The use of Adverse Outcome Pathways (AOPs) in nanotoxicology (SmartNanoTox)

Ulla Vogel, professor
Nanosafety at the National Research Centre for the Working Environment

- Government research institute under the Ministry of Employment
- Nanosafety as strategic research area since 2005
- At present 35 persons in nanosafety research
- Advisors for the Danish Working Environment Authorities, EPA, EU, OECD, WHO
- Past and present partners in 20 EU projects on (nano)particle safety including NanoReg1 and 2
SmartNanoTox has focus on inhalation of nanomaterials

Inhalation

Ingestion

Through the skin

IV: nanomedicine
Inhalation of particles affects health: Soot and death per 1000 person year before and after ban on coal in Dublin

Soot: -70%
Pulmonary related deaths: -15%
All cause deaths: 6%
Heart related deaths: -10%

The vision

SmartNanoTox AOPs & assays for testing of toxicity

Mechanism-based understanding of toxicological effect

Evidence-based risk assessment. Prediction of toxicological effects on the basis of information on physico-chemical properties

Grouping and ranking for regulation Safe-by-design for innovation

Safe use of nanomaterials including high volume nanomaterials
Lung deposition is determined by particle size.
Low clearance of nanoparticles from the lung

Airways

- Fine particles
  - Particle deposition and removal by mucociliary clearance
  - Particle phagocytosis by macrophage

- Nanoparticles
  - Ultrafine particles
  - Inflammation
  - Release of mediators
  - ROS

Inflammatory cells and inflammatory mediators

Marianne Dybdahl
Inhaled TiO$_2$ nanoparticles in the lung are removed very slowly

Mice inhaled 40 mg/m$^3$ nanosized TiO$_2$ 1 hour daily for 11 days.

TiO$_2$ content in lung tissue was measured by ICP-MS.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Days after exposure</th>
<th>N</th>
<th>TiO$_2$ in lung (mg/kg) (mean ± sd)</th>
<th>Procent of deposited dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO$_2$</td>
<td>5</td>
<td>3</td>
<td>63 ± 10</td>
<td>24%</td>
</tr>
<tr>
<td>Air</td>
<td>5</td>
<td>3</td>
<td>&lt; 8</td>
<td></td>
</tr>
<tr>
<td>TiO$_2$</td>
<td>25</td>
<td>3</td>
<td>55 ± 30</td>
<td>21%</td>
</tr>
<tr>
<td>Air</td>
<td>25</td>
<td>3</td>
<td>&lt; 1</td>
<td></td>
</tr>
</tbody>
</table>

Hougaard et al, 2010, PF&T
Inhalation of nano-TiO$_2$ results in long lasting inflammation

After 5 days

After 4 weeks

Types and numbers of cells in lung fluid

Hougaard et al, 2010, PF&T
Known health effects of (nano)particle inhalation exposure

- Inflammation (all insoluble nanoparticles)
- Cardiovascular disease (air pollution, welding fumes)
- Fibrosis (Quartz, carbon nanotubes)
- Lung cancer (carbon black, titanium dioxide, one carbon nanotube)
- Acute lung toxicity (Surface treatment spray products)

- SMARTNANOTOX DEVELOPS ADVERSE OUTCOME PATHWAYS (AOPs) FOR THESE HEALTH OUTCOMES
SmartNanoTox will develop Adverse Outcome Pathways for nanomaterial-induced toxicity

- An **adverse outcome pathway (AOP)** is a structured representation of biological events leading to adverse effects and is considered relevant to risk assessment.

- The AOP links in a linear way existing knowledge along one or more series of causally connected **key events (KE)** between two points — a **molecular initiating event (MIE)** and an **adverse outcome (AO)** that occur at a level of biological organization relevant to risk assessment. The linkage between the events is described by **key event relationships (KER)** that describe the causal relationships between the key events.

https://aopwiki.org/
AOP 173: Increased substance interaction with the resident cell membrane components leading to lung fibrosis

https://aopwiki.org/aops/173
Sabina Halappanavar
AOP 237: Secretion of inflammatory cytokines after cellular sensing of the stressor leading to plaque progression

https://aopwiki.org/aops/237
Sarah Søs Poulsen
SmartNanoTox ambitions

- Submit 5 AOPs to OECD AOP sponsorship program
- AOP 173 has been approved
- AOP 237 has been submitted
- 3 more are under development in SmartNanoTox
- Next step:

**PATROLS:** Focus on in vitro assays to detect KEs

In vitro assay

\[ \text{Nanomaterial Properties} \rightarrow \text{iQSAR} \rightarrow \text{KE/MIE} \rightarrow \text{AOP} \rightarrow \text{Adverse Outcome} \]
An Example: AOP for ENM-induced risk of developing atherosclerotic plaques
Inhalation of TiO$_2$ NP induced inflammation and acute phase response in mice. Acute phase genes were the most differentially expressed genes in lung tissue.

**TABLE II.** List of all Acute Phase Response Genes Showing Fold Changes Higher Than 1.2 in exposed mice

<table>
<thead>
<tr>
<th>Acute phase reactants</th>
<th>P value</th>
<th>Fold change$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum amyloid A1</td>
<td>0.00</td>
<td>2.24</td>
</tr>
<tr>
<td>Serum amyloid A3</td>
<td>0.00</td>
<td>4.71</td>
</tr>
<tr>
<td>Complement protein C3</td>
<td>0.00</td>
<td>1.37</td>
</tr>
<tr>
<td>Complement component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, s (C1s)</td>
<td>0.00</td>
<td>1.28</td>
</tr>
<tr>
<td>Complement component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3a receptor 1 (C3ar1)</td>
<td>0.00</td>
<td>1.15</td>
</tr>
<tr>
<td>Complement component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, q beta polypeptide (C1qb)</td>
<td>0.00</td>
<td>1.30</td>
</tr>
<tr>
<td>Complement component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, r subcomponent (C1r)</td>
<td>0.00</td>
<td>1.31</td>
</tr>
<tr>
<td>Complement component</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1RB (C1rb)</td>
<td>0.00</td>
<td>1.21</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>0.01</td>
<td>2.05</td>
</tr>
<tr>
<td>Coagulation factor II (F2)</td>
<td>0.01</td>
<td>1.72</td>
</tr>
<tr>
<td>Mannose binding protein</td>
<td>0.02</td>
<td>1.70</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.01</td>
<td>1.79</td>
</tr>
<tr>
<td>apoA1</td>
<td>0.01</td>
<td>1.51</td>
</tr>
<tr>
<td>apoAII</td>
<td>0.03</td>
<td>1.61</td>
</tr>
<tr>
<td>alpha2-HS glycoprotein</td>
<td>0.00</td>
<td>1.85</td>
</tr>
<tr>
<td>S100A8 (calgranulin A)</td>
<td>0.01</td>
<td>$-1.85$</td>
</tr>
<tr>
<td>Serpin3n</td>
<td>0.00</td>
<td>1.37</td>
</tr>
</tbody>
</table>

Gene names in bold indicate FDR adjusted $P$ value $>0.05$.

$^a$Average fold change compared with matched controls.
The acute phase response: A risk factor for cardiovascular disease

- The acute phase response is the systemic response to acute and chronic inflammatory states caused by fx bacterial infection, trauma and infarction.

- Conditions that induce acute phase response are associated with risk of cardiovascular disease, including asthma and air pollution exposure.

Figure 1. Characteristic Patterns of Change in Plasma Concentrations of Some Acute-Phase Proteins after a Moderate Inflammatory Stimulus. Modified from Gitlin and Colten with the permission of the publisher.

Gabay and Kushner 1999, NEJM
Acute phase proteins CRP & SAA are associated with risk of CVD in prospective epidemiological studies

Nurses’ Health Study: 120,000 participants

**Table 3. Relative Risk of Cardiovascular Events According to Base-Line Plasma Levels of Markers of Inflammation and Lipids.**

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>QUARTILE OF PLASMA LEVEL</th>
<th><strong>P VALUE FOR TREND</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>High-sensitivity C-reactive protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median — mg/dl</td>
<td>0.06</td>
<td>0.19</td>
</tr>
<tr>
<td>Relative risk (95% CI)</td>
<td>1.0</td>
<td>2.1 (1.0–4.5)</td>
</tr>
<tr>
<td>Serum amyloid A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median — mg/dl</td>
<td>0.25</td>
<td>0.43</td>
</tr>
<tr>
<td>Relative risk (95% CI)</td>
<td>1.0</td>
<td>1.8 (0.9–3.6)</td>
</tr>
</tbody>
</table>

Ridker et al. 2000, NEJM
Proposed mechanism of action

Inhaled particles promote atherosclerosis via acute phase response

Inhalation of (nano)particles → Systemic circulation of acute phase proteins → Inflammatory & acute phase response → Atherosclerosis → Cardiovascular disease

New knowledge

Established fact
### Time- and dose-dependent pulmonary acute phase response in mice

**TABLE 1 | Differential Expression of Murine Acute Phase Genes and Saa3 Expression Levels after Exposure to Different Nanomaterials and at Different Time Points**

<table>
<thead>
<tr>
<th>Post Exposure Day</th>
<th>1</th>
<th>3</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose/Animal</td>
<td>18 μg</td>
<td>54 μg</td>
<td>162 μg</td>
</tr>
<tr>
<td></td>
<td>18 μg</td>
<td>54 μg</td>
<td>162 μg</td>
</tr>
<tr>
<td><strong>TiO$_2$ nanoparticles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N acute phase genes$^1$</td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Fold increase of Saa3 mRNA$^2$</td>
<td>1.8</td>
<td>87</td>
<td>368</td>
</tr>
<tr>
<td><strong>Carbon Black nanoparticles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N acute phase genes$^1$</td>
<td>0</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Fold increase of Saa3 mRNA$^2$</td>
<td>63</td>
<td>237</td>
<td>294</td>
</tr>
<tr>
<td><strong>Multiwalled Carbon nanotubes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N acute phase genes$^1$</td>
<td>5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Fold increase of Saa3 mRNA$^2$</td>
<td>52</td>
<td>151</td>
<td>95</td>
</tr>
</tbody>
</table>

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Saber et al. 2014  
WIREs Nanomed nanobiotech
Close correlation between pulmonary acute phase response and pulmonary inflammation across particles, doses, time points

Saber AT et al, Plos One, 2013
Plasma levels of acute phase protein SAA3 correlates with lung responses

Saa3 mRNA in lung correlates with plasma SAA3

Plasma SAA3 levels and neutrophil influx

\[ y = 0.5148x - 0.0018, \quad R^2 = 0.5435 \]

\[ y = 0.379x + 3.983, \quad R^2 = 0.660, \quad P < 0.0001 \]

Poulsen et al 2015, TAAP

Poulsen et al. 2017, Plos One
SAA: an acute phase protein that directly promotes formation of foam cells

- SAA can replace ApoA-1 as the major HDL protein.
- This inhibits HDLs role in reverse cholesterol transport.
- SAA induces foam cell formation in macrophages [1].

[1] Lee et al, 2013, BBRC
Acute phase protein SAA is causally implicated in plaque progression

- Mice have 3 inducible SAA isogenes (Saa1, Saa2, Saa3)

- Over-expression of SAA3 increases plaque progression (Thompson 2018)

- Inactivation (KO) of all SAA isogenes results in reduced plaque progression (Thompson 2018)
Any Human relevance ?: Yes; inhalation of ZnO induces acute phase response in human volunteers

Study set up:
- 16 volunteers
- Exposed to 0, 0.5, 1 or 2 mg/m$^3$ ZnO particles for 4 h
- OEL: 5 mg/m$^3$ for 8 h
- Acute phase response proteins CRP and SAA

Acute phase response was induced after ZnO inhalation at concentrations well below current OEL

Adapted from Monsé et al Part Fibre Toxicol. 2018 15(1):8
Summary: The Adverse Outcome Pathway for particle-induced cardiovascular disease

Inhaled particles promote atherosclerosis via acute phase response

Inhalation of (nano)particles → Systemic circulation of acute phase proteins → Atherosclerosis → Cardiovascular disease

New knowledge

Inflammatory & acute phase response

Established fact
Acknowledgments and funding

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Sabina Halappanavar
Health Canada

SmartNanoTox
Smart Tools for Gauging Nano Hazards

caLIBRAte
nano risk governance

PATROLS
Advanced Tools for NanoSafety Testing