**sting and **S**afe by design **S**trategies

Coordination: Helmholtz Zentrum München (HMGU)
Partner: 20 (research, governmental, SME, industry) from 12 countries
Budget/ Funding: 8 Mill Euro, (EU NMBP-16)
Duration: 4 years (started 01.01.2021)

WP1: Data Collection and Data Management

WP2: Safety Assessment Strategies

WP3: New Approach Methodologies

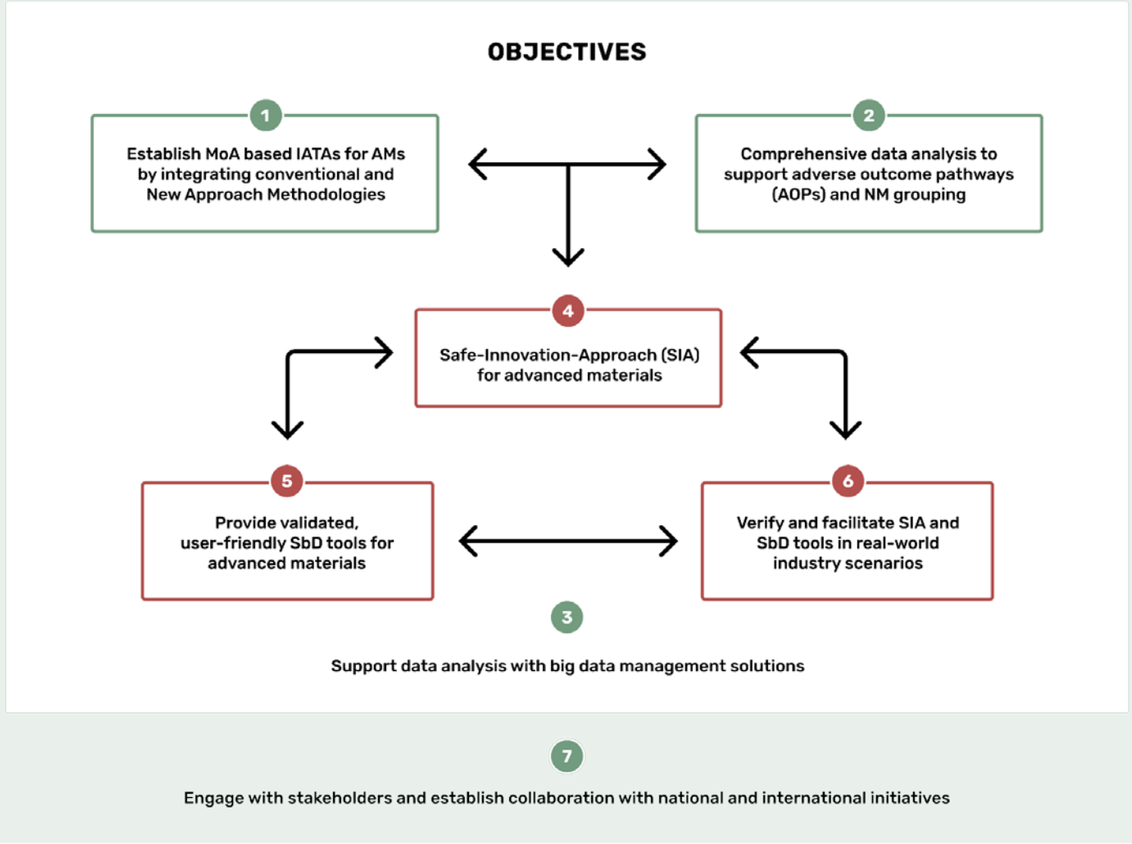
WP4: Safe Innovation Approach

WP5: DSS Tool Development

WP6: SbD Case Studies

WP7: Stakeholder Engagement

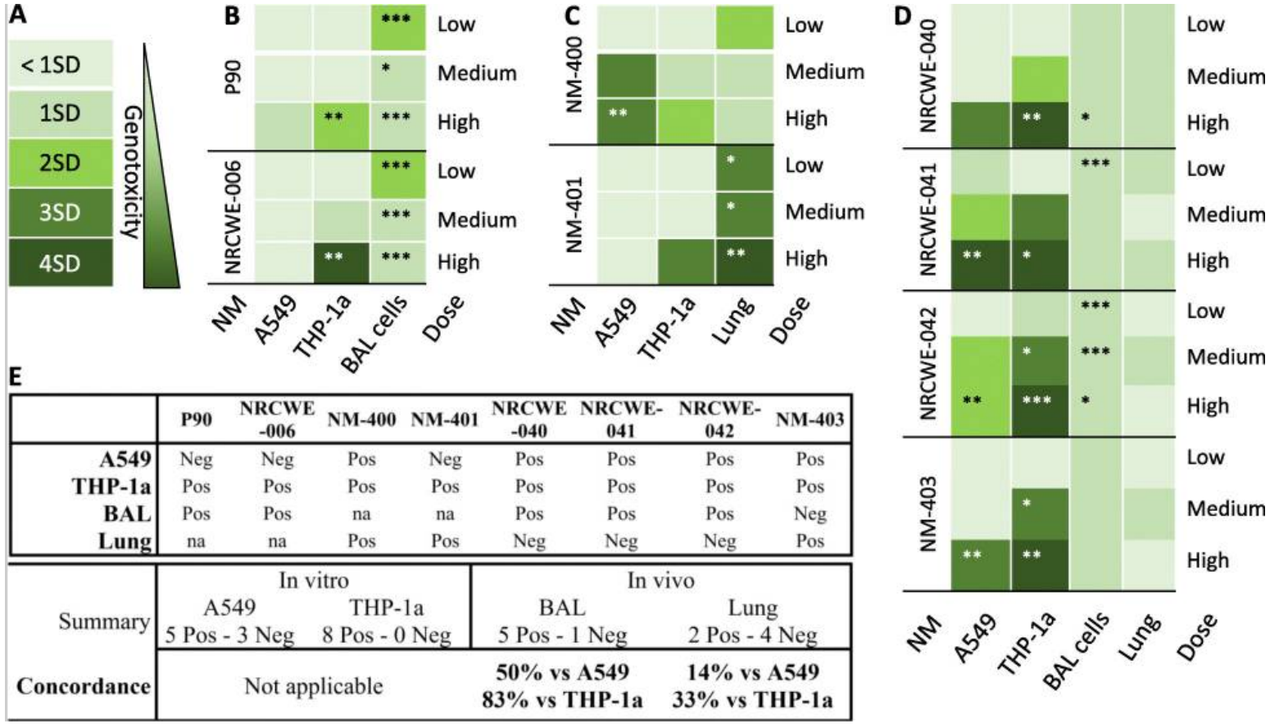
WP8: Coordination and Management



Materials

- **MWCNT**
  - Various morphologies (Thin, Thick, Short)
  - NM-400 / NM-401
  - **Mitsui – 7**
    - Benchmark material for a carcinogenic fiber

- More fibers will be investigated
- Investigation of case study materials (Perovskites, Silicas, Imogolites)



E

	P90	NRCWE -006	NM-400	NM-401	NRCWE -040	NRCWE-041	NRCWE-042	NM-403
A549	Neg	Neg	Pos	Neg	Pos	Pos	Pos	Pos
THP-1a	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
BAL	Pos	Pos	na	na	Pos	Pos	Pos	Neg
Lung	na	na	Pos	Pos	Neg	Neg	Neg	Pos

	In vitro		In vivo	
Summary	A549	THP-1a	BAL	Lung
	5 Pos - 3 Neg	8 Pos - 0 Neg	5 Pos - 1 Neg	2 Pos - 4 Neg
Concordance	Not applicable		**50% vs A549 83% vs THP-1a**	**14% vs A549 33% vs THP-1a**

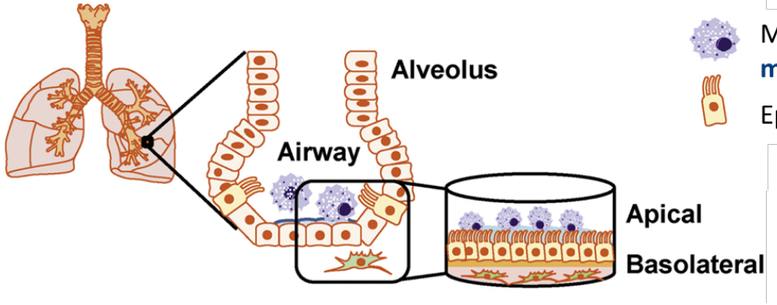
Di Ianni, *Part Fibre Toxicol.*, 2021, doi: 10.1186/s12989-021-00413-2

Project partners provide:
- Physiochemical characterization data
- *In vivo* data

Cell models

The air-blood barrier:

- Alveolar Epithelium acts as a **diffusional barrier**
- Macrophages represent an **immunological barrier**



❖ Step 1: Monocultures *(my task)*

A549: Human, epithelial adenocarcinoma cells

- **Advantages:** Widely used as a TII pulmonary epithelial cell model
- **Disadvantages:** Do not form **functional tight junctions** (no barrier) / Cancer cells

THP-1: Human acute monocytic leukaemia cell line

- **Advantages:** Can be differentiated to macrophages –like cells with phorbol 12-myristate-13-acetate **(PMA) → dTHP-1**
- **Disadvantages:** Cancer cells

hAELVi: Human alveolar epithelial lentivirus immortalized

- **Advantages:** Tight junction formation / Physiologically closer to alveolar cells
  High trans-epithelial electrical resistance (> 1000 Ω*cm²)

Primary monocytic cells

- **Advantages:** Can be differentiated to macrophages like cells
- **Disadvantages:** Slow proliferation / finite lifespan

Proteomics

- Proteomics is applied to mechanistically unravel the mode of action **(MoA)** of HARNs

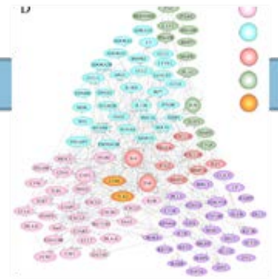
Mechanistic Toxicology Data
(Omics, Biomarkers)

Sample Preparation:

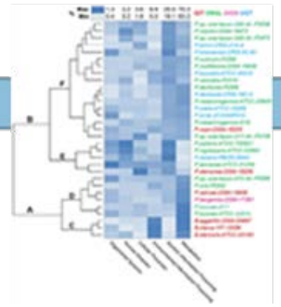
High Throughput Proteomics

Shotgun proteomics

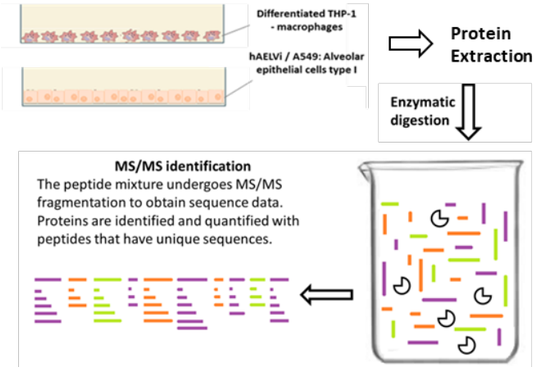
Analysis of protein sets and signatures networks



KEGG Pathway Analysis



Bottom – Up Proteomics



Wikipedia

Outlook: Validation

- Phagocytosis inhibitors: Cytochalasin D
- Western Blots
- Confocal microscope imaging, e.g. of cytoskeletal proteins

Goal:

- Elucidate a biomarker that indicates fiber-specific pathology *in vitro*

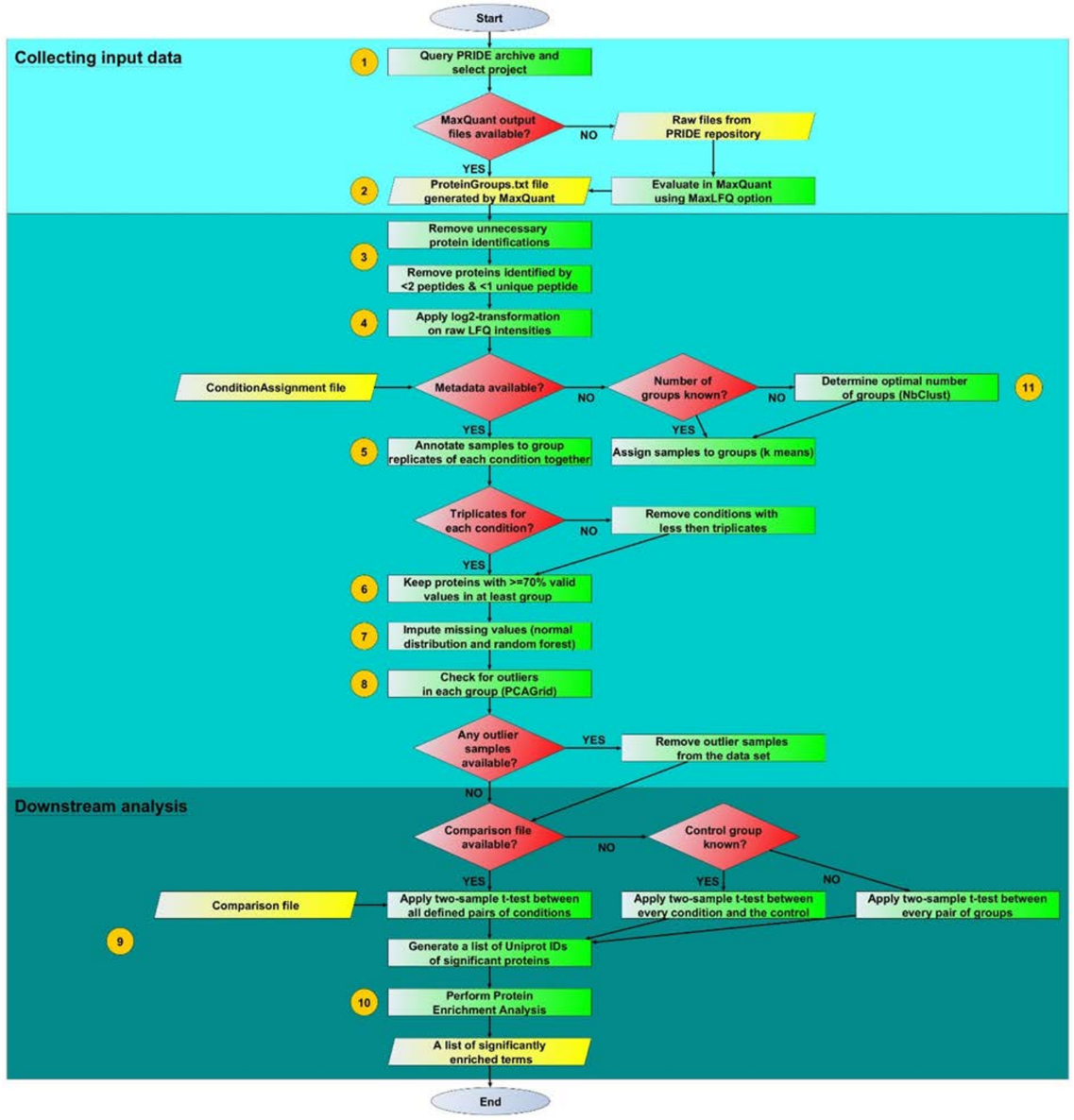
PROTEOMAS workflow

NanoInformaTIX workflow for automated data analysis

Allows for automated handling of:

- Missing meta data
- Data imputation for missing values
- Outlier detection

- First measurements are completed
- Data analysis is ongoing



Outlook

Comparative assessment of altered pathways:

- Regression modelling - Identify the key factors driving changes in the proteome linked to phys. chem. properties
- Analysis of observed proteomic alterations matching to publicly available data (E.g. transcriptomic data / NM proteome data / aggregated lung proteome alterations)
- Comparative analysis to Adverse Outcome Pathways (AOP) for fibrosis and frustrated phagocytosis induced cancer (**AOP 173 / 303**)
  → Substantiate existing key events / identify candidates of additional key events

Acknowledgment

HARMLESS

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The BfR team: staff and technical assistants

Group 76

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Thank you for your attention

Identify Risks –
Protect Health

Rico Ledwith

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