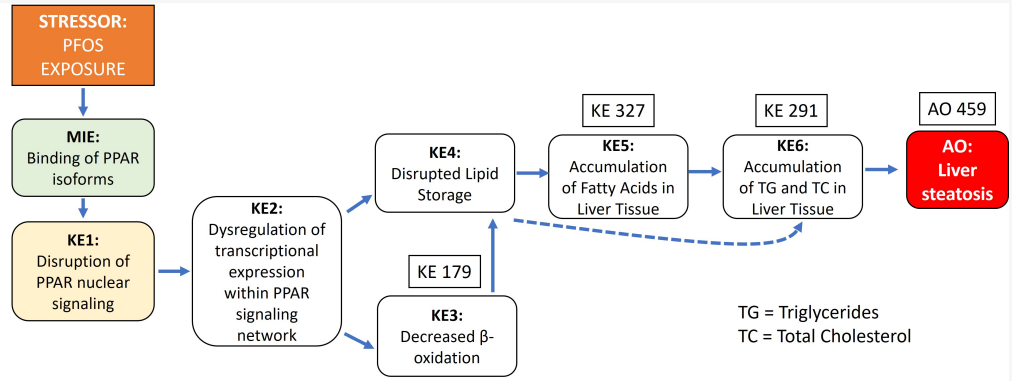
AOP ID and Title:

AOP 529: Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis
Short Title: PFOS binding to PPARs leads to liver steatosis

Graphical Representation



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Abstract

This AOP describes the chain of events where the molecular initiating event (MIE) of perfluorooctanesulfonic acid (PFOS) binding to the ligand-binding domain of the peroxisome proliferator-activated receptor (PPAR) causes a cascade of key events (KEs) including altered transcriptional expression of genes involved in lipid metabolism leading to impacted lipid transport, metabolism, and storage, ultimately leading to lipid accumulation in the liver and the adverse outcome (AO) of liver steatosis. Specifically, ligand binding analyses and molecular modeling studies have indicated the potential for PFOS to bind to the lipid-binding domain of various PPAR isoforms (the MIE) resulting in disruption of PPAR nuclear signaling (KE1). Disruption of PPAR nuclear signaling leads to KE2 in which the activity of PPAR as a transcriptional regulator is altered affecting transcriptional expression of a suite of genes within the PPAR signaling network. Transcriptional studies have shown that exposure to PFOS results in broad dysregulation of gene expression for a suite of genes involved in lipid metabolism which ultimately result in decreased β-oxidation (KE3) and disrupted lipid storage (KE4). Altered expression of β-oxidation related genes (*acox1, acadm, cpt1a, cyp4a1*) have been observed in conjunction with inhibition of β-oxidation in PFOS exposures. Also, transcriptional expression of genes involved in both lipogenesis and lipid transport including, *apoa*, *apoe, acacb, CD36, fabp* isoforms, *Plin* isoforms and *lpl,* have been observed to be affected by PFOS exposure in conjunction with disrupted of lipid storage (KE4). Alterations in fatty acids, triglycerides (TG), and total cholesterol (TC) accumulation and profiles have been observed in the livers of PFOS-exposed vertebrates including fish, reptiles, birds, and mammals and serve as evidence of KE5 (accumulation of fatty acids) and KE6 (accumulation of TG/TC) in liver tissue. KE5 and KE6 thus contribute to hepatocellular vacuolation as seen in multiple histopathological assessments performed on livers of vertebrate species exposed to PFOS, including work funded under SERDP project ER20-1542 (Mylroie et al, manuscript in development). Finally, KE5 and KE6 ultimately drive the adverse outcome (AO) of liver steatosis. Additional, more systemic AOs may also be affected by this MIE and the cascade of KEs that can ultimately alter global energy metabolism, such as AOs of impacted growth and reproduction.

Background

Poly- and perfluoroalkyl substances (PFAS) are a large group of fluorinated compounds that have a wide variety of commercial and industrial applications ranging from use in firefighting foams to non-stick coatings to fishing lines (DeWitt, et al. 2019; Annunziato et al. 2020; Glüge et al. 2020). PFAS exposure can have negative effects on development, growth, reproduction, hepatic function, immune function, neurological function, and lipid metabolism in humans and other vertebrates (Sunderland et al. 2019; Lee et al. 2020; Agency for Toxic Substances and Disease Registry (ATSDR), 2021; Ankley et al. 2021; Bell et al

2021; Fragki et al. 2021; Ho et al. 2021; Boyd et al. 2022). Research in terrestrial and aquatic vertebrates has shown the liver to be a target organ of PFAS accumulation and resulting hepatoxicity (Lee et al. 2020; Costello et al. 2022; Ducatman and Fenton 2022; Huang et al. 2022a; Wang et al. 2022b). Here we propose an adverse outcome pathway (AOP) linking the binding of a specific PFAS, perfluorooctanesulfonic acid (PFOS), to peroxisome proliferator-activated receptors (PPARs) as the molecular initiating event (MIE) causing perturbation of PPAR-linked lipid metabolism which ultimately results in the adverse outcome (AO) of liver steatosis in PFOS-exposed vertebrates.

PPARs are a family of nuclear receptors in vertebrates that bind lipids as signaling molecules resulting in a cascade of transcriptional regulatory events that maintain energy homeostasis (Grygiel-Gorniak 2014). Specifically, PPARα is integral in regulating fatty acid catabolism and energy production through beta-oxidation; PPARγ regulates fatty acid synthesis and storage; and PPARβ/δ plays a key role in glucose homeostasis and beta-oxidation (Varga et al. 2011; Grygiel-Gorniak 2014; Lamas-Bervejillo and Ferreira 2019; Gust et al. 2019). Despite their more discrete roles, the crosstalk between all PPAR isoforms is essential to maintaining energy homeostasis; and therefore, any over-activation or repression of the PPAR signaling network can have deleterious outcomes for the organism.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	2226	Stressor binding PPAR isoforms	Binding PPAR isoforms
	KE	2227	Disrupted PPAR isoform nuclear signaling	Disrupted PPAR isoform nuclear signaling
	KE	2224	Dysregulation of transcriptional expression within PPAR signaling network	Dysregulation of transcriptional expression within PPAR signaling network
	KE	179	Decrease, Fatty acid beta-oxidation	Decrease, Fatty acid β-oxidation
	KE	2225	Disrupted Lipid Storage	Disrupted Lipid Storage
	KE	327	Accumulation, Fatty acid	Accumulation, Fatty acid
	KE	291	Accumulation, Triglyceride	Accumulation, Triglyceride
	AO	459	Increased, Liver Steatosis	Increased, Liver Steatosis

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Stressor binding PPAR isoforms	adjacent	Disrupted PPAR isoform nuclear signaling	High	Moderate
Disrupted PPAR isoform nuclear signaling	adjacent	Dysregulation of transcriptional expression within PPAR signaling network	High	Moderate
Dysregulation of transcriptional expression within PPAR signaling network	adjacent	Disrupted Lipid Storage	High	Moderate
Dysregulation of transcriptional expression within PPAR signaling network	adjacent	Decrease, Fatty acid beta-oxidation	High	Moderate
Decrease, Fatty acid beta-oxidation	adjacent	Disrupted Lipid Storage	Moderate	Moderate
Disrupted Lipid Storage	adjacent	Accumulation, Fatty acid	Moderate	Moderate
Accumulation, Fatty acid	adjacent	Accumulation, Triglyceride	Moderate	Moderate
Accumulation, Triglyceride	adjacent	Increased, Liver Steatosis	High	Moderate
Disrupted Lipid Storage	non-adjacent	Accumulation, Triglyceride	Moderate	Moderate

Stressors

Name	Evidence
Perfluorooctanesulfonic acid	
PPARalpha antagonists	
PPAR agonist	
Per- and Polyfluorinated Substances (PFAS)	

Overall Assessment of the AOP

The weight of evidence from the literature indicates the potential for the molecular initiating even (MIE) of PFOS binding to the lipid-binding site of PPAR isoforms resulting in the key event of dysregulation of PPAR nuclear signaling (KE1). This KE results in the downstream KE of impacted regulation of diverse transcriptional expression pathways (KE2) that subsequently control KEs of altered lipid metabolism and transport. The effects of these KEs thus affect systemic lipid profiles resulting in the KEs of lipid accumulation in livers and hepatocellular vacuolation. Finally, these key events drive the adverse outcome (AO) of liver steatosis. Additional, more systemic AOs may also be affected by this MIE and cascade of KEs that can ultimately alter global energy metabolism, such as AOs of impacted growth and reproduction.

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	Moderate
Adult, reproductively mature	High

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
zebrafish	Danio rerio	High	NCBI

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

The AOP is likely to be relevant for the majority of vertebrate species as an overall phylogenetic group across various lifestages and for both sexes.

Life Stage Applicability

There is evidence of disruption of PPAR isoforms in all life stages and evidence of perturbed lipid accumulation has also been seen at all lifestages across multiple vertebrate species. However, the liver (or proto-liver) is only formed and characterized in a subset of the organisms used for generating experimental data (e.g. zebrafish), and therefore evidence of the AO is limited across all potential vertebrates at the embyo stage. MIE, KE, and AO has been characterized in adults across mutliple vertebrate species types.

Life Stage	Evidence
Embryo	Moderate
Juvenile	Moderate
Adult	High

Taxanomic Applicability

The conservation of PPAR molecular structure and function among vertebrates (Gust et al 2020) indicates the MIE is likely to be conserved among this broad phylogenetic group. Further, evidence for the various KEs and the AO were assembled from investigations in various vertebrate species including mammals, birds, reptiles, amphibians, and fish where responses were largely congruent among the species tested. These observations indicate that the overall AOP is likely be relevant across the

majority of vertebrate species. Further, these observations indicate the potential to use non-animal models, such as zebrafish embryo tests, in the context of this AOP to provide screening-level assessments that have relevance for human health, especially when rapid toxicity screening of diverse PFAS structures remains a critical need.

Sex Applicability

AOP is expected to be applicable across both sexes. However, it is important to note that in many of the fish studies in adults where sex differences were examined, lipid accumulation in liver was more severe in males than in females.

Sex	Evidence
Male	High
Female	Moderate

Essentiality of the Key Events

Essentiality of Key Events

MIE: Stressor binding PPAR isoforms: Numerous studies have shown the ability of synthetic ligands to bind the ligand binding domains of the PPAR isoforms (α, β/δ, γ). Specifically, the prototypical stressor, PFOS, has been shown to bind the three PPAR isoforms with varying degrees of affinity through *in vitro* ligand binding assays (Vanden Heuvel et al. 2006; Takacs and Abbot 2007; Wolf et al. 2008; Behr et al. 2020; Evans et al. 2022; Sun et al. 2023) as well as through computational binding/docking analyses (Li et al. 2018; Yi et al. 2019; Almedia et al. 2021; Garoche et al. 2021; Khazee et al. 2021; Huang et al. 2022b; Wang et al. 2022a, Wang et al. 2022b; Kowalska et al. 2023).

Key Event 1: Disruption of PPAR Isoform Nuclear Signaling: Studies have demonstrated that exposure to the prototypical stressor, PFOS, can have a direct effect on the transcriptional expression of the PPAR isoforms in vertebrates (Lee et al. 2020; Beale et al. 2022) with these studies showing expression changes occurring primarily in the PPARα and PPARγ isoforms. Furthermore, activation of one PPAR isoform can have effects on the expression of other PPAR isoforms. For example, agonism of PPARβ/δ can cause reduced expression of PPARα and PPARγ isoforms (Shi et al. 2002; Kim et al. 2020), and certain coregulators can have effects (sometimes opposite) on different PPAR isoforms (Tahri-Joutey et al. 2021). Finally, omics studies have shown that agonist and antagonist of PPAR isoforms alter PPAR signaling transcripts (Louisse et al. 2020; Heintz et al. 2024). Overall, this evidence displays that disruption of PPAR isoforms stressor chemicals can effect other PPAR isoforms and impact PPAR nuclear signaling.

Key Event 2: Dysregulation of Transcriptional Expression within PPAR Signaling Network: There is abundant evidence of showing how stressors can affect transcriptional expression in the PPAR signaling network and key genes involved in lipid homeostasis. Specifically, pathway and gene ontology (GO) enrichment analyses have identified lipid metabolism, lipid transport, fatty acid degradation, PPAR signaling pathway, and lipid homeostasis as being transcriptionally altered in response to PFOS exposure (Chen et al. 2014; Jacobsen et al. 2018; Rodríguez-Jorquera et al. 2018; Martinez et al. 2019; Christou et al. 2020; Dong et al. 2021; Lee et al. 2021; Mylroie et al. 2021; Beale et al. 2022; Davidsen et al. 2022; Haimbuagh et al. 2022; Wang et al. 2022a; Mylroie et al. IN PREP).

Key Event 3: Decreased β-oxidation: Decreased β-oxidation has been linked to liver steatosis and the PPAR isoforms play a key role in regulating β-oxidation (Cherkaoui-Malki et al. 2012). PPARα knockouts have shown decreased β-oxidation and subsequent lipid accumulation in the liver (Hashimoto et al. 2000; Reddy 2001; Badmann et al. 2007) whereas activation of PPARα has been shown to increase β-oxidation (Tahri-Joutey et al. 2021). PPARβ/δ has also been shown to have a critical role in the regulation β-oxidation (Roberts et al. 2011).

Key Event 4: Disrupted Lipid Storage: Disruption of the PPAR isoforms can have effects on lipid storage and transport (Dixon et al. 2021). PPARγ over expression results in promotes storage of lipids in the liver and thus exacerbates hepatic steatosis (Yu et al. 2003; Patsouris et al. 2006). Conversely, deletion of PPARα resulted in an increased liver lipid (Ptsouris et al. 2006). Wang et al. (2003) demonstrated that PPARβ/δ deficient mice had increased obesity which, while potentially not a function of improper lipid storage, underpins the importance of all PPAR isoforms in proper lipid homeostasis. Evidence of disruption of lipogenesis at the transcriptional level has also been observed across multiple studies using PFAS as the stressor (Tse et al. 2016; Cui et al. 2017; Huck et al. 2018; Liu et al. 2019; Martinez 2019; Yi et al. 2019; Louisse et al. 2020; Wang et al. 2022a).

Key Event 5: Accumulation of Fatty Acids in Liver Tissues: A *Ppara*-null strain in mice exhibited signs of increased fatty acid accumulation during fasting and over time under normal dietary conditions as *Ppara*-null strain mice cannot properly catabolize fatty acids (Montager et al. 2016). Under exposure to a stressor, Sant et al. (2021) observed increased accumulation of fatty acids and changes in fatty acid ratios when PFOS exposed zebrafish embryos were compared to control fish and Yang et al. (2022) observed differing lipid profiles between PFOS and PFOA exposed zebrafish embryos.

Key Event 6: Accumulation of Triglycerides (TG) and Total Cholesterol (TC) in the Liver Tissue: Disruption of the PPAR isoforms can be linked to accumulation of TG and TC in the liver tissue. In a review by Wang et al. (2020), it is explained how increased PPARγ expression can alter triacylglycerol levels. As examples of exposure to a stressor, studies using human cell cultures demonstrated increases in TG levels after exposure to PFOS (Liu et al. 2019; Louisse et al. 2020), and a metadata analysis performed on the blood lipid profiles of adult and juvenile humans showed that PFOS exposure was significantly correlated with an increase in TC levels in the blood and showed a trend of decreased TG levels in the blood (Ho et al. 2021).

Adverse Outcome: Liver Steatosis: The PPAR isoforms are essential for regulation of energy metabolism and specifically

lipid metabolism (Wang et al. 2010). There is significant evidence in the literature demonstrating that repression, overexpression, or complete knock-out (KO) of the various PPAR isoforms can lead to disruptions in lipid metabolism and the adverse outcome of liver steatosis. An extensive review by Wang et al. (2020) presented evidence of how differential repression or activation of the various PPAR isoforms can affect metabolic regulation in mice livers and could lead to lipid accumulation and steatosis in the liver. A *Pparα*-null strain in mice exhibited signs of increased fatty acid accumulation and steatosis during fasting and over time under normal dietary conditions (Montager et al. 2016). Conversely, overexpression of PPARγ in mice increased the rate of hepatosteatosis (Yu et al. 2003; Wang et al. 2020). In fish, Li et al. (2020) demonstrated that a pparα knockout zebrafish, resulted in altered fatty acid oxidation enzymes and an increase in lipid accumulation in zebrafish livers. Conversely as to what was observed in mice, PPARγ KO male zebrafish showed indicators of hepatic steatosis under standard diet conditions (Zhao et al. 2022). Overall, there is evidence in multiple species of vertebrates that repression, overexpression, or complete knock-out of the PPAR isoforms can disrupt lipid metabolism and lead to the AO of liver steatosis even in the absence of a stressor such as PFOS.

Weight of Evidence Summary

Evidence of PFOS/PPAR Interaction as the Molecular Initiating Event (MIE)

Perflouroalkyl substances like PFOS have structural similarities to fatty acids which are natural ligands of PPARs. Binding analyses and molecular docking models have shown that PFOS and other PFAS can bind to the ligand binding site of PPARs in both the agonist and antagonistic confirmations of the PPARs (Li et al. 2018; Yi et al. 2019; Almedia et al. 2021; Garoche et al. 2021; Khazee et al. 2021; Huang et al. 2022b; Wang et al. 2022a, Wang et al. 2022b; Kowalska et al. 2023) representing the molecular initiating event (MIE) of the present AOP. Activity assays in *in vitro* cell assay studies involving expressed PPAR receptors from mammals have also shown activation of PPARs by PFOS (Vanden Heuvel et al. 2006; Takacs and Abbot 2007; Wolf et al. 2008; Behr et al. 2020; Evans et al. 2022; Sun et al. 2023). An omics-based metadata study examining the response to PFAS exposure across multiple terrestrial and aquatic organisms (Beale et al. 2022) identified PPAR receptors as one of the key molecular targets of PFAS after exposure. Investigation of PPARα molecular structure and function indicated a high degree of conservation among vertebrate species including mammals, birds, reptiles, amphibians, and fish, whereas there was little conservation across invertebrates (Gust et al. 2020), which indicates that the MIE is likely to be conserved for majority of vertebrate species as an overall phylogenetic group.

Evidence of Disruption of PPAR Nuclear Signaling (KE1)

Evidence of disruption of PPAR nuclear signaling (KE1) following biding of PFOS to PPAR isoforms can be evidenced by numerous studies demonstrating that exposure to PFOS can have a direct effect on the transcriptional expression of the PPAR isoforms in vertebrates (Lee et al. 2020; Beale et al. 2022) with these studies showing expression changes occurring primarily in the PPARα and PPARγ isoforms. Investigations in human cells (Liu et al. 2019), mice [*Mus musculus*] (Das et al. 2018; Huck et al. 2018), Atlantic salmon [*Salmo salar*] (Arukwe and Mortensen 2011), and zebrafish [*Danio rerio*] (Olivares-Rubio and Vega Lopez 2016; Christou et al. 2020; Mylroie et al. 2021; Sant et al. 2021; Wang et al. 2022a) have shown both up- and down-regulation of PPAR transcriptional expression. In some cases, the expression of different PPAR isoforms can be regulated in opposite directions in the same exposure as was observed in Rodríguez-Jorquera et al. (2018) after fathead minnows [*Pimephales promelas*] were exposed to PFOS. Finally, studies in zebrafish have indicated that modulation of PPAR isoform signaling by PPAR agonist and antagonist results in apical toxicity outcomes similar to those seen as a result of PFOS and other PFAS exposures (Venezia et al. 2021). Given the sum of these observations, it is reasonable to hypothesize that PFASs, such as PFOS, can directly interact with PPARs through receptor binding and thus affect the downstream transcriptional signaling cascade and resultant enzymatic expression events that control lipid homeostasis with implications for all vertebrate species, with the best described outcomes associated with mammals.

Evidence of Disruption in PPAR Pathway Causing Early Key Events (KE2, KE3, & KE4)

Evidence of the overall dysregulation of transcriptional expression within the PPAR signaling network (KE2) can been observed in global and pathway-centered gene expression analyses in vertebrate embryos, larvae, and adult tissues which have shown that exposure to PFOS and other PFAS disrupts gene expression in multiple PPAR pathway-associated genes. Specifically, pathway and gene ontology (GO) enrichment analyses have identified lipid metabolism, lipid transport, fatty acid degradation, PPAR signaling pathway, and lipid homeostasis as being transcriptionally altered in response to PFOS exposure (Chen et al. 2014; Jacobsen et al. 2018; Rodríguez-Jorquera et al. 2018; Martinez et al. 2019; Christou et al. 2020; Dong et al. 2021; Lee et al. 2021; Mylroie et al. 2021; Beale et al. 2022; Davidsen et al. 2022; Haimbuagh et al. 2022; Wang et al. 2022a; Mylroie et al. IN PREP).

In addition to observations of dysregulation in transcriptional expression of the PPAR receptors, there is ample evidence that PFOS exposure results in transcriptional expression changes in downstream genes involved in the specific process of fatty acid metabolism (KE3), lipid storage (KE4), and lipid transport. For example, in mammal models, up-regulation of β -oxidation related genes *Thiolase B* and *cyp4a1* have been observed in rats [*Rattus norvegicus*] (Davidsen et al. 2022) and with *cyp4a14* and *acadm* observed as upregulated in mice (Rossen et al. 2010). At a cellular level, Wan et al. (2012) and Geng et al. (2019) demonstrated decreases in overall mitochondrial β -oxidation rates in liver tissue from PFOS exposed mice and chicken [*Gallus gallus*] embryos. In zebrafish, Cheng et al. (2016) observed increased transcriptional expression for genes related to β-oxidation (*acox1, acadm, cpt1a*) which is suggestive of a compensatory response to β-oxidation inhibition caused by PFOS exposure. Similarly, Wang et al. (2022a) also observed trends of increased transcriptional expression of genes in the β -oxidation pathway in zebrafish after PFOS exposure, and Yi et al. (2019) observed increased transcriptional expression of genes within the β -oxidation pathway including *acox1* and *acadm* in response to PFOS. However, other investigations using zebrafish have observed genes in the β -oxidation pathway having decreased expression or mixed profiles of both increased and decreased expression (Tu et al. 2019; Mylroie et al. 2021).

Disruption of lipid storage (KE4) can occur when the genes involved in lipogenesis and/or lipid transport experience dysregulation and can be exacerbated by simultaneous effects on lipid metabolism such as altered β-oxidation (KE3). Evidence of disruption of lipogenesis at the transcriptional level has also been observed across multiple studies (Tse et al. 2016; Cui et al. 2017; Huck et al. 2018; Liu et al. 2019; Martinez 2019; Yi et al. 2019; Louisse et al. 2020; Wang et al. 2022a). Changes in lipogenesis could result in an accumulation of lipids in liver cells if lipogenesis is increased or transport is perturbed. Huck et al. (2018) saw a decrease expression in *apoa1* and *apoa2* in mice which has been associated with increased risk of liver steatosis (Karavia et al. 2012). Liu et al. (2019) and Louisse et al. (2020) saw an increase in expression in perilipin (*Plin*) family genes in human liver and stem cells exposed to PFOS, but Rodríguez-Jorquera et al. (2018) saw a decrease in *Plin* expression in livers from exposed fathead minnows. *Plin* family genes are involved in the formation and degradation of lipid droplets and thus dysregulation of these genes may impact proper lipid storage in the liver (Carr and Ahima 2016). Tse et al. (2016) saw an increase in *apoe* expression in zebrafish, which can signal a shift towards accumulation of lipids in hepatocytes. Furthermore, Wang et al. (2022a) saw a trend of decreased transcriptional expression of genes involved in lipid synthesis in zebrafish in response to PFOS; whereas Yi et al. (2109) saw PFOS exposure result in an increase in *acacb* transcriptional expression, a gene involved in fatty acid synthesis.

Disruption in lipid transport in and out of liver cells can result in excess lipid accumulation in cells which can ultimately lead to liver steatosis. Specifically, previous work has shown that along with disruptions to β-oxidation and lipogenesis, PFOS exposure can result in transcriptional changes to lipid transport genes in terrestrial vertebrates and fish (Cheng et al. 2016; Tse et al. 2016; Cui et al. 2017; Rodríguez-Jorquera et al. 2018; Sant et al. 2018; Martinez 2019; Christou et al. 2020; Mylroie et al. 2021; Davidsen et al. 2022; Wang et al. 2022a). Studies in mice (Huck et al. 2018; Liu et al. 2019), rats (Davidsen et al. 2022), and human cells (Wan et al. 2012), showed increases in *CD36* expression in response to PFOS exposure. *CD36* is responsible for transport of lipids in liver cells and an increase in *CD36* expression due to PFOS exposure has been linked in increased TG levels in the liver (Jai et al. 2023). Dysregulation in *fabp* isoforms, which are responsible for the transport of fatty acids for fates such as β-oxidation and lipogenesis, was observed in mammals and fish exposed to PFOS (Rossen et al. 2010; Jacobsen et al. 2018; Sant et al. 2018; Mylroie et al. 2021; Wang et al. 2022a). Furthermore, *lpl*, which is involved in the proper transport of triglycerides was shown to be upregulated in studies in human cells (Wan et al. 2012) and mice (Liu et al. 2019); conversely Cheng et al. (2016) and Tse et al. (2016) showed *lpl* to be downregulated in response to PFOS exposure in zebrafish. Finally, Rodríguez-Jorquera et al. (2018) saw an overall decrease in lipid transport related genes in livers from PFOS exposed fathead minnow.

Overall, the results from these transcriptional studies show that PFOS exposure caused various disruptions of gene-transcript expression within the PPAR nuclear signaling network which are involved in fundamental processes that control lipid homeostasis and lipid profiles in liver tissue. Further, evidence for these KEs span multiple vertebrate species suggesting conservation of responses throughout vertebrates as a phylogenetic group.

Evidence of Changes in Lipid Profiles Indicative of Downstream Key Events (KE5 & KE6)

The observed dysregulation in β-oxidation and lipid storage should ultimately result in observable alterations in fatty acid, triglyceride, and total cholesterol profiles and accumulation as evidence of KE5 and KE6. Studies examining, whole body, serum, and liver lipid profiles have shown that PFOS exposure results in disrupted lipid profiles and accumulation in vertebrates, including humans. For example, Geng et al. (2019) saw increases in multiple types of lipid classes, including TGs, in developing chicken embryo livers after exposure to PFOS. Wan et al. (2012) and Huck et al. (2018) observed that mice had increased levels of TG in hepatic tissues after exposure to PFOS. Two studies using human cell cultures demonstrated increases in TG levels after exposure to PFOS (Liu et al. 2019; Louisse et al. 2020), and a metadata analysis performed on the blood lipid profiles of adult and juvenile humans showed that PFOS exposure was significantly correlated with an increase in TC levels in the blood and showed a trend of decreased TG levels in the blood (Ho et al. 2021).

Similar patterns of lipid alterations have been observed in fish. Sant et al. (2021) observed increased accumulation of fatty acids and changes in fatty acid ratios when PFOS exposed zebrafish embryos were compared to control fish and Yang et al. (2022) observed differing lipid profiles between PFOS and PFOA exposed zebrafish embryos. Cheng et al. (2016) observed a decrease in serum triglyceride (TG) and total cholesterol (TC) levels in the serum of male fish and an accumulation of TG in male and female livers (with males have significant increased TC levels as well). Cui et al. (2017) also observed a decrease in serum TG and TC levels in male fish and observed an increase in TC levels in female fish exposed to the highest PFOS concentration. Wang et al. (2022a) observed a significant increase in TC levels in adult zebrafish livers. The decrease in serum TC and TG levels combined with the increase in those same parameters in the liver tissue suggest a dysregulation of lipid homeostasis and preferential deposition of TC and TG in liver tissues.

These measured observations in fatty acid, TG, and TC profiles and accumulation provide further evidence of PPAR pathway dysregulation and are the downstream Key Events of the disruption of lipid metabolism and storage, a response conserved across the vertebrate species that were investigated.

Evidence of Lipid Accumulation in the Liver and the Adverse Outcome (AO) of Liver Steatosis

The contribution of KE5 and KE6 to lipid accumulation and lipid-induced hepatocellular vacuolation has been observed in various vertebrate species exposed to PFOS and other PFASs (Lee et al. 2020; Beale et al. 2022). In mice and rats, multiple studies have observed that PFOS exposure resulted in lipid-style hepatocellular vacuolation, lipid accumulation, or liver steatosis (Cui et al. 2009; Rosen et al. 2010, Zhang et al. 2016; Huck et al. 2018; Salter et al. 2021; Davidsen et al. 2022). Meanwhile in amphibians, results from Lin et al. (2022) showed that black-spotted frogs [*Rana nigromaculata*] exposed to PFOS had increased levels of hepatocellular vacuolation when compared to control frogs. Numerous studies have shown that increased lipid accumulation and/or hepatocellular vacuolation occurs in the developing liver of zebrafish beginning at the embryo/larval stage (Tse et al. 2016; Yi et al. 2019; Sant et al. 2021). At the adult stage, Mylroie et al. (IN PREP) found significant incidences of hepatocellular vacuolation in male zebrafish after exposure to 100 µg/L PFOS with other studies reporting similar outcomes at differing concentrations of PFOS exposure (Du et al. 2009; Keiter et al. 2012; Cheng et al. 2016; Cui et al. 2017; Huang et al. 2022a; Wang et al. 2022a). It is important to note that in many of the studies in adults where

sex differences were examined, lipid accumulation in liver was more severe in males than in females. Excess accumulation of lipids in the liver, as seen by the evidence here, is a key factor in the ultimate adverse outcome (AO) of liver steatosis.

Considerations for Potential Applications of the AOP (optional)

This AOP is likely to be applicable to chemicals, such as PFAS, that have been shown to interact with and disrupt the signaling of more than one PPAR isoform. The risk for this AOP is likely dependent on the concentrations of the chemical stressor and the duration of the exposure. It is possible that co-factors such as diet, genetic predisposition, and lack of physical activity could exacererbate or hasten the onset of the adverse outcome.

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Appendix 1

List of MIEs in this AOP

Event: 2226: Stressor binding PPAR isoforms

Short Name: Binding PPAR isoforms

Key Event Component

Process	Object	Action
receptor binding		occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:529 - Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	MolecularInitiatingEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term
eukaryotic cell

Organ term

Organ term

Organ term

liver

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

The conservation of PPAR molecular structure and function among vertebrates (Gust et al 2020) indicates this key event is likely to be conserved among this broad phylogenetic group. Furthermore, PPAR isoforms play a crucial role in lipid metabolism across representative vertebrate species. However, given that species to species variation does exist in structure and specific function, it is important to exercise care when looking to extrapolate across species.

Key Event Description

Both natural and synthetic ligands can interact with all 3 main PPAR isoforms with unsaturated fatty acids and other lipid-derived molecules being the primary natural ligands the PPAR isoforms (Ferré 2004). This Key Event describes the binding of stressor ligands to the PPAR isoforms with either agonist or antagonist interactions. Numerous studies have shown the ability of synthetic ligands to bind the ligand binding domains of the PPAR isoforms (α, β/δ, γ). Some of these synthetic ligands can be PPAR isoform specific whereas others, like bezafibrate, can bind and activate all 3 main PPAR isoforms (Grygiel-Górniak 2014). Specifically, the prototypical stressor, PFOS, has been shown to bind the three PPAR isoforms with varying degrees of affinity through *in vitro* ligand binding assays (Vanden Heuvel et al. 2006; Takacs and Abbot 2007; Wolf et al. 2008; Behr et al. 2020; Evans et al. 2022; Sun et al. 2023) as well as through computational binding/docking analyses (Li et al. 2018; Yi et al. 2019; Almedia et al. 2021; Garoche et al. 2021; Khazee et al. 2021; Huang et al. 2022b; Wang et al. 2022a, Wang et al. 2022b; Kowalska et al. 2023).

How it is Measured or Detected

Nuclear signaling assays, affinity assays, x-ray crystallography, and *in silico* analyses can all be used to assess the affinity and location of binding by known or potential ligands to the PPAR isoforms (Vanden Heuvel et al. 2006; Takacs and Abbot 2007; Capelli et al. 2016; Rajapaksha et al. 2017; Li et al. 2018; Behr et al. 2020; Almedia et al. 2021; Garoche et al. 2021; Evans et al. 2022; Sun et al. 2023). *In silico* analyses are a powerful screening tool to determine if a molecule of interest may be able to bind to one or more of the PPAR isoforms; however, confirmation of binding location should be done via x-ray crystallography. Nuclear signaling assays can be used to determine if a potential ligand of interest acts as an agonists or antagonists. A comprehensive example of *in silico* primary analyses coupled with confirmation steps using cell-based report assays and x-ray crystallography for PPAR isoforms can be found in Capelli et al. (2016).

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List of Key Events in the AOP

Event: 2227: Disrupted PPAR isoform nuclear signaling

Short Name: Disrupted PPAR isoform nuclear signaling

Key Event Component

Process	Object	Action
peroxisome proliferator activated receptor signaling pathway		disrupted

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:529 - Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term
eukaryotic cell

Organ term

Organ term
liver

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

The conservation of PPAR molecular structure and function among vertebrates (Gust et al 2020) indicates this key event is likely to be conserved among this broad phylogenetic group. Furthermore, PPAR isoforms play a crucial role in lipid metabolism across representative vertebrate species. However, given that species to species variation does exist in structure and specific function, it is important to exercise care when looking to extrapolate across species.

Key Event Description

This Key Event describes disruption of PPAR isoform nuclear signaling following the binding of stressor ligands to the PPAR isoforms with either agonist or antagonist interactions. Following binding with an activating ligand, PPAR isoforms heterodimerize with the retinoid X receptor (RXR) with this complex then recognizing the peroxisome proliferator response elements (PPRE) of the PPAR isoform target genes promoting gene expression (Capelli et al. 2016). Therefore, non-native ligands that bind the PPAR isoforms either agonistically or antagonistically can disrupt proper PPAR activity and signaling of either expression or repression of target genes. Results from activity assays, nuclear signaling assays, and transcriptomic analyses using PPAR isoform agonist and antagonist have demonstrate that PPAR ligands directly affect PPAR activity, nuclear signaling, and the transcription of PPAR mediated target genes (Kojo et al. 2003; Behr et al. 2020; Gao et al. 2020; Evans et al. 2022; Murase et al. 2023; Ardenkjær-Skinnerup et al. 2024). Moreover, studies have demonstrated that exposure to the prototypical stressor, PFOS, can have a direct effect on the transcriptional expression of the PPAR isoforms in vertebrates (Lee et al. 2020; Beale et al. 2022) with these studies showing expression changes occurring primarily in the PPARα and PPARγ isoforms.

Beyond the direct effects of stressor ligands on PPAR isoforms, activation of one PPAR isoform can have effects on the expression of other PPAR isoforms. For example, agonism of PPARβ/δ can cause reduced expression of PPARα and PPARγ isoforms (Shi et al. 2002; Kim et al. 2020), and certain coregulators can have effects (sometimes opposite) on different PPAR isoforms (Tahri-Joutey et al. 2021). Finally, omics studies have shown that agonist and antagonist of PPAR isoforms alter PPAR signaling transcripts (Louisse et al. 2020; Heintz et al. 2024). Overall, this evidence displays that disruption of PPAR isoforms via stressor chemicals can affect other PPAR isoforms and impact PPAR nuclear signaling.

How it is Measured or Detected

Activity assays, nuclear signaling assays, and transcriptomic or proteomic analyses can identify disrupted nuclear signaling as

the result of ligand binding to PPAR isoforms (Kojo et al. 2003; Li et al. 2017; Gao et al. 2020; Murase et al. 2023; Ardenkjær-Skinnerup et al. 2024). These assays can be used to determine if a potential ligand of interest acts as an agonists or antagonists either via direct activity assays or by analysis of gene targets in the PPAR isoform pathways.

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Event: 2224: Dysregulation of transcriptional expression within PPAR signaling network

Short Name: Dysregulation of transcriptional expression within PPAR signaling network

Key Event Component

Process	Object	Action
regulation of gene expression		disrupted

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:529 - Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term
eukaryotic cell

Organ term

Organ term
liver

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Juvenile	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

The conservation of PPAR molecular structure and function among vertebrates (Gust et al 2020) indicates this key event is likely to be conserved among this broad phylogenetic group. Furthermore, PPAR isoforms play a crucial role in lipid metabolism across representative vertebrate species. However, given that species to species variation does exist in structure and specific function, it is important to exercise care when looking to extrapolate across species.

Key Event Description

This Key Event describes dysregulation of PPAR mediated transcriptional expression within the PPAR signaling network following the binding of stressor ligands to the PPAR isoforms with either agonist or antagonist interactions. There is abundant evidence of showing how synthetic ligands can affect transcriptional expression in the PPAR signaling network and of key genes involved in lipid homeostasis (Meierhofer et al. 2014; Li et al. 2020; Cariello et al. 2021; Heintz et al. 2022; Eide et al. 2023; Heintz et al. 2024). Specifically, pathway and gene ontology (GO) enrichment analyses have identified lipid metabolism, lipid transport, fatty acid degradation, PPAR signaling pathway, and lipid homeostasis as being transcriptionally altered in response to PFOS exposure (Chen et al. 2014; Jacobsen et al. 2018; Rodríguez-Jorquera et al. 2018; Martinez et al. 2019; Christou et al. 2020; Dong et al. 2021; Lee et al. 2021; Mylroie et al. 2021; Beale et al. 2022; Davidsen et al. 2022; Haimbuagh et al. 2022; Wang et al. 2022; Mylroie et al. IN PREP).

How it is Measured or Detected

Targeted gene expression assays along with “omic” tools such as transcriptomics or proteomics can be used to determine if known or suspected ligands of the PPAR isoforms disrupt gene expression in the PPAR pathway. There are abundant resources

available describing methodologies to assess disruption of 1 or more of the PPAR isoform pathways (Meierhofer et al. 2014; Li et al. 2020; Cariello et al. 2021; Mylroie et al. 2021; Heintz et al. 2022; Eide et al. 2023; Heintz et al. 2024).

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Event: 179: Decrease, Fatty acid beta-oxidation

Short Name: Decrease, Fatty acid β-oxidation

Key Event Component

Process	Object	Action
fatty acid beta-oxidation	fatty acid	decreased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:36 - Peroxisomal Fatty Acid Beta-Oxidation Inhibition Leading to Steatosis	KeyEvent
Aop:60 - NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis	KeyEvent
Aop:497 - ERa inactivation alters mitochondrial functions and insulin signalling in skeletal muscle and leads to insulin resistance and metabolic syndrome	KeyEvent
Aop:529 - Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	KeyEvent

Biological Context

Level of Biological Organization
Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	High	NCBI

See review for Human PPARalpha signaling in (Evans et al 2004).

Key Event Description

Fatty acid oxidation in liver tissue is controlled by PPARalpha signaling networks (Evans et al 2004). The PPARalpha signaling network controls expression of the genes within metabolic pathways that catalyze fatty acid oxidation reactions (Desvergne and Wahli 1999).

How it is Measured or Detected

A variety of approaches establishing the effects of PPARalpha signaling on fatty acid oxidation are reviewed in Evans et al (2004).

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Event: 2225: Disrupted Lipid Storage

Short Name: Disrupted Lipid Storage

Key Event Component

Process	Object	Action
lipid storage		disrupted

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:529 - Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
eukaryotic cell

Organ term

Organ term
liver

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

The conservation of PPAR molecular structure and function among vertebrates (Gust et al 2020) indicates this key event is likely to be conserved among this broad phylogenetic group. Furthermore, PPAR isoforms play a crucial role in lipid metabolism across representative vertebrate species. However, given that species to species variation does exist in structure and specific function, it is important to exercise care when looking to extrapolate across species.

Key Event Description

This Key Event describes the disruption of normal lipid storage in liver cells. Disruption of lipid storage and transport can be identified by excess accumulation of fatty acids or other lipids in the liver or altered ratios of expected lipid species which can ultimately lead to liver steatosis (Ipsen et al. 2018). An example of an event that can cause disrupted lipid storage is the binding of stressor ligands to the PPAR isoforms with either agonist or antagonist interactions which can lead to effects on lipid storage and transport (Dixon et al. 2021). PPARγ over expression results in promotes storage of lipids in the liver and thus exacerbates hepatic steatosis (Yu et al. 2003; Patsouris et al. 2006). Conversely, deletion of PPARα resulted in an increased liver lipid (Patsouris et al. 2006). Wang et al. (2003) demonstrated that PPARβ/δ deficient mice had increased obesity which, while potentially not a function of improper lipid storage, underpins the importance of all PPAR isoforms in proper lipid homeostasis. Evidence of disruption of lipogenesis at the transcriptional level has also been observed across multiple studies using PFAS as the stressor (Tse et al. 2016; Cui et al. 2017; Huck et al. 2018; Liu et al. 2019; Martinez 2019; Yi et al. 2019; Louisse et al. 2020; Wang et al. 2022a).

How it is Measured or Detected

There are numerous methodologies available for measuring disrupted lipid storage in the liver cells. Fatty acids and other lipid species can be measure directly or measured globally using lipidomic methodologies (Wang et al. 2022; Albers et al. 2024), and histopathology can confirm lipid deposits in liver sections (Huck et al. 2018; Wang et al. 2022). Also, targeted or global gene expression analyses can reveal disruptions in key genes responsible for proper lipid storage and transport (Tse et al. 2016; Yi et al. 2019; Louisse et al. 2020).

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Event: 327: Accumulation, Fatty acid

Short Name: Accumulation, Fatty acid

Key Event Component

Process	Object	Action
	fatty acid	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:36 - Peroxisomal Fatty Acid Beta-Oxidation Inhibition Leading to Steatosis	KeyEvent
Aop:57 - AhR activation leading to hepatic steatosis	KeyEvent
Aop:58 - NR1I3 (CAR) suppression leading to hepatic steatosis	KeyEvent

AOP ID and Name	Event Type
Aop:60 - NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis	KeyEvent
Aop:529 - Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	KeyEvent

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

liver

Event: 291: Accumulation, Triglyceride

Short Name: Accumulation, Triglyceride

Key Event Component

Process	Object	Action
	triglyceride	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:34 - LXR activation leading to hepatic steatosis	KeyEvent
Aop:57 - AhR activation leading to hepatic steatosis	KeyEvent
Aop:318 - Glucocorticoid Receptor activation leading to hepatic steatosis	KeyEvent
Aop:517 - Pregnane X Receptor (PXR) activation leads to liver steatosis	KeyEvent
Aop:518 - Liver X Receptor (LXR) activation leads to liver steatosis	KeyEvent
Aop:529 - Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	KeyEvent
Aop:580 - Mineralocorticoid Receptor Activation Leading to Increased Body Mass Index	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

hepatocyte

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	High
Juvenile	Moderate

Sex Applicability

Sex	Evidence
Unspecific	High

Life Stage: Older individuals are more likely to manifest this key event (adults > juveniles) due to accumulation of triglycerides.

Sex: Applies to both males and females.

Taxonomic: Appears to be present broadly in vertebrates, with most representative studies in mammals (humans, lab mice, lab rats). Likely pervasive in many animal taxa.

Key Event Description

Triglycerides are important building blocks for a wide variety of compounds found in organisms, with cellular concentrations reflecting the relative rate of influx and efflux, as well as the relative rate of synthesis and breakdown. However, excess accumulation leads to Fatty Liver Cells *and steatosis*.

In this key event we focus on excessive accumulation of triglycerides in mammalian systems. Four major pathways for triglyceride accumulation are: 1. Increased fatty acid uptake; 2. Increased De Novo FA and Lipid Synthesis; 3. Decreased FA Oxidation; 4. Decreased Lipid Efflux (Angrish et al. 2016). Chemical stressors can increase gene expression of key genes involving these pathways, leading to increased accumulation of triglycerides (Aguayo-Orozco et al. 2018). In addition, excessive dietary compounds of fatty compounds can also increase likelihood of accumulation of triglycerides (Nguyen et al. 2008). Nuclear receptors that have been implicated in causing excessive accumulation of triglycerides leading to steatosis, when overexpressed, include (Mellor et al. 2016): Aryl hydrocarbon receptor (AHR), Constitutive androstane receptor (CAR), Oestrogen receptor (ER), Farnesoid X receptor (FXR), Glucocorticoid receptor (GXR), Liver X receptor (LXR), Peroxisome proliferator-activated receptor (PPAR), Pregnane X receptor (PXR), and Retinoic acid receptor (RAR or RXR).

How it is Measured or Detected

Concentrations of triglycerides, cholesterols, fatty acids, and related compounds are measured biochemically to assess levels in control versus potentially affected individuals; common techniques include high throughput enzymatic analyses, analytical ultracentrifuging, gradient gel electrophoresis, Nuclear Magnetic Resonance, lipidomics, and other direct assessment techniques (Schaefer et al. 2016; Yang and Han 2016). Analysis is often performed to look at gene expression levels to see which pathway(s) have increased expression levels, to attribute plausibility to changes in influx, eflux, synthesis, and/or breakdown pathways (Nguyen et al. 2008; Mellor et al. 2016, Aguayo-Orozco et al. 2018). Assessment of cellular components including mitochondria and membrane integrity can also be used as evidence of alteration of normal function within cells.

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NOTE: Italics symbolize edits from John Frisch

List of Adverse Outcomes in this AOP

Event: 459: Increased, Liver Steatosis

Short Name: Increased, Liver Steatosis

Key Event Component

Process	Object	Action
Hepatic steatosis		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:58 - NR1I3 (CAR) suppression leading to hepatic steatosis	AdverseOutcome
Aop:60 - NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis	AdverseOutcome
Aop:61 - NFE2L2/FXR activation leading to hepatic steatosis	AdverseOutcome
Aop:62 - AKT2 activation leading to hepatic steatosis	AdverseOutcome
Aop:36 - Peroxisomal Fatty Acid Beta-Oxidation Inhibition Leading to Steatosis	AdverseOutcome
Aop:213 - Inhibition of fatty acid beta oxidation leading to nonalcoholic steatohepatitis (NASH)	KeyEvent
Aop:285 - Inhibition of N-linked glycosylation leads to liver injury	KeyEvent
Aop:318 - Glucocorticoid Receptor activation leading to hepatic steatosis	AdverseOutcome
Aop:517 - Pregnane X Receptor (PXR) activation leads to liver steatosis	AdverseOutcome
Aop:518 - Liver X Receptor (LXR) activation leads to liver steatosis	AdverseOutcome
Aop:529 - Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	AdverseOutcome
Aop:232 - NFE2/Nrf2 repression to steatosis	AdverseOutcome
Aop:57 - AhR activation leading to hepatic steatosis	AdverseOutcome
Aop:494 - AhR activation leading to liver fibrosis	KeyEvent

Biological Context

Level of Biological Organization

Organ

Organ term

Organ term

liver

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Steatosis is the result of perturbations in well-known metabolic pathways that are well-studied and well-known in many taxa.

Life Stage: The life stage applicable to this key event is all life stages with a liver. Older individuals are more likely to manifest this adverse outcome pathway (adults > juveniles) due to accumulation of triglycerides.

Sex: This key event applies to both males and females.

Taxonomic: This key event appears to be present broadly in vertebrates, with most representative studies in mammals (humans, lab mice, lab rats).

Key Event Description

Biological state: liver steatosis is the inappropriate storage of fat in hepatocytes. *Four major pathways for triglyceride accumulation are: 1. Increased fatty acid uptake; 2. Increased De Novo FA and Lipid Synthesis; 3. Decreased FA Oxidation; 4. Decreased Lipid Efflux (Angrish et al. 2016). Chemical stressors can increase gene expression of key genes involving these pathways, leading to increased accumulation of triglycerides (Aguayo-Orozco et al. 2018). In addition, excessive dietary compounds of fatty compounds can also increase likelihood of accumulation of triglycerides (Nguyen et al. 2008).*

Biological compartment: steatosis is generally an organ-level diagnosis; however, the pathology occurs within the hepatocytes.

Role in biology: steatosis is an adverse endpoint.

Consequences: Liver steatosis, or fatty liver, serves as a pivotal factor in the development of liver fibrosis by triggering a cascade of pathological events. According to the two-strikes hypothesis (Day and James, 1998), liver damage progresses in two stages: the first strike involves the accumulation of lipids in hepatocytes, often due to metabolic disturbances such as insulin resistance, excess free fatty acids, or oxidative stress. This stage, though asymptomatic, increases liver vulnerability by inducing mild oxidative stress and inflammation. The second strike introduces additional insults, such as inflammatory mediators or cellular damage, exacerbating liver injury and promoting fibrogenesis. The accumulation of fat sensitizes the liver to oxidative stress and triggers mechanisms like the activation of hepatic stellate cells (HSCs) and hepatocyte apoptosis or necrosis, central to the fibrotic process. While early-stage steatosis is reversible, chronic steatosis perpetuates a cycle of inflammation and fibrosis, creating a feedback loop that amplifies liver damage (Pafili K et al, 2021). Consequently, liver steatosis is not only a precursor but also a critical driver of fibrosis progression.

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Description from EU-ToxRisk:

Activation of stellate cells results in collagen accumulation and change in extracellular matrix composition in the liver causing fibrosis. (Landesmann, 2016; Koo et al 2016)

How it is Measured or Detected

Steatosis is measured by lipidomics approaches *(e.g. Yang and Han 2016)*that measure lipid levels, or by histology. *Concentrations of triglycerides, cholesterols, fatty acids, and related compounds are measured biochemically include high throughput enzymatic analyses, analytical ultracentrifuging, gradient gel electrophoresis, Nuclear Magnetic Resonance, and other direct assessment techniques (Schaefer et al. 2016).*

Regulatory Significance of the AO

Steatosis is a regulatory endpoint and has been used as an endpoint in many US EPA assessments, including IRIS

assessments.

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 3220: Binding PPAR isoforms leads to Disrupted PPAR isoform nuclear signaling

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate

Life Stage	Evidence
Juvenile	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

The conservation of PPAR molecular structure and function among vertebrates (Gust et al 2020) indicates this key event is likely to be conserved among this broad phylogenetic group. Furthermore, PPAR isoforms play a crucial role in lipid metabolism across representative vertebrate species. However, given that species to species variation does exist in structure and specific function, it is important to exercise care when looking to extrapolate across species.

Key Event Relationship Description

Both natural and synthetic ligands can interact with all 3 main PPAR isoforms (α, β/δ, γ) with unsaturated fatty acids and other lipid-derived molecules being the primary natural ligands the PPAR isoforms (Ferré 2004). Numerous studies have shown the ability of synthetic ligands to bind the ligand binding domains of the PPAR isoforms (Ferré 2004; Grygiel-Górniak 2014). This Key Event Relationship describes the binding of stressor ligands to the PPAR isoforms with either agonist or antagonist interactions which then disrupts downstream PPAR isoform nuclear signaling. The ligands that bind the PPAR isoforms either agonistically or antagonistically can disrupt proper PPAR activity and nuclear signaling for the either expression or repression of target genes.

Evidence Supporting this KER

Biological Plausibility

Natural and synthetic ligands can interact with all 3 main PPAR isoforms (α, β/δ, γ) with unsaturated fatty acids and other lipid-derived molecules being the primary natural ligands the PPAR isoforms (Ferré 2004). Following binding with an activating ligand, PPAR isoforms heterodimerize with the retinoid X receptor (RXR) with this complex then recognizing the peroxisome proliferator response elements (PPRE) of the PPAR isoform target genes and promoting gene expression (Capelli et al. 2016). Therefore, ligands that act either agonistically or antagonistically beyond or more persistently than the normal biological range can disrupt proper nuclear signaling and subsequent gene expression.

Empirical Evidence

Synthetic ligands can be PPAR isoform specific whereas others, like bezafibrate, can bind and activate all 3 main PPAR isoforms (Grygiel-Górniak 2014). Specifically, the prototypical stressor, PFOS, has been shown to bind the three PPAR isoforms with varying degrees of affinity through *in vitro* ligand binding assays (Vanden Heuvel et al. 2006; Takacs and Abbot 2007; Wolf et al. 2008; Behr et al. 2020; Evans et al. 2022; Sun et al. 2023) as well as through computational binding/docking analyses (Li et al. 2018; Yi et al. 2019; Almedia et al. 2021; Garoche et al. 2021; Khazee et al. 2021; Huang et al. 2022; Wang et al. 2022a, Wang et al. 2022b; Kowalska et al. 2023).

Results from activity assays, nuclear signaling assays, and transcriptomic analyses using PPAR isoform agonist and antagonist have demonstrate that PPAR ligands directly affect PPAR activity, nuclear signaling, and the transcription of PPAR mediated target genes (Kojo et al. 2003; Behr et al. 2020; Gao et al. 2020; Evans et al. 2022; Murase et al. 2023; Ardenkjær-Skinnerup et al. 2024). Moreover, studies have demonstrated that exposure to the prototypical stressor, PFOS, can have a direct effect on the transcriptional expression of the PPAR isoforms in vertebrates (Lee et al. 2020; Beale et al. 2022) with these studies showing expression changes occurring primarily in the PPARα and PPARγ isoforms.

Beyond the direct effects of stressor ligands on PPAR isoforms, activation of one PPAR isoform can have effects on the expression of other PPAR isoforms. For example, agonism of PPARβ/δ can cause reduced expression of PPARα and PPARγ isoforms (Shi et al. 2002; Kim et al. 2020; Kim et al. 2023), and certain coregulators can have effects (sometimes opposite) on different PPAR isoforms (Tahri-Joutey et al. 2021). Finally, omics studies have shown that agonist and antagonist of PPAR isoforms alter PPAR signaling transcripts (Louisse et al. 2020; Heintz et al. 2024). Overall, this evidence displays that disruption of PPAR isoforms via stressor chemicals can affect other PPAR isoforms and impact PPAR nuclear signaling.

Uncertainties and Inconsistencies

While the PPAR molecular structure and function among vertebrates is largely conserved (Gust et al 2020), species to species variation does exist in structure and specific function; and therefore, it is important to exercise care when looking to extrapolate across species. The binding affinity of certain ligands and the magnitude of response in PPAR nuclear signaling may differ from species to species due to variations in PPAR molecular structure.

Quantitative Understanding of the Linkage

Response-response relationship

Unknown.

Time-scale

Rapid Molecular Interactions.

Known Feedforward/Feedback loops influencing this KER

As PPAR signaling is essential for maintaining energy homeostasis, there is a complex network of feedforward/feedback loops influencing PPAR nuclear signaling via ligands, products, and the PPAR isoforms acting on each other. Due to extensive detail needed to properly describe all potential feedforward/feedback loops that could influence this KER, the authors direct readers to reviews by Ament et al. (2012) and Lamichane et al. (2018).

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Relationship: 3221: Disrupted PPAR isoform nuclear signaling leads to Dysregulation of transcriptional expression within PPAR signaling network

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

The conservation of PPAR molecular structure and function among vertebrates (Gust et al 2020) indicates this key event is likely to be conserved among this broad phylogenetic group. Furthermore, PPAR isoforms play a crucial role in lipid metabolism across representative vertebrate species. However, given that species to species variation does exist in structure and specific function, it is important to exercise care when looking to extrapolate across species.

Key Event Relationship Description

This Key Event Relationship describes how the disruption of PPAR isoform nuclear signaling affects transcriptional expression within the PPAR signaling network. The ligands that bind the PPAR isoforms either agonistically or antagonistically can disrupt proper PPAR activity and nuclear signaling for the either expression or repression of target genes in the PPAR signaling network.

Evidence Supporting this KER

Biological Plausibility

Following binding with an activating ligand, PPAR isoforms heterodimerize with the retinoid X receptor (RXR) with this complex then recognizing the peroxisome proliferator response elements (PPRE) of the PPAR isoform target genes and promoting gene expression (Capelli et al. 2016). Therefore, ligands that act either agonistically or antagonistically beyond or more persistently than the normal biological range can disrupt proper nuclear signaling and subsequent gene expression in the PPAR signaling pathway.

Empirical Evidence

Results from activity assays, nuclear signaling assays, and transcriptomic analyses using PPAR isoform agonist and antagonist have demonstrate that PPAR ligands directly affect PPAR activity, nuclear signaling, and the transcription of PPAR mediated target genes (Kojo et al. 2003; Behr et al. 2020; Gao et al. 2020; Evans et al. 2022; Murase et al. 2023; Ardenkjær-Skinnerup et al. 2024). Moreover, studies have demonstrated that exposure to the prototypical stressor, PFOS, can have a direct effect on the transcriptional expression of the PPAR isoforms in vertebrates (Lee et al. 2020; Beale et al. 2022) with these studies showing expression changes occurring primarily in the PPARα and PPARγ isoforms.

Beyond the direct effects of stressor ligands on PPAR isoforms, activation of one PPAR isoform can have effects on the expression of other PPAR isoforms. For example, agonism of PPARβ/δ can cause reduced expression of PPARα and PPARγ isoforms (Shi et al. 2002; Kim et al. 2020; Kim et al. 2023), and certain coregulators can have effects (sometimes opposite) on different PPAR isoforms (Tahri-Joutey et al. 2021). Finally, omics studies have shown that agonist and antagonist of PPAR isoforms alter PPAR signaling transcripts (Louisse et al. 2020; Heintz et al. 2024). Overall, this evidence displays that disruption of PPAR isoforms via stressor chemicals can affect other PPAR isoforms and impact PPAR nuclear signaling.

Dysregulation of gene expression follows disrupted nuclear signaling as can be seen from abundant evidence of showing how synthetic ligands can affect transcriptional expression in the PPAR signaling network and of key genes involved in lipid homeostasis (Meierhofer et al. 2014; Li et al. 2020; Cariello et al. 2021; Heintz et al. 2022; Eide et al. 2023; Heintz et al. 2024). Specifically, pathway and gene ontology (GO) enrichment analyses have identified lipid metabolism, lipid transport, fatty acid degradation, PPAR signaling pathway, and lipid homeostasis as being transcriptionally altered in response to PFOS exposure (Chen et al. 2014; Jacobsen et al. 2018; Rodríguez-Jorquera et al. 2018; Martinez et al. 2019; Christou et al. 2020; Dong et al. 2021; Lee et al. 2021; Mylroie et al. 2021; Beale et al. 2022; Davidsen et al. 2022; Haimbuagh et al. 2022; Wang et al. 2022; Mylroie et al. IN PREP).

Uncertainties and Inconsistencies

While the PPAR molecular structure and function among vertebrates is largely conserved (Gust et al 2020), species to species variation does exist in structure and specific function; and therefore, it is important to exercise care when looking to extrapolate across species. The binding affinity of certain ligands and the magnitude of response in PPAR nuclear signaling may differ from species to species due to variations in PPAR molecular structure. Furthermore, the direction and magnitude of gene expression response may differ from species to species or even within species depending on the ligand assayed and the concentration used.

Quantitative Understanding of the Linkage

Response-response relationship

Unknown

Time-scale

Rapid Molecular Interactions

Known Feedforward/Feedback loops influencing this KER

As PPAR signaling is essential for maintaining energy homeostasis, there is a complex network of feedforward/feedback loops influencing PPAR nuclear signaling and gene expression via ligands, products, and the PPAR isoforms acting on each other. Due to the extensive detail needed to properly describe all potential feedforward/feedback loops that could influence this KER, the authors direct readers to reviews by Ament et al. (2012) and Lamichane et al. (2018).

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Wang, Q., Huang, J., Liu, S., Wang, C., Jin, Y., Lai, H. and Tu, W., 2022. Aberrant hepatic lipid metabolism associated with gut microbiota dysbiosis triggers hepatotoxicity of novel PFOS alternatives in adult zebrafish. *Environment International*, *166*, p.107351.

Relationship: 3224: Dysregulation of transcriptional expression within PPAR signaling network leads to Disrupted Lipid Storage

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

The conservation of PPAR molecular structure and function among vertebrates (Gust et al 2020) indicates this key event is likely to be conserved among this broad phylogenetic group. Furthermore, PPAR isoforms play a crucial role in lipid metabolism across representative vertebrate species. However, given that species to species variation does exist in structure and specific function, it is important to exercise care when looking to extrapolate across species.

Key Event Relationship Description

This Key Event Relationship describes how the dysregulation of transcriptional expression within the PPAR signaling network results in disrupted lipid storage, specifically in liver cells. All 3 PPAR isoforms and the genes they regulate are essential for proper lipid storage and transport; and therefore, dysregulation in the expression profiles of any or all of the PPAR isoform controlled signaling networks can disrupt the proper storage of lipids in cells (Ament et al. 2012; Dixon et al. 2021; Xiao et al. 2021).

Evidence Supporting this KER

Biological Plausibility

Ligands that act either agonistically or antagonistically beyond or more persistently than the normal biological range can disrupt proper nuclear signaling and subsequent gene expression in the PPAR signaling pathway. The complex control of lipid metabolism means dysregulation of gene expression in the PPAR signaling network can have a disruptive effect on lipid storage and transport as all 3 PPAR isoforms and the genes they modulate play essential roles in the delicate control of lipid homeostasis (Dixon et al. 2021; Xiao et al. 2021).

Empirical Evidence

Dysregulation of gene expression follows disrupted nuclear signaling as can be seen from abundant evidence of showing how synthetic ligands can affect transcriptional expression in the PPAR signaling network and of key genes involved in lipid homeostasis (Meierhofer et al. 2014; Li et al. 2020; Cariello et al. 2021; Heintz et al. 2022; Eide et al. 2023; Heintz et al. 2024). Specifically, pathway and gene ontology (GO) enrichment analyses have identified lipid metabolism, lipid transport, fatty acid degradation, PPAR signaling pathway, and lipid homeostasis as being transcriptionally altered in response to PFOS exposure (Chen et al. 2014; Jacobsen et al. 2018; Rodríguez-Jorquera et al. 2018; Martinez et al. 2019; Christou et al. 2020; Dong et al. 2021; Lee et al. 2021; Mylroie et al. 2021; Beale et al. 2022; Davidsen et al. 2022; Haimbuagh et al. 2022; Wang et al. 2022; Mylroie et al. IN PREP).

When a stressor ligand binds to the PPAR isoforms with either agonist or antagonist interactions which can lead to effects on lipid storage and transport (Dixon et al. 2021). PPARγ over expression results in promotes storage of lipids in the liver and thus exacerbates hepatic steatosis (Yu et al. 2003; Patsouris et al. 2006). Conversely, deletion of PPARα resulted in an increased liver lipid (Patsouris et al. 2006). Wang et al. (2003) demonstrated that PPARβ/δ deficient mice had increased obesity which, while potentially not a function of improper lipid storage, underpins the importance of all PPAR isoforms in proper lipid homeostasis. Evidence of disruption of lipogenesis at the transcriptional level has also been observed across multiple studies using PFAS as the stressor (Tse et al. 2016; Cui et al. 2017; Huck et al. 2018; Liu et al. 2019; Martinez 2019; Yi et al. 2019; Louisse et al. 2020; Wang et al. 2022). Changes in lipogenesis could result in an accumulation of lipids in liver cells if lipogenesis is increased or transport is perturbed. Huck et al. (2018) saw a decrease expression in *apoa1* and *apoa2* in mice which has been associated with increased risk of liver steatosis (Karavia et al. 2012). Liu et al. (2019) and Louisse et al. (2020) saw an increase in expression in perilipin (*Plin*) family genes in human liver and stem cells exposed to PFOS, but Rodríguez-Jorquera et al. (2018) saw a decrease in *Plin* expression in livers from exposed fathead minnows. *Plin* family genes are involved in the formation and degradation of lipid droplets and thus dysregulation of these genes may impact proper lipid storage in the liver (Carr and Ahima 2016). Tse et al. (2016) saw an increase in *apoe* expression in zebrafish, which can signal a shift towards accumulation of lipids in hepatocytes. Furthermore, Wang et al. (2022) saw a trend of decreased transcriptional expression of genes involved in lipid synthesis in zebrafish in response to PFOS; whereas Yi et al. (2019) saw

PFOS exposure result in an increase in *acacb* transcriptional expression, a gene involved in fatty acid synthesis.

Disruption in lipid transport in and out of liver cells can result in excess lipid accumulation in cells which can ultimately lead to liver steatosis. Specifically, previous work has shown that along with disruptions to β-oxidation and lipogenesis, PFOS exposure can result in transcriptional changes to lipid transport genes in terrestrial vertebrates and fish (Cheng et al. 2016; Tse et al. 2016; Cui et al. 2017; Rodríguez-Jorquera et al. 2018; Sant et al. 2018; Martinez 2019; Christou et al. 2020; Mylroie et al. 2021; Davidsen et al. 2022; Wang et al. 2022). Studies in mice (Huck et al. 2018; Liu et al. 2019), rats (Davidsen et al. 2022), and human cells (Wan et al. 2012), showed increases in *CD36* expression in response to PFOS exposure. *CD36* is responsible for transport of lipids in liver cells and an increase in *CD36* expression due to PFOS exposure has been linked in increased TG levels in the liver (Jai et al. 2023). Dysregulation in *fabp* isoforms, which are responsible for the transport of fatty acids for fates such as β-oxidation and lipogenesis, was observed in mammals and fish exposed to PFOS (Rosen et al. 2010; Jacobsen et al. 2018; Sant et al. 2018; Mylroie et al. 2021; Wang et al. 2022). Furthermore, *lpl*, which is involved in the proper transport of triglycerides was shown to be upregulated in studies in human cells (Wan et al. 2012) and mice (Liu et al. 2019); conversely Cheng et al. (2016) and Tse et al. (2016) showed *lpl* to be downregulated in response to PFOS exposure in zebrafish. Finally, Rodríguez-Jorquera et al. (2018) saw an overall decrease in lipid transport related genes in livers from PFOS exposed fathead minnow.

Uncertainties and Inconsistencies

While the PPAR molecular structure and function among vertebrates is largely conserved (Gust et al 2020), species to species variation does exist in structure and specific function; and therefore, it is important to exercise care when looking to extrapolate across species. The binding affinity of certain ligands and the magnitude of response in PPAR nuclear signaling may differ from species to species due to variations in PPAR molecular structure. Furthermore, the direction and magnitude of gene expression response may differ from species to species or even within species depending on the ligand assayed and the concentration used.

Quantitative Understanding of the Linkage

Response-response relationship

Unknown

Time-scale

Rapid Molecular Interactions

Known Feedforward/Feedback loops influencing this KER

As PPAR signaling is essential for maintaining energy homeostasis, there is a complex network of feedforward/feedback loops influencing PPAR nuclear signaling and gene expression via ligands, products, and the PPAR isoforms acting on each other. Due to the extensive detail needed to properly describe all potential feedforward/feedback loops that could influence this KER, the authors direct readers to reviews by Ament et al. (2012) and Lamichane et al. (2018).

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Relationship: 3223: Dysregulation of transcriptional expression within PPAR signaling network leads to Decrease, Fatty acid β-oxidation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	adjacent	High	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

The conservation of PPAR molecular structure and function among vertebrates (Gust et al 2020) indicates this key event is likely to be conserved among this broad phylogenetic group. Furthermore, PPAR isoforms play a crucial role in lipid metabolism and β-oxidation across representative vertebrate species. However, given that species to species variation does exist in structure and specific function, it is important to exercise care when looking to extrapolate across species.

Key Event Relationship Description

This Key Event Relationship describes how the dysregulation of transcriptional expression within the PPAR signaling network results in disrupted β-oxidation and specifically cause a decrease in β-oxidation. All 3 PPAR isoforms and the genes they regulate are essential for proper energy homeostasis of which β-oxidation is a key component; and therefore, dysregulation in the expression profiles of any or all of the PPAR isoform controlled signaling networks can disrupt fatty acid β-oxidation in cells (Ament et al. 2012; Liu et al. 2020; Dixon et al. 2021; Xiao et al. 2021).

Evidence Supporting this KER

Biological Plausibility

Ligands that act either agonistically or antagonistically beyond or more persistently than the normal biological range can disrupt proper nuclear signaling and subsequent gene expression in the PPAR signaling pathway. The complex control of lipid metabolism means dysregulation of gene expression in the PPAR signaling network can have a disruptive effect on β-oxidation (Dixon et al. 2021; Xiao et al. 2021) as the PPAR isoforms play a key role in regulating β-oxidation (Cherkaoui-Malki et al. 2012). PPARα knockouts have shown decreased β-oxidation and subsequent lipid accumulation in the liver (Hashimoto et al. 2000; Reddy 2001; Badman et al. 2007) whereas activation of PPARα has been shown to increase β-oxidation (Tahri-Joutey et al. 2021). PPARβ/δ has also been shown to have a critical role in the regulation β-oxidation and PPARγ activation promotes lipid storage and decreases fatty acid β-oxidation (Reddy 2001; Roberts et al. 2011).

Empirical Evidence

Dysregulation of gene expression follows disrupted nuclear signaling as can be seen from abundant evidence of showing how synthetic ligands can affect transcriptional expression in the PPAR signaling network and of key genes involved in lipid homeostasis (Meierhofer et al. 2014; Li et al. 2020; Cariello et al. 2021; Heintz et al. 2022; Eide et al. 2023; Heintz et al. 2024). Specifically, pathway and gene ontology (GO) enrichment analyses have identified lipid metabolism, lipid transport, fatty acid β-oxidation, PPAR signaling pathway, and lipid homeostasis as being transcriptionally altered in response to PFAS exposure (Chen et al. 2014; Tse et al. 2016; Cui et al. 2017; Huck et al. 2018; Jacobsen et al. 2018; Rodríguez-Jorquera et al. 2018; Liu et al. 2019; Martinez et al. 2019; Christou et al. 2020; Louisse et al. 2020; Dong et al. 2021; Lee et al. 2021; Mylroie et al. 2021; Beale et al. 2022; Davidsen et al. 2022; Haimbuagh et al. 2022; Wang et al. 2022; Mylroie et al. IN PREP).

The proper control of mitochondrial β -oxidation is reliant on PPAR induced transcription of the enzymes integral to carrying out fatty acid oxidation (Fan and Evans 2015; Hong et al. 2019). Agonist of PPARα increase gene expression of genes involved in

mitochondrial fatty acid β -oxidation (Bougarne et al. 2018) whereas PPARα null mice have a decreased expression of fatty acid oxidation genes with the same being seen in PPARβ/δ knockouts (Wang 2010).

Stressors can impact the expression of genes involved in β -oxidation. For example, in mammal models, up-regulation of β -oxidation related genes *Thiolase B* and *cyp4a1* have been observed in rats [*Rattus norvegicus*] (Davidsen et al. 2022) and with *cyp4a14* and *acadm* observed as upregulated in mice (Rosen et al. 2010) after exposure to PFAS. At a cellular level, Wan et al. (2012) and Geng et al. (2019) demonstrated decreases in overall mitochondrial β -oxidation rates in liver tissue from PFOS exposed mice and chicken [*Gallus gallus*] embryos. In zebrafish, Cheng et al. (2016) observed increased transcriptional expression for genes related to β-oxidation (*acox1, acadm, cpt1a*) which is suggestive of a compensatory response to β-oxidation inhibition caused by PFOS exposure. Similarly, Wang et al. (2022) also observed trends of increased transcriptional expression of genes in the β -oxidation pathway in zebrafish after PFOS exposure, and Yi et al. (2019) observed increased transcriptional expression of genes within the β -oxidation pathway including *acox1* and *acadm* in response to PFOS. However, other investigations using zebrafish have observed genes in the β -oxidation pathway having decreased expression or mixed profiles of both increased and decreased expression (Tu et al. 2019; Mylroie et al. 2021).

Uncertainties and Inconsistencies

While the PPAR molecular structure and function among vertebrates is largely conserved (Gust et al 2020), species to species variation does exist in structure and specific function; and therefore, it is important to exercise care when looking to extrapolate across species. The binding affinity of certain ligands and the magnitude of response in PPAR nuclear signaling may differ from species to species due to variations in PPAR molecular structure. Furthermore, the direction and magnitude of gene expression response may differ from species to species or even within species depending on the ligand assayed and the concentration used. Finally, the fed state of the organism being assayed is important as food availability can have a direct effect on β-oxidation in the target organism.

Quantitative Understanding of the Linkage

Response-response relationship

Unknown

Time-scale

Rapid Molecular Interactions

Known Feedforward/Feedback loops influencing this KER

As PPAR signaling is essential for maintaining energy homeostasis, there is a complex network of feedforward/feedback loops influencing PPAR nuclear signaling and gene expression via ligands, products, and the PPAR isoforms acting on each other. Due to the extensive detail needed to properly describe all potential feedforward/feedback loops that could influence this KER, the authors direct readers to reviews by Ament et al. (2012) and Lamichane et al. (2018).

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Yi, S., Chen, P., Yang, L. and Zhu, L., 2019. Probing the hepatotoxicity mechanisms of novel chlorinated polyfluoroalkyl sulfonates to zebrafish larvae: Implication of structural specificity. *Environment international*, *133*, p.105262.

Relationship: 3209: Decrease, Fatty acid β-oxidation leads to Disrupted Lipid Storage

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	High
Adult, reproductively mature	High

Sex Applicability

Sex	Evidence
Male	High
Female	Moderate

β-oxidation is a crucial biological function maintained across representative vertebrate species. However, given that species to species variation does exist in gene sequences and enzyme specific structures; therefore, it is important to exercise care when looking to extrapolate across species.

Key Event Relationship Description

This Key Event Relationship describes how a decrease in β-oxidation can disrupt proper lipid storage. Disruption of lipid storage and transport can be identified by excess accumulation of fatty acids or other lipids in the liver or altered ratios of expected lipid species which can ultimately lead to liver steatosis (Ipsen et al. 2018). Decreased or impaired mitochondrial β-

oxidation has been linked to the accumulation of lipids and potentially liver steatosis (Cherkaoui-Malki et al. 2012; Fromenty 2019).

Evidence Supporting this KER

Biological Plausibility

Mitochondrial fatty acid β-oxidation is an important biochemical mechanism that is vital in maintaining energy homeostasis in the liver (Houten and Wanders 2010; Naguib et al. 2019). It is important in whole organism energy production during fasting but also serves as the main mechanism for fatty acid degradation and removal (Houten and Wanders 2010; Cherkaoui-Malki et al. 2012; Naguib et al. 2019). When fatty acid β-oxidation is decreased in the liver, lipids are not able to be eliminated as efficiently and can to begin to accumulate in the liver (Fromenty 2019; He et al. 2019; Naguib et al. 2019). Therefore, a decrease in or complete inhibition of mitochondrial fatty acid β-oxidation can result in disrupted lipid storage in the liver.

Empirical Evidence

There is ample evidence showing how the decrease or inhibition of mitochondrial fatty acid β-oxidation can cause disrupted lipid storage in the liver. Fromenty et al. (2019) present a comprehensive review of multiple examples of drug-induced inhibition of mitochondrial fatty acid β-oxidation disruptions in lipid liver storage resulting in steatosis. Specifically, drugs such as acetaminophen, linezolid, and traglitazone that decrease or inhibit fatty acid β-oxidation causes triglycerides to accumulate as small or large droplets in liver tissue. He et al. (2019) showed that cadmium (Cd) exposure in mice inhibited mitochondrial fatty acid oxidation via a suppression of SIRT1 and PPARα signaling resulting in excess lipid accumulation in the liver. Finally, Massart et al. (2019) presented multiple modes of actions for drug-induced inhibition of mitochondrial fatty acid oxidation and disrupted lipid storage in the liver with direct inhibition of mitochondrial fatty acid β-oxidation and disruptions of PPARα activity as two pathways for disruption of fatty acid β-oxidation.

Uncertainties and Inconsistencies

Energy homeostasis is a complex system in vertebrates and controlled via the cross-talk of numerous pathways. Therefore, it is important to understand that factors like age, sex, and the fed state of the organism could all have a direct effect on lipid storage and subsequent fatty acid accumulation in the liver of the target organism.

Quantitative Understanding of the Linkage

Response-response relationship

Unknown

Time-scale

Hours to days.

Known Feedforward/Feedback loops influencing this KER

Mitochondrial fatty acid β-oxidation is a well-studied biological process integral to energy homeostasis. The feedforward/feedback loops involved in regulating mitochondrial fatty acid β-oxidation are extensive and present a challenge to properly represent in this KER summary. The authors suggest reading the reviews by Houten and Wanders (2010) and Morris et al. (2011) for a comprehensive summary of feedforward/feedback loops influencing this KER.

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Relationship: 3210: Disrupted Lipid Storage leads to Accumulation, Fatty acid

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	Moderate
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate
Female	Moderate

Lipid storage and transport is a crucial biological function maintained across representative vertebrate species. However, given that species to species variation in genes and specific regulatory mechanisms do exist it is important to exercise care when looking to extrapolate across species.

Key Event Relationship Description

This Key Event Relationship describes how disrupted lipid storage in the liver results in the accumulation of fatty acids. Disruption of lipid storage and transport can be identified by excess accumulation of fatty acids or other lipids in the liver or altered ratios of expected lipid species which can ultimately lead to liver steatosis (Ipsen et al. 2018). Disruption of lipid metabolism through dysregulation of transcriptional control and/or decreased or impaired mitochondrial β-oxidation can result in improper lipid storage and an accumulation of fatty acids in liver cells (Ament et al. 2012; Cherkaoui-Malki et al. 2012; Fromenty 2019; Dixon et al. 2021; Xiao et al. 2021).

Evidence Supporting this KER

Biological Plausibility

Proper lipid homeostasis is controlled by the balance of lipid influx and efflux as well as the balance between lipogenesis and lipid catabolism (Ipsen et al. 2018; Kloska et al. 2020; Geng et al. 2021; Yoon et al. 2021). Therefore, disruption of this balance via diet, disease, or environmental stressor can lead to the improper storage and transport of lipids in the liver and the subsequent accumulation of fatty acids (Ipsen et al. 2018).

Empirical Evidence

There is ample evidence outlining how improper lipid storage and transport can result in the accumulation of fatty acids in the liver (Ipsen et al. 2018). For example, overexpression of a fatty acid transport gene CD36 in mice increased fatty acid uptake and accumulation in livers (Koonen et al. 2007). The over expression of human hepatic lipase (hHL) in mice resulted in increased *de novo* synthesis of fatty acids and upregulation of fatty acid synthesis genes such as *Srebf1*, *Fasn*, *Acaca*, and *Nr1h3* (Cedó et al. 2017). Finally, overexpression of sterol regulatory element–binding proteins (SREBP), which is one of the key regulatory elements in lipid synthesis, resulted in an increase in fatty acid synthesis and fatty acid synthase (Fas) gene expression in mouse livers (Horton et al. 2002).

Uncertainties and Inconsistencies

Energy homeostasis is a complex system in vertebrates and controlled via the cross-talk of numerous pathways. Therefore, it is important to understand that factors like age, sex, and the fed state of the organism could all have a direct effect on lipid storage and subsequent fatty acid accumulation in the liver of the target organism.

Quantitative Understanding of the Linkage

Response-response relationship

Unknown

Time-scale

Hours to Days

Known Feedforward/Feedback loops influencing this KER

Lipid homeostasis is a well-studied biological process integral to vertebrates and invertebrates. The feedforward/feedback loops involved in regulating lipid storage and transport are extensive and present a challenge to properly represent in this KER summary. The authors suggest reading the reviews by Ipsen et al. (2018) and Geng et al. (2021) for comprehensive summaries of feedforward/feedback loops influencing this KER.

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Relationship: 472: Accumulation, Fatty acid leads to Accumulation, Triglyceride

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	Moderate
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate
Female	Moderate

Lipid storage and transport is a crucial biological function maintained across representative vertebrate species. However, given that species to species variation in genes and specific regulatory mechanisms do exist it is important to exercise care when looking to extrapolate across species.

Key Event Relationship Description

This Key Event Relationship describes how the accumulation of fatty acids in the liver results in an increase in and accumulation of triglycerides (TG) in the liver. Disruption of lipid storage and transport can be identified by excess accumulation of fatty acids followed by an accumulation of triglycerides and other lipids which can ultimately lead to liver steatosis (Ipsen et al. 2018).

Evidence Supporting this KER

Biological Plausibility

Proper lipid homeostasis is controlled by the balance of lipid influx and efflux as well as the balance between lipogenesis and lipid catabolism (Ipsen et al. 2018; Kloska et al. 2020; Geng et al. 2021; Yoon et al. 2021). Disruption of this balance via diet, disease, or environmental stressor can lead to the improper storage and transport of lipids in the liver and the subsequent accumulation of fatty acids (Ipsen et al. 2018). When an excess of accumulation of fatty acid occurs in the liver via increased import, de novo synthesis, and/or reduced β-oxidation TG synthesis increases for storage and export and to also protect cells from lipotoxicity under periods of extremely high free fatty acid accumulation (Listenberger 2003; Reddy and Rao 2006; Rada et al. 2020). Therefore, it is plausible to assume that an increase in fatty acid accumulation would lead to an increase in TG accumulation especially under conditions of greater lipid homeostasis perturbation due to a stressor.

Empirical Evidence

There is ample evidence outlining how accumulation of fatty acids in the liver results in an increased accumulation of triglycerides (Reddy and Rao 2006; Angrish et al. 2016; Ipsen et al. 2018). For example, overexpression of sterol regulatory element–binding proteins (SREBP), which is one of the key regulatory elements in lipid synthesis, resulted in an increase in fatty acid synthesis and an accumulation of TG species in the liver (Horton et al. 2002). Selen et al. (2021) demonstrate that mice with a KO in a key gene involved in β-oxidation showed increased fatty acid accumulation and increased TG content when fed a high-fate diet. Finally, Koonen et al. (2007) showed that overexpression of CD36 in mice resulted in an influx of fatty acids and increased triglyceride levels.

Uncertainties and Inconsistencies

Energy homeostasis is a complex system in vertebrates and controlled via the cross-talk of numerous pathways. Therefore, it is important to understand that factors like age, sex, and the fed state of the organism could all have a direct effect on fatty

acid accumulation and subsequent triglyceride accumulation in the liver of the target organism/species.

Quantitative Understanding of the Linkage

Response-response relationship

Unknown

Time-scale

Hours to Days

Known Feedforward/Feedback loops influencing this KER

Lipid homeostasis is a well-studied biological process integral to vertebrates and invertebrates. The feedforward/feedback loops involved in regulating lipid storage and transport are extensive and present a challenge to properly represent in this KER summary. The authors suggest reading the reviews by Ipsen et al. (2018) and Geng et al. (2021) for comprehensive summaries of feedforward/feedback loops influencing this KER.

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Relationship: 2265: Accumulation, Triglyceride leads to Increased, Liver Steatosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Glucocorticoid Receptor activation leading to hepatic steatosis	adjacent		
Pregnane X Receptor (PXR) activation leads to liver steatosis	adjacent	High	Not Specified
Liver X Receptor (LXR) activation leads to liver steatosis	adjacent	High	Not Specified
Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	adjacent	High	Moderate
AhR activation leading to hepatic steatosis	adjacent	High	High

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Mus musculus	Mus musculus	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Adult	High
Juvenile	Moderate

Sex Applicability

Sex	Evidence
Unspecific	Moderate

Life Stage: All life stages with a liver. Older individuals are more likely to manifest this adverse outcome pathway (adults > juveniles) due to accumulation of triglycerides.

Sex: Applies to both males and females.

Taxonomic: Appears to be present broadly in vertebrates, with most representative studies in mammals (humans, lab mice, lab rats).

Key Event Relationship Description

Steatosis is a key event representing increased accumulation of fat in liver cells. In this key event relationship we are focused on accumulation of triglycerides leading to steatosis. Increased accumulation of triglycerides in cells is evidence of imbalance in the influx and synthesis versus metabolism or breakdown of lipid compounds. Increased accumulation of triglycerides can be enhanced by chemical stressors, or alteration of regulation by gene expression.

Evidence Supporting this KER

Biological Plausibility

The biological plausibility linking accumulation of triglycerides to steatosis is strong. Increased accumulation of triglycerides represents an imbalanced influx and synthesis of compounds versus normal function, resulting in liver steatosis.

Empirical Evidence

Species	Duration	Dose	Accumulated triglycerides?	Liver steatosis	Summary	Citation
Human (*Homo sapiens)*	14 days	In vitro exposure of 20 mM amiodarone, 50 mM tetracycline.	yes	yes	HepG2 human cells showed correlated increases in triglycerides and other lipid compounds and steatosis oxidation after 14 days of tetracycline exposure and after both 1 and 14 days of amiodarone exposure.	Antherieu *et al.* (2011)

Human (*Homo sapiens)*	24 hours	In vitro exposure of at least 6 concentrations to 28 compounds selected for steatogenic potential.	yes	yes	HepG2 human cells exposed to fialuridine, sodium valproate, doxycycline, amiodarone, tetracycline showed corresponding increases in lipid accumulation, with higher doses exhibiting greater lipid accumulation and correlated steatosis.	Donato *et al.* (2009)
Human (*Homo sapiens)* and mouse (*Mus musculus*)	16 weeks	Transgenic and wild-type mice with normal and high cholesterol diet.	yes	yes	Human subjects with liver steatosis had increased RBP4 gene expression. Transgenic mice with human RBP4 gene had correlated increases in triglycerides associated with steatosis, in comparison to wild-type mice.	Liu *et al.* (2016)

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References
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List of Non Adjacent Key Event Relationships

Relationship: 3211: Disrupted Lipid Storage leads to Accumulation, Triglyceride

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	non-adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	Moderate	NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	Moderate
Juvenile	Moderate
Adult, reproductively mature	Moderate

Sex Applicability

Sex	Evidence
Male	Moderate
Female	Moderate

Lipid storage and transport is a crucial biological function maintained across representative vertebrate species. However, given that species to species variation in genes and specific regulatory mechanisms do exist it is important to exercise care when looking to extrapolate across species.

Key Event Relationship Description

This Key Event Relationship describes how disrupted lipid storage in the liver results in the accumulation of triglycerides. Disruption of lipid storage and transport can be identified by excess accumulation of triglycerides or other lipids in the liver or altered ratios of expected lipid species which can ultimately lead to liver steatosis (Alves-Bezerra and Cohen 2017; Ipsen et al. 2018; Dixon et al. 2021).

Evidence Supporting this KER

Biological Plausibility

Proper lipid homeostasis is controlled by the balance of lipid influx and efflux as well as the balance between lipogenesis and lipid catabolism (Ipsen et al. 2018; Kloska et al. 2020; Geng et al. 2021; Yoon et al. 2021). Therefore, disruption of this balance via diet, disease, or environmental stressor can lead to the improper storage and transport of lipids in the liver and the subsequent accumulation of triglycerides (Alves-Bezerra and Cohen; Ipsen et al. 2018).

Empirical Evidence

There is ample evidence outlining how improper lipid storage and transport can result in the accumulation of TG in the liver (Ipsen et al. 2018). For example, overexpression of diacylglycerol acyltransferases (DGAT2) in the liver resulted in increased levels of TG in mice livers (Monetti et al. 2007). Disruption of G3P acyltransferase (GPAT) enzymes in the liver, which is necessary for maintaining the balance between lipid storage and fatty acid oxidation, can result in increased TG levels in hepatocytes (Lewin et al. 2005; Alves-Bezerra and Cohen). Impaired secretion of TG as TG-enriched very low-density lipoprotein (VLDL) can result in increased TG accumulation in the liver. This connection has been demonstrated via inhibition of microsomal triglyceride transfer protein (MTP), which is critical for proper TG-VLDL packing and export, which was shown to increase TG content in liver in mice where expression was inhibited (Josekutty et al. 2013). Finally, lipid droplets (LD) are TG are stored temporarily in the liver for use in fatty acid oxidation; and thus, a disruption in regulation of the formation of LD can thus result in accumulation of TG in the liver (Alves-Bezerra and Cohen 2017). Perilipin proteins (PLIN) are critical for formation of LD and Trevino et al. (2015) demonstrated that overexpression of PLIN5 resulted an increase of TG and other lipids in mouse livers.

Uncertainties and Inconsistencies

Energy homeostasis is a complex system in vertebrates and controlled via the cross-talk of numerous pathways. Therefore, it is important to understand that factors like age, sex, and the fed state of the organism could all have a direct effect on lipid storage and subsequent fatty acid accumulation in the liver of the target organism.

Quantitative Understanding of the Linkage

Response-response relationship

Unknown

Time-scale

Hours to Days

Known Feedforward/Feedback loops influencing this KER

Lipid homeostasis is a well-studied biological process integral to vertebrates and invertebrates. The feedforward/feedback loops involved in regulating lipid storage and transport are extensive and present a challenge to properly represent in this KER summary. The authors suggest reading the reviews by Alves-Bezerra and Cohen (2017) and Ipsen et al. (2018) for comprehensive summaries of feedforward/feedback loops influencing this KER.

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