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## COMMENTARY

Cytochrome P450 2E1 should not be neglected for acetaminophen-induced liver injury in metabolic diseases with altered insulin levels or glucose homeostasis

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### **Key words:**

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*Abbreviations: APAP, acetaminophen; CYP2E1, cytochrome P450 2E1; CYP3A4, cytochrome P450 3A4; DAMP, damage-associated molecular pattern; NAC, N-acetylcysteine; NAFLD,*

*nonalcoholic fatty liver disease; NEFA, non-esterified fatty acid; ROS, reactive oxygen species; STZ, streptozotocin; T1D, type 1 diabetes; T2D, type 2 diabetes*

## **Summary**

Acetaminophen (APAP) hepatotoxicity is mediated by N-acetyl-*p*-benzoquinone imine (NAPQI), a highly toxic metabolite generated by cytochrome P450 2E1 (CYP2E1). Thus, pathological conditions increasing CYP2E1 activity can favour APAP-induced liver injury, which is characterized by massive hepatocellular necrosis and secondary sterile inflammation. In a recent work, Wang et al. showed that APAP-induced hepatotoxicity was exacerbated in a murine model of type 1 diabetes induced by the administration of streptozotocin (STZ). Higher hepatotoxicity was in particular associated with a stronger pro-inflammatory response of the resident macrophages. Although the authors carried out numerous investigations, they did not study hepatic CYP2E1, nor discussed the possible role of this enzyme in the exacerbation of APAP hepatotoxicity. However, numerous investigations reported hepatic CYP2E1 induction in STZ-treated rodents, which could be secondary to insulinopenia and ketosis. This commentary also discusses the role of insulin resistance in CYP2E1 induction observed in obesity and nonalcoholic fatty liver disease. Investigators studying APAP-induced liver injury in the context of insulinopenia or hyperinsulinemia are thus encouraged to consider CYP2E1 as a significant player in the observed phenotypic changes.

Acetaminophen (APAP), also known as paracetamol, is one of the most popular painkillers used worldwide, even though its mode of action is still not fully understood. The current maximum recommended dosage of APAP is 4000 mg/d in adults although the Food and Drug Administration (FDA) suggested a reduction of this dose to 3250 or 3000 mg/day [1]. This recommendation was actually driven by a concern regarding the incidence of acute liver failure (ALF) due to APAP overdose. Indeed, APAP intoxication after intentional or accidental overdoses can lead to massive hepatocellular necrosis and acute liver failure, which can be fatal if liver transplantation is not performed [2,3]. Different predisposing factors are known, or suspected, to enhance the risk and severity of APAP-induced hepatotoxicity such as fasting, chronic alcohol intoxication, hepatitis C virus infection, nonalcoholic fatty liver disease (NAFLD) and diabetes, as discussed below [2,4]. At the moment, N-acetylcysteine (NAC) is the only approved drug to treat APAP-induced liver injury in patients after APAP overdose [5].

Although there are still some uncertainties regarding the precise mechanisms whereby APAP overdose can induce liver injury, numerous studies pointed to a major role of cytochrome P450 2E1 (CYP2E1), at least in mice [2,6,7]. Indeed, this enzyme catalyzes the oxidation of APAP to N-acetyl-*p*-benzoquinone imine (NAPQI), a highly reactive metabolite inducing severe mitochondrial dysfunction at the respiratory chain level, overproduction of reactive oxygen species (ROS), c-jun N-terminal kinase (JNK) activation, mitochondrial permeability transition, ATP depletion and massive hepatocellular necrosis [8,9]. In human, CYP3A4 might be an important enzyme catalyzing NAPQI generation in addition to CYP2E1 [2,10]. Importantly, CYP2E1 is located not only in the endoplasmic reticulum (like most of the CYPs) but also in mitochondria [7,11]. Previous investigations from our group reported that mitochondrial CYP2E1 could play a major role in APAP-induced cytotoxicity [12].

APAP-induced massive liver necrosis subsequently leads to the passive release of several intracellular molecules (the so-called damage-associated molecular patterns or DAMPs) that are able to activate macrophages [13,14]. Activated macrophages thus produce different cytokines and chemokines, which induce the recruitment of neutrophils, monocytes and other leukocytes [13,14]. Notably, this sterile inflammation might have a dual role during APAP-induced hepatotoxicity. Indeed, while the inflammatory response cleans up necrotic cell debris and promotes tissue repair, it can also aggravate liver injury [14]. Finally, the ability of the damaged liver to properly regenerate also plays a major role in the outcome of APAP-induced hepatotoxicity [15].

A few clinical investigations suggested that diabetes increases the risk and severity of acute liver failure, in particular related to drugs [16,17]. Because a significant proportion of acute liver failure is linked to APAP intoxication [18,19], it is important to determine the mechanisms whereby diabetes might favor APAP hepatotoxicity. In this context, Wang and collaborators carried out investigations in hyperglycemic mice treated once with 400 mg/kg APAP and sacrificed 24 hours after the intoxication [20]. Notably, hyperglycemia was induced by repeated injections of streptozotocin (STZ), a fungal antibiotic able to induce apoptosis of the pancreatic  $\beta$ -cells mainly by alkylating nuclear DNA [21,22]. Hence, STZ-induced  $\beta$ -cell destruction and hypoinsulinemia leads to type 1 diabetes (T1D) with hyperglycemia, ketosis, polyuria and dehydration (Fig. 1A).

By performing many investigations in serum, liver tissue and isolated Kupffer cells, Wang and collaborators showed that APAP-induced hepatotoxicity was more severe in STZ-treated mice compared with control mice, as assessed by plasma transaminases (ALT and AST), histopathology and TUNEL assay [20]. In addition, STZ-treated mice presented more hepatic infiltration of different types of macrophages and neutrophils as well as higher serum levels of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6) [20]. Administration of the antioxidant NAC prior to APAP intoxication alleviated many APAP-induced alterations in Kupffer cells including overproduction of ROS, TNF- $\alpha$  and IL-6, high expression of the inducible form of nitric oxide synthase (iNOS) and hyperphosphorylation of signal transducer and activator of transcription 1 (STAT1) [20], a transcription factor playing a key role in macrophage M1 (pro-inflammatory) polarization [23]. Moreover, administration of the AMP-activated protein kinase (AMPK) activator AICAR prior to APAP intoxication alleviated APAP-induced liver injury with less hepatic cytolysis and TUNEL-positive hepatocytes as well as reduced macrophage and neutrophil infiltration and other pro-inflammatory markers [20]. Of note, AMPK activators such as metformin and canagliflozin are currently used for the treatment of type 2 diabetes (T2D), in particular for their ability to improve glucose uptake and utilization in different tissues [24].

Altogether, these investigations might suggest that hyperglycemia *per se* exacerbates APAP-induced liver injury by favoring an intrahepatic pro-inflammatory response. However, Wang and collaborators did not investigate the role of CYP2E1 [20], which could have played a significant role in most of their results. Indeed, numerous investigations in rats and mice showed that the  $\beta$ -cell poison STZ enhanced hepatic CYP2E1 protein expression and activity [25-31]. In one of these studies, increased hepatic CYP2E1 protein expression in diabetic rats was observed not only in microsomes (i.e. in isolated endoplasmic reticulum) but also in mitochondria [28]. In the T1D

murine model set up by Wang and collaborators [20], higher hepatic CYP2E1 activity might thus have favored APAP-induced mitochondrial dysfunction, ROS overproduction, hepatocellular necrosis and subsequent sterile inflammation. In addition, the protective effect afforded by NAC pretreatment against APAP-induced activation of Kupffer cells [20] is possibly related to milder oxidative stress and hepatic cytolysis.

The mechanisms whereby STZ treatment can enhance hepatic CYP2E1 expression and activity are not fully understood. In rat, low insulin levels increase hepatic CYP2E1 mRNA expression possibly via transcriptional and post-transcriptional mechanisms [32,33]. Notably, an opposite effect was observed in human hepatoma HepaRG cells [34], thus suggesting that regulation of CYP2E1 mRNA levels by insulin is not conserved across species. Ketosis related to insulin deficiency seems also to play a significant role. Indeed, the ketone bodies acetone and acetoacetate were shown in rodents to enhance hepatic CYP2E1 protein expression by reducing CYP2E1 protein degradation [35-37]. In addition, acetoacetate increases translation of Cyp2e1 mRNA in rat [37]. Finally, the effect of high glucose levels on CYP2E1 expression is still unclear. Indeed, investigations in human cells showed that high glucose levels enhanced CYP2E1 protein expression [38], while others reported reduced CYP2E1 mRNA levels [39]. Hence, hepatic CYP2E1 induction in T1D could be secondary to different metabolic and hormonal factors (Fig. 1A). However, it should be underlined that most of the aforementioned studies were carried out *in vivo* in rodents or in rodent hepatocytes, and thus further investigations should be done in human hepatocytes.

Importantly, the extent of hyperglycemia and ketosis after STZ treatment depends on numerous factors including species and strains, gender, diet, time of injection in the day and time period after last administration [21,40-42]. These variations might explain that in a few studies higher hepatic CYP2E1 expression was not detected in STZ-treated rodents and that T1D diabetes protected against APAP-induced liver injury [43,44]. In these studies, reduced APAP hepatotoxicity resulted from increased APAP detoxification into its nontoxic glucuronide conjugate and higher urine elimination secondary to polyuria [43,44]. Of note, STZ-induced T1D was shown to favor hepatotoxicity of thioacetamide [45] and carbon tetrachloride [26], two toxins also generating highly reactive and toxic metabolites via CYP2E1. The description and characterization of the chemically-induced T1D model should be carefully examined in order to better understand APAP hepatotoxicity severity.

Although Wang and collaborators performed numerous investigations in STZ-treated mice, they actually did not provide sufficient data that might support a significant role of hyperglycemia in the exacerbation of APAP-induced liver injury. For instance, the experiment in mice pretreated with

AICAR was not fully informative because blood glucose levels were not reported [20]. Notably, we recently found that AICAR protects against APAP-induced cytotoxicity in human HepaRG cells [34], thus indicating that this AMPK activator has a protective effect which can be, at least in part, independent of its hypoglycemic effect. It would also have been interesting to determine APAP-induced hepatotoxicity in nonchemically-induced models of T1D. Importantly, STZ can be hepatotoxic in rats and mice, and thus STZ toxicity is not fully specific to the pancreatic  $\beta$ -cells [22,46]. Finally, it might have been informative to carry out *in vitro* investigations in cultured hepatocytes in order to determine whether high glucose levels might exacerbate APAP-induced cytotoxicity in the absence or the presence of co-cultured macrophages. Of note, CYP2E1 is also expressed in Kupffer cells and monocytes-derived macrophages [47,48]. While in absolute amount, CYP2E1 abundance is higher in hepatocytes than in macrophages, its induction by ethanol is of the same relative magnitude in both cell types [49]. Increased expression of CYP2E1 in macrophages results in TNF- $\alpha$  secretion and sensitization to lipopolysaccharide (LPS) stimuli [47]. Furthermore, CYP2E1 activity is increased in macrophages treated with APAP for 2 hours [50]. Hence, CYP2E1 induction in macrophages might contribute to the early phase of APAP-induced hepatotoxicity.

Hyperglycemia itself promotes multiple cellular events that could trigger APAP-induced toxicity independently of CYP2E1. *In vitro* studies revealed that hyperglycemia induces TNF- $\alpha$  and IL-6 secretion in Kupffer cells (Fig. 2), thus inhibiting M2 polarization via activation of CHOP-mediated endoplasmic reticulum stress [51]. Similarly, cultures of bone marrow-derived macrophages (BMDM) under high glucose induced a pro-inflammatory phenotype through alteration of sphingosine-1-phosphate pathway [52]. Hyperglycemia induces the expression of nitric oxide synthase 2 (NOS2, also referred to as iNOS) while reduces arginase-1 (Arg1) expression and IL-10 secretion in human monocytes-derived macrophages and BMDM [52,53]. Within the liver, hepatocytes are an additional source of cytokines. Exposure to high glucose of mouse hepatocytes activates the nuclear factor kappa B (NF $\kappa$ B) pathway [54]. This pro-inflammatory response is accompanied by an increased monocyte chemoattractant protein 1 (MCP-1) secretion [54], which could favor recruitment and activation of monocytes to the liver (Fig. 2). Likely, in HepG2 cells, high level of glucose induced the expression of TNF- $\alpha$ , IL-6 and plasminogen activator inhibitor-1 (PAI-1) already 6 hours after exposure and sustaining over the next 24 hours. Hyperglycemia promotes the expression of these pro-inflammatory cytokines through ROS production leading to the activation of mitogen-activated protein kinase (MAPK) and NF $\kappa$ B signaling pathways [55]. Overall, *in vitro* data support that hyperglycemic state generates a pro-inflammatory response directly within hepatocytes and macrophages through different pathways. However, these effects were not observed in the hyperglycemic STZ-treated mice until APAP treatment was administered

[20]. Thus, in vivo, APAP treatment could aggravate the preexisting hyperglycemic state to reach a point where glucose levels promote a hepatic pro-inflammatory response (Fig. 2). Indeed, APAP could affect hepatic glucose production, as in mice, administration of a single dose of APAP increases blood glucose levels due to increased glycogenolysis [56]. In addition to the effect of APAP on hepatic glycogen depletion to increase glycemia, in vivo APAP administration alters pancreatic cells structure and damages  $\beta$ -cells [57], potentially further damaging pancreatic cells in the STZ model. While a direct toxic effect of APAP on the sensitized hepatic inflammatory cells cannot be ruled out, the role of APAP on glycemia and the subsequent exacerbation of a hepatic pro-inflammatory phenotype remain to be considered.

A more frequent form of diabetes is T2D, whose prevalence parallels the surge of overweight and obesity worldwide. T2D is preceded by insulin resistance, which induces a compensatory increase in insulin secretion by the pancreatic  $\beta$ -cells, thus resulting in hyperinsulinemia [58]. In turn, both insulin resistance and hyperinsulinemia favor the occurrence of NAFLD, respectively via increased non-esterified fatty acid (NEFA) delivery to the liver and enhanced *de novo* lipogenesis (Fig. 1B) [59,60]. Many experimental and clinical investigations consistently reported higher hepatic CYP2E1 expression and activity in obesity and NAFLD [61-64]. Although the mechanisms of CYP2E1 induction in NAFLD are still unclear, some investigations suggest the role of insulin resistance and some fatty acids such as stearic acid (C18:0) (Fig. 1B) [34,61]. Interestingly, investigations in rodents and patients suggest that NAFLD increases the risk and severity of APAP-induced hepatotoxicity, at least in part via CYP2E1 induction [2,34,62]. Hence, both hypoinsulinemia and hyperinsulinemia appear to enhance hepatic CYP2E1 activity (Fig. 1), which might favor APAP-induced mitochondrial dysfunction and liver damage.

Although hepatic CYP2E1 plays a major role in APAP-induced hepatotoxicity [2,6,7], we should recall that other factors can play a significant role in the occurrence of hepatic cytolysis and subsequent sterile inflammation. These factors include for instance APAP absorption by the gastrointestinal tract, gut microbiota, hepatic APAP conjugation into the nontoxic glucuronide and sulfate conjugates, diurnal variation of NAPQI formation and detoxification in liver and possibly APAP volume of distribution [2,65,66]. While some of these factors favor APAP hepatotoxicity, others protect against such toxicity [2,67]. Because obesity, diabetes and NAFLD significantly affect these factors [2,68-70], investigators studying liver injury induced by APAP or other xenobiotics in these metabolic diseases should consider the main factors that are likely to explain higher hepatotoxicity, or a protective effect.

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## Legends of figures:

### Figure 1:

**Consequences of insulinopenia or hyperinsulinemia on CYP2E1 activity in liver.** Panel A. Destruction of the pancreatic  $\beta$ -cells, for instance by streptozotocin, leads to insulinopenia. In turn, low insulin levels induce an increase in ketone bodies (ketosis), hyperglycemia and polyuria (not shown). In rodents, hypoinsulinemia increases CYP2E1 mRNA levels, whereas ketosis enhances CYP2E1 protein expression by reducing CYP2E1 protein degradation. The effect of insulin on CYP2E1 mRNA and protein levels seems to be different in human hepatocytes (not shown), as discussed in the text. It is still unclear whether hyperglycemia affects CYP2E1 mRNA and protein expression. Overall, insulinopenia and type 1 diabetes enhance CYP2E1 activity in rodents. Panel B. Multiple factors, such as obesity, promote the development of insulin resistance, which induces a compensatory hyperinsulinemia and high level of circulating non-esterified fatty acids (NEFAs) secondary to unabated peripheral lipolysis. In turn, both hyperinsulinemia and high NEFAs promote nonalcoholic fatty liver disease (NAFLD), in particular by increased triglyceride synthesis. Some fatty acids such as stearic acid seem to enhance CYP2E1 activity, yet the mechanisms remain unknown. Other factors linked to insulin resistance might also increase hepatic CYP2E1 activity. Overall, insulin resistance, hyperinsulinemia and NAFLD are associated with higher CYP2E1 activity in rodents and humans.

### Figure 2:

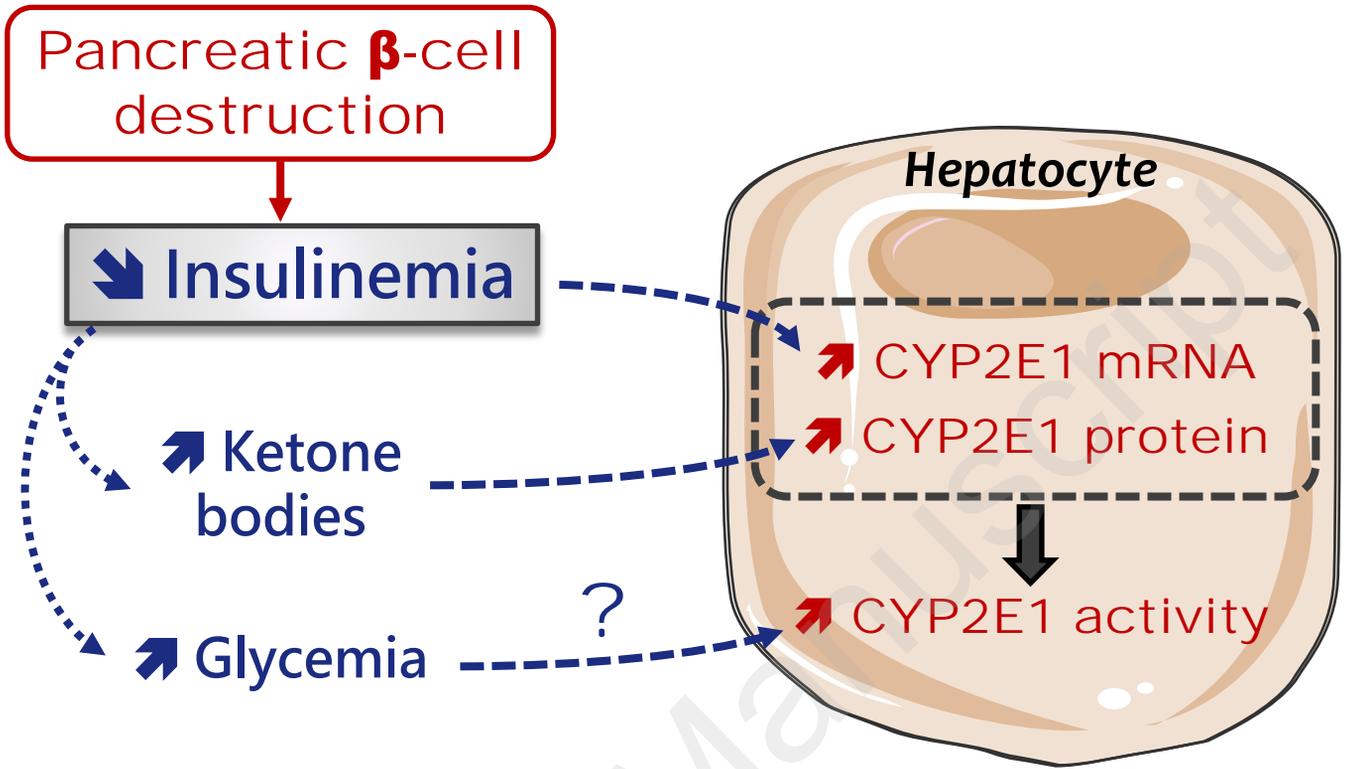
Role for hyperglycemia in hepatic inflammatory responses and potential mechanisms contributing to APAP-induced hepatotoxicity. Chronic hyperglycemia as a cause of insulinopenia or insulin resistance is a source of liver disease. High glucose level promotes a pro-inflammatory response in liver resident macrophages (Kupffer cells). In hepatocytes, high glucose induces ROS overproduction and oxidative stress, which promote, at least partly, the expression of pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6. Secretion of monocyte chemoattractant protein 1 (MCP-1) is increased in hepatocytes exposed to high glucose, and could favor the recruitment of inflammatory cells to the liver. If exposed to chronic hyperglycemia, recruited monocytes exhibit a pro-inflammatory M1-like phenotype and contribute to hepatic inflammation. In this context,

hyperglycemia could prime hepatocytes to APAP toxicity by promoting oxidative stress and the pro-inflammatory environment within the liver, thus favoring APAP-induced liver injury. However, whether APAP could directly affect activated Kupffer cells or aggravate the preexisting hyperglycemia to further worsen the pro-inflammatory response remain unclear in the exacerbated APAP-induced hepatotoxicity.

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**A**

## Type 1 diabetes and insulinopenia

**B**

## Insulin resistance, hyperinsulinemia and NAFLD

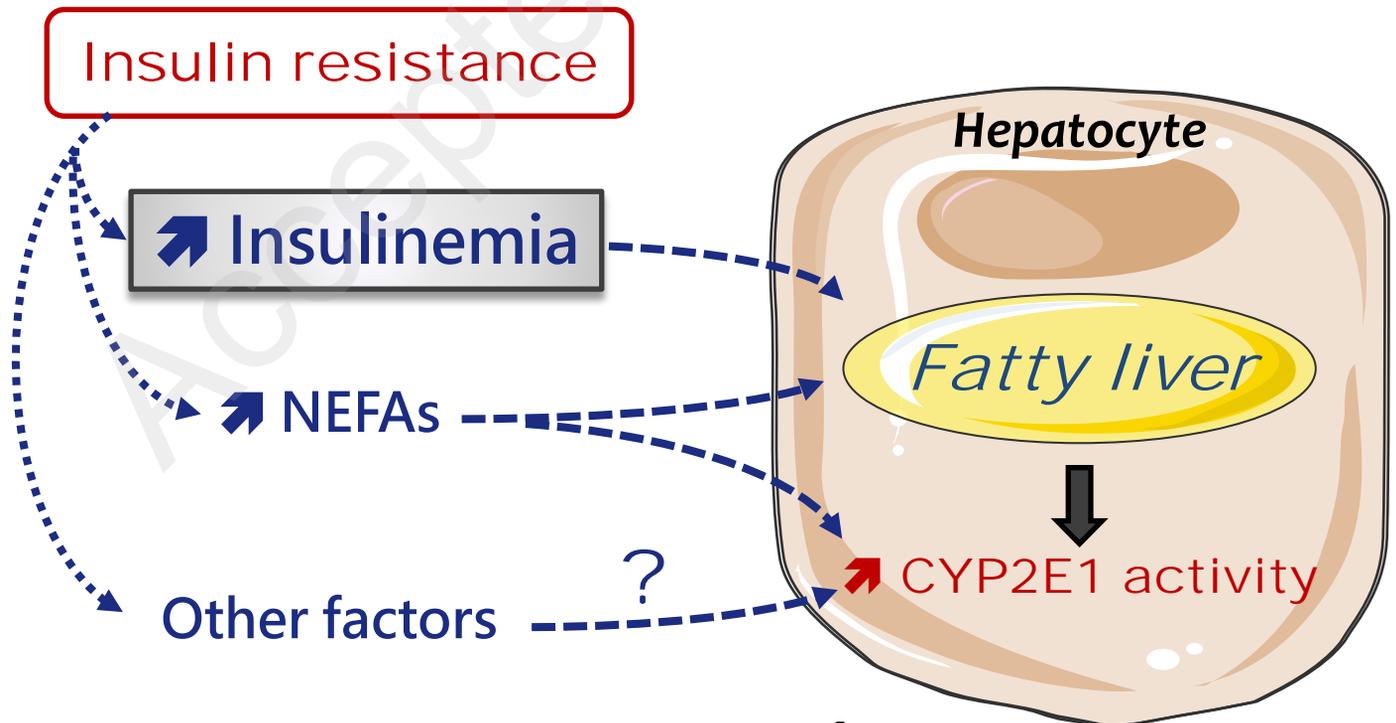


Figure 1

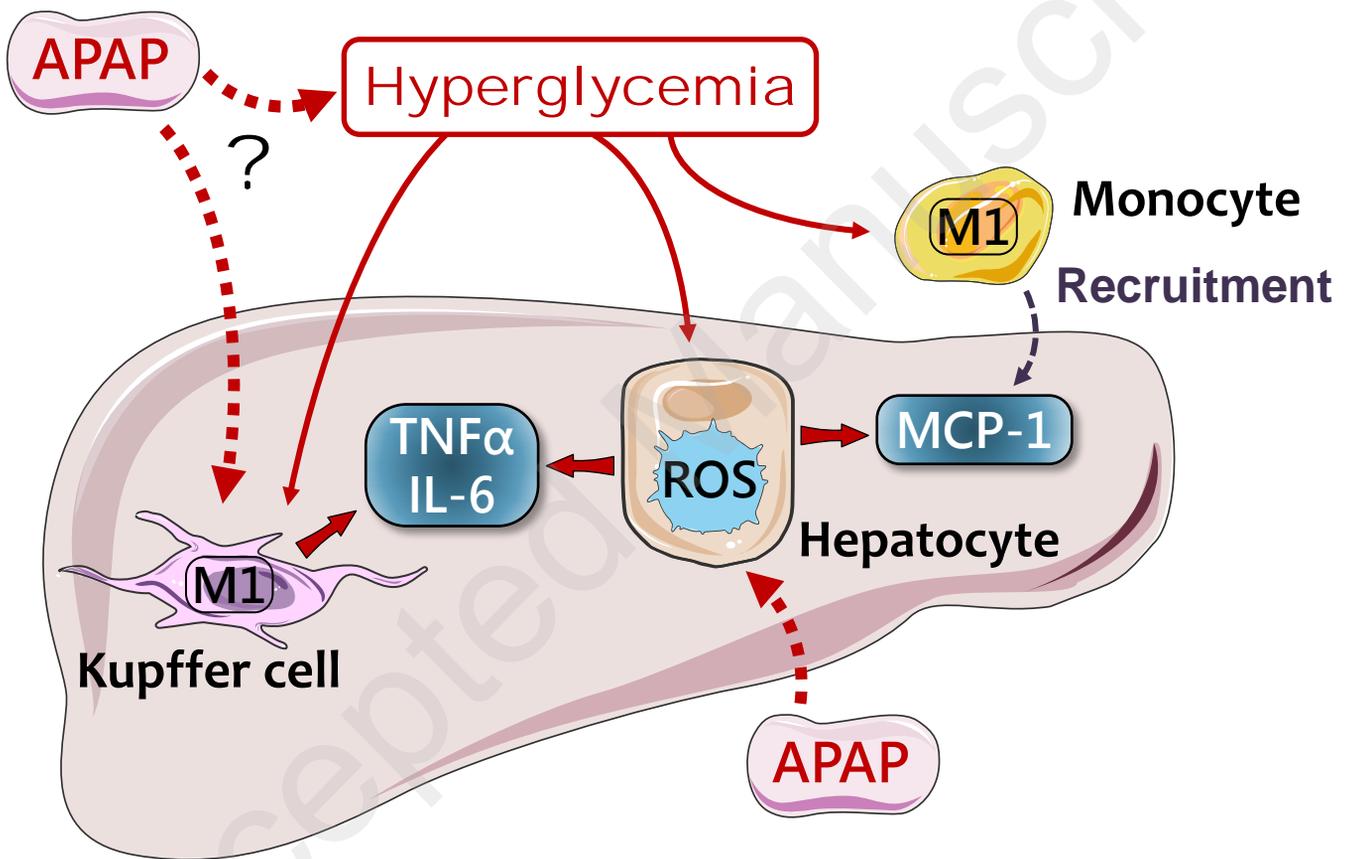


Figure 2