NAM TO IDENTIFY (LOW, MEDIUM, HIGH) CONCERN OF CARBOXYLIC ACID-INDUCED HEPATIC STEATOSIS

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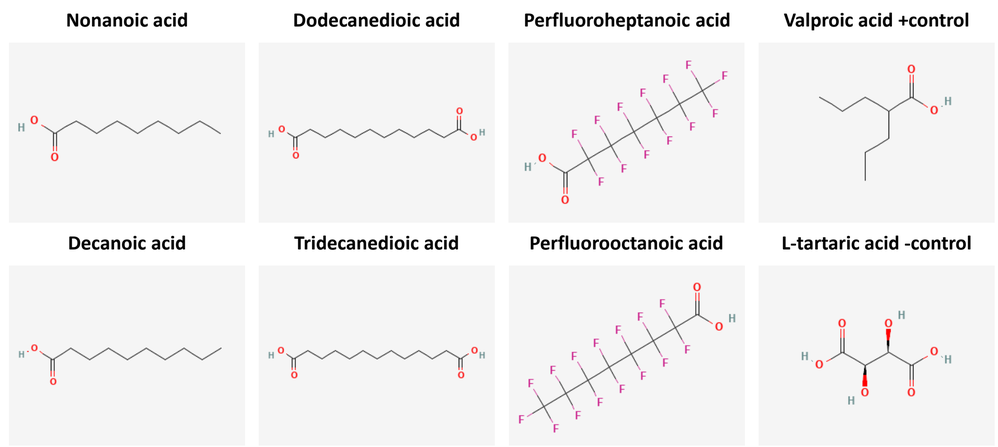
Proposed solution

One of the most frequent manifestations of hepatotoxicity is steatosis (accumulation of lipids within hepatocytes), which can be considered an early event in hepatotoxicity. Xenobiotic carboxylic acids have been associated with a high risk of steatosis. We propose an innovative NAM able to classify xenobiotic carboxylic acids into high, medium and low concern of liver steatosis. To this end, we combined two human steatosis-responsive *in vitro* models with a pioneering QSAR *in silico* multimodel to provide a classification based on toxicodynamic outputs. This dimension was then combined with a toxicokinetic dimension based on the prediction of systemic availability that relies on a PBK-modelling approach. The two-dimension NAM matrix provided a sound classification of 7 xenobiotic carboxylic acids included in the EPAA reference list. Six of them (nonanoic, decanoic, dodecanedioic, tridecanedioic, perfluoroheptanoic and perfluorooctanoic acid) were categorized as high concern chemicals, whereas tartaric acid was classified as low concern.



Methods and data integration

1-Toxicodynamics *In vitro*

- **UV: *In vitro* model. Human upcyte® hepatocytes**: derived from primary human hepatocytes by an innovative technology that force cells to a limited proliferation, without immortalization. Unlike hepatic cell lines, they express and preserve a phenotype close to primary human cultured hepatocytes (Levy 2015; Tolosa 2016) (upcyte technologies, ClinicSciences Group).
- **UV: Cytotoxicity. MTT test:** The conversion of the tetrazolium salt MTT into formazan crystals reflects the mitochondrial activity of the cells. Maximal non-toxic concentrations and concentrations decreasing cell viability by 10% were calculated.
- **UV: Steatosis assay. Triglyceride accumulation:** Hepatocytes were pre-incubated for 14 h with a 62 µM mixture of oleate and palmitate (2:1 ratio). After medium renewal and chemical exposure, cellular lipids were extracted from hepatocyte homogenates with a methanol-chloroform mixture. The dried lipid pellets were resuspended in isopropyl alcohol. Triglycerides were analysed with a colorimetric kit (Spinreact, Barcelona, Spain).

- **VUB: *In vitro* model. Human skin stem cell-derived hepatic cells:** hSKP-HPC (Verhoeven, 2024).
- **VUB: Cytotoxicity. CellTiter-Glo® Luminescent Cell Viability Assay:** CellTiter-Glo® Luminescent Cell Viability Assay upon 72 hours of 24-hour repeated exposure. Subsequently, the concentration at which 10% of the cells died (CC10) was determined.
- **VUB: Steatosis assay: BODIPY 493/503.** After chemical exposure, cells were detached and exposed to 2 µM BODIPY 493/503 for 15 min. The fluorescence of >20,000 cells was measured by flow cytometry upon adding Hoechst123

2-Toxicodynamics *In silico*

PROTO: QSAR multimodel:

- Fatty acid (FA) accumulation binary QSAR model as recently described in (Ortega-Vallbona 2024).
- Models used to predict pKa:
  - ✓ QSAR toolbox (https://qsartoolbox.org/) model for pKa;
  - ✓ MolGpKa (https://xundrug.cn/molgpka/) model for pKa (Xiaolin, 2021);
  - ✓ ChemAxon (https://chemaxon.com) model for pKa (Lee,2009).
- Models used to predict lipophilicity (LogP and LogKow):
  - ✓ ADMETLab v2.0 (admetmesh.scbdd.com) model for logD (pH 7.4) (Xiong, 2021);
  - ✓ ProtoPRED (protopred.protoqsar.com) models for logKow and logD (pH 7.4);
  - ✓ BAYER in-house models for logD (ph 7.5) (Göller, 2020).

3-Toxicokinetics

ESQ: Kinetic modelling for Systemic Availability: It relies on a high-throughput physiologically-based kinetic (HT-PBK) modelling approach.

Its core method is the PK-Sim (William, 2003), as well as its corresponding R package (R Core Team, 2022). The HT-PBK approach is parameterised from various *in silico* tools that predict different ADME relevant chemical properties. Further details are outlined in the independent report submission ("ONTOX HT-PBK").

Integration & interpretation

Chemical-induced steatosis was categorized as Low concern (0-25% increase), medium concern (25-100% increase) or high concern (≥100% increase).

On top of that, if time- and/or dose-dependency was observed we reinforced the level of confidence with a '+'.

Results from the two different human *in vitro* models (UV & VUB) were combined by using the same criteria as in the combination table provided by the EPAA.

Similarly, in *in silico* modeling, compounds were classified into High, Medium, and Low concern of steatosis based on three criteria, which followed these rules: High: positive in all 3 criteria, Medium: 1 or 2 positive criteria, Low: all criteria negative.

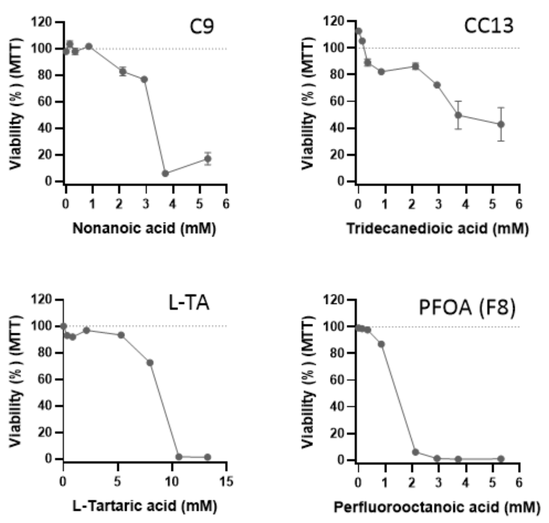
Next, the *in vitro* and *in silico* classifications were combined following the EPAA categorization rules. This resulted in the definitive toxicodynamics classification.

Finally, we combined the result of toxicodynamics with that of ESQ kinetic modeling (toxicokinetics) following the proposed EPAA NAM designathon 2D (tabular) combination, giving the definitive classification.

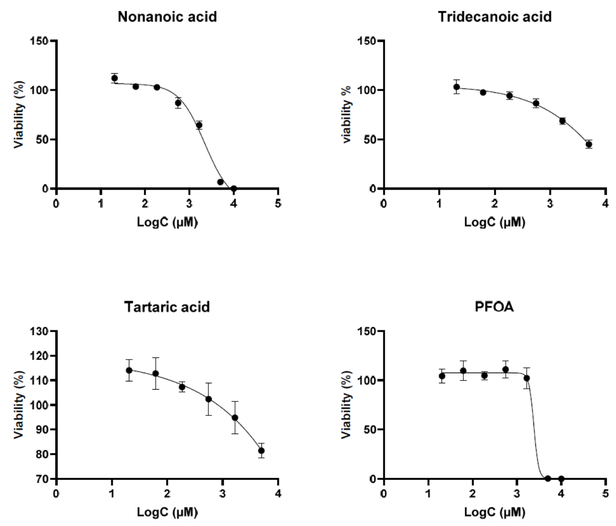
Results & Chemical classification

1a- *In vitro* models → Cell death > Cytotoxicity

UV: Cultured human upcytes® hepatocytes.



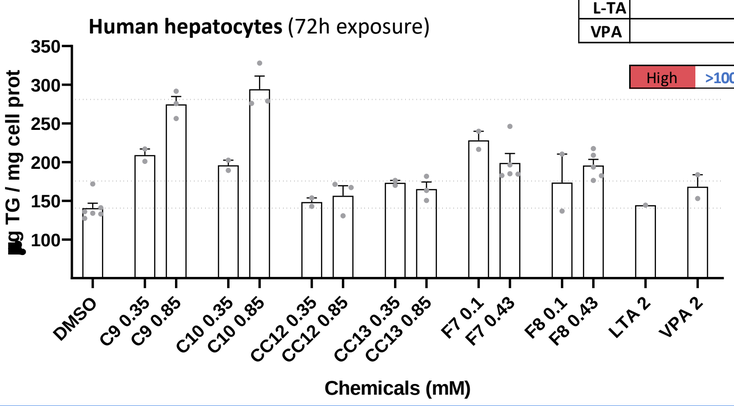
VUB: Cultured hSKP-HPCs



1b- In vitro models → Steatosis assay > Triglyceride accumulation

Steat Conc	Conc (mM) 0.1	0.3-0.35	0.43	0.85	0.6	1.2	2-2.4	4.8
C10		72		72	24	24		
C9		72		72	24	24		
F7	72	24	72		24			
F8	72	24	72		24			
CC13		72		72		24		
CC12		72		72		24		
L-TA							24	
VPA							72	24

High >100% | Medium 25-100% | Low 0-25%



2- Toxicodynamics *in silico*→ QSAR multimodel

Compound	FA prediction	pKa (mean)	LogKow	LogD (mean)	Classification
Dodecanedioic acid	+	4,48	2,92	1,53	High
Tridecanedioic acid	+	4,48	3,23	1,66	High
Decanoic acid	+	4,76	4,09	1,40	High
Nonanoic acid	+	4,85	3,42	1,15	High
Valproic acid	+	4,71	2,64	0,95	High
Perfluorooctanoic acid	Outside AD	-1,26	3,80	2,45	Medium
Perfluoroheptanoic acid; Tridecafluoroheptanoic acid;	Outside AD	-0,57	3,64	1,90	Medium
L-Tartaric acid	-	2,79	-2,19	-1,34	Low

3-Toxicokinetics → HT-PBK modelling approach

Compound Cas	Compound Name	Mean Final Score	Class
335-67-1	Perfluorooctanoic	60,08	High
375-85-9	Perfluoroheptanoic	33,58	High
693-23-2	Dodecanedioic	13,7	High
505-52-2	Tridecanedioic	6,56	High
334-48-5	Decanoic	1,11	Medium
112-05-0	Nonanoic	0,53	Medium
87-69-4	L-Tartaric	0,46	Medium

	TOXICODYNAMICS IN VITRO UV	VUB	UV+VUB	IN SILICO PROTO	IN VITRO + IN SILICO	TOXICOKINETICS (ESQ) C10	C9	F7	F8	CC13	CC12	L-TA
						M	M	H	H	H	H	M
C10	H+	L	M	H	H	H						
C9	H+	L	M	H	H		H					
F7	M+	M	M	M	M			H				
F8	M+	M	M	M	M				H			
CC13	L	L	L	H	M					H		
CC12	L	L	L	H	M						H	
LTA	L	L	L	L	L							L

Scientific basis & chemicals

The liver is a key systemic-toxicity target organ, and steatosis is a common early event in hepatotoxicity. In this NAM, we assess a KE in which all the pathways of the steatosis AOP come together: intracellular fat accumulation. By using two different *in vitro* systems the false-positive and false-negative rates are reduced. Given that steatosis precedes worse hepatotoxic outcomes it is important to test this adverse event at subcytotoxic concentrations. Regarding *in silico* modelling, the integration of a specialized binary QSAR model for carboxylic acids, focused on predicting FA accumulation, along with the assessment of pKa and lipophilicity, enhances the robustness of the risk classification, resulting in a three-tiered system.

We have only addressed 7 chemicals of the reference list for practical reasons, given the limited amount of time and resources. Moreover, we focused on xenobiotic carboxylic acids because the availability of SAR and QSAR models by PROTO able to identify relevant molecular substructures and descriptors that contribute to carboxylic acid-induced steatosis.

Uncertainty & Limitations

The reliability of QSAR predictions with steatosis models was analysed based on the applicability domain (AD) of the model. The AD was evaluated by four different methods (Jaworska, 2005). Regarding kinetic modelling, the proposed classification does not include any expression of uncertainty. If desired, an expression of the parameterization uncertainty could be included by integrating not only the mean of predictions from the different PBK variants but also the range of these predictions.

Regarding limitations: Human upcyte hepatocytes and hSKP-HPC cells may not fully replicate the complexity of the human liver. QSAR model for steatosis was developed on organic chemicals and consequently is not applicable to inorganic/organometallic compounds or chemicals including 'unusual' atoms. The HT-PBK model in general represents assumptions that are reasonable for most "small molecules" (<1000 g/mol). Moreover, whole body availability was assessed which should mostly be sensitive to oral availability and clearance.

References

Göller et al. Drug Discovery Today. **2020**, 25(9): 1702-1709; Jaworska et al. Altern Lab Anim. **2005**, 33(5):445-59; Lee & Crippen. Journal of Chemical Information and Modeling. **2009**, 49(9):2013-2033; Levy et al. Nat. Biotechnol. **2015**, 33:1264–1271; Ortega-Vallbona et al. Toxicology **2024**, 28:153764; Tolosa et al. Toxicol Sci. **2016**, 152(1):214-29; Verhoeven et al. Toxicology. **2024** (under review); Xiaolin et al. Journal of Chemical Information and Modeling. **2021**, 61:3159; Xiong G et al. Nucleic Acids Research. **2021**, 49(W1):W5-W14.

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