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Abstract

Adverse outcome pathways (AOPs) and their networks are important tools for the development of mechanistically based non-animal testing approaches, such as *in vitro* and/or *in silico* assays, to assess toxicity induced by chemicals. In the present study, an AOP network connecting 14 linear AOPs related to human hepatotoxicity, currently available in the AOP-Wiki, was derived according to established

criteria. The derived AOP network was characterised and analysed with regard to its structure and topological features. In-depth analysis of the AOP network showed that cell injury/death, oxidative stress, mitochondrial dysfunction and accumulation of fatty acids are the most highly connected and central key events. Consequently, these key events may be considered as the rational and mechanistically anchored basis for selecting, developing and/optimising *in vitro* and/or *in silico* assays to predict hepatotoxicity induced by chemicals in view of animal-free hazard identification.

Key words

Adverse outcome pathway; predictive toxicology; network derivation; hepatotoxicity.

Abbreviations

ACC-1 Acetyl-CoA carboxylase; AO Adverse outcome; AOP Adverse outcome pathway; AKT2 RAC-β serine/threonine-protein kinase; BSEP Bile salt export pump; CAR Constitutive androstane receptor; CD36 Cluster of differentiation 36; FA Fatty acid; FXR Farnesoid X receptor; GR Glucocorticoid Receptor; HSC Hepatic stellate cells; KE Key event; KER Key event relationship; MIE Molecular initiating event; LXR Liver X receptor; OECD Organisation for Economic Cooperation and Development; OSTα/β Organic solute transporter α/β; PXR Pregnane X receptor; ROS Reactive oxygen species; SCD-1 Stearoyl-CoA desaturase-1; TG Triglyceride

1. Introduction

The adverse outcome pathway (AOP) is a conceptual framework used to aggregate and organise biological knowledge, which can be employed to translate mechanistic data into outcomes relevant to chemical safety assessment (Ankley et al. 2010). AOPs are modular and consist of a molecular initiating event (MIE), one or more key event(s) (KEs) and an adverse outcome (AO), which represent responses at different levels of biological organisation. Key event relationships (KERs) specify the causal linkages between each KE across multiple biological levels of organisation (Vinken 2013; Vinken et al. 2017). In 2012, the Organisation for Economic Cooperation and Development (OECD) launched

a programme for the development of AOPs, and later developed a guidance document to standardise the construction and assessment of AOPs (OECD 2017). The OECD, along with a number of other stakeholders, also introduced the AOP Knowledgebase (https://aopkb.oecd.org/), which serves as the primary repository for AOPs. The AOP-Wiki (https://aopwiki.org/) represents one of the five modules of the AOP Knowledgebase. At present, the AOP-Wiki contains 320 AOPs, more than 1390 KEs and as many as 1860 KERs.

Although AOPs are assumed to be chemical agnostic, they can support the use of a mode (and/or mechanism) of action basis for understanding adverse effects of chemicals and other stressors. In recent years, emphasis has been placed on AOPs as a conceptual support in the construction of mechanistically based non-animal testing approaches consisting of a combination of *in vitro* and *in silico* methods (Burden et al. 2015; Kleinstreuer et al. 2016; Sakuratani et al. 2018; Vinken 2018; Parish et al. 2020). The general principle of the use of these approaches is that a limited set of (measurable) KEs can sufficiently describe or predict a toxicological response (Worth and Patlewicz, 2016). If AOPs are to be useful for safety assessment purposes, rather than merely serving hazard identification or characterisation, it is crucial that the KERs in an AOPs reflect quantitative elements of the toxicity pathway (Carusi et al. 2018; Sewell et al. 2018). However, linear AOPs may be of limited use for the purpose of safety assessment, as biological processes are in most cases complex (Sewell et al. 2018). Indeed, interaction and crosstalk between biological pathways are acknowledged to be the norm rather than the exception. So-called AOP networks provide the actual tools for real-life applications of AOPs, as they more realistically represent interactions occurring in a systems biology context (Villeneuve et al. 2014). An AOP network is defined as an assembly of two or more linear AOPs that share one or more KEs, including the MIE and AO. In an AOP network, KEs represent nodes, while KERs are reflected as directed edges that link those nodes together (OECD 2018). AOP networks open the possibility of linking toxicological pathways, thus highlighting areas where one assay could predict multiple outcomes or converge multiple MIEs (Knapen et al. 2018). Network science provides the means to quantitatively analyse AOP networks and identify such KEs of interest (Sturla et al. 2014;

Hartung et al. 2017; Villeneuve et al. 2018). In this respect, an AOP network for neurotoxicity was previously introduced (Spinu et al. 2019) based on established guidelines regarding AOP network derivation, characterisation and analysis (Knapen et al. 2018; Villeneuve et al. 2018). In the present study, a similar exercise was undertaken focused on hepatotoxicity with the aim to identify relevant KEs that could form the basis for setting up a battery of *in vitro* and/or *in silico* assays for predictive toxicity screening of chemicals.

2. Materials and Methods

2.1. Data set

The OECD AOP-Wiki 2.0 was manually searched to identify linear AOPs related to hepatotoxicity and to derive the AOP network according to previously published criteria (Spinu et al. 2019). For each individual AOP, the following information was extracted: KE title, KE type (*i.e.* MIE, KE, AO), KER (*i.e.* linkage between upstream and downstream KEs), adjacency of the relationship between a pair of KEs, qualitative weight of evidence (WoE) (*i.e.* low, moderate, high), AOP development stage as reported by the AOP developer, and progress through the OECD review and endorsement processes. The information regarding individual AOPs was extracted 1 September 2020. All data collected are available as supplementary material (Appendix 1).

2.2. AOP network derivation

The process of deriving the hepatotoxicity AOP network was performed following four steps as described elsewhere (Spinu et al. 2019).

Step 1 - Definition of purpose

The present study aimed to identify the most common and the most highly connected KEs in a hepatotoxicity AOP network that could be measured in *in vitro* assays to assist in the prediction of adverse effects of chemicals on the liver. Accordingly, the scope of the study included AOPs developed for hepatotoxicity and published in the AOP-Wiki.

Step 2 - Definition of criteria for AOP selection

AOPs were selected based on the following criteria: the taxonomic applicability and the AOP development stage in terms of the progress of the AOP through the OECD review and endorsement processes. Taxonomy was considered the main criterion for collecting individual AOPs (*i.e.* those applicable and relevant for humans). The AOP development stage was considered, as it indicates the level of maturity of the AOPs used to derive the AOP network. The difference between adjacent KERs and non-adjacent KERs, previously termed "indirect KERs", was taken into consideration. Additionally, the WoE for the relationships between the KEs, which relied on the assessment performed by the AOP developers, described as low, medium or high, was considered.

Step 3 - Identification of appropriate AOPs from the AOP-Wiki and data curation

AOPs identified according to the criteria in step two were evaluated and collected manually in an Excel spreadsheet. In the cases where KEs were given different titles, while having the same meaning and/or referring to the same process, they were grouped and renamed under a common KE title. Wherever possible, abbreviations have been used. All amendments to KE titles are described in the Excel spreadsheet available as supplementary material (Appendix 1).

Step 4 - Generation and analysis of the network

Cytoscape 3.8.0 (https://cytoscape.org/), which is an open source software platform, was used to model the AOP network. NetworkAnalyzer 4.4.6 App, pre-installed in the Cytoscape software, was used to analyse the derived network. The nodes (*i.e.* KEs) were manually positioned to maximise readability. Information regarding WoE, adjacency and type of KE were added to further define the visual attributes of the AOP network. KERs shared by more than one AOP are represented by a single arrow, though such multiple relationships between KEs were considered during network analysis.

2.3. Network analysis

The derived network was analysed using Cytoscape NetworkAnalyzer 4.4.6 App. The level of degree, betweenness centrality and eccentricity were used to characterised the network analytically due to

their ability to quantify the position of a KE in relation to its neighbour KEs in the network (Spinu et al. 2019). By analysing the network as a directed graph, the level of indegree and outdegree was used to identify points of convergence and divergence, as well as to analyse the overall connectivity of the KEs across the network. The most upstream and downstream KEs were assessed based on the analysis of the eccentricity and betweenness centrality. With the non-directed analysis of the AOP network, the eccentricity was used to identify the most central KEs in the AOP network. The combined consideration of all parameters was used to identify the most highly connected and central KEs.

3. Results and discussion

3.1. Derivation of an AOP network for human hepatotoxicity

By manually searching the AOP-Wiki, 30 AOPs related to human hepatotoxicity were identified. Of those, 16 were considered suitable to be included in the network based on the defined criteria. As AOP ID 58 (NR1I3 (CAR) suppression leading to hepatic steatosis) was believed to be a more comprehensive version of AOP ID 34 (LXR activation leading to hepatic steatosis), it was decided to exclude the latter from the data set. Detailed information on the included AOPs is provided in Table 1. To aid readability, only the AOP ID and a note to indicate the MIE (*i.e.* AOP ID 58/CAR) will be used throughout the text. Differences in KE annotation (*i.e.* discrepancies between KE titles albeit the action refers to the same process) was previously identified as a challenge in the development of AOP networks (Spinu et al. 2019). In the present study, multiple KEs relating to the same process were identified, albeit being titled differently. In this respect, KE 327 (accumulation, fatty acid), KE 838 (induction, microvesicular fat) and KE 1305 (increase, cytosolic fatty acid) all refer to the accumulation of fatty acids (FAs), but they are titled differently. These inconsistencies in KE annotation were expected to have a major impact on the construction of the AOP network. For this reason, discrepancies in annotation have been reviewed and KEs addressing the same process have been grouped or renamed under common KE titles. Annotation changes are provided in the supplementary material (Appendix 1).

Non-adjacent KERs describe the relationship between KEs that are not next to one another in the sequence defined for the AOP and help to more thoroughly capture the WoE supporting an AOP while maintaining the modular structure (Villeneuve et al. 2018). Non-adjacent KERs have been reported likely to be associated with more than one biological process (Spinu et al. 2019). Hence, an AOP network containing both adjacent and non-adjacent KERs implies more connections, thereby representing a higher level of biological complexity. However, inclusion of non-adjacent KERs in the analysis of an AOP network has been suggested to inflate node degree and betweenness centrality or deflate distance-based calculations like eccentricity (Villeneuve et al. 2018). Moreover, if a KE is to be useful for the prediction of adverse effects and be fit to serve as a basis for *in vitro* and/or *in silico* assay selection and/or development, a detailed quantitative description of the relationship between KEs is required (Carusi et al. 2018). Consequently, it is called for that the AOP network relies on directly connected KEs to facilitate an accurate quantitative simulation of the AOP network. The difference between adjacent and non-adjacent KERs was considered accordingly and a total of nine non-adjacent KERs from five different AOPs, including AOP ID 38/PROTEIN ALKYLATION, AOP ID 58/CAR, AOP ID 60/PXR, AOP ID 220/CYP2E1 and AOP ID 278/IKK, were excluded. In addition, one AOP (AOP ID 62 AKT2 activation to steatosis) containing (solely) three non-adjacent KERs was omitted from the dataset. Due to the removal of the non-adjacent KERs, including AOP ID 62, there was a discrepancy of four KEs between the two AOP networks, namely, activation of RAC-β serine/threonine-protein kinase (AKT2), chronic inflammation, increased tumour necrosis factor α (TNFα) and liver cancer.

A network of 14 AOPs (Table 1), all of which were found to share common KEs, was ultimately derived (Figure 1). A hepatotoxicity AOP network including all KERs (*i.e.* non-adjacent KERs and adjacent KERs) is available as supplementary material (Figure S1, Appendix 2). The derived hepatotoxicity AOP network consists of 14 linear AOPs related to various AOs, including cholestasis (1), steatosis (4), fibrosis (3), steatohepatitis (1), liver injury, described as the altered state of the liver wherein the normal homeostasis of all processes in the liver are perturbed, (3) and hepatotoxicity, defined as cell death (1). Not surprisingly, there is no adjacent relationships between any KE and the AO cancer (AOP

ID 220/CYP2E1). Therefore, albeit AOP ID 220/CYP2E1 is present in the network, its AO, defined as liver cancer, lost its connection to the network as it is connected to the upstream KEs (*i.e.* increased reactive oxygen species (ROS), cell injury/death and sustained cell proliferation) exclusively through non-adjacent KERs. For this reason, the AO liver cancer is not present in the network containing only adjacent KERs (Figure 1).

3.2. Characterisation and analysis of the AOP network for human hepatotoxicity

AOP networks can contribute to the knowledge regarding interactions among linear AOPs and analysis thereof can reveal unexpected or overlooked connections between toxicity pathways. The analysis was performed on the derived AOP network for hepatotoxicity involving 14 AOPs (Table 1) with a total of 82 unique KEs and their adjacent relationships (Appendix 1). No single KE was present in all 14 AOPs. The most common KE across all AOPs is cell injury/death, which is shared by six out of the 14 AOPs (*i.e.* AOP ID 36/PPAR, AOP ID 144/LYSOSOMAL UPTAKE, AOP ID 209/SREBF2, AOP ID 220/CYP2E1, AOP ID 273/MITOCHONDRIAL INHIBITION and AOP ID 278/IKK) (Figure 2a&b), thereby connecting linear AOPs leading to steatosis, fibrosis, hepatotoxicity, cancer and liver injury. In the network, cell injury/death is triggered by multiple mechanisms, including perturbation of cholesterol, altered glutathione homeostasis, protein alkylation, mitochondrial dysfunction, increased ROS, impaired proteostasis and activation of the caspase 8 pathway. Perturbation of this KE leads to tissue resident cell activation, release of pro-inflammatory mediators, sustained cell proliferation and presence of necrotic tissue.

FA accumulation, decreased β-oxidation and steatosis are all shared by five AOPs (Figure 2a&b). Steatosis and decreased β-oxidation are shared by the same five AOPs (*i.e.* AOP ID 36/PPAR, AOP ID 58/CAR, AOP ID 60/PXR, AOP ID 213/B-OXIDATION and AOP ID 318/GR). Of those, steatosis is the AO in all but one AOP (AOP ID 213/B-OXIDATION) in which the AOP is defined as steatohepatitis. In the network, steatosis is prompted by increased triglyceride (TG) formation, FA accumulation, and decreased β-oxidation. In AOP ID 213/B-OXIDATION, decreased β-oxidation is considered the MIE.

Additionally, decreased β-oxidation is triggered by decreased 3-hydroxyacyl-CoA dehydrogenase type-2 activity, downregulation of CPT1A, activation of glucocorticoid receptor (GR) and decreased PPAR-α activation. Downstream KEs include FA accumulation and steatosis. FA accumulation is present in AOP ID 36/PPAR, AOP ID 58/CAR, AOP ID 60/PXR, AOP ID 130/PHOSPHOLIPASE A and AOP ID 213/B-OXIDATION. It is not surprising that FA accumulation is a common event, since it is a hallmark of hepatic steatosis (Ipsen et al. 2018) which is the defined AO in four AOPs, and a KE in one AOP, included in the network. FA accumulation is prompted by increased *de novo* FA synthesis, increased FA influx and decreased β-oxidation all of which are indicative of disruption of the FA metabolism (Alves-Bezerra and Cohen 2018), as well as ballooning and vacuolisation of hepatocytes, and vacuolisation of Kupffer and bile duct cells. FA accumulation results in increased TG formation and steatosis. KEs shared by three AOPs include fibrosis, liver injury, activation of hepatic stellate cells (HSC), decreased PPAR-α activation, increased ROS, release of pro-inflammatory mediators and mitochondrial dysfunction. Mitochondrial dysfunction is involved in a feedback loop mechanism together with increased mitochondrial ROS (Figure S1, Appendix 2). KEs shared by two AOPs include accumulation of collagen, increased *de novo* FA synthesis, increased FA influx, increased TG formation, presence of necrotic tissue, up-regulation of cluster of differentiation 36 (CD36) and up-regulation of stearoyl-CoA desaturase-1 (SCD-1).

The MIE defined as decreased PPAR-α activation is the single MIE shared by multiple AOPs (*i.e.* AOP ID 58/CAR, AOP ID 36/PPAR and AOP ID 318/GR), which all are leading to steatosis. Conversely, several AOPs have multiple MIEs (*i.e.* AOP ID 36/PPAR (2), AOP ID 58/CAR (4) and AOP ID 273/MITOCHONDRIAL INHIBITION (8)). The latter AOP, ultimately leading to liver injury, stands out in terms of number of MIEs, which are all connected downstream to the KE decreased oxidative phosphorylation. However, this may be due to an unconventional or even incorrect structure of the AOP, rather than a true representation of the pathway. Furthermore, the structure of AOP ID 209/SREBF2 deviates, as this particular AOP is lacking a defined MIE. Both AOP ID

273/MITOCHONDRIAL INHIBITION and AOP ID 209/SREBF2 are currently stated as to be under development in the AOP-Wiki.

By using the edge count, it was found that the most hyperlinked KEs in the AOP network are accumulation of FA, cell injury/death, mitochondrial dysfunction, decreased β-oxidation and the AO steatosis, with a level of degree of 17, 12, 11, 9 and 9, respectively. The majority of the least connected KEs are defined as MIEs. Exceptions include the AO steatohepatitis for which only one AOP exist, and the KEs upregulation of acetyl-CoA carboxylase (ACC-1), decreased ketogenesis and sustained cell proliferation, all of which were connected to other KEs with non-adjacent KERs that were omitted from the analysis.

The number of shared AOPs by a KE varies between one and six AOPs. The interconnectivity between the AOPs in the network is fairly limited, as 78 % of the 82 KEs are included in merely one AOP (Figure 3a). Nevertheless, 22 % (*i.e.* absolute number: 18) KEs are shared by two or more AOPs. The directed eccentricity score showed that almost half (*i.e.* 45 %) of the KEs are categorised as downstream KEs, whilst 20 % should be regarded as upstream KEs. Accordingly, 35 % of the KEs cannot be categorised as either upstream or downstream due to their level of interconnectivity (Figure 3b).

The point at which the effects of separate stressors may converge to influence a common downstream KE can be the basis for predicting multiple stressors jointly, while measuring only one perturbation. *Vice versa*, divergent KEs indicate where AOPs are branching off from a common MIE or KE. Thus, identified points of convergence and divergence may indicate the most promising KEs for development of *in vitro* and/or *in silico* assays that can encapsulate all the pathways upstream from that particular KE (Knapen et al. 2018; Villeneuve et al. 2018). Because the KERs in an AOP network are directed, the degree of a node in the network (*i.e.* KE) can be broken down to indegree and outdegree, indicating KEs upstream and downstream, respectively, of any specific KE (Knapen et al. 2018; Villeneuve et al. 2018). By using the ratio between the indegree and outdegree, KEs can be attributed to be either convergent or divergent. In the derived hepatotoxicity AOP network,

accumulation of FA is the KE with the highest number of connections to upstream KEs with a value of 11, while being connected to six downstream KEs. This suggests that FA accumulation is a point of convergence, which demonstrates the importance of metabolism and transport in liver disease aetiology. As a matter of fact, FA accumulation is one of the KEs with the highest number of downstream relationships albeit being a point of convergence. Again, this makes sense, as steatosis is the defined AO in several AOPs in the network. Similarly, cell injury/death is a highly connected KE and point of convergence, with seven upstream and five downstream connections. The AO steatosis is regarded as a point of convergence as AOP ID 213/B-OXIDATION, leading to steatohepatitis, uses this AO as a KE. In contrast, activation of the liver X receptor (LXR), defined as a MIE, has one upstream and six downstream connections, of which all ultimately lead to steatosis, and therefore identified as a point of divergence. Mitochondrial dysfunction, shared by AOP ID 144/LYSOSOMAL UPTAKE, AOP ID 130/PHOSPHOLIPASE A and AOP ID 273/MITOCHONDRIAL INHIBITION, is a diverging KE triggered by four upstream events, including disruption of lysosomes, inhibition of phospholipase A, decreased oxidative phosphorylation and increased levels of mitochondrial ROS. Mitochondrial dysfunction leads to cell injury/death, vacuolisation of hepatocytes and Kupffer cells, ballooning of hepatocytes, increased levels of mitochondrial ROS and impaired proteostasis.

The eccentricity of a node in a directed graph measures the maximum distance from one node to any other node in the network. A lower eccentricity score implies a more downstream KE and *vice versa*. Conversely, in a non-directed analysis of a network, the eccentricity score depicts the distance from the centre of the network to that of a given node. Here, a low eccentricity score in an undirected graph therefore implies a more central KE, which in turn indicates what KEs are most easily influenced by other KEs with which they are interconnected. The calculation of eccentricity depends on path length, which is to some extent a subjective result of the number of KEs the AOP developer decides to include. This could vary depending on the AOP's level of maturity and detail. This means that the results can be misleading if the AOPs included in the network vary greatly in length (*i.e.* the numbers of KEs included in the network) (Villeneuve et al. 2018). The non-directed eccentricity score suggests that

cell injury/death, mitochondrial dysfunction, FA accumulation, bile accumulation, increased ROS, collagen accumulation, Mallory body formation, liver fibrosis and perturbation of cholesterol are the most centrally located KEs in the network with the score of seven (Figure S2, Appendix 2). The most upstream KEs, and therefore least sensitive to influence, are decreased oxidative phosphorylation and inhibition of N-linked glycosylation with a score of 12. Similarly, betweenness centrality measures the number of shortest paths between any KEs in the AOP network that passes through a particular KE, and thus could help to identify important KEs in the network. The KEs with the highest betweenness centrality scores were cell injury/death, steatosis, increased ROS, release of pro-inflammatory mediators, mitochondrial dysfunction and FA accumulation with scores of 3.71, 2.97, 2.96, 1.91, 1.78 and 1.59, respectively (Figure S3, Appendix 2).

The combined consideration of all parameters included in the topology analysis suggested that cell injury/death, increased ROS, mitochondrial dysfunction and FA accumulation are highly connected and central KEs in an AOP hepatotoxicity network. Table 3 provides an overview of available assays that can be used to measure endpoints, as well as a selection of representative stressors, associated with these four KEs.

All derived networks, including AOP networks, are sensitive to errors in the data underlying the network, which can affect the analysis and thus the conclusions drawn. A network with a high level of qualitative and quantitative evidence will contribute to the confidence in the selected *in vitro* and/or *in silico* assays used to predict hepatotoxicity. Therefore, the qualitative level of understanding of the relationship between pairs of KEs in the network was examined in the present study. The level of understanding, as reported by the AOP developers, is described as to be moderate or higher for 65 % of the KERs in the network. The qualitative level of understanding for 57 % of the KERs in the network is high (Figure 4). One AOP, AOP ID 130/PHOSPHOLIPASE A, which is still under development in the AOP-Wiki, stands out among the KERs described as low. Indeed, this particular AOP contributes to 20 out of the 22 KERs where the level of understanding is low. Moreover, the AOPs in the AOP-Wiki may

at a given time be at different stages of development, and thus some AOPs may be only partially complete. It has been demonstrated that the user-defined fields, such as title and description in the majority of the AOPs, KEs and KERs in the AOP-Wiki are only partially complete (Pollesch et al. 2019). AOPs, KEs and KERs only contained on average information in 49.2%, 47% and 26.3%, respectively, of the possible fields. The fields with the lowest amount of information were those associated with describing the supporting evidence. In the derived hepatotoxicity AOP network, AOP ID 209/SREBF2, AOP ID 273/MITOCHONDRIAL INHIBITION and AOP ID 285/GLYCOSYLATION, which are all under development in the AOP-Wiki, contain most cases (*i.e.* 23 out of 26) where the qualitative evidence is unspecified. Nevertheless, all AOPs, regardless of their stage of development, were included in the network characterisation and analysis.

4. Conclusions

Next generation non-animal safety assessment of chemicals is focused on hypothesis-driven approaches tailored to the characteristics and intended use of the chemical compound of interest. This paradigm shift in toxicology implies a need to move towards pathway-based strategies that are based on knowledge of the biological mechanisms underlying toxicity in potentially exposed organisms (Mahony et al. 2020). In this context, AOP networks, rather than individual linear AOPs, have emerged as tools for real-life applications, as they provide a more reliable reflection of the mechanistic complexity underlying chemical adversity (Edwards et al. 2016; Sakuratani et al. 2018; Sewell et al. 2018; Coady et al. 2019; Hecker and LaLone 2019). However, a detailed molecular understanding of all possible adversities may not be necessary, nor practical, in chemical safety assessment. Apart from highly specifically acting chemicals, the majority of chemical compounds are likely to perturb more than one AOP. AOP networks are therefore envisaged to be the functional units of prediction for most chemically induced AOs (Worth and Patlewicz, 2016). Hence, it appears logical to make use of KEs that are crucial and conserved across multiple AOPs related to the organ system of interest. In the current study, a previously introduced workflow to develop and analyse an AOP

network for neurotoxicity (Spinu et al. 2019) was applied to the case of hepatotoxicity. Although the derived AOP network almost certainly does not contain all underlying mechanisms of hepatotoxicity, this exercise provided guidance for the prioritisation of KEs for testing. A testing strategy derived from AOP networks includes *in vitro* and/or *in silico* assays that address the KEs capable of serving as alternatives to the measurement of apical AOs in animals. There is a large variety of mechanisms known to be involved in hepatotoxicity (Jaeschke et al. 2002; Russmann et al. 2009; Vinken et al. 2013b) which is reflected in the limited interconnectivity within the derived AOP network in the present study. Analytical characterisation of the AOP network based on individual AOPs currently available in the AOP-Wiki suggests that cell injury/death, increased ROS, mitochondrial dysfunction and FA accumulation are the most highly connected and central KEs. As such, these KEs may be considered for selecting, developing and optimising *in vitro* and/or *in silico* assays to predict hepatotoxicity induced by chemicals. It should be noted that *in vitro* assays must be selected carefully as overlap and redundancies between assays may exist. Nevertheless, there are AOPs that do not pass through any of these most common KEs. Thus, AOP ID 318/GR (GR activation leading to hepatic steatosis) is connected to the network solely through its AO steatosis. However, one may argue that FA accumulation always occurs *prior* the development of steatosis (Ipsen et al. 2018), even though FA accumulation is not present as a KE in AOP ID 318/GR. This may be attributed to the varying level of detail in the linear AOPs included in the network. Another AOP that does not pass through the identified KEs is AOP ID 285/GLYCOSYLATION (Inhibition of N-linked glycosylation leads to liver injury), which is connected to the AOP network through the KEs activation HSC and apoptosis. Again, this may be attributed to a varying level of detail in the KE descriptions. Despite the large efforts in the present study undertaken to harmonise KEs under common KE titles, the derived network contains four KEs that to some extent are related to cell death (cell injury/death, apoptosis, presence of necrotic tissue and increased oncotic necrosis). Although it is well known that cell death can manifest in different ways, this is not consistently reflected in the KEs descriptions. Specifically, the KE cell injury/death does not distinguish between apoptosis and necrosis, albeit the two elicit considerably different

cellular responses (Fink and Cookson, 2005). This greatly implicates KE harmonisation, and ultimately, AOP network derivation. Moreover, the KE annotations and descriptions impact the construction of an AOP network. When KEs and KERs are shared among multiple AOPs, the AOP-Wiki automatically generate connections between them. This allows information that has been used to build an AOP to be reused in subsequently developed AOPs, whilst revealing their interconnections. However, as seen in this exercise, there are many KEs titled differently, while having the same meaning and/or referring to the same event, thereby hampering this process. Moreover, attention should be paid to the nomenclature used for KE titles to ensure a sufficient level of detail. In this respect, 'FA accumulation' may oversimplify the more generalised process of disruption in FA metabolism and transport that can ultimately lead to compensatory changes in multiple mechanisms of the FA metabolism processes. Further harmonisation and review of the present and future contributions to the AOP-Wiki is warranted. In this regard, there is an opportunity to take advantage of efforts made in the field of ontology-based semantic mapping (Wang et al. 2019). It has been shown that semantic analysis may assist in developing future AOPs by selecting candidate events from the AOP-Wiki based on ontology-terms that are semantically similar to the MIE or AO of interest (Wang 2020), which could bypass the current challenge with differences in KE titles. Finally, the discussion on incomplete AOPs and KE descriptions should not be taken as critique of the specific AOP developers. It is merely intended to highlight areas in need of further improvement to facilitate advancement of the use of AOPs for risk assessment purposes.

It is important to recognise that the current 'AOP mining' approach is limited to the knowledge presently incorporated in the AOPs available in the AOP-Wiki. To illustrate, many nuclear hormone receptors, including pregnane X receptor (PXR) and constitutive androstane receptor (CAR), both included in the linear AOPs making up this AOP network, are known to have overlapping ligands whilst having a high degree of overlap in target genes related to the regulation of cholesterol homeostasis and energy metabolism (Krasowski et al. 2011). However, activation of CAR inhibits lipogenesis, whereas PXR activation promotes lipid accumulation (Mackowiak et al. 2018; Daujat-Chavanieu and

Gerbal-Chaloin 2020). Furthermore, as shown in Figure 1, one aspect that is missing in the derived AOP network is the well-established role of farnesoid X receptor (FXR) in the regulation of bile acid and lipid homeostasis (Stofan and Guo 2020). FXR is a ligand-activated transcription factor known to be involved in the control of bile acid synthesis and is critical in regulating the enterohepatic circulation of bile acids by inducing the expression of bile acid efflux transporters, such as the bile salt export pump (BSEP) and organic solute transporter (OST)α/β, and suppressing influx transporters (Stofan and Guo 2020; Sun et al. 2021). It has been shown that inhibition of BSEP alone may not be sufficient to induce cholestasis as also other efflux transporters (*i.e.* OSTα/β) play an important role in the compensatory mechanism (Jackson et al. 2016; Jackson et al. 2018). Actually, this has been published in regards to the AOP on cholestasis (AOP ID 27/BSEP) as part of an adaptive response to counteract the accumulation of bile acids (Gijbels et al. 2020). However, these mechanisms are not included in the AOP-Wiki. These nuclear receptor compensatory mechanisms and/or feedback loops are not well reflected in the present network. Despite the fact that AOPs are not intended to be chemical agnostic, such types of ligand- and/or target gene-overlap, as well as feedback-mechanisms, should be included when the application of AOPs shifts towards risk assessment, rather than solely hazard identification.

Qualitative AOPs inform on biological plausibility and can, for example, be used to guide the prioritisation of assays for inclusion in testing strategies and screening of chemicals. However, they typically do not determine the probability of the AO to occur under a specified exposure scenario and are thus not fit for quantitative risk assessment. Quantitative AOPs incorporate knowledge on the required level of perturbation needed to transition from one KE to the next, as well as informing on the modulating factors that can influence those relationships. Ultimately, a quantitative AOP should be precise enough to allow for a quantitative prediction of under what conditions, and to what degree, and AO is likely to occur for a given activation of a MIE (Villeneuve et al. 2014; Conolly et al. 2017; Wittwehr et al. 2017). In particular, quantitative AOPs may determine biological tipping points along a toxicity pathway (Spinu et al. 2020). In respect to the herein derived network, it is worth investigating

if the selected KEs become initiated simultaneously or as a step-wise process. Such discoveries could enable identification of common toxicity limiting steps for high priority focus in toxicology screening.

The AOP network proposed herein was evaluated in the context of any available AOP networks that study hepatotoxicity. At present, eleven AOP networks have been developed for several liver endpoints, including hepatic steatosis, hepatic fibrosis and hepatic cholestasis, as summarised in the supplementary material (Appendix 1). Their applications vary from development and WoE assessment (Vinken et al. 2013a; Angrish et al. 2016) to quantification of MIEs and/or KEs of an AOP network (Gadaleta et al. 2018; Perkins et al. 2019; Burgoon et al. 2020) In this respect, additional MIEs have been studied for their potency of inducing liver steatosis (Mellor et al. 2016) and a bioassay toolbox has been compiled for the *in vitro* assessment of KEs leading to liver steatosis (Luckert et al. 2018). However, none of the AOP networks has studied multiple outcomes. Hence, the AOP network developed herein offers the most advanced mechanistic representation given that it described linkages between several hepatic diseases and adverse effects, including liver cancer. In addition, the available AOP networks differ in terms of the level of details the construction contained, *e.g.* mechanistic knowledge, phenotype, genomics, proteomics, metabolomics. Thus, a combination of information was utilised to develop an AOP network for hepatic steatosis modelled as a Bayesian network (Burgoon et al. 2020). This underlines the challenge for harmonisation and integration of the diverse nature of information towards improved decision making in chemical risk assessment. The OECD AOP-Wiki represents an excellent repository in this sense, allowing for the curation, evaluation, and validation of linear AOPs and additionally safeguards the quality of the mechanistic data and facilitates the identification of knowledge gaps and prioritisation of testing strategies. Notably, solely two of the previously developed AOP networks (Appendix 1) included linear AOPs available in the OECD AOP-Wiki, as opposed to the AOP network *de novo* formulated. Two identified AOP networks were available in Cytoscape AOPXplorer (Burgoon 2021). This database is part of the AOP Knowledgebase and becomes essential for the development, analysis and storage of resultant AOP networks for real-world applications.

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Declarations

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Availability of data and material

All data analysed and generated during this study are included in this published article and its supplementary information files.

Code availability

Cytoscape, version 3.8.0 (https://cytoscape.org/), RRID:SCR_003032.

Microsoft® Office Excel Version 16.0.13906.35904, RRID:SCR_016137

Compliance with ethical standards

The manuscript does not contain clinical studies or patient data.

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Table 1

ID	TITLE	MOLECULAR INITIATING EVENT	ADVERSE OUTCOME	AUTHOR STATUS	OECD STATUS	AVAILABLE VIA
27	Cholestatic liver injury induced by inhibition of the bile salt export pump (ABCB11)	Inhibition, Bile salt export pump	Cholestasis	Under development: Not open for comment. Do not cite	Under Development	https://aopwiki.org/aops/27
36	Peroxisomal fatty acid beta-oxidation inhibition leading to steatosis	Decreased PPARα/β/γ activation	Steatosis	Under Development: Contributions and Comments Welcome	-	https://aopwiki.org/aops/36
38	Protein alkylation leading to liver fibrosis	Alkylation, Protein	Fibrosis	Open for citation & comment	TFHA/WNT Endorsed	https://aopwiki.org/aops/38
58	NR1I3 (CAR) suppression leading to hepatic steatosis	Suppression, CAR (multiple)	Steatosis	Under Development: Contributions and Comments Welcome	-	https://aopwiki.org/aops/58
60	NR1I2 (pregnane X receptor, PXR) activation leading to hepatic steatosis	Activation, PXR	Steatosis	Under Development: Contributions and Comments Welcome	-	https://aopwiki.org/aops/60
130	Phospholipase A inhibitors lead to hepatotoxicity	Inhibition, Phospholipase A	Fibrosis	Under Development: Contributions and Comments Welcome	Under Development	https://aopwiki.org/aops/130
144	Endocytic lysosomal uptake leading to liver fibrosis	Endocytic lysosomal uptake	Fibrosis	Under development: Not open for comment. Do not cite	EAGMST Under Review	https://aopwiki.org/aops/144
209	Perturbation of cholesterol and glutathione homeostasis leading to hepatotoxicity: Integrated multi-OMICS approach for building AOP	No MIE defined (Upregulation, SREBF2)	Hepatotoxicity	Under development: Not open for comment. Do not cite	-	https://aopwiki.org/aops/209
213	Inhibition of fatty acid beta oxidation leading to non-alcoholic steatohepatitis (NASH)	Decreased, β-oxidation	Steatohepatitis	Open for adoption	-	https://aopwiki.org/aops/213
220	CYP2E1 activation leading to liver cancer	Activation, CYP2E1	Cancer	Open for citation & comment	EAGMST Under Review	https://aopwiki.org/aops/220
273	Mitochondrial complex inhibition leading to liver injury	Mitochondrial inhibition (multiple)	Liver injury	Under development: Not open for comment. Do not cite	-	https://aopwiki.org/aops/273
278	IKK complex inhibition leading to liver injury	Inhibition, IKK complex	Liver injury	Under development: Not open for comment. Do not cite	-	https://aopwiki.org/aops/278
285	Inhibition of N-linked glycosylation leads to liver injury	Inhibition, N-linked glycosylation	Liver injury	Under development: Not open for comment. Do not cite	-	https://aopwiki.org/aops/285
318	Glucocorticoid receptor activation leading to hepatic steatosis	Activation, GR (multiple)	Steatosis	Under Development: Contributions and Comments Welcome	-	https://aopwiki.org/aops/318

Table 1 Linear AOPs for hepatotoxicity included in the AOP network. CAR Constitutive Androstane Receptor; CYP2E1 Cytochrome P450 2E1; GR Glucocorticoid Receptor; MIE Molecular initiating event; OECD Organisation for Economic Co-operation and Development.

Convergent KEs

KE type	*KE name*
KE	Accumulation, FA
KE	Activation, SREBF1
KE	Ballooning (hepatocyte)
AO	Cell injury/death
AO	Cholestasis, Pathology
KE	Decreased, HSD17B10 expression
KE	Decreased, Ketogenesis
KE	Decreased, Oxidative phosphorylation
KE	Formation, Mallory body
KE	Increased, De novo FA synthesis
KE	Increased, TG formation
AO	Liver fibrosis
AO	Liver injury
KE	Perturbation of cholesterol
AO	Steatohepatisis
AO	Steatosis
KE	Sustained cell proliferation
KE	Up Regulation, ACC-1
KE	Up Regulation, SCD-1
KE	Vacuolization (bile duct cell)
KE	Vacuolization (hepatocyte)
KE	Vacuolization (kupffer cell)

Divergent KEs

KE type	*KE name*
MIE	Activation, CYP2E1
MIE	Activation, GR
MIE	Activation, LXR
MIE	Activation, PXR
KE	Apoptosis
KE	Bile accumulation
MIE	Binding of inhibitor, mitochondrial complex III
MIE	Binding of inhibitor, mitochondrial complex IV
MIE	Binding of inhibitor, mitochondrial complex V
MIE	Binding of inhibitor, NADH-ubiquinone oxidoreductase (complex I)
KE	Damage, Lipid bilayer
MIE	Decreased, PPAR-α activation
MIE	Decreased, PPAR-β activation
MIE	Decreased, PPAR-γ activation
KE	Decreased, β-oxidation
MIE	Demethylation, PPAR-γ promoter
KE	Disturbance, Lysosomal function
KE	Down Regulation, GSS and GSTs gene
MIE	Endocytotic lysosomal uptake
MIE	Inhibition of N-linked glycosylation
MIE	Inhibition, BSEP
KE	Inhibition, FoxA2
MIE	Inhibition, IKK complex
MIE	Inhibition, Phospholipase A
KE	Mitochondrial dysfunction
MIE	Protein Alkylation
MIE	Suppression, CAR
KE	Up Regulation, SREBF2

Table 2 The analysis of the in-degree and out-degree confirmed 22 convergent KEs and 28 divergent KEs.

Table 3

Key event	Representative stressors	Endpoint measured	Methodology	References
Cell injury/ death	Isoniazid, paracetamol, phenobarbital, propranolol, verapamil*	Oxidoreductase enzyme activity	Detection of formazan product derived from reduction in viable cells of tetrazolium substrates MTT, MTS, XTT.	Riss et al. (2016)
		Lactate dehydrogenase leakage	Bioluminescent representation of LDH activity in extracellular medium, indicating its effluence from damaged cells.	Chan et al. (2013)
		Neutral red uptake	Quantification of the internalisation of fluorescent neutral red dye, occurring exclusively within viable cells.	Repetto et al. (2008)
		Protease enzyme activity	Assaying of protease enzyme activity, through cleavage of peptide (GF-AFC) in functional cells.	Riss et al. (2016)
Oxidative stress	Chloroform, 1,2-dichlorobenzene, furan, menadione, valproic acid	ROS presence	Quantification of fluorescence of dihydroethidium or fluorescein derivatives.	Kalyanaraman et al. (2012)
		Oxidation of DNA	Detection of 8-hydroxydeoxyguanosine formation (as ELISA).	Dasgupta and Klein (2014)
		Peroxidation of lipids	Detection of malondialdehyde presence, through reactivity with TBARS.	Dasgupta and Klein (2014)
		Protein oxidation	Identification of markers of oxidative damage, such as protein carbonylation and advanced glycation end products.	Dasgupta and Klein (2014)
Mitochondrial dysfunction	2,4-dinitrophenol, HCN, pentachlorophenol rotenone, zidovudine	Respirometry	Monitoring response in oxygen consumption in order to infer efficiency of OXPHOS, either through polarimetry or extracellular flux analysis.	Horan et al. (2012)
		Membrane potential depletion	Assaying of fluorescent rhodamine dye uptake into polarised mitochondria.	Perry et al. (2011)
		ATP production	Luciferase-based bioluminescent determination of ATP concentration (also used as proxy for general cell viability).	Riss et al. (2016)
		Reduction in glycolysis dependence	Promotion of OXPHOS reliance in cancer cell lines, enabling mitochondrial liability to be emphasised.	Marroquin et al. (2007)
Fatty acid accumulation	Amiodarone, fenofibrate, tetracycline, ticlodipine, valproic acid	Intracellular lipid content	Quantification of BODIPY, Nile red or Oil Red O fluorescence in treated, cultured cells (HepG2, HepaRG).	Muller and Sturla (2019), Donato et al. (2012), Gomez-Lechon et al. (2007), Tolosa et al. (2016)

Table 3 Overview of assays that can be used to measure endpoints associated with the four KEs (cell injury/death, increased ROS, mitochondrial dysfunction and FA accumulation) identified as the most highly connected and central KEs in an AOP hepatotoxicity network. Abbreviations: ELISA Enzyme-linked immunosorbent assay; GF-AFC Glycylphenylalanyl-aminofluorocoumarin; MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MTT 3,-4,5 dimethyithiazol-2,5 diphenyl tetrazolium bromide; ROS Reactive oxygen species; OXPHOS Oxidative phosphorylation; TBARS Thiobarbituric acid reactive substance assay; XTT 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

Figures

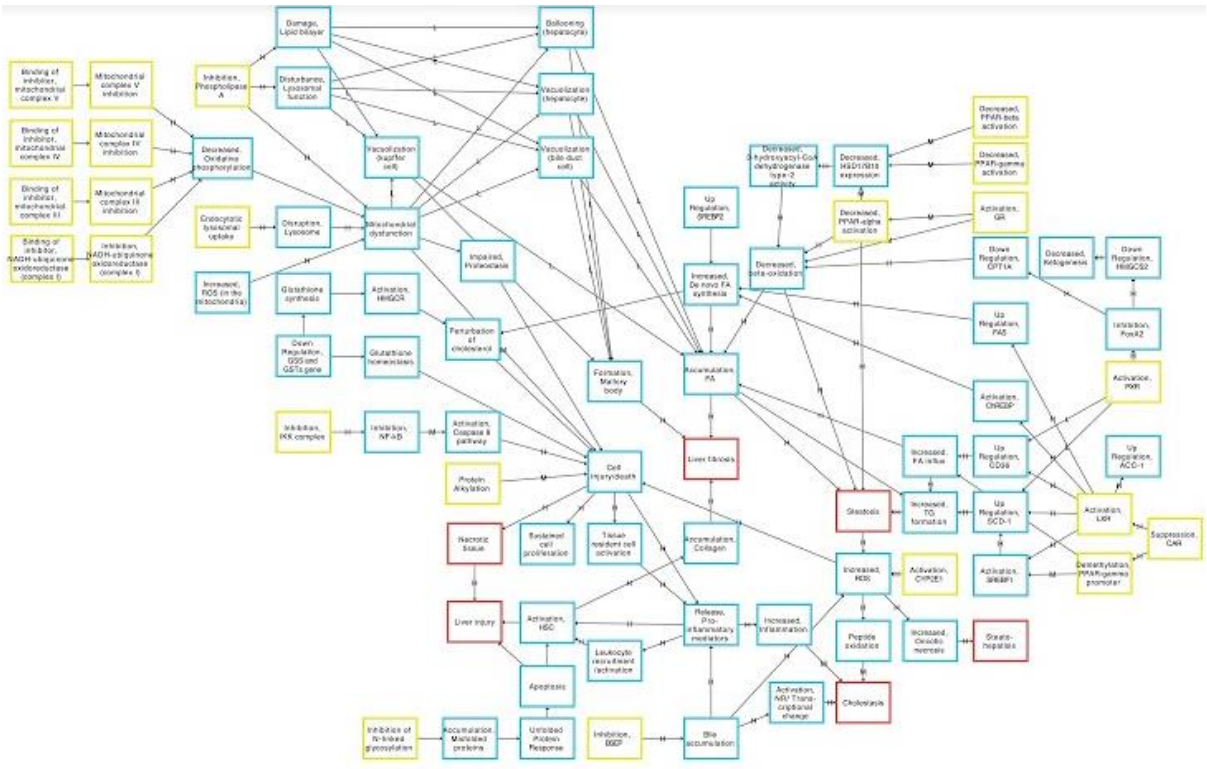
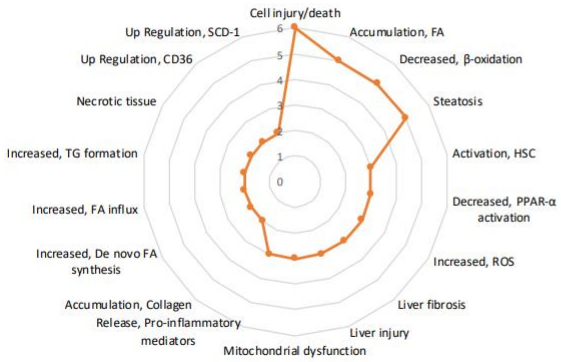


Figure 1 The network consisting of 14 AOPs available in the AOP-Wiki related to hepatotoxicity (extracted 1 September 2020) that share at least one KE. MIEs, KEs and AOs are coloured yellow, blue and red, respectively. Solid arrows indicate adjacent KER, with the arrow emanating from the upstream KE and into the downstream KE. KERs shared by more than one AOP are represented by a single arrow. KER label indicates strength of evidence as defined by the AOP author in the AOP-Wiki where H=high, M=medium, L=low. No label indicates lack of information in the AOP-Wiki. Curated KE titles, including abbreviations, are available as supplementary information (Appendix 1). KE Key event; KER Key event relationship; MIE molecular initiating event; AO Adverse outcome.

a



b

KE Title	Included in AOPs (AOP ID)
Cell injury/death	36/PPAR; 144/LYSOSOMAL UPTAKE; 209/SREBF2; 220/CYP2E1; 273/MITOCHONDRIAL INHIBITION; 278/IKK
Accumulation, FA	36/PPAR; 58/CAR; 60/PXR; 130/PHOSPHOLIPASE A; 213/B-OXIDATION
Decreased, β-oxidation	36/PPAR; 58/CAR; 60/PXR; 213/B-OXIDATION; 318/GR
Steatosis	36/PPAR; 58/CAR; 60/PXR; 213/B-OXIDATION; 318/GR
Activation, HSC	38/PROTEIN ALKYLATION; 144/LYSOSOMAL UPTAKE; 285/GLYCOSYLATION
Decreased, PPAR-α activation	36/PPAR; 58/CAR; 318/GR
Increased, ROS	27/BSEP; 213/B-OXIDATION; 220/CYP2E1
Liver fibrosis	38/PROTEIN ALKYLATION; 130/PHOSPHOLIPASE A; 144/LYSOSOMAL UPTAKE
Liver injury	273/MITOCHONDRIAL INHIBITION; 278/IKK; 285/GLYCOSYLATION
Mitochondrial dysfunction	130/PHOSPHOLIPASE A; 144/LYSOSOMAL UPTAKE; 273/MITOCHONDRIAL INHIBITION;
Release, Pro-inflammatory mediators	27/BSEP; 38/PROTEIN ALKYLATION; 144/LYSOSOMAL UPTAKE
Accumulation, Collagen	38/PROTEIN ALKYLATION; 144/LYSOSOMAL UPTAKE
Increased, De novo FA synthesis	58/CAR; 209/SREBF2
Increased, FA influx	58/CAR; 60/PXR
Increased, TG formation	58/CAR; 60/PXR
Necrotic tissue	273/MITOCHONDRIAL INHIBITION; 278/IKK
Up Regulation, CD36	58/CAR; 60/PXR
Up Regulation, SCD-1	58/CAR; 60/PXR

Figure 2 (a) KEs shared by more than one AOP. The score indicates the number of AOPs in which the KE is present. Cell injury/death is included in the highest number of AOPs, followed by FA accumulation, decreased, β-oxidation and steatosis. (b) The distribution of the shared KEs among the linear AOPs included in the network. AOP Adverse outcome pathway; CD36 Cluster of differentiation 36; FA Fatty acid; HSC Hepatic stellate cell; KE Key event; SCD-1 Stearoyl-CoA desaturase-1; ROS Reactive oxygen species; TG Triglyceride.

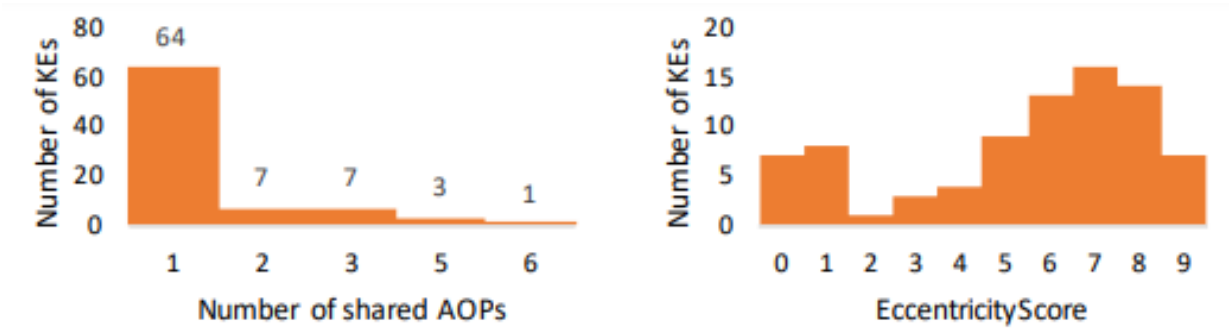


Figure 3 (a) The distribution of KEs in shared AOPs show that the interconnectivity between the AOPs in the network is fairly limited. (b) The distribution of KEs according to the directed eccentricity score shows that 35 % of the KEs cannot be categorised as either upstream or downstream due to their level of interconnectivity (*i.e.* eccentricity score between 3-6).

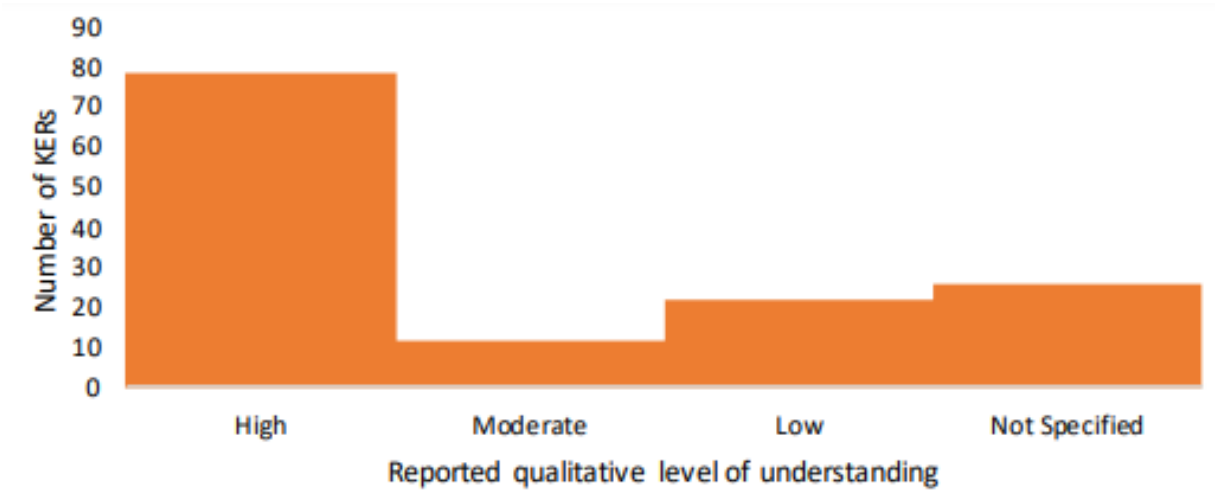


Figure 4 The distribution of the weight of evidence for KERs as reported by the AOP developers.

Supplementary material

Appendix 1

This Excel file contains all collected material used to derive the network, including KE annotation changes described in section 2.2. of the Materials and Methods, as well as NetworkAnalyzer output.

Appendix 2

This Word file contains supplementary figures describing the topological analysis of the derived AOP network for hepatotoxicity.