Deliverable Report for Grant Agreement Number 760813

Deliverable D2.5

Identification of key events with predictive value for effects due to chronic ENM exposure

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Additional beneficiary members: NRCWE, MISVIK, BASF, HC

Dissemination Level:		
PU	Public	X
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1. Description of task

An adverse outcome pathway (AOP) is a linear description of a toxicological process linking a molecular initiating event (MIE), in which a stressor first perturbs the biological system (e.g., a molecular interaction between a xenobiotic and a specific biomolecule), through a series of intermediate or key events (KEs), to the adverse outcome (AO), i.e. an adverse effect, on health for example [1]. AOPs are based on existing knowledge (*in vivo*, *in vitro*, or computational systems), are generally a sequential series of events and represent plausible hypotheses of important events, relevant to risk assessment [2, 3]. AOPs are substance-agnostic and KEs are not AOP-specific. KEs must be essential for the AO to occur, thus describing causal relationships, and are defined as measurable changes in a biological state [4].
In AOPs, knowledge is organized in a way that the information can be used for risk assessment, from which key uncertainties and research priorities can be identified, and through which predictive approaches needed to advance regulatory (eco)toxicology can be improved [2].

Task 2.5 Identification of mechanistic key events linked to AOPs; (LTAP, HC, NRCWE, MISVIK, BASF); M2-28
"This task will identify mechanistic key events (KE) for the design and refinement of AOPs induced by the chronic oral and/or inhalation exposure to ENM (engineered nanomaterials). Existing *in vivo* toxicogenomics and toxicity data will be curated from partners (LTAP, HC) and mined in the literature and databases (Misvik). Meta-analyses will be conducted using integrative bioinformatics and predictive toxicology workflows, including advanced clustering algorithms, large-scale pathway analysis and network methods based on the use of various R/Bioconductor packages and gene set sources (e.g. KEGG, WikiPathways, Gene Ontology, transcriptional networks, Comparative Toxicogenomics Database and the MsigDB). This information will be connected to toxicity endpoints for validating established and putative AOPs, including the OECD AOP for liver fibrosis (AOP: 38 - Protein Alkylation leading to Liver Fibrosis) and the recently published AOPs for lung fibrosis built by partners involved in this task. An AOP workshop will be organized at M17 to inform Tasks 3.3 and 4.3 on the identified KEs/pathways enabling development of targeted *in vitro* bioassays with a high predictive value. In addition, novel AOP-targeted pathway analysis concepts based on the WikiPathways/ PathVisio platform will be explored using the data and pathways/KE identified by the meta-analyses to further refine our understanding, particularly in relation to data generated from studies applying realistic exposure scenarios. Archived tissue and tissues from Tasks 2.2 and 2.3 will be utilised to generate new complementary transcriptomics (HC) and toxicological (LTAP, BASF, NRCWE) data where gaps of knowledge are identified. Novel economical targeted omics strategies, such as tempO-seq (used within EU-ToxRisk) will be considered to reduce costs and increase throughput. Based on the AOPs and on the mechanistic KEs identified above, analyses will be performed on target tissues, mainly lung and liver. Candidate pathways include ENM-induced pro- and anti-inflammatory and fibrotic responses, and the carcinogenicity axis associated with hypoxic-like responses (e.g. HIF-1 and its target genes). New data will then be integrated in meta-analyses to further support existing hypotheses or identify new KEs of importance for chronic ENM exposure (Misvik). This information will be continually fed to Tasks 3.3 and 4.3 as it becomes available to refine the bioassays being developed in WP3 and 4. Additionally, pathways with a predictive potential will be validated by large-scale omics-based toxicity prediction analysis and will support the IVIVE work in WP6 (Task 6.5)."

2. Description of work & main achievements

2.1 Summary

AOPs are part of the current paradigm shift taking place within toxicology, i.e. to move from the assessment of apical endpoints in animals to the use of upstream molecular mechanisms and pathways tested in alternative cell-based systems and predictive of toxicity and disease in humans [5]. In 2012, the OECD launched a programme on the development of AOPs and established the AOPwiki (https://aopwiki.org/), which provides an open source platform for researchers to collaborate. OECD endorsement of the AOP concept has greatly facilitated implementation into regulatory thinking, which means that alternative methods developed in line with and linked to AOPs will be more likely to reach new levels of importance and recognition in regulatory risk assessment procedures. The AOP concept was originally developed in order to predict chemical risks to humans and the environment [2] and more recent efforts have aimed at application of the concept within nanosafety [6]. Even if the AOP concept is inherently compound agnostic, specific attention to the peculiarities and challenges associated with assessment of nanomaterial-induced toxicity is needed. An example includes particular attention to the MIE, which may manifest itself as less “molecular” for NMs as compared to chemicals. Nanomaterials are known to be involved in physical or mechanical damage of cell components, in contrast to chemicals which may initiate the AOP cascade through molecular interactions, such as ligand-receptor binding processes [6]. In addition, *in vitro* assays targeting specific KEs need to consider nanosafety-specific aspects.

Objective: The main objective of T2.5 was to develop/identify AOPs for ENM-induced AOs relevant for human health, to identify key events (KEs) useful as predictive markers of the AO and to feed WP3 and 4 (*in vitro* WPs) with this information to help decide which *in vitro* assays to prioritize.

Summary of the methodological approach: first, all partners worked on the strategy for completing the task. The first step was to identify AOs:

- (potentially) induced by ENM
- after inhalation or oral exposure,
- supported by sufficient evidence in regulatory (collected in T2.1) and other experimental studies,
- and relevant in the frame of PATROLS.

Existing AOPs (related to NM or not) and KEs were then identified on the AOP-Wiki (https://aopwiki.org/). According to their expertise, partners volunteered to work on specified AOs and used relevant literature to develop or refine new/existing putative AOPs and identify KEs and potentially predictive *in vitro* biomarkers/assays that could be prioritized for *in vitro* testing. This information was organized in tables and the selection of biomarkers was justified by the type of evidence available in the literature to suggest predictive value.

Summary of results: we identified relevant (potential) AOs for ENM toxicity and AOPs/KEs/potentially predictive *in vitro* markers to guide WP3 and 4 partners in their *in vitro* experiments. This was presented and discussed during the “AOP workshop” (Milestone 4) organized by LTAP in Brussels (May 14-15, 2019). A crosstalk was then established between T2.5 and WP3 and 4 partners to determine what KEs and

biomarkers are planned to be tested, how the current PATROLS work covers the AOPs/KEs, and to identify gaps. Data generated *in vitro* and further analyses conducted by PATROLS partners will allow a constant refinement of the AOPs, and will help to identify which biomarker(s) can be predictive of the AOs.

2.2 Methodological approach

2.2.1 Identification of ENM-relevant AOs
We first identified AOs (potentially) induced by ENM after inhalation or oral exposure (routes of exposure considered in PATROLS), supported by evidence in regulatory (collected in T2.1 and presented in D2.1) or other experimental studies.

2.2.2 Identification of nano-relevant AOPs
Existing AOPs (related to ENM or not) were then identified on AOPWiki (https://aopwiki.org/). A first list of publications was also proposed by experts to serve for AOP enrichment and identification of KE.

2.2.3 Identification of MIEs/KEs, biomarkers and assays potentially predictive of selected AOPs

According to their expertise, partners volunteered to work on some of the selected AOs (Table 1). Table 1 indicates which partners contributed to which AO (contributing partners), how they proposed to work on the identification of AOP(s), KEs and potentially predictive assays (methodological approach) and which partner led the work (leading partner).

Partners selected relevant literature/information based on their expertise to define or refine new/existing putative AOPs and identify KEs and potentially predictive *in vitro* biomarkers/assays that could be prioritized for *in vitro* testing. Information on the *in vitro* model and/or cell type to use to perform the assays was also included. The justification for the selection of biomarkers was given (type of evidence to suggest a predictive potential of the marker). The methodological approaches used by each leading partner are described below.

Table 1: Contribution of partners to the definition of nano-relevant AOPs.

Organ	AO	Contributing partners	Methodological approach(es) based on	Leading partner
Lung	**inflammation**	BASF	literature and correlation with in-house immunohistological staining for M1 and M2 macrophages	BASF
		NRCWE	own ongoing research and literature	
		MISVIK	data mining/integration and transcriptomics	
	fibrosis	LTAP	own past/ongoing research and literature	LTAP
		HC	development of AOP173; gene signature/classifier (enrichment method)	
		MISVIK	data mining/integration and transcriptomics	
	mesothelioma	LTAP	literature	LTAP
	cancer	NRCWE	literature	NRCWE
Liver	**inflammation**	MISVIK	data mining/integration and transcriptomics	MISVIK
	fibrosis	MISVIK	data mining/integration and transcriptomics	MISVIK
	cancer	NRCWE	own ongoing work and literature	NRCWE

2.2.3.1 AOP for lung inflammation (leading partner: BASF)

Inflammation is an important biological process involved in many target organ toxicities and it should be recognized as a highly connected, central node within the global AOP network [7]. In rodent inhalation studies with engineered nanoparticles, parameters indicative for inflammatory processes were often observed already after short-term inhalation exposure [8], while other AOs like fibrosis and tumor formation could only be observed in long-term studies. Within this task, inflammation in the lung was processed in detail as an AO because not all inflammatory processes result in fibrosis and tumor.

The AOP for lung inflammation was mostly based on the first KEs of AOP173 (https://aopwiki.org/aops/173, “Substance interaction with the lung resident cell membrane components leading to lung fibrosis”) that are linked to inflammatory processes (KEs 1493 “Increased Pro-inflammatory mediators”, 1496 “Increased, secretion of proinflammatory and profibrotic mediators”, 1497 “Increased, recruitment of inflammatory cells”, 1498 “Loss of alveolar capillary membrane integrity” and 1499 “Increased, activation of T (T) helper (h) type 2 cells”). BASF reviewed the literature [9-14] and identified several parameters that were also observed in *in vivo* inhalation studies in rats (Annex 2). Most of these parameters can be assigned to the KE 1496 ”Increased, secretion of proinflammatory and profibrotic mediators” as presented in AOP 173.

in vivo inhalation studies showed that alveolar macrophages (AMs) have a crucial role in pulmonary clearance as well as in orchestrating pulmonary immune responses. Data published during the past 3 decades suggest that these various activities are mediated by distinct subpopulations of macrophages, which are induced by signals they encounter in their local tissue microenvironment. In a rather simplistic view, these

subpopulations can be divided into 2 major distinct macrophage phenotypes, which have been categorized broadly as pro-inflammatory/cytotoxic M1 macrophages and anti-inflammatory/wound repair M2 macrophages. Increasing evidence suggests that nanomaterials are capable of activating macrophages to the M1 phenotype, leading to the expression of pro-inflammatory mediators and recruitment of inflammatory cells. In order to refine the existing AOP, we identified AM phenotype as M1 or M2 upon short-term inhalation exposure to different (nano)materials followed by a post-exposure period. AM phenotyping was retrospectively performed using immunohistochemistry. M1 (CD68+iNOS+) and M2 (CD68+CD206+ and CD68+ArgI+) AMs were characterized in formalin-fixed paraffin-embedded lung tissue of rats exposed for 6 h/day for five days to air, 100 mg/m³ nano-TiO_2, 25 mg/m³ nano-CeO_2, 32 mg/m³ multi-walled carbon nanotubes, or 100 mg/m³ micron-sized quartz. Figure 1 and 2 show examples of the immunohistological stain for M1 and M2 AMs, respectively [15].

During acute inflammation, relative numbers of M1 AMs were markedly increased, whereas relative numbers of M2 were generally decreased compared to control. Following an exposure-free period, changes in iNOS or CD206 expression correlated with persistence, regression or progression of inflammation, suggesting a role of M1/M2 AMs in the pathogenesis of pulmonary inflammation. However, no clear correlation of AM subpopulations with qualitatively distinct histopathological findings caused by different (nano)materials was found. A more detailed understanding of the processes underlying these morphological changes is needed, to identify biomarkers for different histopathological outcomes [15]. At the current stage, identifying AM subpopulation did not contribute to the refinement of the AOP.

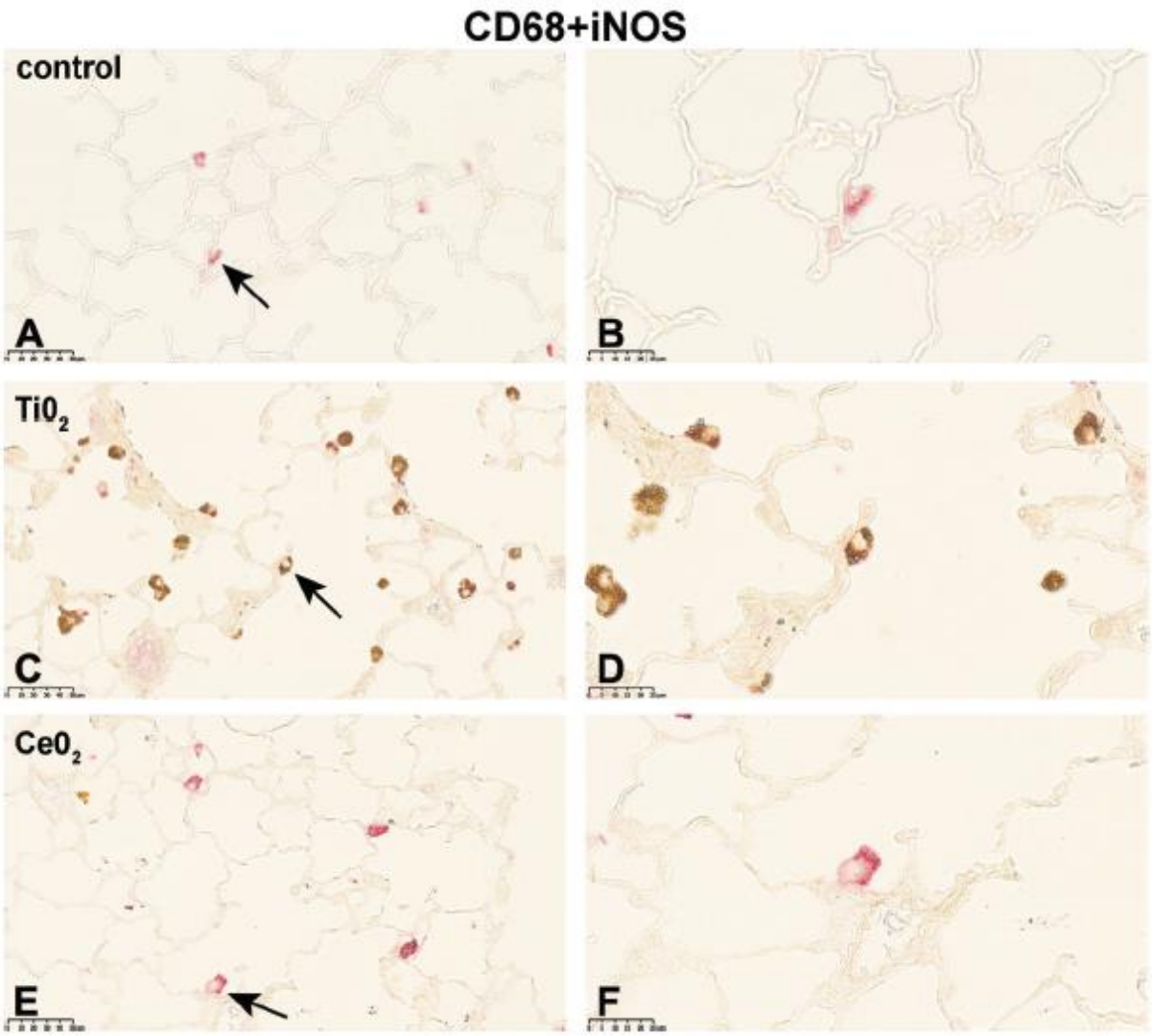

Figure 1 Micrographs of left lung sections of rats exposed to different (nano)materials for 5 days. Expression of iNOS (M1 marker) shortly after the last exposure was visualized by immunohistochemistry. Binding of antibodies was visualized using a red chromogen for the AM marker CD68 and a brown chromogen for the M1 marker iNOS (A, C, E). Arrows indicate AMs shown with higher magnification in (B, D, F). Representative sections from each treatment group are shown. Controls were exposed to air only. AM: alveolar macrophages.

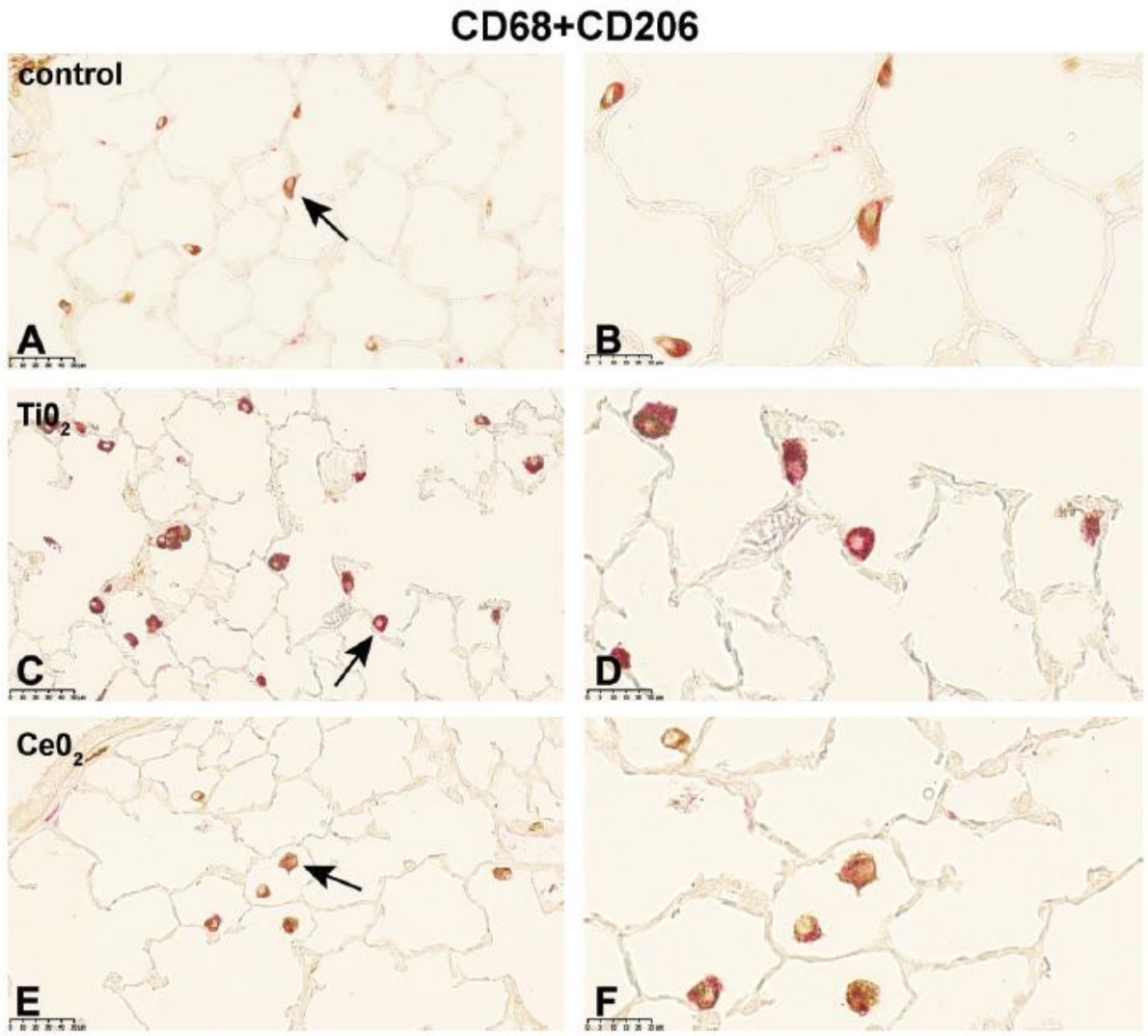

Figure 2. Micrographs of left lung sections of rats exposed to different (nano)materials for 5 days. Expression of CD206 (M2 marker) shortly after the last exposure was visualized by immunohistochemistry. Binding of antibodies was visualized using a red chromogen for the AM marker CD68 and a brown chromogen for the M2 marker CD206 (A, C, E). Arrows indicate AMs shown with higher magnification in (B, D, F). Representative sections from each treatment group are shown. Controls were exposed to air only. AM: alveolar macrophages.

2.2.3.2 AOP for lung fibrosis (leading partner: LTAP)

A qualitative AOP for lung fibrosis was developed by Sabina Halappanavar (HC), Monita Sharma, Hakan Wallin, Ulla Vogel (NRCWE), Kristie Sullivan and Amy J. Clippinger and published on AOPWiki: Aop 173, Substance interaction with the lung resident cell membrane components leading to lung fibrosis (https://aopwiki.org/aops/173). This AOP is included in the OECD Work Plan and has completed the external review facilitated by the OECD WPHA (Working Party on Hazard Assessment)/WNT (Working Group of the National Coordinators for the Test Guidelines Programme). Thus, HC mainly contributed to this part of the work. The MIE/KEs for the lung fibrosis AOP were strictly based on this AOP since it is at an advanced level of development (detailed description of KEs and relationship between KEs, stressors, applicability, etc). Most KEs match the tentative AOP proposed by Vietti et al. [9]. Identification of KEs and biomarkers were based on Vietti et al. and Nymark et al. [9, 16]. Vietti et al. [9] mainly focused on the roles of immune cells (macrophages) and structural cells (epithelial cells and fibroblasts) in the development of lung fibrosis

and review potentially pro-fibrotic mediators/biomarkers according to the producing cell type. The type of evidence supporting the role of the biomarkers in the development of lung fibrosis is described and gives a good indication on their predictive potential. Nymark et al. [16] used a data mining approach to identify pathways and genes potentially involved in lung fibrosis that could also be used to assess the pro-fibrotic potential of NMs.

2.2.3.3 AOP for lung cancer (leading partner: NRCWE)

The KEs proposed for the lung cancer AOP are based on Modrzynska et al. and Jacobsen et al. [17, 18] and AOP296 “Oxidative DNA damage leading to chromosomal aberrations and mutations” (https://aopwiki.org/aops/296). The AOP was based on chronic inhalation studies showing that inhalation of TiO_2 and carbon black nanoparticles induced lung cancer in chronic inhalation studies in rats [19]. Lung cancer was observed after exposure to an average 10 mg/m^3 of P25 TiO_2 NPs and Printex90 carbon black with very similar potency. Notably, lung clearance rates were assessed and half-lives were in the order of 360 days.

There are different possible mechanisms of genotoxicity. Carbon black nanoparticles are mutagenic *in vitro* and *in vivo*. *In vitro*, increased levels of DNA strand breaks and oxidative DNA damage have been demonstrated, as well as increased mutation rates in the *cII* gene [20]. Carbon black is an efficient generator of reactive oxygen species [18] and the spectrum of mutations induced by carbon black suggests that the mutations are likely caused by oxidative DNA damage [21]. In addition, secondary genotoxicity caused by chronic inflammation upon inhalation of carbon black nanoparticles may also contribute to genotoxicity [22].

Diesel exhaust is carcinogenic in chronic inhalation studies in rats and has the same carcinogenic potential as carbon black and TiO_2 nanoparticles of similar size [19, 23]. The mutagenic potential of diesel exhaust particles and carbon black nanoparticles is similar *in vitro* [24]. Diesel exhaust particles consist of an insoluble carbon core and adsorbed polyaromatic hydrocarbons, some of which are genotoxic. Thus, both the insoluble carbon core and associated polyaromatic hydrocarbons may contribute to formation of DNA damage, mutations and subsequently cancer.

2.2.3.4 AOP for lung mesothelioma (leading partner: LTAP)

For lung mesothelioma, AOP 171 (Chronic cytotoxicity of the serous membrane leading to pleural/peritoneal mesotheliomas in the rat, https://aopwiki.org/aops/171) is under development. This AOP is tentative and currently lists potential MIE and KEs, without detailed information on KEs, relationship between KEs, stressors, applicability, etc. Moreover, this AOP does not include any KE on genotoxicity, which is an essential process in the development of tumours [25]. Therefore, publications on the general understanding of the pathogenesis of lung mesothelioma [26, 27] and on the carcinogenic potential of carbon nanotubes (CNT) and/or nanofibers [28, 29] were identified. These references propose a sequence of events (that could be considered as tentative AOPs) and include genotoxicity and/or genome instability, that were included as KEs to refine AOP 171. Biomarkers were identified from the same literature.

2.2.3.5 AOP for liver inflammation (leading partner: MISVIK)

At the start of the task an AOP named “Lysosomal damage leading to liver inflammation” (https://aopwiki.org/wiki/index.php/Aop:144) had been established in the AOPwiki. Since then the AOP has been refined and further developed towards describing liver fibrosis and is now named “Endocytic lysosomal uptake leading to liver fibrosis” (AOP:144, https://aopwiki.org/aops/144). Both the legacy AOP and the updated version aimed at liver fibrosis prediction are developed to be applicable to nanomaterials.

The original legacy AOP describing liver inflammation was used as a basis for mapping biomarkers related to the adverse outcome and assays for testing. A previously developed toxicogenomic tool predictive of liver injury was used to map four gene sets representative of diverse toxicity mechanisms and pathways to the KEs in the AOP ([30], data from Supplemental Data 5). Based on bioinformatics assessment on pathway-level, the four gene sets were associated with four of seven KEs in the AOP. The relevance level assigned (C - strongly associated with the AO) was based on the fact that the gene sets (referred to as components G, H, N and I in the original publication) had been validated for their prediction of 17 different liver pathologies induced by chemicals in rats and of human drug-induced liver injury, based on data from rat and human primary hepatocytes.

Assays recommended for *in vitro* testing of the gene sets include high-throughput whole-genome or targeted transcriptomics techniques such as Affymetrix microarrays (PrimeView U219 Array Plate (up to 96x)), L1000 (uses Luminex™ beads), or TempO-Seq (BioSpyder). The information in the gene sets mapped to the AOP (including both genes and pathways) may also be used as a basis for development of other types of assays.

For some of the KEs in the AOP there are other gene sets that relate to them, in addition to the ones that have been mapped in this task, but that have not been validated for liver pathology prediction. Nevertheless, these may become relevant based on further ongoing work on refinement and validation of the tool for nanomaterials. The suggested cell types (hepatocytes, e.g. cell lines HepG2 and HepaRG) are based on knowledge gained from chemicals, but other cell types may become more relevant for nanomaterials, e.g. immune cells or 3D/co-culture systems.

2.2.3.6 AOP for liver fibrosis (leading partner: MISVIK)

AOP:144 describing “Endocytic lysosomal uptake leading to liver fibrosis” (https://aopwiki.org/aops/144) was used as a basis for mapping biomarkers and assays for testing, similarly as described above for liver inflammation. Briefly, the four gene sets previously identified to be predictive of liver injury, as described above, were mapped to five of nine KEs in the AOP. The same assays (high-throughput whole-genome or targeted transcriptomics) and cell types are recommended as for the liver inflammation AOP (please refer to section 2.2.3.5).

2.2.3.7 AOP for liver cancer (leading partner: NRCWE)

The KEs proposed for the liver cancer AOP are based on Modrzynska et al. and Jacobsen et al. [17, 18] and AOP296 “Oxidative DNA damage leading to chromosomal

aberrations and mutations" (https://aopwiki.org/aops/296). The AOP for liver cancer is based on the key observation that inhalation exposure to carbon black nanoparticles induces DNA damage in liver [31, 32].

The observed DNA damage was shown to be likely caused by particle-induced generation of reactive oxygen species. Carbon black nanoparticles are mutagenic *in vitro* and *in vivo*. *In vitro*, increased levels of DNA strand breaks and oxidative DNA damage have been demonstrated, as well as increased mutation rates in the *cII* gene [20]. Carbon black is an efficient generator of reactive oxygen species [18] and the mutation spectrum of carbon black-induced mutations suggest that the mutations are likely caused by oxidative DNA damage [21].

It was shown that pulmonary inflammation was not the cause of the DNA damage induced by carbon black nanoparticles since pulmonary dosing with CeO_2 and TiO_2 NPs of similar size induced similar inflammation but no DNA damage [17]. All three types of NPs translocated to liver. Translocation likely occurred via blood, as translocation to the liver following oral exposure was below the level of detection [17]. In the study, carbon black but not CeO_2 or TiO_2 generated high levels of reactive oxygen species and DNA damage in liver following intravenous injection as well as following translocation of nanoparticles from lung. This suggests that liver genotoxicity is caused by primary genotoxicity in terms of particle-induced ROS, leading to mutations [20] and cancer.

2.2.4 Interaction between identified AOPs and in vitro *work*

The strategy for T2.5, selection of AOs and identification of AOPs/KEs/assays were presented and discussed during the "AOP workshop" (Milestone 4) organized by LTAP in Brussels (May 14-15, 2019) with the *in vitro* partners and to establish an interaction between T2.5 and WP3 and 4 to exchange information. Assays planned by *in vitro* partners were cross-checked with the assays proposed by T2.5.

2.3 Results

2.3.1 Identification of ENM-relevant AOs

We identified the following AOs (potentially) induced by ENMs, supported by evidence in regulatory (collected in T2.1 and presented in D2.1) and other experimental studies, after inhalation and oral exposure:

- lung inflammation,
- lung emphysema,
- lung fibrosis,
- lung cancer including mesothelioma,
- cardiovascular diseases,
- liver inflammation,
- liver fibrosis,
- liver cancer,
- gut inflammation and cancer and,
- kidney fibrosis.

Based on the available expertise of partners and limited evidence for induction of some of the pre-selected AOs by ENMs, **lung inflammation, fibrosis, mesothelioma and**

cancer, and **liver inflammation, fibrosis and cancer** were consensually considered relevant in the framework of PATROLS and selected for further work (Annex 1: list of potential ENM-induced AOs and related (tentative) AOPs).

2.3.2 Identification of nano-relevant AOPs

Existing AOPs (related to NM or not) were identified on AOPWiki (https://aopwiki.org/) and a first list of publications was proposed by experts to serve for AOP enrichment and identification of KEs (Annex 1). AOPs (on AOPWiki) exist at different stages of development for lung inflammation (as part of the AOP for lung fibrosis), fibrosis, mesothelioma and liver inflammation (as part of the AOP for liver fibrosis), fibrosis and cancer.

2.3.3 Identification of MIEs/KEs, biomarkers and assays potentially predictive of selected AOs

Information was organized in an Excel document (one sheet per AOP; see Annex 2). All Excel sheets are included in this document in Annex 2:

- Sheet 1: MIEs/KEs, biomarkers and assays for lung inflammation.
- Sheet 2: MIEs/KEs, biomarkers and assays for lung fibrosis.
- Sheet 3: MIEs/KEs, biomarkers and assays for lung cancer.
- Sheet 4: MIEs/KEs, biomarkers and assays for lung mesothelioma.
- Sheet 5: MIEs/KEs, biomarkers and assays for liver inflammation.
- Sheet 6: MIEs/KEs, biomarkers and assays for liver fibrosis.
- Sheet 7: MIEs/KEs, biomarkers and assays for liver cancer.

Each excel sheets contains:

- Information about the leader partner who organized the information about the AOP.
- AOPWiki URL and literature used to identify putative MIE/KEs, biomarkers and assays.
- A left table that includes MIE/KEs URL (KE number) and denomination (KE), potential biomarkers (markers), proposed cell types (cell type) and assays (assay) to measure the biomarker, and finally the type of evidence (type of evidence) found in the literature that suggested a predictive potential of the marker.
  Types of evidence was coded as follows :
  A Association between *in vitro* and *in vivo* data
  B Implication in the AO (deficient or transgenic mice, inhibitors, etc)
  C Strongly associated with the AO
  D *In vivo* transcriptomics
  E Data mining
  F Other (specified)
- A right table (green, see below for further details) filled by WP3 and 4 *in vitro* partners (partner) with the biomarkers (markers), cell types or models (cell type) and assays (assay) they plan to use in PATROLS.

Based on the identified AOPs and MIE/KEs, figures 3 and 4 show the schematic representations of (tentative) AOPs defined/refined in this task for lung and liver AOs, respectively, after inhalation or oral exposure.

Some KEs are shared by several AOPs. As expected, many KEs are common/identical for (i) lung inflammation and fibrosis (KEs 1495, 1496, 1497, 1498 and 1499) and (ii) liver inflammation and fibrosis (KEs 1539, 898, 177 and 55) since they derive from the same AOPs (AOP 173 for lung and AOP144 for liver). Similar KEs (with a different AOPWiki number but a similar description) are found in lung cancer and mesothelioma (inflammation and genotoxicity), lung and liver cancer (mutations), lung and liver inflammation and fibrosis (increased pro-inflammatory mediators KEs 1496/1493 and cytokine release KE87; recruitment of inflammatory cells KE1497, inflammatory cell infiltration KE901 and leukocyte recruitment KE1494), lung and liver fibrosis (extracellular matrix deposition KE1501 and accumulation collagen KE68).

Except for liver cancer, where primary genotoxicity is proposed as the mechanism of action, inflammatory processes (“secretion of proinflammatory mediators”, “particle surface area-dependent inflammation”, “inflammation”, “cytokine release” and “Increased pro-inflammatory mediators”) appear to be essential for the development of all AOs. Thus, it is proposed that these KEs (inflammation and genotoxicity) and associated biomarkers should be tested in a first tier, in a screening approach to identify NMs that need prioritization for further testing. The second tier would include KEs downstream of inflammation or genotoxicity or “specific” to (some of) the AOP(s) (e.g. fibroblast proliferation and myofibroblast differentiation for lung fibrosis).

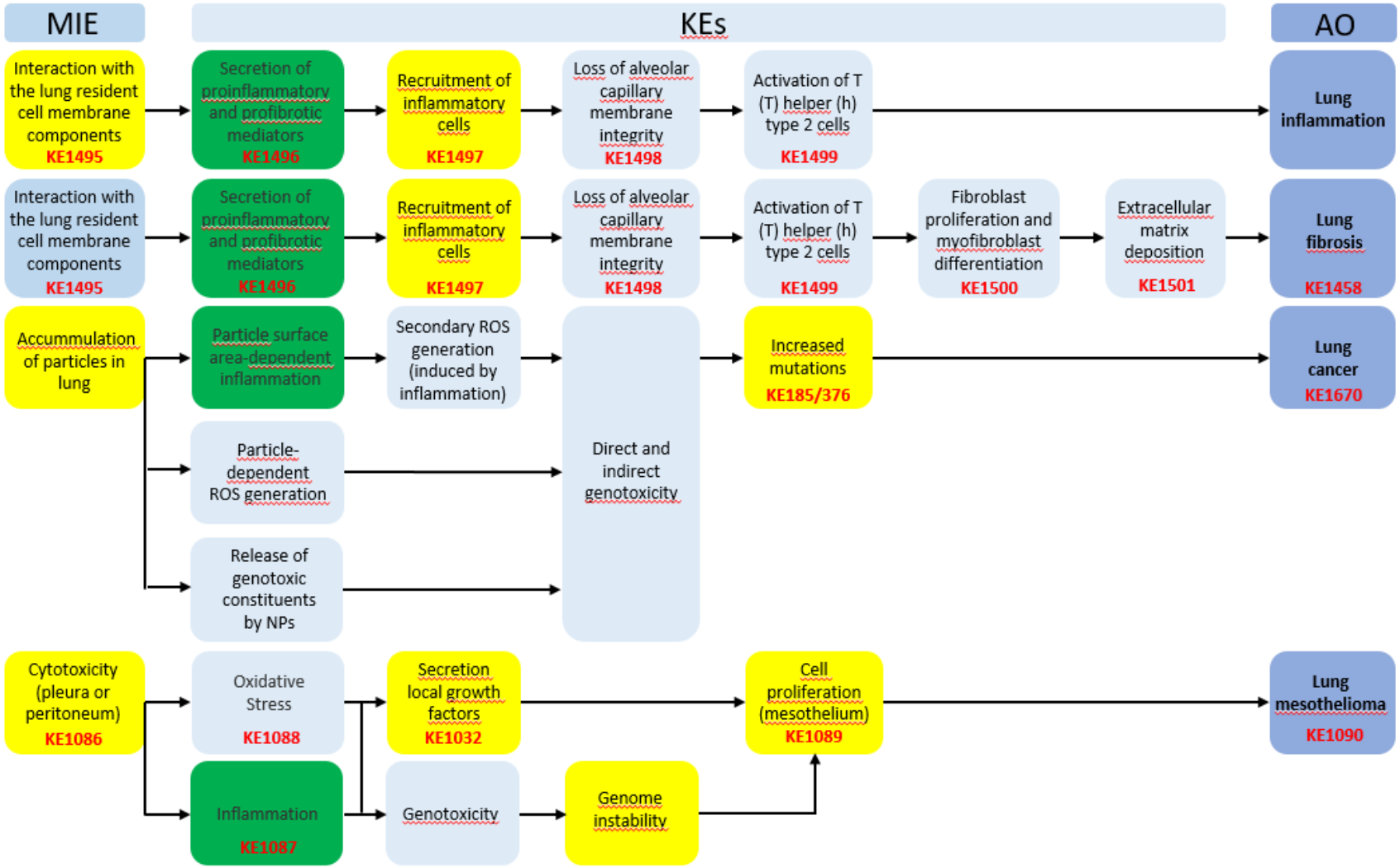

Figure 3: Nano-relevant AOPs/networks of MIEs/KEs leading to lung AOs identified in PATROLS. When available, AOPwiki IDs for MIEs/KEs are indicated in red. AOP for lung inflammation is a part of AOP173; AOP for lung fibrosis is AOP173; AOP for lung mesothelioma is partially based on AOP171. Common inflammatory KEs are indicated in green; KEs not covered by PATROLS *in vitro* testing are indicated in yellow.

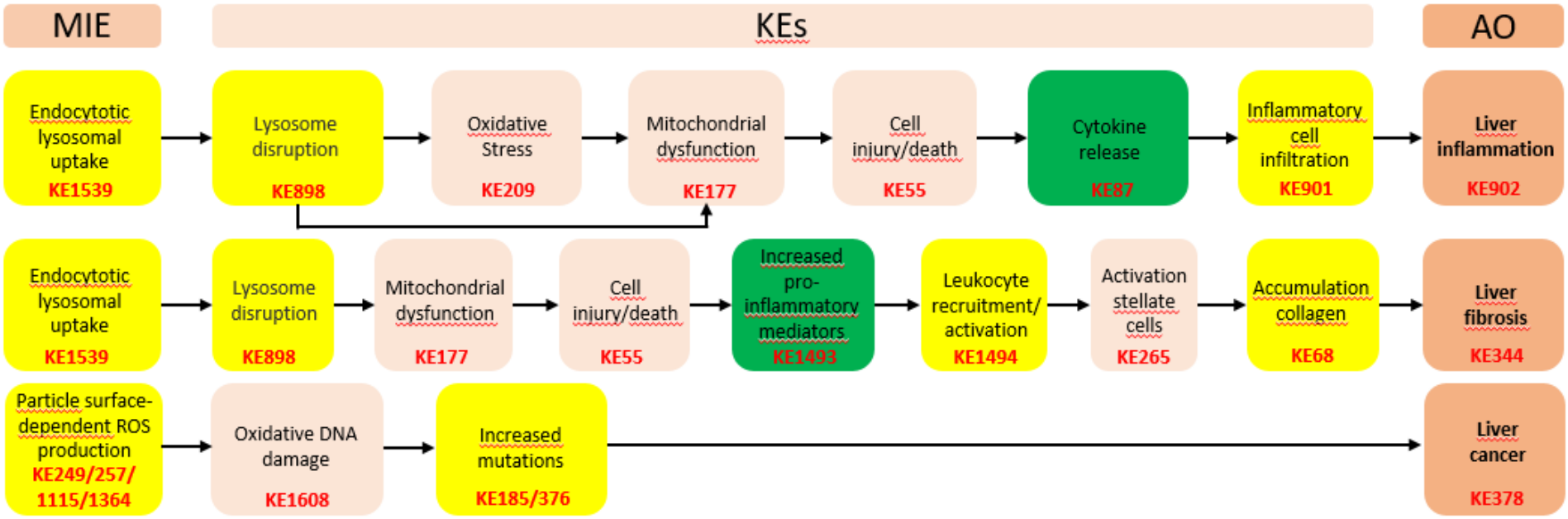

Figure 4: Nano-relevant AOPs/networks of MIEs/KEs leading to liver AOs identified in PATROLS. When available, AOPwiki IDs for MIEs/KEs are indicated in red. AOP for liver inflammation is partially based on AOP144; AOP for liver fibrosis is AOP144. Common inflammatory KEs are indicated in green; KEs not covered by PATROLS *in vitro* testing are indicated in yellow.

2.3.4 Anchorage of in vitro *testing in the AOP approach*

The strategy for T2.5, selected AOs and identified AOPs/KEs/assays were presented and discussed during the "AOP workshop" (Milestone 4) organized by LTAP in Brussels (May 14-15, 2019). The objective was to inform partners from other WP about the selection of endpoints in the *in vitro* assays for toxicity testing and to establish a crosstalk between T2.5 and WP3 and 4 partners. KEs and biomarkers planned to be tested by each group were discussed. How the current strategy covers the AOPs/KEs and gaps were identified. T2.5 partners presented their work on individual AOPs.

PATROLS also co-organized an OECD workshop, the WPMN Workshop on "Advancing Adverse Outcome Pathway Development for Nanomaterials Risk Assessment and Categorization", held at OECD on 11-12th September 2019, where AOP173 was presented together with relevant PATROLS *in vitro* assays.

The information collected and formatted by T2.5 on the identification of AOPs, MIEs/KEs, biomarkers and assays potentially predictive of selected AOs was distributed to WP3 and 4 *in vitro* partners. They were asked to complete the table with their own tests. The objective was to cross-check biomarkers and assays identified in T2.5 with assays planned by WP3 and 4 *in vitro* partners (see annex 2 where the right tables (in green) were filled by WP3 and 4 with the biomarkers, cell types or models and assays they selected for PATROLS *in vitro* testing). This document allows, on the one hand, to guide *in vitro* partners in the choice of assays and cell models that should be prioritized and, on the other hand, to get an insight on how PATROLS *in vitro* testing covers the AOP KEs. This document is a living document that can constantly be fed/refined by T2.5, if new knowledge and data (relevant to the AOPs) or AOPs become available during the project. *In vitro* partners will also contribute, if they plan to test other biomarkers or experimentally identify one or a set of assays predictive of an AO. Annex 2 illustrates how PATROLS *in vitro* testing covers identified KEs and identifies KEs not (currently) evaluated *in vitro* (in yellow in annex 2). Annex 2 indicates that a large part of KEs (excluding AOs, 25 on a total of 44 KEs) will be assessed *in vitro*, suggesting that the current plans are appropriate to identify predictive biomarkers. Moreover, although the recruitment of inflammatory cells (common to KEs found in 4 AOPs: lung and liver inflammation and fibrosis) is not directly testable *in vitro*, it is the consequence of the upstream KEs (pro-inflammatory mediators or cytokine release) that will be covered.

2.4 Refinement of selected AOPs

As mentioned before, AOPs can constantly be refined if new knowledge or data (relevant to the AOPs) or AOPs become available during the project and if *in vitro* partners experimentally identify one or a set of assays predictive of an AO.

Previously published gene expression microarray data from mouse lungs exposed to a variety of stressors (bleomycin, bacterial infections, overexpression of cytokines, welding fumes, etc) available from the public domain that are known to cause lung diseases (lung inflammation, emphysema, chronic obstructive pulmonary disease (COPD), lung fibrosis and lung cancer) were used by HC to develop a 17-gene pro-fibrotic biomarker panel (PFS17) that is specifically predictive of lung fibrosis ([33-35] and Rahman et al, under revision in *Small*). The Table 2 lists the 17 genes in PFS17. HC is currently validating this 17-gene pro-fibrotic signature with new transcriptomics

data from lungs exposed to CNTs and on PCLS (precision-cut lung slices) model exposed to NMs. The results will indicate (i) whether the signature is predictive of lung pro-fibrotic activities of NMs and could be used as biomarker and (ii) whether the 17 genes could be included in AOP173 for refinement.

Table 2: 17-gene pro-fibrotic biomarker panel (PFS17).

Gene	Accession Code	Title
Arg1	NM_007482.3	Mus musculus arginase, liver (Arg1), mRNA
C1qb	NM_009777.2	Mus musculus complement component 1, q subcomponent, beta polypeptide (C1qb), mRNA
Ccl9	NM_011338.2	Mus musculus chemokine (C-C motif) ligand 9 (Ccl9), mRNA
Ccr5	NM_009917.5	Mus musculus chemokine (C-C motif) receptor 5 (Ccr5), mRNA
Ch25h	NM_009890.1	Mus musculus cholesterol 25-hydroxylase (Ch25h), mRNA
Clec4a2	NM_001170333.1	Mus musculus C-type lectin domain family 4, member a2 (Clec4a2), transcript variant 1, mRNA
Ctss	NM_021281.3	Mus musculus cathepsin S (Ctss), transcript variant 2, mRNA
Fcgr2b	NM_001077189.1	Mus musculus Fc receptor, IgG, low affinity IIb (Fcgr2b), transcript variant 1, mRNA
Fxyd4	NM_001173372.1	Mus musculus FXYD domain-containing ion transport regulator 4 (Fxyd4), transcript variant 2, mRNA
Itgb2	NM_008404.4	Mus musculus integrin beta 2 (Itgb2), mRNA
Lpxn	NM_134152.3	Mus musculus leupaxin (Lpxn), mRNA
Ly86	NM_010745.2	Mus musculus lymphocyte antigen 86 (Ly86), mRNA
Retnla	NM_020509.3	Mus musculus resistin like alpha (Retnla), mRNA
S100a4	NM_011311.2	Mus musculus S100 calcium binding protein A4 (S100a4), mRNA
Serpina3g	NM_009251.2	Mus musculus serine (or cysteine) peptidase inhibitor, clade A, member 3G (Serpina3g), transcript variant 1, mRNA
Serpina3n	NM_009252.2	Mus musculus serine (or cysteine) peptidase inhibitor, clade A, member 3N (Serpina3n), mRNA
Slc26a4	NM_011867.4	Mus musculus solute carrier family 26, member 4 (Slc26a4), mRNA

2.5 Conclusions – contribution to PATROLS

In this task, partners identified:

- (potential) AOs relevant to NM exposure via inhalation and oral routes, which are the routes considered in PATROLS and
- associated AOPs/KEs/biomarkers with a predictive potential on AOPWiki and in the literature.

This information was shared with *in vitro* partners to identify potentially predictive assays, to decide which assays should be prioritized for *in vitro* testing, how KEs are covered by their strategies and identify gaps. An overview of the selected AOPs also identified inflammatory processes and genotoxicity as probably priority endpoints to address in the first instance.

Knowing that AOPs are interesting at different levels i) to develop testing strategies, ii) to identify (knowledge) gaps, iii) to prioritize substances for testing and iv), for regulatory risk assessment, this work contributed to improve/strengthen the PATROLS *in vitro* testing strategy which aims at identifying predictive assays for NM toxicity that could be used in the frame of risk assessments.

3. Deviations from the Workplan

Although archived tissues and tissues identified in Tasks 2.2 and 2.3 were planned to be used to generate new complementary transcriptomics (HC) and toxicological (LTAP, BASF, NRCWE) data, additional experiments are not planned for the moment. According to Task 2.2 and 2.3 (PATROLS Task 2.2 2.3_BASF NRCWE in https:\\patrolsproject.webdav.hidrive.strato.com\users\patrolsproject\3 WP Space\WP2\Task2.2), organs are available as paraffin blocks that could be used for additional histology and immunohistology. Very few organs are available as deep frozen tissues (required for transcriptomics analysis) for inhalation and intratracheal studies. Lungs are available from a 28 days inhalation study (BASF) with $BaSO_4$ (NM-220) and CeO_2 (NM-212). Transcriptomics analysis are already published for pulmonary exposure of the strongly pro-fibrotic NM401 and Mitsui-7 [33, 36-41]. For oral studies, frozen kidneys and livers are available from a study with CeO_2 and SiO_2 and kidneys, livers and spleens from a study with TiO_2 and Ag-PVP. These frozen tissues are proposed to HC for new transcriptomics analysis but time and resources are not available for the moment. Due to the type of samples available and the very few NMs (at least for inhalation studies), toxicological experiments are not foreseen for the moment.

4. Performance of the partners

All partners contributed to the task as requested and fulfilled their requirements in a satisfactory time period. The report was drafted by LTAP with input from NRCWE, BASF, HC and MISVIK.

5. Conclusions

The Steering Board deems this deliverable to be fulfilled satisfactorily.

6. Annexes

Annex 1: List of (potential) NM-induced AOs and related (tentative) AOPs and publications.

Organ	(Potential) AO	Evidence for the AO	AOPWiki AOPs	AOPWiki URL	Publications proposed for AOP enrichment and KE identification
Lung	**Inflammation**	inhalation long-term/sub-chronic studies	part of lung fibrosis AOP (aop173)	https://aopwiki.org/aops/173	Williams and Halappanavar, 2017 (https://doi.org/10.1016/j.dib.2017.10.060); Nymark et al, 2017 (doi: 10.1093/toxsci/kfx252)
	Cardio-vascular diseases	induced by ambient air pollution PM and CNT (Poulsen et al, 2015 DOI: 10.1016/j.taap.2015.01.011)	Secretion of inflammatory cytokines after cellular sensing of the stressor leading to plaque progression	https://aopwiki.org/aops/237	Saber et al, 2014 (PMID: 24920450)
	Emphysema	TiO_2, Metals and MeOx. Bomhard, 2018 (doi: 10.1016/j.etap.2018.02.003)			Draft developed by HC for OECD review (HC, Sabina Halappanavar))
	Fibrosis	inhalation long-term/sub-chronic studies (mainly CNT)	Substance interaction with the lung resident cell membrane components leading to lung fibrosis (aop173)	https://aopwiki.org/aops/173; https://aopwiki.org/aops/206; https://www.wikipathways.org/instance/WP3624; https://www.wikipathways.org/instance/WP3632	Nikota et al, 2016 (DOI 10.1186/s12989-016-0137-5); Labib et al, 2016 (DOI 10.1186/s12989-016-0125-9); Vietti et al, 2016 (DOI 10.1186/s12989-016-0123-y); Nikota et al, 2017 (DOI 10.1186/s12989-017-0218-0); Clippinger et al. 2016 (doi: 10.1007/s00204-016-1717-8); Williams and Halappanavar, 2017 (https://doi.org/10.1016/j.dib.2017.10.060); Nymark et al, 2017 (doi: 10.1093/toxsci/kfx252)
	Cancer	inhalation long-term/sub-chronic studies (mainly CNT)			
	Mesothelioma	inhalation long-term/sub-chronic studies (mainly CNT)	Chronic cytotoxicity of the serous membrane leading to pleural/peritoneal mesotheliomas in the rat (aop171)	https://aopwiki.org/aops/171	
Liver	**Inflammation**	oral long-term/sub-chronic studies	Lysosomal damage leading to liver inflammation (aop144)	https://aopwiki.org/wiki/index.php/Aop:144	Kohonen et al, 2017 (DOI: 10.1038/ncomms15932); Nymark et al, 2017 (doi: 10.1093/toxsci/kfx252)
	Fibrosis	Van der Zande et al, 2014 (https://doi.org/10.1186/1743-8977-11-8); Zhuravskii et al, 2016	Protein Alkylation leading to Liver Fibrosis (aop38)	https://aopwiki.org/wiki/index.php/Aop:38	Gerloff et al, 2017 (https://doi.org/10.1016/j.comtox.2016.07.001); Kohonen et al. 2017 (DOI: 10.1038/ncomms15932);

		(DOI: 10.3109/15376516.2016.1169341)	Endocytic lysosomal uptake leading to liver fibrosis (aop144)	https://aopwiki.org/aops/144	Nymark et al. 2017 (doi: 10.1093/toxsci/kfx252); Diwan et al. 2014 (https://doi.org/10.1371/journal.pone.0112193)
	Cancer	mainly based on epidemiological studies (association with air pollution)	PPARalpha-dependent liver cancer (aop37)	https://aopwiki.org/wiki/index.php/Aop:37	Modrzynska J et al (2017) (doi: 10.1186/s12989-017-0238-9)
			Sustained AhR Activation leading to Rodent Liver Tumours (aop41)	https://aopwiki.org/wiki/index.php/Aop:41	
			Tumorigenesis, Hepatocellular carcinoma (aop378)	https://aopwiki.org/events/378	
Gut	Inflammation	Bettini et al, 2017 (DOI: 10.1038/srep40373)			
	Cancer				
Kidney	Fibrosis	Huang et al, 2014 (DOI: 10.1021/tx500287f)			

AOPs identified in the frame of PATROLS are indicated in bold.

Annex 2: MIEs/KEs, biomarkers and assays for PATROLS-relevant AOPs.

1) Sheet 1: MIEs/KEs, biomarkers and assays for lung inflammation.

Lung inflammation	Person of contact	Lan Ma-Hock (lan.ma-hock@basf.com)
	KE based on:	https://aopwiki.org/aops/173
	Markers based on:	Vietti et al, 2016 (DOI 10.1186/s12989-016-0123-y) Pavan and Fubini 2017 (doi: 10.1021/acs.chemrestox.6b00409) Nikota et al. Particle and Fibre Toxicity (2017) 14:37 He et al. J Clin Toxicol (2012) S5:005 Sohaebuddin et al. Particle and Fibre Toxicity (2010) 7:22 Hussain et al. Particle and Fibre Toxicity (2010) 7: 10

KE that are not covered by PATROLS in vitro strategy

KE number	**KE**	**markers**	**cell type**	**assay**	**Type of evidence**
1495	Increased, interaction with the resident cell membrane components	lysosome membrane permeabilization	3T3, ht bronchial epithelial cells, RAW macrophage, Mouse peritoneal macrophages, 16HBE14o cells, human bronchial epthelia cells, THP-1 cells, human monocytic cell line, human macrophages	Acridine orange staining (change from lysosomal red to cytosolic green fluorescence)	C
		membranolysis	red blood cells (RBC)	RBC lysis assay	A
		membranolysis	lipid vesicles	?	?
1496	Increased, secretion of proinflammat	ROS	macrophage, fibroblast	EPR (acellular), heme oxygenase I	C

To be filled by partners			
markers	**cell type**	**assay**	**partner**
ROS	A549, NCI-H441	CM-H2DCFDA - Invitrogen (Cat#C6827)	SU

	ory and profibrotic mediators			(cellular, ELISA, RT-PCR), …					
		p38 MAPK	fibroblast	WB	C				
		NFK-B	macrophage	WB, immunofluo	C				
		NADPH oxidase	macrophage	measure IL-1β in presence of inhibitors (ex: DPI)	B				
		inflammasome	macrophage, epithelial cell	measure IL-1β in presence of inhibitors	B, C	IL-1β (+ IL-18)	THP-1	WST-1 (Roche), IL-1β (ELISA); IL-18 (ELISA)	RIVM
		MAP kinase	epithelial cell	WB	C				
		IL-1β	macrophage, epithelial cell	ELISA, WB (RT-PCR)	A, B	IL-1β	EpiAlveolar™ (MatTek) without macrophages	ELISA, R&D Systems (Cat#DY201)	AMI
							EpiAlveolar™ (MatTek) with macrophages		
							co-culture epithelial cells (A549)-macrophages (THP-1)-fibroblasts (MRC-5)		
						IL-1β	Calu-3 and Calu-3 + MDM	ELISA	RIVM
		TNF-α	macrophage	ELISA, WB (RT-PCR)	A, B, C	TNF-α	EpiAlveolar™ (MatTek) without macrophages	ELISA, R&D Systems (Cat#DY210)	AMI

		IL-18	epithelial cell	ELISA, WB (RT-PCR)	C
		IL-8	epithelial cell	ELISA, WB (RT-PCR)	C

	EpiAlveolar™ (MatTek) with macrophages		
	co-culture epithelial cells (A549)-macrophages (THP-1)-fibroblasts (MRC-5)		
TNF-α	Calu-3	ELISA	RIVM
TNF-α	A549, NCI-H441	R&D Systems (Cat#DY210)	SU
IL-18	THP-1	eBioscience (Thermo Fisher)	RIVM
IL-8	EpiAlveolar™ (MatTek) without macrophages	ELISA, R&D Systems (Cat#DY208)	AMI
	EpiAlveolar™ (MatTek) with macrophages		
	co-culture epithelial cells (A549)-macrophages (THP-1)-fibroblasts (MRC-5)		
IL-8	Calu-3 and Calu-3 + MDM	ELISA	RIVM

		TGF-β	macrophage, fibroblast, epithelial cell	ELISA, WB (RT-PCR)	B, C	TGF-β	EpiAlveolar™ (MatTek) without macrophages	ELISA, R&D Systems (Cat#DY240)	AMI
							EpiAlveolar™ (MatTek) with macrophages		
							co-culture epithelial cells (A549)-macrophages (THP-1)-fibroblasts (MRC-5)		
		PDGF	macrophage, fibroblast, epithelial cell	ELISA, WB (RT-PCR)	C	PDGF-AA	co-culture epithelial cells (A549)-macrophages (THP-1)-fibroblasts (MRC-5)	ELISA, R&D Systems (Cat#DY221)	AMI
		MCP-1	macrophage	ELISA	A	MCP-1	Calu-3 and Calu-3 + MDM	ELISA	RIVM
		GM-CSF	macrophage, T-cells, mast cells, endothelia cells, fibroblasts	ELISA	A				
		IL-6	macrophage	ELISA	A, B	IL-6	EpiAlveolar™ (MatTek) without macrophages	ELISA, R&D Systems (Cat#DY206)	AMI
							EpiAlveolar™ (MatTek) with macrophages		

							co-culture hAELVi epithelial cells-macrophages (MDMs)		
						IL-6	Calu-3 and Calu-3 + MDM	ELISA	RIVM
						IL-6	A549, NCI-H441	R&D Systems (Cat#DY206)	SU
		IL-10	macrophage	ELISA	A, B	IL-10	primary monocytes	eBioscience (Thermo Fisher)	RIVM
						IL-10	Calu-3 + MDM	ELISA	RIVM
						All	A549	RNA-seq	Misvik (samples from SU)
1497	Increased, recruitment of inflammatory cells								
1498	Increased, loss of alveolar capillary membrane integrity					TEER	A549, NCI-H441	EVOM2 + electrodes (https://www.wpi-europe.com/products/cell-and-tissue/teer-measurement/evom2.aspx)	SU
						Membrane integrity	Calu-3 and Calu-3 + MDM	TEER, LDH release	RIVM
1499	Increased, activation of T (T) helper (h) type 2 cells	STAT-6	mediated Th2 response		B	STAT-6	A549	RNA-seq	Misvik (samples from SU)

	Lung inflammation								

2) Sheet 2: MIEs/KEs, biomarkers and assays for lung fibrosis.

Lung fibrosis	Person of contact	sybille van den Brule (sybille.vandenbrule@uclouvain.be)	
	KE based on:	https://aopwiki.org/aops/173	
	Markers based on:	Vietti et al, 2016 (DOI 10.1186/s12989-016-0123-y)	
		Nymark et al. 2018 (DOI: 10.1093/toxsci/kfx252)	https://www.wikipathways.org/instance/WP3624

KE that are not covered by PATROLS in vitro strategy

KE number	**KE**	**markers**	**cell type**	**assay**	**Type of evidence**
1495	Interaction with the resident cell membrane components	Toll-like receptor signaling WP75 (CXCL8, CCL3, CCL4, CCL5)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics, whole genome or reduced feature high-throughput transcriptomics	E
		DAMPS/alarmins (IL-1α)	macrophages	ELISA, qRT-PCR	C
1496	Secretion of proinflammatory and profibrotic mediators	ROS	macrophage, fibroblast	EPR (acellular), heme oxygenase I (cellular, ELISA, RT-PCR), …	C
		p38 MAPK	fibroblast	WB	C
		NFK-B	macrophage	WB, immunofluo, transcriptomics	C
		NADPH oxidase	macrophage	measure IL-1β in presence of inhibitors (ex: DPI)	B

To be filled by partners			
markers	**cell type**	**assay**	**partner**
All	A549	RNA-seq	Misvik (samples from SU)
ROS	A549, NCI-H441, d_THP-1/primary macrophages	CM-H2DCFDA - Invitrogen (Cat#C6827)	SU

		inflammasomme	macrophage, epithelial cell	measure IL-1β in presence of inhibitors	B, C	IL-1β (+ IL-18)	THP-1	WST-1 (Roche), IL-1β (ELISA); IL-18 (ELISA)	RIVM
		MAP kinase	epithelial cell	WB	C				
		IL-1β	macrophage, epithelial cell	ELISA, WB (RT-PCR), transcriptomics	A, B	IL-1β	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
						IL-1β	EpiAlveolar™ (MatTek) without macrophages	ELISA, R&D Systems (Cat#DY201)	AMI
							EpiAlveolar™ (MatTek) with macrophages		
							co-culture epithelial cells (A549)-macrophages (THP-1)-fibroblasts (MRC-5)		
		TNF-α	macrophage	ELISA, WB (RT-PCR)	C	TNF-α	EpiAlveolar™ (MatTek) without macrophages	ELISA, R&D Systems (Cat#DY210)	AMI
							EpiAlveolar™ (MatTek) with macrophages		
							co-culture epithelial cells (A549)-macrophages		

							(THP-1)-fibroblasts (MRC-5)		
						TNF-α	d_THP-1/Primary macrophages	R&D Systems (Cat#DY210)	SU
		IL-18	epithelial cell	ELISA, WB (RT-PCR)	C	IL-18	THP-1	eBioscience (Thermo Fisher)	RIVM
		IL-8	epithelial cell	ELISA, WB (RT-PCR)	C	IL-8	EpiAlveolar™ (MatTek) without macrophages	ELISA, R&D Systems (Cat#DY208)	AMI
							EpiAlveolar™ (MatTek) with macrophages		
							co-culture epithelial cells (A549)-macrophages (THP-1)-fibroblasts (MRC-5)		
						IL-8	A549, NCI-H441	R&D Systems (Cat#DY208)	SU
		TGF-β	macrophage, fibroblast, epithelial cell	ELISA, WB (RT-PCR), transcriptomics	B, C	TGF-β1, 2, 3, R1, R2	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
						TGF-β1	EpiAlveolar™ (MatTek) without macrophages	ELISA, R&D Systems (Cat#DY240)	AMI
							EpiAlveolar™ (MatTek) with macrophages		

							co-culture epithelial cells (A549)-macrophages (THP-1)-fibroblasts (MRC-5)		
		PDGF	macrophage, fibroblast, epithelial cell	ELISA, WB (RT-PCR), transcriptomics	C	PDGFA and B	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
		Cytokine and inflammatory response WP530 (PDGFA, CXCL2, CSF3, CSF2, IL12B, IL13, IL4, IL5, IL6)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E	-			
		Chemokine signaling WP3929 (CCL2, CCL11, CCR2, CCR3)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E	CCL2	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
						All	A549	RNA-seq	Misvik (samples from SU)
1497	Recruitment of inflammatory cells								
1498	Loss of alveolar capillary membrane integrity	Transepithelial/transendothelial electrical resistance (TEER)	endothelial and epithelial cell	measuring ohmic resistance or impedance	C	TEER	EpiAlveolar™ (MatTek) without macrophages	EVOM2 + electrodes (https://www.wpi-europe.com/products/cell-and-tissue/teer-	AMI

								measurement/ evom2.aspx)	
							EpiAlveolar™ (MatTek) with macrophages		
						TEER	A549, NCI-H441	EVOM2 + electrodes (https://www.wpi-europe.com/products/cell-and-tissue/teer-measurement/evom2.aspx)	SU
		ROS	macrophage, fibroblast	EPR (acellular), heme oxygenase I (cellular, ELISA, RT-PCR), …	C				
1499	Activation of T (T) helper (h) type 2 cells	Chondrocyte differentiation WP474 (CTGF, TGFA, GREM1, ATP11A)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E	CTGF	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
		Matrix metalloproteinases WP129 (MMP9, MMP2, TIMP1)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E	MMP-9, TIMP-1	human lung fibroblasts (MRC-5 and CRL1490)	quantitative RT-PCR	LTAP

						MMP-2 and 9, TIMP-1	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
		TGFB signaling WP560 (SKIL, SPP1)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E				
		Differentiation pathway WP2848 (EFG, IGF1, HGF, FGF1, FGF2, FGF7)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E				
		Cytokine and inflammatory response WP530 (PDGFA, CXCL2, CSF3, CSF2, IL12B, IL13, IL4, IL5, IL6)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E				
		Chemokine signaling WP3929 (CCL2, CCL11, CCR2, CCR3)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E	CCL2, CCL11	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
		Leukocyte/Myeloid cell differentiation GO: 0045637/GO: 1902105 (CALCA, CEBPB)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E				
		TGF-β	macrophage, fibroblast, epithelial cell	ELISA, WB (RT-PCR), transcriptomics	B, C, E	TGF-β1, 2, 3, R1, R2	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
						TGF-β	epithelial cells monocultures, co-cultures of epithelial cells-	ELISA, R&D Systems (Cat#DY240)	AMI

							macrophages-(fibroblasts)		
		PDGF	macrophage, fibroblast, epithelial cell	ELISA, WB (RT-PCR), transcriptomics	C, E	PDGFA and B	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
1500	Fibroblast proliferation and myofibroblast differentiation	Smad	fibroblast, epithelial cell	WB	C	Smad2, 3, 4, 6, 7	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
		ERK1/2	fibroblast	WB	A				
		fibroblast proliferation	fibroblast	WST-1, CyQUANT, MTT, Trypan blue exclusion,	A	Proliferation	human lung fibroblasts (MRC-5 and CRL1490)	WST-1 (Roche)	LTAP
		fibroblast differentiation (α-SMA)	fibroblast	RT-PCR, WB, immunofluo	C	α-SMA	human lung fibroblasts (MRC-5 and CRL1490)	quantitative RT-PCR	LTAP
						α-SMA	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
		EMT (ZO-1, SP-C, E-Cad, fibronectin, FSP-1, α-SMA, vimentin)	epithelial cells	RT-PCR, WB, immunofluo	C	Vimentin	EpiAlveolar (with or without macrophages) and co-culture (A549+MRC-5+THP-1)	Vimentin (Immunostaining, chicken polyclonal anti-vimentin antibody, abcam, ab24525)	AMI

						Fibronectin	EpiAlveolar™ (MatTek) without macrophages	Fibronectin ELISA, R&D Systems (Cat#DY1918-05)	AMI
							EpiAlveolar™ (MatTek) with macrophages		
							co-culture epithelial cells (A549)-macrophages (THP-1)-fibroblasts (MRC-5)		
		MAPK signaling WP382	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E				
		p38 MAPK WP400	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E				
		TGFB signaling WP560 (SKIL, SPP1)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E				
		TGF-β	macrophage, fibroblast, epithelial cell	ELISA, WB (RT-PCR), transcriptomics	B, C, E	TGF-β1, 2, 3, R1, R2	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
						TGF-β	epithelial cells monocultures, co-cultures of epithelial cells-macrophages-(fibroblasts)	ELISA, R&D Systems (Cat#DY240)	AMI
		PDGF	macrophage, fibroblast, epithelial cell	ELISA, WB (RT-PCR), transcriptomics	C, E	PDGFA and B	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays	LTAP

								human fibrosis (Qiagen)	
		Chondrocyte differentiation WP474 (CTGF, TGFA, GREM1, ATP11A)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E	CTGF	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
		Differentiation pathway WP2848 (EFG, IGF1, HGF, FGF1, FGF2, FGF7)	epithelial cell (e.g. BEAS-2B, A549)	transcriptomics	E	HGF	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
		-				All	A549	RNA-seq	Misvik (samples from SU)
1501	Extracellular matrix deposition	Collagen production	fibroblast	Collagen I and III (RT-PCR and WB), Sircol assay	A	Collagen I and III	human lung fibroblasts (MRC-5 and CRL1490)	quantitative RT-PCR	LTAP
						Collagen I and III	human lung fibroblasts (MRC-5 and CRL1490)	RT2 Profiler PCR Arrays human fibrosis (Qiagen)	LTAP
						Collagen I	EpiAlveolar™ (MatTek) without macrophages	ELISA, R&D Systems (Cat#DY6220-5)	AMI
							EpiAlveolar™ (MatTek) with macrophages		
							co-culture epithelial cells (A549)-macrophages (THP-1)-fibroblasts (MRC-5)		

1458	Pulmonary fibrosis								

3) Sheet 3: MIEs/KEs, biomarkers and assays for lung cancer.

Lung cancer	Person of contact	Ulla Birgitte Vogel (UBV@nfa.dk)
	KE based on:	https://aopwiki.org/events/378
	Markers based on:	PMID: 18618583

KE that are not covered by PATROLS in vitro strategy

KE number	KE	markers	cell type	assay	Type of evidence
	KE0: agglomerate-size dependent alveolar deposition of insoluble particles		lung tissue	size distribution during aerosolisation, physico-chemical properites including solubility, specific surface area, size, shape	B
	MIE: accummulation of particles in lung	Lung burden	lung tissue	modelling	B
	KE1A: particle surface area dependent inflammation	increased neutrophil influx in BAL, increased expression of proinflammatory cytokines		in vitro assays for cytokine expression and release	B

To be filled by partners			
markers	**cell type**	**assay**	**partner**
Cytokines/Chemokines	A549, NCI-H441, macrophages (co-cultures of both)	ELISA	SU

	KE 1B: particle-dependent generation of ROS	particle-surface generated ROS	all cell types from lung	in vitro and acellular assays of ROS	A,B	Oxygen centred radicals	A549, NCI-H441, macrophages (co-cultures of both)	DCFH-DA (cells and cell-free), PCR	SU
	KE1C: release of genotoxic constitutients from NPs ie PAH, toxic metals ect	DNA adducts, oxidative DNA damage, DNA strand breaks	all cell types from lung	DNA adducts	B	8OHGG	A549, NCI-H441	ELISA	SU
						8OHGG	A549, Beas-2B	8OHG staining	Misvik
						gamma-H2AX	A549, Beas-2B	gamma-H2AX staining	Misvik
						All	A549	RNA-seq	Misvik (samples from SU)
	KE2: secondary ROS generation (induced by inflammation)	cellular ROS			A, B	ROS	A549, NCI-H441	CM-H2DCFDA - Invitrogen (Cat#C6827)	SU
	KE3: genotoxicity (direct genotoxicity, ROS-mediated genotoxicity (direct and indirect))	DNA adducts, oxidative DNA damage, DNA strand breaks	all cell types from lung including macrophages, epithelial cells	comet assay: **OECD TG488:** In vivo alkaline single-cell gel electrophoresis assay for DNA strand breaks (comet assay), (2014, 2016)	B	DNA Damage	A549, NCI-H441	COMET assay	SU
185	increase, Mutations	Mutations	all cell types from lung	OECD TG 489: In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene	A,B				

		Mutations	all cell types from lung	OECD: 490 (2015, 2016), Transgenic rodent (TGR) somatic and germ cell gene mutation assays	A,B				
						DNA Damage	A549, NCI-H441	Micronucleus assay	SU
376	Increased, Induced Mutations in Critical Genes	Mutations	all cell types from lung	OECD TG 489: In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene	A,B				
		Mutations	all cell types from lung	OECD: 490 (2015, 2016), Transgenic rodent (TGR) somatic and germ cell gene mutation assays	A,B				
	Lung cancer								

4) Sheet 4: MIEs/KEs, biomarkers and assays for lung mesothelioma.

Lung mesothelioma	Person of contact	sybille van den Brule (sybille.vandenbrule@uclouvain.be)
	KE based on:	https://aopwiki.org/aops/171 Kuempel et al 2017 (doi: 10.1080/10408444.2016.1206061) Chernova et al 2017 (doi: 10.1016/j.cub.2017.09.007)
	Markers based on:	Chernova et al 2017 (doi: 10.1016/j.cub.2017.09.007) Kuempel et al 2017 (doi: 10.1080/10408444.2016.1206061) Bononi et al 2015 (DOI:10.1586/17476348.2015.1081066) Mohr et al 2005 (PMID: 16316830)

KE that are not covered by PATROLS in vitro strategy

KE number	KE	markers	cell type	assay	Type of evidence
1086	Cytotoxicity (pleura or peritoneum)	cell viability and damage, apoptosis?	mesothelial cells	WST-1, LDH activity, apoptotic markers?	?
1088	Oxidative Stress	HMOX-1 induction	mesothelial cells	qRT-PCR	C
		8-hydroxy-2'-deoxyguanosine (8-OHdG)	mesothelial cells, fibroblasts, macrophages?	modified comet assay with enzymes, ELISA	C
1087	inflammation	alarmins (HMGB-1)	mesothelial cells	ELISA	C
		pro-inflammatory cytokines (IL-6, IL-1β, TNF-α)	mesothelial cells, fibroblasts, inflammatoy cells (macrophages and lymphocytes)	ELISA	C

To be filled by partners			
markers	**cell type**	**assay**	**partner**
8OHGG	A549, NCI-H441, macrophages (co-cultures of both)	COMET	SU
IL-6, IL-1β, TNF-α	fibroblasts, macrophages	ELISA R&D Systems (Cat#DY206, DY201, DY210)	AMI

		inflammatory signature (Chernova 2017, not clearly described)	mesothelial cells, fibroblasts, inflammatoy cells (macrophages and lymphocytes)	mRNA array, NGS	D, F (observed in mouse asbestos mesothelioma)				
		STAT3 activation (phosphorylation)	fibroblasts, inflammatoy cells (macrophages and lymphocytes)	Antibody-based array, Western blot, …	C, F (observed in human mesothelioma)				
		STAT3 expression	mesothelial cells, fibroblasts, inflammatoy cells (macrophages and lymphocytes)	qRT-PCR, …	C				
		Src kinases activation (phosphorylation)	mesothelial cells, fibroblasts, inflammatoy cells (macrophages and lymphocytes)	Antibody-based array, Western blot, …	C, F (observed in human mesothelioma)				
		Akt activation (phosphorylation)	mesothelial cells, fibroblasts, inflammatoy cells (macrophages and lymphocytes)	Antibody-based array, Western blot, …	C, F (observed in human mesothelioma)				

		mTOR activation (phosphorylation)	mesothelial cells, fibroblasts, inflammatoy cells (macrophages and lymphocytes)	Antibody-based array, Western blot, …	C, F (observed in human mesothelioma)				
		ERK1/2 activation (phosphorylation)	mesothelial cells	Antibody-based array, Western blot, …	C, F (observed in human mesothelioma)				
		IL-6 expression	mesothelial cells, fibroblasts, inflammatoy cells (macrophages and lymphocytes)	qRT-PCR, …	C	IL-6	A549, NCI-H441, macrophages (co-cultures of both)	qRT-PCR	SU
1032	Secretion local growth factors	EGF?, PDGF, VEGF	mesothelial cells	ELISA	C, F (observed in human mesothelioma)				
1089	Cell Proliferation (mesothelium)	cell proliferation	mesothelial cells, fibroblasts?	WST-1, other assays?	C				
		EGF receptor	mesothelial cells	Western blot	F (Pache 1998, PMID: 9466557)				
		Histone H3 phosphorylation	mesothelial cells, fibroblasts?	Western blot (relevant in vitro?)	C				

	Genotoxicity	8-hydroxy-2'-deoxyguanosine (8-OHdG)	mesothelial cells, fibroblasts, macrophages?	modified comet assay with enzymes, ELISA	C	8OHGG	A549, NCI-H441, macrophages (co-cultures of both)	COMET	SU
		DNA damage	mesothelial cells	comet assay	C, A (Kuempel 2017)				
		mutagenicity	mesothelial cells	micronuclei	C				
	Genome instability	p16 and p19 expression (products of the tumor suppressor gene Cdkn2a)	mesothelial cells, fibroblasts?	Antibody-based array, Western blot, qRT-PCR	C, F (observed in human mesothelioma)				
		Cdkn2a (Ink4a/Arf) hypermethylation	mesothelial cells, fibroblasts?	Bisulfite sequencing	C, F (observed in human mesothelioma)				
1090	mesothelioma								

5) Sheet 5: MIEs/KEs, biomarkers and assays for liver inflammation.

Liver fibrosis	Person of contact	Penny Nymark (penny.nymark@ki.se)
	KE based on:	https://aopwiki.org/aops/144 Gerloff et al. 2016, doi.org/10.1016/j.comtox.2016.07.001
	Markers based on:	Kohonen et al. 2017, doi: 10.1038/ncomms15932 (Pathways related to the PTGS components are derived from Supplemental Data 4b. Genes for each components are available in Supplemental Data 2)

Red pathways indicate overlap with AOPwiki description of the KE.

KE that are not covered by PATROLS in vitro strategy

KE number	KE	markers	cell type	assay	Type of evidence
1539	Endocytotic lysosomal uptake				
898	Lysosome, Disruption				
209	Oxidative Stress, Increase	**PTGS component G, H and N (in total 242 genes related to the following IPA ToxList pathways:** Cardiac Hypertrophy Liver Necrosis/Cell Death Liver Proliferation Cardiac Fibrosis Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa Renal Necrosis/Cell Death Increases Liver Hyperplasia/Hyperproliferation	Hepatocyte (e.g. HepG2, HepRG)	transcriptomics, whole genome or reduced feature high-throughput transcriptomics of PTGS	C

To be filled by partners			
markers	**cell type**	**assay**	**partner**
Oxidative Stress	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	RT-PCR Biorad Hepatocarcinoma Panel (AOP Genes of Interest: JUN, NFKB1, SOD, HIF-1α and MAPK)	SU
ROS	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	CM-H2DCFDA - Invitrogen (Cat#C6827)	SU

		Primary Glomerulonephritis Biomarker Panel (Human) RAR Activation Hepatic Cholestasis Cardiac Necrosis/Cell Death VDR/RXR Activation **Oxidative Stress (ICAM1,JUN,NFKB2,NFKB1)** Increases Cardiac Dysfunction Acute Renal Failure Panel (Rat) Increases Liver Damage NRF2-mediated Oxidative Stress Response p53 Signaling Hepatic Stellate Cell Activation NF-kB Signaling Hypoxia-Inducible Factor Signaling Aryl Hydrocarbon Receptor Signaling Increases Heart Failure PPARa/RXRa Activation LXR/RXR Activation Hepatic Fibrosis)		(1331 genes)		Oxidative stress response	HepG2 BAC-GFP reporters (SRXN1, HMOX1, NQO1, NRF2, KEAP1)	Confocal microscopy	Leiden
						Lipid peroxidation (TBARS)	3D human primary multicellular MT	Abcam	HWU

177	Mitochondrial dysfunction	**PTGS component I (in total 76 genes related to the following IPA ToxList pathways:** Increases Liver Damage Renal Necrosis/Cell Death Cardiac Hypertrophy Hepatic Fibrosis Cardiac Fibrosis VDR/RXR Activation TGF-b Signaling Liver Proliferation Cardiac Necrosis/Cell Death Increases Renal Damage Hepatic Stellate Cell Activation Liver Necrosis/Cell Death **Decreases Transmembrane Potential of Mitochondria and Mitochondrial Membrane (TGM2,IFNG,BNIP3,FGF2,MAPK9)** Increases Renal Proliferation Cell Cycle: G1/S Checkpoint Regulation Increases Cardiac Dilation Anti-Apoptosis Hepatic Cholestasis Increases Cardiac Dysfunction Increases Glomerular Injury)	Hepatocyte (e.g. HepG2, HepRG)	transcriptomics, whole genome or reduced feature high-throughput transcriptomics of PTGS (1331 genes)	C	Mitochondrial Dysfunction	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	RT-PCR Biorad Hepatocarcinoma Panel (AOP Genes of Interest: IFNG and FGF2)	SU
						Caspase 3/7 assay	3D human primary multicellular MT	Promega	HWU

55	Cell injury/death	**PTGS component G, H, N and I (in total 299 genes related to the following IPA ToxList pathways:** Cardiac Hypertrophy **Liver Necrosis/Cell Death (ADM,IFNG,NFKBIA,SMAD3,CDKN1A,MAPK9,PTGS2,SERPINE1)** Liver Proliferation Cardiac Fibrosis Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa Renal Necrosis/Cell Death Increases Liver Hyperplasia/Hyperproliferation Primary Glomerulonephritis Biomarker Panel (Human) RAR Activation Hepatic Cholestasis Cardiac Necrosis/Cell Death VDR/RXR Activation Oxidative Stress Increases Cardiac Dysfunction Acute Renal Failure Panel (Rat) Increases Liver Damage NRF2-mediated Oxidative Stress Response p53 Signaling Hepatic Stellate Cell Activation NF-kB Signaling Hypoxia-Inducible Factor Signaling Aryl Hydrocarbon Receptor Signaling	Hepatocyte (e.g. HepG2, HepRG)	transcriptomics, whole genome or reduced feature high-throughput transcriptomics of PTGS (1331 genes)	C	Cell Death	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	RT-PCR Biorad Hepatocarcinoma Panel (AOP Genes of Interest: IFNG, CDKNIA and PTGS2)	SU
						Cell Death/Cytotoxicity	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	Trypan Blue exclusion (Sigma - T8154)	SU
						Necrosis/apoptosis	HepG2	Propidium iodide / AnnexinV staining with Confocal microscopy	Leiden

		Increases Heart Failure PPARa/RXRa Activation LXR/RXR Activation Hepatic Fibrosis TGF-b Signaling Increases Renal Damage Decreases Transmembrane Potential of Mitochondria and Mitochondrial Membrane Increases Renal Proliferation Cell Cycle: G1/S Checkpoint Regulation Increases Cardiac Dilation Anti-Apoptosis Increases Glomerular Injury)				Adenylate kinase AND live/dead staining AND hiostology	3D human primary multicellular MT	Lonza AND abcam AND NA	HWU
87	Cytokine, Release					IL-8, IL-6 & TNF-α	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	R&D Systems (Cat#DY208) R&D Systems (Cat#DY206) R&D Systems (Cat#DY210)	SU
						IL1B, IL8, IL10, IFN-ϒ, TNF, IL6	3D human primary multicellular MT	Biotechne flex sets	HWU
901	Inflammatory cells, Infiltration								

902	Liver, Inflammation	**PTGS component G and N* (in total 162 genes related to the following IPA ToxList pathways:** Cardiac Hypertrophy Liver Necrosis/Cell Death Liver Proliferation Cardiac Fibrosis Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa Renal Necrosis/Cell Death Increases Liver Hyperplasia/Hyperproliferation Primary Glomerulonephritis Biomarker Panel (Human) RAR Activation Hepatic Cholestasis Cardiac Necrosis/Cell Death VDR/RXR Activation Oxidative Stress Increases Cardiac Dysfunction Acute Renal Failure Panel (Rat) Increases Liver Damage NRF2-mediated Oxidative Stress	Hepatocyte (e.g. HepG2, HepRG)	transcriptomics, whole genome or reduced feature high-throughput transcriptomics of PTGS (1331 genes)	C	Liver Inflammation	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	RT-PCR Biorad Hepatocarcinoma Panel (AOP Genes of Interest: TNFAIP3, IL1B and NFKB1)	SU
						IL1B, IL8, IL10, IFN-ϒ, TNF, IL6	3D human primary multicellular MT	Biotechne flex sets	HWU

		Response p53 Signaling Hepatic Stellate Cell Activation **NF-kB Signaling (TNIP1,NFKBIA,NFKBIE,RELB,TNFAIP3,IL1B,NFKB2,NFKB1)** Hypoxia-Inducible Factor Signaling Aryl Hydrocarbon Receptor Signaling Increases Heart Failure PPARa/RXRa Activation Hepatic Fibrosis LXR/RXR Activation)				NFkB signaling	HepG2 BAC-GFP reporters for NFkB signaling (ICAM1, A20, RelA)	Confocal microscopy	Leiden

*strongly related to the probability of the final AO happening in vivo

6) Sheet 6: MIEs/KEs, biomarkers and assays for liver fibrosis.

Liver fibrosis	Person of contact	Penny Nymark (penny.nymark@ki.se)
	KE based on:	https://aopwiki.org/aops/144 Gerloff et al. 2016, doi.org/10.1016/j.comtox.2016.07.001
	markers based on:	Kohonen et al. 2017, doi: 10.1038/ncomms15932. (Pathways related to the PTGS components are derived from Supplemental Data 4b. Genes for each components are available in Supplemental Data 2)

Red pathways indicate overlap with AOPwiki description of the KE.

KE that are not covered by PATROLS in vitro strategy

KE number	KE	markers	cell type	assay	Type of evidence	To be filled by partners: markers	cell type	assay	partner
1539	Endocytotic lysosomal uptake								
898	Disruption, Lysosome								
177	N/A, Mitochondrial dysfunction 1	**PTGS component I (in total 76 genes related to the following IPA ToxList pathways:** Increases Liver Damage Renal Necrosis/Cell Death Cardiac Hypertrophy Hepatic Fibrosis	Hepatocyte (e.g. HepG2, HepRG)	transcriptomics, whole genome or reduced feature high-	C	Mitochondrial Dysfunction	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	RT-PCR Biorad Hepatocarcinoma Panel (AOP Genes of Interest: IFNG and FGF2)	SU

		Cardiac Fibrosis VDR/RXR Activation TGF-b Signaling Liver Proliferation Cardiac Necrosis/Cell Death Increases Renal Damage Hepatic Stellate Cell Activation Liver Necrosis/Cell Death **Decreases Transmembrane Potential of Mitochondria and Mitochondrial Membrane (TGM2,IFNG,BNIP3,FGF2,MAPK9)** Increases Renal Proliferation Cell Cycle: G1/S Checkpoint Regulation Increases Cardiac Dilation Anti-Apoptosis Hepatic Cholestasis Increases Cardiac Dysfunction Increases Glomerular Injury)		throughput transcriptomics of PTGS (1331 genes)		Caspase 3/7 assay	HepG2	Caspase-3/7-glo assay	Misvik

55	N/A, Cell injury/death	**PTGS component G, H, N and I (in total 299 genes related to the following IPA ToxList pathways:** Cardiac Hypertrophy **Liver Necrosis/Cell Death (CXCL3,TNIP1,JUN,NFKBIA,IER3,CEBPB,CFLAR,RXRA,NFKB1)** Liver Proliferation Cardiac Fibrosis Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa Renal Necrosis/Cell Death Increases Liver Hyperplasia/Hyperproliferation Primary Glomerulonephritis Biomarker Panel (Human) RAR Activation Hepatic Cholestasis Cardiac Necrosis/Cell Death VDR/RXR Activation Oxidative Stress Increases Cardiac Dysfunction Acute Renal Failure Panel (Rat) Increases Liver Damage NRF2-mediated Oxidative Stress Response p53 Signaling Hepatic Stellate Cell Activation NF-kB Signaling Hypoxia-Inducible Factor Signaling Aryl Hydrocarbon Receptor Signaling Increases Heart Failure PPARa/RXRa Activation	Hepatocyte (e.g. HepG2, HepRG)	transcriptomics, whole genome or reduced feature high-throughput transcriptomics of PTGS (1331 genes)	C	Cell Death	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	RT-PCR Biorad Hepatocarcinoma Panel (AOP Genes of Interest: JUN, RXRA and NFKB1)	SU
						Cell Death/Cytotoxicity	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	Trypan Blue exclusion (Sigma - T8154)	SU
						Necrosis /apoptosis	HepG2	Propidium iodide / AnnexinV staining with	Leiden

		LXR/RXR Activation Hepatic Fibrosis TGF-b Signaling Increases Renal Damage Decreases Transmembrane Potential of Mitochondria and Mitochondrial Membrane Increases Renal Proliferation Cell Cycle: G1/S Checkpoint Regulation Increases Cardiac Dilation Anti-Apoptosis Increases Glomerular Injury)			

		Confocal microscopy	
Cell viability	HepG2	CellTiter-Glo assay	Misvik
Cell Number	HepG2	Dapi staining	Misvik
Nucleic acid oxidative stress	HepG2	8OHG staining	Misvik
DNA damage	HepG2	gamma-H2AX staining	Misvik
Apoptosis	HepG2	Caspase-3/7-glo assay	Misvik

1493	Increased Pro-inflammatory mediators	**PTGS component G and N (in total 162 genes related to the following IPA ToxList pathways:** Cardiac Hypertrophy Liver Necrosis/Cell Death Liver Proliferation Cardiac Fibrosis Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa Renal Necrosis/Cell Death Increases Liver Hyperplasia/Hyperproliferation Primary Glomerulonephritis Biomarker Panel (Human) RAR Activation Hepatic Cholestasis Cardiac Necrosis/Cell Death VDR/RXR Activation Oxidative Stress Increases Cardiac Dysfunction Acute Renal Failure Panel (Rat) Increases Liver Damage NRF2-mediated Oxidative Stress Response p53 Signaling Hepatic Stellate Cell Activation **NF-kB Signaling**	Hepatocyte (e.g. HepG2, HepRG)	transcriptomics, whole genome or reduced feature high-throughput transcriptomics of PTGS (1331 genes)	C	Liver Inflammation	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	RT-PCR Biorad Hepatocarcinoma Panel (AOP Genes of Interest: TNFAIP3, IL1B, IL8 and NFKB1)	SU
						IL-8, IL-6 & TNF-α	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	R&D Systems (Cat#DY208) R&D Systems (Cat#DY206) R&D Systems (Cat#DY210)	SU

		(TNIP1,NFKBIA,NFKBIE,RELB,TNFAIP3,IL1B,NFKB2,NFKB1) Hypoxia-Inducible Factor Signaling Aryl Hydrocarbon Receptor Signaling Increases Heart Failure PPARa/RXRa Activation Hepatic Fibrosis LXR/RXR Activation)				NFkB signaling	HepG2 BAC-GFP reporters for NFkB signaling (ICAM1, A20, RelA)	Confocal microscopy	Leiden
1494	Leukocyte recruitment/activation								

265	Activation, Stellate cells	**PTGS component G, N and I (in total 226 genes related to the following IPA ToxListpathways:** Cardiac Hypertrophy Liver Necrosis/Cell Death Liver Proliferation Cardiac Fibrosis Mechanism of Gene Regulation by Peroxisome Proliferators via PPARa Renal Necrosis/Cell Death Increases Liver Hyperplasia/Hyperproliferation Primary Glomerulonephritis Biomarker Panel (Human) RAR Activation Hepatic Cholestasis Cardiac Necrosis/Cell Death VDR/RXR Activation Oxidative Stress Increases Cardiac Dysfunction Acute Renal Failure Panel (Rat) Increases Liver Damage NRF2-mediated Oxidative Stress Response p53 Signaling **Hepatic Stellate Cell Activation (IL8,PDGFA,NFKB2,NFKB1)** NF-kB Signaling Hypoxia-Inducible Factor Signaling Aryl Hydrocarbon Receptor Signaling Increases Heart Failure	Hepatocyte (e.g. HepG2, HepRG)	transcriptomics, whole genome or reduced feature high-throughput transcriptomics of PTGS (1331 genes)	C	Stellate Cell Activation	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	RT-PCR Biorad Hepatocarcinoma Panel (AOP Genes of Interest: IL8 and NFKB1)	SU
						Stellate activation	3D human primary multicellular MT containing stellate cells	alpha-SMA ELISA, LOX activity, Col1A1 expression (qPCR), p3np (procollagen III N-terminal peptide) ELISA	HWU / Insphero

		PPARa/RXRa Activation Hepatic Fibrosis **TGF-b Signaling (SMAD3,TGFB2,MAPK9,MAP2K3, SMURF2,SERPINE1)** Increases Renal Damage Decreases Transmembrane Potential of Mitochondria and Mitochondrial Membrane Increases Renal Proliferation Cell Cycle: G1/S Checkpoint Regulation Increases Cardiac Dilation Anti-Apoptosis Increases Glomerular Injury LXR/RXR Activation)				Stellate activation and Pathology	3D human primary multicellular MT containg stellate cells	Histology - Trichrome Masson staining, Siriusred staining with dark field microscopy; collagen 1 and 4 staining	HWU / Insphero
68	Accumulation, Collagen								

344	N/A, Liver fibrosis	**PTGS component N and I* (in total 106 genes related to the following IPA ToxList pathways:** Increases Liver Damage Renal Necrosis/Cell Death Cardiac Hypertrophy **Hepatic Fibrosis (IL8,ICAM1,PDGFA,IL1B,CXCL2)** Cardiac Fibrosis VDR/RXR Activation TGF-b Signaling Liver Proliferation Cardiac Necrosis/Cell Death Increases Renal Damage Hepatic Stellate Cell Activation Liver Necrosis/Cell Death Decreases Transmembrane Potential of Mitochondria and Mitochondrial Membrane Increases Renal Proliferation Cell Cycle: G1/S Checkpoint Regulation Increases Cardiac Dilation Anti-Apoptosis Hepatic Cholestasis Increases Cardiac Dysfunction Increases Glomerular Injury PPARa/RXRa Activation Mechanism of Gene Regulation by Peroxisome Proliferators via	Hepatocyte (e.g. HepG2, HepRG)	transcriptomics, whole genome or reduced feature high-throughput transcriptomics of PTGS (1331 genes)	C	Fibrosis	HepG2 hepatocyte monoculture HepG2/Kupffer cell co-culture	RT-PCR Biorad Hepatocarcinoma Panel (AOP Genes of Interest: IL8 and IL1B)	SU

		PPARa NF-kB Signaling Aryl Hydrocarbon Receptor Signaling Oxidative Stress LXR/RXR Activation RAR Activation)							

*strongly related to the probability of the final AO happening in vivo

7) Sheet 7: MIEs/KEs, biomarkers and assays for liver cancer.

Liver cancer	Person of contact	Ulla Birgitte Vogel (UBV@nfa.dk)
	KE based on:	https://aopwiki.org/events/378
		PMID: 29298701 Modrzynska et al, Part Fibre Toxicol. 2018 Jan 3;15(1):2. doi: 10.1186/s12989-017-0238-9.
	Markers based on:	PMID: 18618583; Jacobsen et al, Environ Mol Mutagen. 2008 Jul;49(6):476-87. doi: 10.1002/em.20406

KE that are not covered by PATROLS in vitro strategy

KE number	KE	markers	cell type	assay	Type of evidence
KE249, KE257, KE1115, KE1364	MIE: particle surface dependent ROS generation				
1608	Oxidative DNA damage	oxidative DNA damage/DNA adducts/DNA strand breaks in liver tissue	liver cells	oxidative DNA damage/DNA adducts/comet assay/micronucleus asssay	B

To be filled by partners

markers	cell type	assay	partner
DNA damage, Genotoxicity	HepG2 monoculture HepG2/Kuppfer cell co-culture	Cytokinesis block micronucleus assay	SU
DNA damage response	HepG2 BAC-GFP reporters for DNA damage response (P21, BTG2, MDM2, P53)	Confocal microscopy	Leiden
Oxidative DNA damage	3D human primary multicellular MT	FPG modified Comet assay	HWU
Oxidative DNA damage	HepG2 monoculture	8OHG staining	Misvik

						DNA strand breaks	HepG2 monoculture	gamma-H2AX staining	Misvik
185 376	Increased mutations	Mutations	liver cells	in vitro assay of mutation: OECD TG 488: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays	A, B, C				
378	Tumorigenesis, Hepatocellular carcinoma								

7. References

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