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Review article: drug-induced liver injury in the context of nonalcoholic fatty liver disease – a physiopathological and clinical integrated view

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Summary

Background: Nonalcoholic fatty disease (NAFLD) is the most common liver disease, since it is strongly associated with obesity and metabolic syndrome pandemics. NAFLD may affect drug disposal and has common pathophysiological mechanisms with drug-induced liver injury (DILI); this may predispose to hepatoxicity induced by certain drugs that share these pathophysiological mechanisms. In addition, drugs may trigger fatty liver and inflammation *per se* by mimicking NAFLD pathophysiological mechanisms.

Aims: To provide a comprehensive update on (a) potential mechanisms whereby certain drugs can be more hepatotoxic in NAFLD patients, (b) the steatogenic effects of drugs, and (c) the mechanism involved in drug-induced steatohepatitis (DISH).

Methods: A language- and date-unrestricted Medline literature search was conducted to identify pertinent basic and clinical studies on the topic.

Results: Drugs can induce macrovesicular steatosis by mimicking NAFLD pathogenic factors, including insulin resistance and imbalance between fat gain and loss. Other forms of hepatic fat accumulation exist, such as microvesicular steatosis and phospholipidosis, and are mostly associated with acute mitochondrial dysfunction and defective lipophagy, respectively. Drug-induced mitochondrial dysfunction is also commonly involved in DISH. Patients with pre-existing NAFLD may be at higher risk of DILI induced by certain drugs, and polypharmacy in obese individuals to treat their comorbidities may be a contributing factor.

Conclusions: The relationship between DILI and NAFLD may be reciprocal: drugs can cause NAFLD by acting as steatogenic factors, and pre-existing NAFLD could be a predisposing condition for certain drugs to cause DILI. Polypharmacy associated with obesity might potentiate the association between this condition and DILI.

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1 | INTRODUCTION

Obesity is an increasingly growing epidemic, with its worldwide prevalence having nearly doubled over the past three decades.[1] Its hepatic manifestation is nonalcoholic fatty liver disease (NAFLD), a term that includes a broad range of liver disease ranging from simple steatosis, present in 70% of obese and diabetic patients, to nonalcoholic steatohepatitis (NASH), described in 10%-20% of patients with steatosis.[2,3] In the case of obese patients, metabolic syndrome and its more common associated consequences, insulin resistance (IR) and dyslipidaemia, are major causal factors of NAFLD, and this explains the high prevalence of this disease among them.

NAFLD and its main associated comorbidity, obesity, may be intertwined with drug-induced liver injury (DILI): there is increasing awareness of potential DILI risk factors in NAFLD patients for a growing number of drugs,[4] and DILI may present as lesions that resemble those of NAFLD.[5] This review will summarise the main clinical and experimental evidence supporting these two aspects of DILI in the fatty liver context, ie, how DILI can influence the development or accelerate progression of the fatty liver disease and how the susceptibility to and outcome of DILI may be influenced by NAFLD. The pathogenic alterations underlying these relationships will be also discussed in detail. Special emphasis will be paid to the mechanisms by which certain drugs induce hepatic steatosis (DIS) and/or steatohepatitis (DISH) by triggering pathological events similar to those occurring in NAFLD development and progression. They involve exacerbation of predisposing factors (eg, obesity, diabetes), steatotic factors (eg, exacerbated lipid hepatic synthesis/uptake), inflammatory factors (eg, accumulation of lipotoxic fatty acids and oxidative stress), and fibrogenic factors (eg, enhanced collagen deposition). These harmful effects not only can mimic the hits that trigger the development and/or progression of NAFLD in the normal liver, but also aggravate similar alterations pre-existing in a fatty liver. In addition, the hepatotoxic potential of drugs can be influenced by the alterations in drug-metabolic systems that occur in the NAFLD context, and they will be also reviewed here. Finally, obesity as a risk factor for DILI in the NAFLD context will be discussed in this review. Although controversial, there is accumulating evidence relating obesity to an increased risk of DILI; this has been reported not only in experimental models but also in some observational studies and clinical trials.[4] Whether this elevated risk reflects higher exposure to medication (polypharmacy) or the existence of sharing/exacerbating pathophysiological pathways in obese patients with pre-existing NAFLD or NAFLD risk factors will be further discussed.

2 | PHYSIOPATHOLOGICAL MECHANISMS OF NAFLD

Nonalcoholic fatty disease has a varied histological spectrum, from simple steatosis, a somewhat benign manifestation related to hepatic accumulation of relatively inert triglycerides (TGs), to overt necro-inflammation, hepatocyte ballooning and fibrosis, a disorder referred to as NASH. This latter condition may further evolve to cirrhosis with liver failure and, eventually, to hepatocellular carcinoma.[6-9]

This progression is currently explained by a "parallel multiple-hit theory".[10] According to this view, IR would be the "first hit" that triggers the disease, by leading to deleterious hepatocellular elevations of free fatty acids (FFAs) and the further formation of FFA-derived toxic metabolites (eg, diacylglycerol, ceramides).[11] This initial damage makes the liver vulnerable to further hits, such as oxidative stress, activation of inflammatory pathways by gut-derived bacterial endotoxin and endogenous inflammatory cytokines, microbial infections, dysregulated hepatocyte apoptosis, activation of hepatic stellate cells (HSCs), and the presence of genetic polymorphisms or pharmacological/dietary factors that trigger or potentiate these deleterious mechanisms.[2,3] These "multiple hits" would cause a progression from pure fatty liver to NASH to occur, as these parallel hepatic injuries slowly accumulate over time.[10] These damaging factors are responsible for the development of inflammation, apoptosis, and fibrosis, the hallmarks of NASH.

2.1 | Metabolic bases of NAFLD pathogenesis

Excessive hepatic FFA accumulation in NAFLD reflects an imbalance between enhanced hepatic FFA uptake and synthesis and, on the other hand, reduced FFA removal via deficits in very low density lipoprotein (VLDL) exportation, mitochondrial β-oxidation, and lipophagy.[12-14]

Dietary fat is the main source of adipose tissue FFAs, which are subsequently released and re-esterified in the liver.[15] In addition, IR, predisposing to both lipolysis of peripheral fat with mobilisation of FFAs to the liver and exacerbated hepatic lipogenesis, is the most important and frequent underlying pathogenic factor involved in hepatic FFA accumulation.[16,17]

When IR predominates in extrahepatic tissues over liver, hepatocytes receive an overload of glucose (not internalised by peripheral tissues) and insulin (due to compensatory hyperinsulinaemia).[17] Under such a condition, insulin and glucose stimulate hepatic synthesis and FFA uptake, via activation of the transcription factors "sterol regulatory binding protein-1c" (SREBP-1c) and "carbohydrate response element binding protein" (ChREBP), respectively. Exacerbated hepatic insulin signalling also impairs the FFA metabolic utilisation. The synthesis of apolipoprotein (apo) B, the main VLDL protein cofactor, is repressed by insulin, and this reduces exportation of hepatic lipids via VLDL.[18] In addition, in advanced NAFLD, there is an impairment of mitochondrial β-oxidation, a metabolic process that converts FFAs into disposable ketone bodies.[19]

Excessive liver FFA accumulation can trigger pro-oxidising, pro-inflammatory, pro-fibrotic, and pro-apoptotic signal pathways that account for the characteristic features of NASH, ie, oxidative stress, inflammation, apoptosis, and fibrosis.

2.2 | Oxidative stress in NAFLD

Oxidative stress is a critical factor in NAFLD pathogenesis, by being a main "second hit" leading to NAFLD progression.[20] It is mainly associated with early mitochondrial overfunction,[21,22] followed by mitochondrial damage and late dysfunction.[23] Initial enhanced mitochondrial β-oxidation is aimed to compensate excessive liver FFA content.[21,22] For this purpose, FFAs are transformed into acyl-coenzyme A (CoA) derivatives and further transported inside the mitochondrial matrix by the carnitine shuttle carnitine acetyl transferase, after its transformation into acyl-carnitine. Once inside the mitochondria, they undergo β-oxidation; this process yields one molecule of acetyl-CoA per oxidation cycle. The further metabolism of acetyl-CoA by the tricarboxylic acid cycle produces nicotinamide adenine dinucleotide and the hydroquinone form of flavin adenine dinucleotide (FADH2), and these molecules are, in turn, oxidised by transferring their electrons to the mitochondrial respiratory chain, to ultimately produce adenosine triphosphate (ATP). However, at I and III mitochondrial complexes, electrons can prematurely escape and interact with oxygen molecules, resulting in exacerbated radical oxygen species (ROS) production under elevated FFA fueling.[24] This oxidative burst further impairs mitochondrial function, thus establishing a vicious circle where ROS generation by the impaired respiratory chain is augmented, which further impairs respiratory chain functional integrity.[24,25]

Another main source of ROS in NASH is "cytochrome P450 (CYP) family 2 subfamily E member 1" (CYP2E1), which metabolises FFAs, and is induced by them.[26-30] CYP2E1 performs futile cycles, with release of electrons to the cytosol and the consequent formation of highly reactive carbonyl free radicals.[26-28]

Oxidative stress results from an imbalance between excessive ROS formation and limited antioxidant defences. Antioxidant enzymes, such as superoxide dismutase and catalase, are overexpressed in NAFLD in an attempt to reestablish this balance.[31] However, a progressive diminution of the antioxidant capacity occurs in NASH patients by both over-consume-induced depletion of antioxidant molecules (eg, glutathione, coenzyme Q10) and oxidative inhibition of the activity of antioxidant enzymes (eg, superoxide dismutase, catalase);[32,33] eventually, not only activity but also expression is decreased, as shown in patients with cirrhotic-stage NASH.[34]

2.3 | Liver inflammation in NASH

Non-alcoholic fatty disease is a pro-inflammatory condition, and inflammation is critical for its development and progression.[35] Inflammation is associated with the exacerbated production of inflammatory factors originated from both extrahepatic sites (eg, adipose tissue, gut) and hepatic sites, the latter being mainly associated with the production of pro-inflammatory cytokines by Kupffer cells. Hepatocytes are also a source of pro-inflammatory mediators, as a secondary consequence of the hepatic lipotoxic attack.[10]

Adipose tissue produces and releases several pro-inflammatory cytokines (eg, tumor necrosis factor-α [TNF-α], interleukin-1ß [IL-1ß] and interleukin-6 [IL-6]) and chemokines (eg, monocyte chemoattractant protein-1 [MCP-1]), collectively known as adipokines.[36] These mediators have receptors in liver parenchyma that traduce signalling cascades involved in IR (through suppressors of cytokine signalling activation) and pro-inflammatory cytokine production (through nuclear factor-κB [NF-κB] activation).[10]

Certain dietary and microbiota factors that reach the liver after disruption of intestinal barrier have also a pro-inflammatory role in NASH.[37] They include pro-inflammatory, gut-derived dietary products[10] and endotoxin, a wall component of Gram-negative microbiota.[38] These gut-derived mediators reach the liver via the portal vein and are sensed in liver mainly by Kupffer cells via toll-like receptor 4,[39] whose expression is enhanced in NAFLD.[40] LPS activates different signalling cascades that lead to pro-inflammatory cytokine synthesis via activation of pro-inflammatory transcription factors, such as NF-κB and "adaptor protein-1" (AP-1).[39-42]

In hepatocytes, accumulated FFAs are the main culprit mediators of the inflammatory response. FFAs may act as "danger-associated molecular patterns", thus activating the "nucleotide oligomerisation domain-like receptor protein 3" (NLRP3) inflammasome. NLRP3 is a large multiprotein complex that senses FFAs and activates caspase 1, a protease that promotes the cleavage and further maturation of pro-inflammatory cytokines to promote and sustain inflammation.[43] In addition, increased levels of FFAs induce Bax translocation to lysosomes; this triggers lysosomal destabilisation and cytosolic release of the protease cathepsin B, which enhances TNF-α expression via NF-κB activation.[44] NF-κB upregulates the expression of several pleiotropic cytokines, including "transforming growth factor" (TGF-ß), IL-6, IL-8, TNF-α, and Fas ligand (FasL), which are considered the primary mediators of the inflammatory and fibrogenic responses that drive NASH progression.[45]

2.4 | Hepatocellular death in NASH

Apoptosis is an important morphological and pathogenic feature in patients with NASH.[46] It has been also implicated in NASH progression, since gradual hepatocyte death triggers a compensatory progenitor cell expansion, thus promoting cirrhosis and predisposing to hepatocellular carcinoma.[47,48] The strong association between excess lipid accumulation and hepatocellular apoptosis makes the latter a form of "lipoapoptosis", with FFAs and their toxic derivatives being the main triggering factors.[49,50] The mechanisms of apoptosis in NASH are multiple, and virtually involve all known pathways leading to this cell death type, namely the intrinsic (mitochondrial) pathway, the endoplasmic reticulum pathway, the extrinsic pathways, and the lysosomal pathway.[49,50]

The intrinsic, mitochondrial pathway is triggered by FFA-induced expression of the pro-apoptotic members of the "B-cell lymphoma-2" (Bcl-2) protein family, "Bcl-2-interacting mediator of cell death" (Bim),[51] "Bcl-2-associated X protein" (Bax),[52] and "p53 upregulated modulator of apoptosis" (PUMA);[53] Bax forms pores in the

mitochondrial outer membrane, whereas Bim and PUMA relieve the inhibitory effect on Bax of pro-survival members of the Bcl-2 family.[54] This results in cytosolic release of cytochrome *c*, among other pro-apoptotic factors. Once in cytosol, cytochrome *c* associates with "apoptotic protease activating factor 1" (Apaf-1) to form the "apoptosome"; this complex recruits pro-caspase 9 to facilitate its autoactivation and the further cleavage of the executioner caspases 3 and 7.[55]

Oxidative stress is another major triggering factor of apoptosis via increased mitochondrial permeabilisation, by facilitating assembly of "mitochondrial permeability transition pores" (MPTPs) in the inner membrane permeability;[56] this induces mitochondrial swelling, rupture of the outer membrane and further release of cytochrome *c*.[57] Oxidative stress-induced MPTP formation critically depends on Ca^{2+}, through a Ca^{2+}/calmodulin/calmodulin-dependent protein kinase II-mediated mechanism.[58] An important mitochondrial source of Ca^{2+} is the endoplasmic reticulum, a neighbour organelle that releases Ca^{2+} under stress conditions.[59-61] MPTP formation may lead to either apoptosis or necrosis, depending on the number of mitochondria affected. Since apoptosis is an energy-demanding process, it occurs when the number of mitochondria affected is low, so that ATP levels are preserved.[62] Contrarily, necrosis is a passive process triggered by massive mitochondrial affectation, which leads to cytolysis by impairment of plasma membrane integrity.[62]

Endoplasmic reticulum stress-induced apoptosis is another major mechanism of hepatocyte death in NASH. Regeneration secondary to liver injury in NASH requires exacerbated protein synthesis, which leads to endoplasmic reticulum stress.[59,63] In addition, saturated FFAs induce endoplasmic reticulum stress in a direct manner.[64] The endoplasmic reticulum-mediated pro-apoptotic pathway involves sequentially: (a) disruption of endoplasmic reticulum Ca^{2+} homeostasis, (b) endoplasmic reticulum Ca^{2+} release, (c) Ca^{2+}-dependent activation of calpains, (d) calpain-dependent caspase 12 activation, and (e) caspase 12-induced activation of executioner caspases.[65,66] Additionally, the stressed endoplasmic reticulum can activate the pro-apoptotic "c-Jun N-terminal kinase" (JNK)-mediated signalling pathway, thus inducing expression of the pro-apoptotic transcription factor "CCAAT/enhancer-binding protein homologous protein" (CHOP) and dimerisation with phosphorylated-c-Jun to render the AP-1 complex; AP-1 enhances PUMA expression, with subsequent apoptosis via the intrinsic pathway.[67]

The extrinsic apoptotic pathway is activated by soluble cytokines released during the inflammatory process, such as FasL and "tumor necrosis factor-related apoptosis-inducing ligand" (TRAIL), or by their plasma membrane-associated forms in inflammatory cells. These cytokines activate their respective hepatocellular plasma-membrane cytokine receptors, ie, Fas and TRAIL receptor (TRAILR). Activation of these receptors leads in turn to apoptosis through activation of caspases 8 and 10, followed by activation of the executioner caspases 3, 6, and 7.[68] Caspases 8 and 10 also cleave Bid, and truncated Bid recruits Bax and Bak to the mitochondria, with the consequent activation of the intrinsic apoptosis pathway.[69,70] Fas/FasL and TRAILR/TRAIL systems have been critically implicated in the pathogenesis of NASH, since Fas[71] and TRAILR[72,73] expressions are increased in livers of patients with NASH, via direct FFA-mediated mechanisms.

As for the lysosomal pathway of apoptosis, long-chain, saturated FFAs induce lysosomal destabilisation and cytosolic release of cathepsin B,[44,74] via Bax translocation to lysosomes.[75] This leads to apoptosis through the intrinsic, mitochondrial pathway,[74] via cathepsin B-dependent proteolytic activation of caspase 2, which induces mitochondrial outer membrane permeabilisation.[76]

Autophagy is a lysosomal, self-degradation mechanism of cell death aimed to promote cell survival of the remaining cells by supplying energy in response to nutrient stress via utilisation of degradation products.[77] In addition, autophagy plays a beneficial role after tissue injury, by removing damaged organelles and proteins.[77] This pathway also regulates the breakdown of lipids contained in hepatocellular droplets, and hence its impairment may result in hepatic steatosis.[78]

During autophagy, cellular organelles such as mitochondria ("mitoautophagy") or lipid droplets ("lipophagy") are sequestered in autophagic vacuoles, including autophagosomes with double-membrane structures and multilamellar bodies, which fuse to lysosomes for degradation.[77,78]

In response to fasting, the number of autophagic vacuoles containing lipid cargo for degradation increases, and the FFAs thus released fuel mitochondrial ß-oxidation for energy supply.[79] On the contrary, inhibition of hepatocellular autophagy results in reduced lipolytic breakdown and excessive TG accumulation.[80,81] Furthermore, fasting-induced formation of lipid droplets, an adaptive event thought to be beneficial by preventing FFAs from inducing IR,[82] depends directly on autophagy integrity, as suggested by studies in autophagy-deficient mice.[83]

A decreased autophagy has been shown to occur in human NAFLD,[83-85] and in genetically and nutritionally generated obese experimental animals.[79,86,87] This has been attributed to downregulation of autophagy genes and decreased levels of lysosomal enzymes,[88] as well as impaired fusion of autophagosomes with lysosomes.[89,90] Hyperinsulinaemia occurring in the NAFLD context seems to be a causal factor.[88]

Reduced macroautophagy may contribute to other features of NAFLD of true pathogenic importance, such as IR and endoplasmic reticulum stress, the latter being due to a reduced ability to remove damaged proteins. In addition, autophagy dysfunction may aggravate NASH injury by lack of removal and further replacement of damaged organelles or proteins that contribute to the hepatocellular dysfunction in this hepatopathy.[78] Furthermore, autophagy dysfunction in NASH may be a causal factor in the progression to hepatocellular carcinoma due to accumulation of the oncogenic protein p62, a selective substrate for autophagy; this would involve (a) a shift of macrophage polarisation to the M1, pro-inflammatory phenotype when NAFLD severity increases, (b) aggregation of M1-polarised macrophages around hepatocytes containing large lipid droplets, (c) formation of Mallory-Denk bodies containing p62, and (d) p62-mediated survival of hepatocellular carcinoma-initiating cells.[91]

2.5 | Fibrogenesis in NASH

Liver injury in NAFLD triggers hepatic fibrosis, a reversible wound healing process consisting of the rapid synthesis and deposition of extracellular matrix components, mainly collagen fibers, aimed to maintain tissue integrity during repair.[92] Fibrogenesis requires the concerted action of many different cell types, with HSCs being the main collagen-producing cells, and Kupffer cells and hepatocytes being the main regulatory actors.

In the normal liver, hepatic HSCs are quiescent, but in response to liver injury, they activate to become the major extracellular matrix-producing cell type.[93] Kupffer cells trigger fibrogenesis by secreting the profibrogenic cytokine TGF-β. This cytokine promotes both HSC proliferation and maintenance of the myofibroblastic phenotype, as well as collagen synthesis via the "TGF-ß/small mothers against decantaplegic homolog 3" (SMAD3)-signalling pathway.[94]

ROS derived from both outside and inside the HSCs can directly activate these cells. Inside the HSCs, ROS are produced via TGF-β-dependent activation of "NADPH oxidase 4" (NOX4), a membrane-integrated enzyme that generates ROS from molecular oxygen. The so-formed ROS activate the redox-sensitive Ras/"extracellular signal-regulated kinase" (Erk) pathway, which stimulates, in turn, HSC proliferation, migration, and pro-collagen type I expression.[95-99]

3 | IMPACT OF NAFLD ON DILI SUSCEPTIBILITY AND OUTCOME

Pre-existing liver disease seems not to be associated with increased risk of idiosyncratic hepatotoxicity when assessed for the bulk of medications.[100] However, there is growing evidence that certain drugs can induce liver injury more often and with higher severity when exposed to a pre-existing fatty liver, whereas other drugs can induce aggravation of the pre-existing NAFLD, with accelerated transition from steatosis to steatohepatitis.[4] Examples of drugs whose hepatotoxicity could be more frequent and severe in NAFLD patients include acetaminophen,[101,102] several antibiotics (fosinopril, piperacillin/tazobactam, telithromycin),[5] the antithrombotic agent ticlopidine,[5] and the antihypertensive agent losartan.[5] This susceptibility has been suggested to be associated with the existence of a differentiated metabolic environment in NAFLD, with higher ROS levels, reduced ATP synthesis, inflammation, and overproduction of CYP-generated toxic drug metabolites, among many other putative predisposing factors.[4] These events may shift the dose-response curve for liver toxicity of certain drugs to the left, so as to produce a hepatotoxic response in the range of therapeutic drug doses.[103]

Incidence of liver steatosis clearly increases with obesity, with values increasing from 10% to 24% for the general population to 58%-74% for obese individuals.[104] Therefore, the increased susceptibility of several drugs reported in obese patients may be related to the fact that their steatotic livers are more vulnerable to certain toxicological insults. For example, hepatotoxicity by halothane[105-109] and methotrexate (MTX)[110] was found to be more prevalent and severe in obese patients. It should be noted, however, that the enhanced susceptibility to DILI in obesity might not be related to steatosis *per se*, but to other mechanisms associated with drug availability/metabolism or inflammatory changes in adipose tissue affecting indirectly the liver.

Similarly, the presence of diabetes mellitus, a disease highly associated with both prevalence and severity of NAFLD,[111] was shown to be an independent risk factor for severe DILI.[112-114] Again, diabetes mellitus may impact on the liver through pathogenic mechanisms others than fatty liver accumulation, eg, hyperglycaemia-induced oxidative stress and further triggering of inflammation.[115]

It should be acknowledged that the evidence on the increased susceptibility of NAFLD patients to the abovementioned forms of DILI is still preliminary, since it is based on few reports with few events, and with potential methodological flaws that preclude a robust analysis.[116] Clearly, further investigations would be needed to confirm these observations. Susceptibility risk factors for DILI will require comparing enrolled patients with control patients who took the same medication without developing liver injury. This will only be achieved when the number of recorded DILI cases caused by individual medications increases, and appropriate controls are enrolled in highly standardised DILI databases.

4 | DRUGS AS PATHOGENIC FACTORS IN FATTY LIVER DEVELOPMENT AND PROGRESSION

4.1 | Drug-induced "pure" steatosis: common pathophysiological pathways with NAFLD

Many drugs can mimic features of the metabolic syndrome and, therefore, can reproduce the hepatic pathogenic mechanisms involved in NAFLD development and progression, even acting as the "first hit" that prompts the fatty liver disease. In other cases, the drug may do so by precipitating or aggravating risk factors for NAFLD, such as central obesity, diabetes, and hypertriglyceridaemia (Figure 1). The more common drugs known to induce different forms of fat accumulation in liver are listed in Table 1, whereas the main mechanisms that lead to these alterations are summarised in Table 2.

Steatosis occurs in two major forms, namely macrovesicular and microvesicular steatosis, depending on the severity of the mitochondrial injury. In many cases, however, macrovesicular steatosis coexists with at least a low degree of microvesicular steatosis (mixed steatosis), particularly in intermediate and severe forms of steatohepatitis. For example, a minor component of microvesicular steatosis coexisting with macrovesicular steatosis was reported to occur in 10% of "pure" NAFLD cases; as expected, it correlated with more advanced histological features of NAFLD, including presence of megamitochondria in microsteatotic hepatocytes.[117]

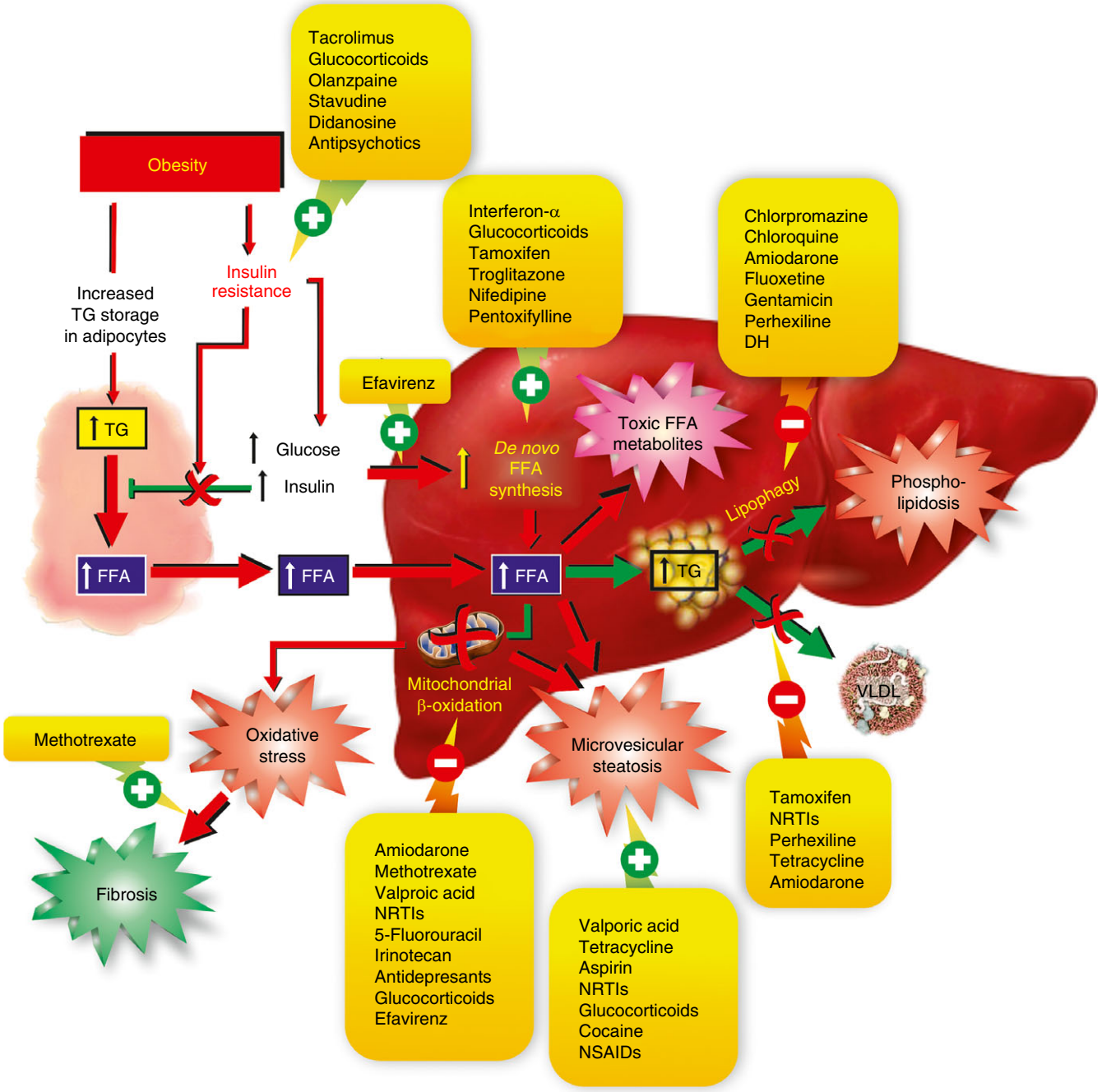

FIGURE 1 Mechanisms of drug-induced steatosis/steatohepatitis and examples of drugs instrumental in activating them. Drugs can act as the "first hit" for the development of steatosis/steatohepatitis by inducing insulin resistance, or by mimicking many pathomechanisms associated with these conditions relevant to NAFLD development and progression, such as enhancement of *de novo* lipid synthesis or free fatty acid (FFA) hepatic uptake, which leads to accumulation of TG in macrovesicular structures. Drugs can also worsen pre-existing steatogenic pathomechanisms in NAFLD patients, such as impairment of lipid degradation via lipophagy (leading to phospholipidosis) or lipid exportation via very low density lipoprotein (VLDL), thus acting as the "second hits" that drive NAFLD to advanced stages of the disease. Drugs can also prompt mitochondrial dysfunction, leading to exacerbated generation of oxidative stress from mitochondrial origin; when this dysfunction occurs acutely in highly overloaded mitochondria metabolising FFAs via β-oxidation, acute accumulation of nonmetabolised FFAs occurs, leading to microvesicular steatosis. Finally, drugs can exacerbate the oxidative stress-dependent activation of hepatic stellated cells, leading to enhanced fibrosis. Red arrows represent damaging pathways and green arrows protective pathways. DH, diethylamino-ethoxyhexestrol; NSAID, nonsteroidal anti-inflammatory drug; NRTI, nucleoside reverse transcriptase inhibitor; TG, triglyceride

TABLE 1 Prototypical drugs involved in macroesteatosis, microesteatosis, phospholidosis, and DISH

Drugs inducing DISH	
Methotrexate	Tamoxifen
Amiodarone	Irinotecan
Drugs inducing microesteatosis	
Aspirin	Didanosine
Valproic acid	Tetracycline
Cocaine	Ibuprofen
Zidovudine	Naproxen
Drugs inducing phospholipidosis	
Chlorpromazine	Gentamicin
Chloroquine	Perhexiline
Amiodarone	Ketoconazole
Fluoxetine	Diethylamino-ethoxyhexestrol
Drugs associated with risk of DILI in obese patients (or in obese rodent models)	
Acetaminophen	Rosiglitazone
Halothane	Stavudine
Methotrexate	Tamoxifen
Drugs associated with a potential DILI in obese patients (more studies are needed)	
Pentoxifylline	
Phenobarbitone	
Omeprazole	

DILI, drug-induced liver injury; DISH, drug-induced steatohepatitis.

TABLE 2 Different mechanisms by which prototypical drugs induce macrosteatosis, microsteatosis, and phospholidosis

Mechanisms of macrosteatosis
Decrease in hepatic triglyceride secretion, increase in lipid peroxidation, and inhibition of mitochondrial β-oxidation (eg, glucocorticoid-induced macroesteatosis)
Decrease in FFA ß-oxidation, ROS generation, and increase in lipogenesis (eg, amiodarone)
Inhibition of mitochondrial electron transport chain (eg, methotrexate)
Promotion of *de novo* FFA synthesis and inhibition of FFA ß-oxidation (eg, tamoxifen)
Mechanisms of microsteatosis
Blockage of β-oxidation by consuming CoA and carnitine, and by prompting MPTP formation (eg, aspirin)
Sequestration of CoA and carnitine, arrest of ATP synthesis, and promotion of weight gain (eg, valproic acid)
Inhibition of β-oxidation and *n*-oxidation, inducing hepatotoxic products (eg, cocaine)
mtDNA depletion and autophagy stimulation induced by mitochondrial dysfunction (eg, zidovudine, didanosine)
Inhibition of FFA β-oxidation and VLDL secretion (eg, tetracycline)
Inhibition of β-oxidation of short- and medium-chain FFAs (eg, ibuprofen and naproxen)
Mechanisms of phospholipidosis
Direct binding of cationic, amphipathic drugs to phospholipids in lysosomes, rendering indigestible phospholipid-drug complexes (eg, amiodarone, gentamicin, perhexiline, diethylamino-ethoxyhexestrol)
Inhibition of phospholipase activity, either directly or mediated by interaction of the drug at the lysosomal phospholipid bilayer (*eg*, chlorpromazine, chloroquine)
Lipophagy inhibition?

ATP, adenosine triphosphate; CoA, coenzyme A; FFA, free fatty acid; MPTP, mitochondrial permeability transition pore; ROS, radical oxide species; VLDL, very low density lipoprotein.

4.1.1 | Macrovesicular steatosis

This form of lipid accumulation is the predominant finding observed in biopsy specimen of NAFLD patients, and in fatty liver induced by drugs as well. Histologically, macrovesicular steatosis consists of a single, large fat vacuole per hepatocyte that fills up most of the cytoplasm, inducing peripheral displacement of the nucleus (Figure 2). "Pure", macrovesicular steatosis by drugs occurs mainly by inducing mild mitochondrial dysfunction, since decreased mitochondrial ß-oxidation of FFAs leads to increased esterification into TGs, which accumulate in hepatocytes as large lipid droplets. Drugs may also alter hepatic lipid homeostasis, either by promoting FFA uptake or by impairing metabolic TG processing via lysosomal hydrolysis or VLDL-mediated TG exportation.[118] Alternatively, some drugs can trigger or exacerbate factors involved in NAFLD pathogenesis, such as acquisition of systemic IR with compensatory hyperinsulinaemia. They can also act at the level of predeterminants of these pathogenic factors, such as central obesity and diabetes, or even cause directly hyperinsulinaemia, either by enhancing insulin pancreatic release or by impairing insulin clearance.[119,120] Macrovesicular steatosis associated with all these iatrogenic causes often appears early after drug exposure, and is considered a benign disease in the short term follow-up.[121]

A single drug may alter one or several lipid metabolic pathways leading to macrovesicular steatosis. For instance, amiodarone, glucocorticoids, and certain antidepressant drugs (eg, amineptine and tianeptine) may induce moderate inhibition of mitochondrial FFA β-oxidation.[24] The non-nucleoside reverse transcriptase inhibitor (NRTI) efavirenz impairs mitochondrial function as well,[122] but can also promote FFA cellular uptake. Interferon-α,[123] glucocorticoids,[124] tamoxifen,[125] troglitazone (via "peroxisome proliferator-activated receptor-γ" activation),[126] and nifedipine[118] can all increase *de novo* FFA synthesis. The mechanisms by which these latter drugs enhance lipogenesis are not completely known, but some of them are activators of lipogenic transcription factors, such as "pregnane X receptor" (PXR) (troglitazone, tamoxifen, nifedipine), "peroxisome proliferator-activated receptor-γ" (troglitazone), and the glucocorticoid receptor (glucocorticoids).[118] In addition, other drugs alter the levels of, or the sensitivity to, different hormones that modulate intrahepatic lipid content and metabolism. For example, direct hypoinsulinaemia

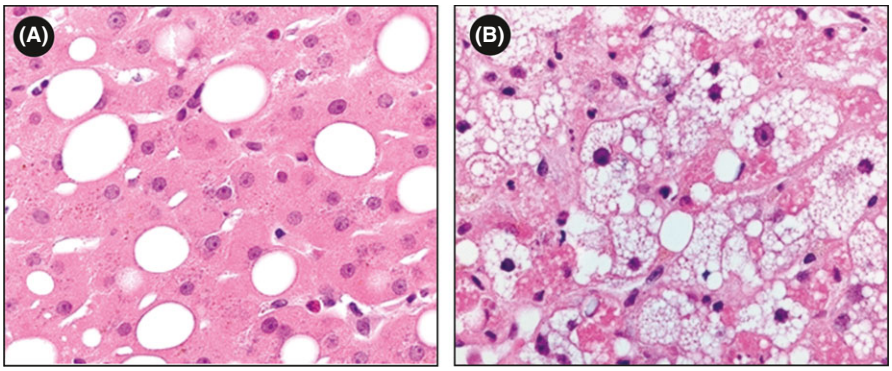

FIGURE 2 Macrovesicular and microvesicular steatosis induced by drugs. A, Macrovesicular steatosis induced by steroids: one fat vacuole displaces the nucleus to the edge of the cell. B, Microvesicular steatosis induced by valproic acid: multiple cytoplasmic microvacuoles surround the nucleus without altering its location

induced by impairment of pancreatic insulin secretion may be caused by tacrolimus, obesity and IR can be caused by glucocorticoids and olanzapine, and low leptinaemia may be caused by stavudine and didanosine through the development of lipoatrophy, ie, reduction of fat mass with decrease in leptin secretion by white adipocytes, causing compensatory *de novo* lipogenesis in the liver.[118,127] Hypoleptinaemia can also promote lipid accretion in skeletal muscle and pancreas, thus causing systemic IR and type 2 diabetes.[128,129] In addition, DIS can be the consequence of the inhibition of VLDL synthesis and secretion, as has been shown in amiodarone- and tetracycline-induced fatty liver;[130] the latter compound inhibits "microsomal TG transfer protein", a chaperone required for VLDL formation.[131]

Interestingly, atypical antipsychotic drugs (eg, quetiapine, clozapine, risperidone, sulpiride, sertindole, olanzapine, and quetiapine) can trigger body weight gain, IR, and metabolic syndrome. In this setting, "pure" steatosis and steatohepatitis can be triggered by an increase in *de novo* lipid synthesis.[132] Apart from these iatrogenic causes, IR and diabetes are more often detected in schizophrenic patients due to lifestyle factors, such as lack of exercise and detrimental dietary habits.[133]

Obesity is an independent predisposing factor for drug-induced macrovesicular steatosis, and this is why some drugs cause fatty liver in obese but not in lean people. The haemorheological agent pentoxifylline was found to exacerbate liver steatosis in obese but not in normal mice, through enhanced intestinal glucose absorption and activation of hepatic lipogenesis.[134] Hydrochlorothiazide, a thiazide diuretic lacking hepatotoxicity potential, is another example. It has diabetogenic potential when administered to obese but not to lean individuals by aggravating IR, thus exacerbating liver fat accumulation and visceral fat redistribution;[135] these effects have been suggested to be dose-dependent in nature.[136]

4.1.2 | Microvesicular steatosis

Some drugs can induce microvesicular steatosis in susceptible patients instead of macrovesicular one, or both of them. Unlike macrovesicular steatosis, microvesicular steatosis is characterised by accumulation of multiple very small droplets in the hepatocyte cytoplasm, with the nucleus retaining its central location (Figure 2).[137] This is a more serious form of liver injury induced by drugs, and can be life threatening when extensive or long-lasting. Drugs that can induce microvesicular steatosis include valproic acid,[138] tetracycline,[139] aspirin (Reye's syndrome),[140] NRTIs,[141] glucocorticoids,[142] cocaine,[143] and nonsteroidal anti-inflammatory drugs,[144] among others.

Different patterns of steatosis can be produced by the same drug in different patients depending on patient susceptibility, which suggests contribution of genetic predisposing factors. For example, amiodarone most often provokes macrovacuolar steatosis (occasionally associated with microvesicular steatosis) and steatohepatitis, although it can also induce "pure" microvesicular steatosis in a few patients.[145,146]

Severe impairment of the mitochondrial β-oxidation of FFAs has been the most frequently implicated pathomechanism for microvesicular steatosis;[147] this may explain its association with the presence of megamitochondria in the affected hepatocytes,[148] a feature that reflects functional mitochondrial impairment and lack of FFA metabolisation.[149] These nonmetabolised FFAs, which are amphipathic in nature, can emulsify TGs by embedding their lipophilic tails into a core of neutral TGs, thus explaining TG accumulation in small lipid structures.[150.]

There are other hepatopathies that display microvesicular steatosis,[147] and therefore this histopathological finding must be considered in the whole clinical context. For example, microvesicular steatosis occurs in acute fatty liver of pregnancy, a rare but potentially fatal condition where pericentral microvesicular steatosis is one of the hallmark histological findings.[151] In this case, homozygous enzymatic defects in foetal and placental β-oxidation of FFAs would lead to accumulation of FFAs and toxic metabolic intermediates that are transferred via maternal circulation to a heterozygous mother; this creates a lipotoxic environment that increases hepatocyte ROS, activates inflammatory pathways, and triggers hepatocellular death, thus leading to acute maternal hepatic failure.[152]

Of note, microvesicular steatosis usually does not lead to the characteristic changes on liver ultrasound or hepatomegaly. Its diagnosis can be suspected by clinical and biochemical evidences, but should be only confirmed by liver biopsy. When suspected, it is a medical emergency requiring rapid identification, removal of the cause (if possible), and supportive care for hepatitis and acute liver failure.

4.2 | Drug-induced steatohepatitis: mitochondrial dysfunction and oxidative stress as key pathomechanisms

When a *de novo* steatohepatitis or the worsening of an underlying simple steatosis to steatohepatitis occurs after drug exposure causing intracellular accumulation of lipids, the syndrome is referred to as DISH. It is suspected when a worsening of the patient`s baseline liver function tests is caused by a drug, and confirmed by considering the histological pattern of liver injury, the interval between the beginning of drug therapy and the onset of liver disease (latency), and the evidence of liver function recovery after drug discontinuation.

The mechanisms underlying the progression of steatosis to steatohepatitis in DILI are far from being well understood, and they are mostly inferred from the better known mechanisms of NAFLD progression.

4.2.1 | Pathogenic mechanisms of DISH

Pathogenesis of DISH involves a more severe mitochondrial dysfunction with excessive mitochondrial ROS production and impairment of lipid egress from hepatocytes via alteration of VLDL production and secretion as the main causal factors.[153]

Even though all the aforementioned alterations can occur in a healthy liver, it has also been reported that a pre-existing fatty liver, or even only risk factors for this condition, may contribute to a higher incidence of advanced forms of fatty liver disease caused by certain drugs. An example is the development and worsening of the hepatic steatosis caused by the oestrogen receptor modulators tamoxifen and raloxifene, via impairment of both mitochondrial function and FFA β-oxidation, as well as stimulation of the *de novo* FFA synthesis.[154-157] In these cases, obesity and other metabolic risk factors have been identified as independent predictors.[158,159]

Since the pathogenic mechanisms leading to DISH overlap with those implicated in drug-induced "pure" steatosis, either differential genetic susceptibility or the existence of underlying hepatopathies that favour DILI (including potentially NAFLD) may be involved. Several genetic polymorphisms have been identified with a differential predisposition to DILI, and hence DISH. They mainly comprise: (a) mutations in "phase 1" or "phase 2" metabolising enzymes, with "loss-of-function" variants leading to potentially toxic accumulation of the drug and its metabolites, or "gain-of-function" variants leading to overproduction of toxic metabolites; (b) mutations in antioxidant enzymes that impair ROS detoxification, and (c) mild preexistent mitochondrial dysfunction, which can be aggravated by drug exposure.[118]

Many drugs known to trigger directly steatohepatitis or exacerbate progression of a pre-existing fatty liver to steatohepatitis induce oxidative stress by impairing mitochondrial function via different mechanisms, as described below.[160-163] Mitochondrial toxicity can promote cell death via necrosis (through severe ATP depletion)[62] or apoptosis (via release of pro-apoptotic mitochondrial proteins, such as cytochrome *c*).[56,57] Dysfunctional mitochondria are a main source of ROS. The oxidative stress thus promoted can potentiate the mechanisms of hepatocyte death and drive key process involved in the progression from simple steatosis to steatohepatitis, such as hepatic inflammation and fibrosis/cirrhosis. Since, in patients with NAFLD, oxidative stress is enhanced and the antioxidant status is impaired (see Section 2.2),[164] addition of a drug-related pro-oxidant challenge might trigger and/or potentiate this progression.

Mitochondrial impairment induced by drugs can occur through primary effects on (a) the mitochondrial genome that encodes protein components of the respiratory chain, (b) the function of these components itself, and (c) the coupling of mitochondrial respiration to ATP production.[165] For example, uncoupling of oxidative phosphorylation, via the protonophoric action of the drug in the H^+-impermeable mitochondrial membrane that dissipates H^+ gradients, thus impeding ATP synthesis by using the energy of this H^+ gradient, may occur through hydrophobic interactions of the drug with polypeptides/phospholipids of the mitochondrial inner membrane.[23,165] Alternatively, interference with the synthesis of proteins of the electron transport chain via, for example, depletion of mitochondrial DNA (mtDNA) is also a common pathogenic mechanism, since mtDNA encodes many of these proteins.[166,167] Mitochondrial FFA β-oxidation, an initially protective metabolic process that reduces hepatotoxic levels of FFAs elevated in NAFLD, is another mitochondrial function that is often impaired by steatogenic drugs; this process requires functional integrity of the respiratory chain and, therefore, it is eventually impaired by the inhibition of respiratory chain activity described above.[25] Alternatively, steatogenic drugs can inhibit FFAs mitochondrial uptake via the carnitine shuttle and/or the activation of FFAs with CoA, a process required for FFAs to fuel mitochondrial β-oxidation;[23,165] since patients with NAFLD may have genetic or acquired alterations in FFA β-oxidation, these drugs may precipitate a latent fatty liver.[168] Finally, certain drugs can induce DISH by increasing mitochondrial membrane permeabilisation via MPTP opening.[118] This can produce either apoptosis or necrosis, depending on whether the number of mitochondria affected is low or high, respectively; if necrosis predominates, the condition is referred to as "cytolytic steatohepatitis".[118] Depending on the severity, it may range from isolated transaminasaemia to fulminant hepatitis requiring liver transplantation.[169] Although rare, many drugs can cause this condition, including acetaminophen, valproic acid, diclofenac, amiodarone, salicylic acid, nimesulide, troglitazone, and disulfiram.[118] Drugs may induce this effect by (a) direct interaction with some MPTP components that trigger pore assembly, (b) induction of oxidative stress and elevations of free cytosolic Ca^{2+}, two well

known triggering factors of MPTP formation, and (c) activation of signalling pathways or other endogenous stimuli that promote MPTP generation.[118]

4.2.2 | Clinical and histological features of DISH

Drug-induced liver injury induced by drugs that impair mitochondrial function typically presents as acute hepatitis. The clinical severity of the resulting syndrome (lactic acidosis, encephalopathy, myopathy, hepatic microvesicular steatosis, and cytolysis) led to the withdrawal of several drugs from the market, or to the addition of black box warnings to their labels.[158] Some of these agents were withdrawn during clinical trials (eg, fialuridine and panadiplon), while others were withdrawn after marketing (eg, alpidem, perhexiline, pirprofen, and troglitazone).

In general terms, DISH usually resembles alcoholic fatty liver disease more closely than NASH. For example, DISH and alcoholic fatty liver disease develop cirrhosis more often than NAFLD. Biopsy-based follow-up studies revealed that only 1% of NAFLD patients developed cirrhosis, as compared with 22% of patients with alcoholic fatty liver disease[170] and 15% of patients with DISH.[121] In addition, like in alcoholic liver disease,[171] progression from fibrosis to cirrhosis in DISH often occurs fast (over weeks or months),[120,172,173] whereas in NASH, generally takes decades, and often never occurs.[174] For example, progression from DISH to cirrhosis was reported to occur in patients on tamoxifen in 19-24-months after DISH diagnosis.[175,176] Furthermore, unlike most other DILI cases, liver damage associated with DISH may progress despite discontinuation of the drug,[177] a feature also reported in alcoholic liver disease.[178]

This differential progression rate may reflect differences in histological lesions, as alterations in liver histopathology are more severe in DISH and alcoholic liver disease than in NASH. The two-first conditions show remarkable histological similarities, with polymorphonuclear inflammation, hepatocyte ballooning degeneration, Mallory-Denk bodies, and foamy degeneration (microvesicular steatosis) being common features;[179,180] in DISH, this histological pattern is referred to as "pseudoalcoholic steatohepatitis".[181] On the other hand, in NASH, Mallory hyaline is poorly formed and foamy degeneration has never been described.[182]

4.2.3 | Obesity and DISH

Obese individuals with NAFLD could be more prone to develop certain types of drug-induced acute hepatitis.[163] This is the case of halothane and acetaminophen, two drugs that undergo CYP2E1 mediated biotransformation into the highly toxic metabolites N-acetyl-*p*-benzoquinone imine (NAPQI) and trifluoroacetyl chloride, respectively.[4,106,183] Since patients with underlying NAFLD show enhanced CYP2E1 activity due to the inducing effect of FFAs,[26] this overexpression could potentiate drug toxicity by increasing the production of these toxic metabolites. For halothane, its toxic metabolites may act as haptens after binding to proteins, thus triggering an immune-mediated reaction.[184] In addition, underlying mitochondrial dysfunction has also been suggested. Indeed, the halothane toxic metabolite can bind to different macromolecules in mitochondria, thus causing mitochondrial dysfunction, and can be conjugated with glutathione, thus inducing glutathione depletion and further impairment of detoxification via glutathione conjugation;[185-187] this leads to oxidative stress and the further triggering of an inflammatory response.[188] Similar hepatotoxic mechanisms have been suggested for isoflurane.[189,190] Thus, in obese individuals, the development of acute hepatitis could be mediated by excessive production of CYP2E1-produced toxic metabolites, by the incapacity to remove them efficiently, or both.[4,130,163] However, since not all the drugs capable of generating toxic metabolites are more toxic in the obese population, further investigations are needed to identify the exact mechanism mediating this type of DILI.[101,130,163] In particular, the role for NAFLD *per se* in this enhanced hepatotoxicity needs to be established with certainty, since factors other than NAFLD may be involved in obesity that might directly or indirectly alter the liver. Nevertheless, a study in NASH patients showed that there was a significant increase in hepatocellular CYP2E1 expression even when more than half of them were not obese,[30] a finding further supported by data in NASH animal models not involving weight gain.[191]

4.2.4 | Common drugs causing DISH and mechanisms of action

Many drugs can produce DISH by inducing acute mitochondrial dysfunction, since mitochondrial respiratory chain and ATP synthesis are required for FFA ß-oxidation to occur. The mechanisms of mitochondrial injury are multifactorial, and the same drug can act through more than one of them. Some drugs affect mitochondrial membrane integrity via MPTP formation; this alteration leads to blockage of the respiratory chain, oxidative stress and, eventually, necrosis and/or apoptosis via release of cytochrome *c*, an event that triggers the mitochondrial pathway of cell death. Good examples are the NRTI stavudine,[192,193] the antianginal drugs amiodarone (or its analogues, benzarone, benzbromarone, and dronedarone),[194-197] perhexiline,[198] and aspirin.[199] Tamoxifen, an anti-estrogenic compound that causes steatosis in 43% of recipients,[200] is a cationic drug that can be electrophoretically taken up by mitochondria, and can also trigger cytochrome *c* via mitochondrial oxidative stress elevation, through a mechanisms involving mitochondrial Ca^{2+} increase and further activation of mitochondrial nitric oxide synthase.[156] Diclofenac,[201,202] valproic acid,[203] and aspirin[199] can also trigger MPTP formation, but the mechanism involves in part the direct inhibition of enzymes belonging to the respiratory chain and the primary uncoupling of oxidative phosphorylation, ie, the uncoupling between the electron transport and phosphorylation, which inhibits ATP synthesis without affecting the components of the respiratory chain or ATP synthase;[202,204–206] both mechanisms leads to mitochondrial oxidative stress and further MPTP generation. Tamoxifen can also act via this mechanism, in addition to promoting primarily MPTP formation.[207] Other drugs inhibit mitochondrial FFA ß-oxidation independently of their inhibitory effect on the mitochondrial respiratory chain, by

direct inhibition of key mitochondrial enzymes involved in this process. Examples of drugs acting via this mechanism are amiodarone,[194] benzarone and benzbromarone,[195] perhexiline,[198] tianeptine,[208] some nonsteroidal antiinflammatory drugs (eg, ibuprofen, diclofenac),[209] and valproic acid.[210] Other drugs, such as valproic acid[211] and aspirin,[212,213] can affect availability of cofactors of the FFA ß-oxidation process, such as CoA (required for long-chain FFA activation) and carnitine (required for FFA mitochondrial uptake). Aspirin[213] and valproic acid[214,215] form acyl-CoA-derivatives; this decreases CoA levels and makes CoA no longer available for FFA activation. Similarly, both aspirin[213] and valproic acid[214,216] form esters with carnitine, leading to carnitine depletion; this effect induced by aspirin may be a triggering factor of Reye's syndrome, since patients with this disease usually have underlying genetic disorders affecting mitochondrial FFA ß-oxidation.[217] A final mechanism of mitochondrial injury that can secondarily impair FFA ß-oxidation is inhibition of mtDNA replication or the direct mtDNA damage, which leads eventually to mtDNA depletion; mtDNA is not only critical for the maintenance of the mitochondrial function by encoding several components of the respiratory chain,[166,167] but it is also a factor leading to inhibition of the tricarboxylic acid cycle.[218] mtDNA replication and repair processes involve certain mitochondrial enzymes, such as topoisomerases and mtDNA polymerase-γ. Examples of DNA-intercalating drugs that inhibit topoisomerases are tacrine,[219] tamoxifen,[155] and neocryptolepine,[220] whereas drugs that inhibit mtDNA polymerase-γ are the antiviral medications fialuridine,[221] zidovudine (AZT), d4T,[222] and ddI.[222] Drugs can also induce direct mtDNA damage through the production of reactive metabolites, or the generation of ROS or reactive nitrogen species; this leads eventually to a reduction in mtDNA levels, since damaged mtDNA molecules can be quickly degraded by mitochondrial endonucleases.[223] Troglitazone[224] and acetaminophen[225] can induce mtDNA strand breaks, and this eventually leads to a reduction of mtDNA levels. NRTIs can oxidise mtDNA, thus causing accumulation of the oxidised base 8-hydroxydeoxyguanosine.[222,226] In addition, NRTIs can produce mtDNA point mutations by misreading of 8-hydroxydeoxyguanosine by DNA polymerase-γ during mtDNA replication and/or impairment of DNA polymerase-γ repair capacity.[222,227]

Methotrexate, an antiproliferative and immunosuppressant drug commonly used to treat dermatological, rheumatic, and oncological diseases, has been long considered a classic and paradigmatic example of a drug able to produce DISH, since it can induce hepatic steatosis followed by hepatic fibrosis and, rarely, cirrhosis.[228] MTX has both type 2 diabetes and obesity as recognised risk factors.[228] An international cooperative study published in 1973 in patients with psoriasis was the first to identify these associations.[229] More recent retrospective studies have further supported this association, by showing a striking increase in fibrosis from 9% to 38% when comparing patients without and with these risk factors, respectively.[230,231] Based on these and other studies, the 2009 American Academy of Dermatology guidelines[232] and the consensus document on the use of MTX in psoriasis by Kalb et al[233] recommended the use of a substitute therapy, or alternatively, a strict follow-up, including the lately controversial practice of liver biopsies every 1000-1500 mg of cumulative MTX dose.[234,235] However, further systematic reviews and meta-analysis found no association between MTX and cumulative MTX dose, and this practice is no longer recommended in current guidelines.[236,237] Today, it is recognised that "pure" MTX-induced DISH is an uncommon adverse event, and that most cases of transaminase elevations during MTX treatment in obese and diabetic patients might be due to aggravation of NAFLD *per se* rather than a consequence of MTX toxicity.[4] This is further supported by the similarities in histological features and risk profile (eg, metabolic syndrome, older age, diabetes, and increased body mass index) between "pure" MTX liver disease and NASH, a fact more likely due to the existence of common pathogenic mechanisms.[238] In line with this, the analysis of a subgroup of a large series of more than 150 000 adults listed for liver transplantation due to end-stage liver disease revealed that only 0.07% of them had liver disease related to MTX therapy and that they shared phenotypic characteristics with NASH patients.[238]

The mechanisms by which MTX induces liver injury are not fully understood, but seem to be multifactorial in nature. MTX acts as a folate antagonist, and folate plays an essential role in one-carbon transfer reactions involving the formation of the methyl-group donor, *S*-adenosylmethionine (SAMe); the reduced availability of folate for homocysteine remethylation results in both SAMe deficiency and hyperhomocysteinaemia.[239] Insufficient amount of methyl-group donors as a pathogenic mechanism associated with NAFLD progression has been supported by experimental and clinical evidences. A transgenic mouse model with deletion of genes needed to produce SAMe from methionine develops NAFLD,[240] and a choline/methionine deficient diet leading to SAMe depletion is a common NAFLD model in rodents.[241] In addition, SAMe-mediated methylation of phosphatidylethanolamine is required for the correct VLDL assembly and secretion, a main pathway to export hepatic TGs.[242] Oxidative stress is another major mechanism involved in MTX toxicity associated with folate depletion, since folate bears antioxidant mechanisms. Indeed, folate is a ROS scavenger itself[243] and inhibits NADPH oxidase-mediated superoxide anion production, a process that triggers signalling cascades involved in HSC activation and fibrogenesis (see Section 2.5)[244]; this may explain why a folate-lowering agent like MTX aggravates preferentially hepatic fibrosis.[235,245-247] Folate deficiency also decreases activity of antioxidant enzymes via oxidation by homocysteine,[248,249] which may accelerate the impairment of antioxidant capacity that occurs progressively in NAFLD.[32-34] Homocysteine induces overproduction of hydrogen peroxide, a pro-oxidant that has been associated with apoptosis and activation of the pro-inflammatory transcriptional factor NF-κB.[250] Finally, folate deficiency may impair mitochondrial function, particularly at the mitochondrial respiratory chain level.[118,251] Actually, folate-deficient rats display aberrant changes of mtDNA deletion and mtDNA content, which depend on mitochondrial folate levels and oxidative DNA damage.[252] All these deleterious mechanisms might be potentiated in obese and/or diabetic patients, since some cross-sectional studies have suggested that these patients have *per se* reduced

folate levels,[253,254] a factor that has been suggested (but not proved) to be implicated in NAFLD progression.[255]

4.3 | Drug-induced phospholipidosis: lysosomal lipid metabolic disorder or autophagy dysfunction?

Many drugs cause impairment of lysosomal catabolism of phospholipids in liver, which leads to tissue accumulation of undegraded phospholipids in lysosomal inclusion bodies (lamellar bodies), a condition referred to as "drug-induced phospholipidosis".[256,257] These drugs include antibiotics, antiallergic, antidepressants, antipsychotics, antimalarial, and antiarrhythmic drugs.[256,257] Prototypical drugs that can induce phospholipidosis are chlorpromazine, chloroquine, amiodarone, fluoxetine, gentamicin, perhexiline, diethylamino-ethoxyhexestrol, amitriptyline, imipramine, ketoconazole, sertraline, tamoxifen, and loratadine.[256] Many of them are cationic, amphiphilic drugs containing a hydrophilic ring and hydrophobic regions (eg, amiodarone, gentamicin, perhexiline, and diethylamino-ethoxyhexestrol).[256] Two hypotheses have been proposed to explain this lysosomal abnormality.[257] The first contention suggests that these drugs bind directly to phospholipids in lysosomes, rendering indigestible phospholipid-drug complexes. The high affinity of cationic, amphiphilic drugs for lysosomes can be explained by the lysosomal acidic milieu together with the weak basic properties of these compounds. After entering the lysosome in their unionised form, lysosomotropic drugs are converted from the free base form to its ionised (non-permeable) one by protonation; consequently, the alkalinity of the lysosome rises, thus developing a less favourable pH for acidic lysosomal hydrolases.[258] The second hypothesis proposes the direct drug-induced inhibition of phospholipase A activity. This may be due to the inactivating binding to the enzyme, as has been shown to occur in a dose-dependent fashion with chlorpromazine and chloroquine.[257] Alternatively, phospholipase A may be inhibited indirectly via interaction of the drug with the lysosomal phospholipid bilayer, as shown for the erythromycin A derivatives azithromycin and gentamicin;[259] phospholipase A is activated by negatively charged lipids of the phospholipid bilayer, and embedding of such cationic drugs results in charge neutralisation.

Recently, an association has been proposed between phospholipidosis and NAFLD development, based upon studies in the phospholipase D1 deficient mice, which develop NAFLD associated with defects in hepatocyte autophagy.[260] A link between autophagy and NAFLD is just emerging (see Section 2.4). Dysfunctional autophagy is a common feature among obese, NAFLD patients, and may enhance TG accumulation in droplets due to failure to degrade them (lipophagy).[13,14] In addition, impairment of autophagy induces endoplasmic reticulum stress and mitochondrial dysfunction, two key events involved in hepatocyte apoptosis and ROS formation in NAFLD/NASH, and that can be aggravated by phospholipidosis.[261,262] Therefore, drug-induced phospholipidosis may potentiate lipid accumulation and pro-apoptotic/pro-oxidising pathways that drive NAFLD progression to NASH. In line with this, lysosomotropic basic, lipophilic compounds similar to those causing phospholipidosis impair autophagy.[263] Furthermore, autophagy has been proposed to be an adaptive response aimed to protect cells from drug-induced undue stress through drug clearance via macrophage-mediated removal of lamellar bodies containing lipid-drug complexes,[256] and therefore its inhibition may enhance rather than to attenuate drug hepatotoxicity. However, drug-induced phospholipidosis in humans has apparently few, if any, clinical or biochemical impact, provided it is an isolated feature not associated with other histopathological changes.[150,264]

5 | ALTERATIONS IN DRUG-METABOLISING SYSTEMS AS POTENTIAL FACTORS INFLUENCING NAFLD SUSCEPTIBILITY TO DILI

As a consequence of its pathogenic mechanisms, NAFLD results in altered activity and expression of a number of metabolising enzymes involved in drug disposal, and this could have implications for the safety of xenobiotics in these patients.[265-267] The presence of oxidative stress and inflammatory mediators like TNF-α and IL-6 in NAFLD has been implicated in the alterations of nuclear factors that regulate these metabolising systems, such as CAR, PXR, and PPAR-α. For example, SREBP1, which is upregulated in NAFLD (see Section 2.1), inhibits both PXR and CAR expressions.[268]

CYPs are the major enzymes involved in drug metabolism. Most of the chemical changes catalysed by CYPs deactivate the drug, thus attenuating its potential toxicity. However, CYPs can also "bioactivate" drugs, by converting them to reactive metabolites that can produce cellular damage.[269] Therefore, both upregulations and downregulations of CYP activity can be detrimental in terms of drug toxicity, depending on whether these CYPs are involved in activation or deactivation of the pharmacological compound, respectively.

Conflicting results have often been obtained in human NAFLD for changes in CYP activity/expression, particularly when the polymorphic nature of some of the members of this family was not considered. However, in some cases, a more consistent picture is emerging. In humans, CYP3A is the most abundant human hepatic CYP isoform, and has been implicated in the metabolism of approximately half of the clinically employed drugs.[270] The influence of NAFLD on the expression and activity of this CYP isoform has been studied in several experimental settings and in human beings.[271] CYP3A activity was shown to decrease with both steatosis severity[272] and NAFLD progression[271] in humans. Impairment of CYP3A activity due to reduced protein levels was reported in diabetic patients, a factor likely associated with the occurrence of NAFLD in these subjects.[273] Downregulation of CYP3A4 by the cytokine-mediated activation of the "Janus kinase" (JAK)/"signal transducer and activator of transcription" (STAT) signalling pathway in the course of the inflammatory response,[274] or by the "fibroblast growth factor 21" (FGF21)-mediated activation of the "mitogen-activated protein kinase" (MAPK) pathway,[275] has been proposed.

Enzyme activity/expression of other CYP isoforms relevant to drug metabolism seems to be also impaired in NAFLD. CYP1A2, which constitutes ~15% of total hepatic CYPs and is involved in the metabolisation of many anticoagulants, antidepressants, antihistamines, and anticancer agents,[276] was reported to be downregulated in NAFLD, both in rodent models[277,278] and in human liver tissue.[279-281] A similar finding was reported for CYP2D6, which constitutes only the ~4% of total CYP content, but it is involved in the biotransformation of more than 25% of clinically relevant drugs.[282] In leptin-deficient, obese (*ob*/*ob*) mice, the protein levels of Cyp2d22, the rat ortholog of human CYP2D6, are decreased,[283] and in human liver tissue, CYP2D6 protein levels and activity showed a trend towards lower values in NASH.[280] Conflicting results have been obtained in NAFLD for other CYP isoforms. This particularly holds true for the CYP2C family, which is responsible for the metabolism of ~12% of clinically useful drugs.[282] Either increasing or decreasing trends have been reported.[280,283] Interestingly, FFAs seems to be involved in the downregulation of members of the CYP2 family, since a reduction in activities and mRNA expressions of CYP2A6, CYP2B6, CYP2C9, and CYP2D6 has been reported in primary human cultured hepatocytes exposed to increasing concentrations of a mixture of oleic and palmitic acids.[279]

Unlike most CYP isoforms, CYP2E1 has enhanced activity and expression in NAFLD. Since this CYP isoenzyme is a main source of ROS, it is believed to worsen the oxidative stress associated with NAFLD, as well as the progression of NAFLD to NASH.[2] This preexistent CYP2E1 induction could play a significant role in hypersensitivity of obese individuals to acetaminophen toxicity, since it leads to an increase in the exacerbated production of the toxic acetaminophen metabolite NAPQI.[101] Similarly, halothane, isoflurane, losartan, ticlopidine, and omeprazole cause acute DILI more often in NAFLD, obese patients,[5,118,284] most likely due to CYP2E1 induction and the further formation of reactive intermediates.[2]

Phase II-metabolising enzymes seem to be also altered in NAFLD. UDP-glucuronosyltransferases (UGTs) are involved in glucuronidation of more than 40% of drugs of clinical use, including several nonsteroidal anti-inflammatory drugs, opioids, anxiolytics, antidepressants, and antipsychotics, with the UGT1A and 2B subfamilies being the most relevant in humans.[285] A former work utilising human liver microsomes reported on a decrease in UGT2B7 activity as well as in mRNA and protein levels in diabetic patients,[286] but further reports showed no change in Ugtb1 protein (rat) and UGT2B7 activity (humans) in NASH,[287,288] so that the issue remains controversial.

Glutathione *S*-transferases (GSTs) are responsible for the conjugation of many electrophilic drugs and drug metabolites with nucleophilic glutathione. The impact of NALFD on GST expression/activity appears to be isoform-specific in nature. GST activity was reported to be decreased in human liver samples of NAFLD patients,[289] a factor that may be aggravated by the depletion of glutathione in these patients.[33,289] Overall GST-μ protein expression was decreased in advanced NAFLD.[289] A decrease in GST-μ1 expression with the progression from "pure" steatosis to NASH was also reported.[290] Similarly, GST-μ1, 2, 4, and 5 were among the genes found to be downregulated in both simple steatosis[291] and NASH.[292]

Sulphotransferases (SULTs) account for the metabolism of less than one-fourth of conjugated therapeutic drugs, with acetaminophen, albuterol, terbutaline, and methyldopa being some examples.[293] *SULT1A2* is among the genes downregulated in human NASH.[291] Similarly, *SULT1A1* expression was significantly downregulated with NASH progression, though only in African Americans.[294]

6 | POLYPHARMACY IN OBESITY: A POTENTIAL CONFOUNDER

Although still controversial, there is accumulating evidence relating both obesity and NAFLD, a highly prevalent condition in obese patients, to an increased risk of DILI, at least for some drugs.[130] This has been reported not only in animal models of obesity but also in some observational studies and clinical trials with obese patients. For example, in a prospective study, pre-existing NAFLD in obese middle-aged patients was associated with a nearly fourfold increased risk of DILI.[5] However, obese patients could be more prone to develop DILI not only due to the enhanced intrinsic susceptibility of their steatotic liver but also as a consequence of this population's use, and potential abuse, of multiple drugs. Indeed, obesity is related to several comorbidities, such as metabolic (diabetes, dyslipidaemia), cardiovascular (hypertension), structural (arthrosis, arthritis), and mental health (depression, anxiety) diseases.[295] When compared to the population with normal weight, obese patients are more prone to use a higher number of prescription drugs, especially as they become older. Regarding the type of prescribed medications, anti-hypertensive drugs, lipid-lowering compounds, analgesics, proton pump inhibitors, anti-diabetogenic drugs, and drugs for treating hypothyroidism are among the more prevalent ones.[296-298] Noteworthy, more than 20% of obese adults were found to use ≥5 different drugs in an US study.[299] In addition, the treatment of the illnesses mentioned above usually demands long-term drug dispensation, which increases the risk of adverse effects, including hepatotoxicity.[300]

Polypharmacy represents a challenge to identify drug toxicity, since comedications were found to modify the mechanisms of DILI; certain combinations may decrease or increase the risk of hepatotoxicity, thus making it difficult to identify the causative agent.[301] Beyond this confounding factor, a number of drugs have been suggested to induce DILI more often in obese individuals, including volatile halogenated anaesthetics, acetaminophen, losartan, ticlopidine, omeprazole, and MTX.[5,102,118,130,284,302,303]

Some of these drugs may be more hepatotoxic in the obesity context due to the increased activity of several CYPs that can metabolise de original drug to more toxic metabolites, such as CYP1A2, CYP2C9, CYP2D6, and CYP2E1.[2,304] For example, higher CYP2E1 activity could explain why drugs such as acetaminophen and halothane seem to be more hepatotoxic in the obesity context, since CYP2E1 transforms these drugs into the highly reactive metabolites NAPQI and trichloroacetyl chloride, respectively.[2,305]

Currently, the role for metabolic factors in the pathogenesis of idiosyncratic DILI is poorly understood, and the assessment of large, prospective cohort of patients included in different DILI registries has allowed its study.[306,307] Based on these and other recent studies, evidence is just emerging that components of the metabolic syndrome have an impact on DILI presentation and outcome. For instance, a study based on DILI cases from the Spanish DILI registry showed that, despite dyslipidemic patients have significantly better outcomes in terms of severity and fatality, patients with metabolic risk factors (diabetes and dyslipidaemia) are more prone to suffer from a persistent liver injury, and diabetic patients have longer treatment and latency time periods prior to DILI appearance.[306] In addition, a study by the Drug-Induced Liver Injury Network (DILIN) showed that, whereas diabetes does not appear to increase the risk of hepatotoxicity, it has been associated with an increased risk of DILI-induced mortality.[307] Finally, a study by the Acute Liver Failure Study Group suggested that obese patients would have significantly poorer outcomes when developing acute liver failure associated with DILI.[308] Unfortunately, studies analysing whether the differences in DILI features in obesity and diabetes are indeed explained by NAFLD/NASH as an underlying chronic liver disease are still lacking. At least for DILI outcome, the possibility is, however, likely. A prospective study by DILIN in patients with both DILI and a pre-existing chronic liver disease (included NAFLD among the more prevalent ones) showed that there is a tendency for these patients to develop a more severe liver injury, and that these patients are at increased risk of DILI-related mortality compared to individuals without liver disease (16% vs 5%, $P < 0.001$);[307] the liver-related mortality was, however, not augmented in these patients, suggesting that other comorbidities that are more prevalent in that population, as for example those related to metabolic syndrome, may have been a contributing factor to the extra mortality.[100]

7 | CONCLUSIONS AND FUTURE CHALLENGES

Although still controversial due to the fact that methodological challenges and sample size limitations preclude a robust analysis, there is accumulating clinical evidence linking NAFLD with an increased risk or poorer outcome of DILI, independently of other confusing factors. This seems to apply with more certainty to certain types of intrinsic DILI (eg, that induced by acetaminophen, MTX, and volatile anaesthetics) than to idiosyncratic DILI, where clinical evidence is even more scarce and circumstantial.

On the contrary, it has been well-documented that many drugs can cause a NAFLD/NASH-like syndrome by triggering metabolic and damaging factors similar to those causing NAFLD/NASH, including diabetes mellitus, impairment of lipid exportation, mitochondrial-driven oxidative stress, and fibrogenesis, among other harmful effects. When a drug cause "pure" steatosis via these mechanisms, the phenomenon is referred to as DIS, whereas when the aggravation of an underlying steatosis or *de novo* steatohepatitis occurs after drug exposure, the syndrome is known as DISH.

Nonalcoholic fatty liver disease patients are often obese, and polypharmacy is a common practice among these individuals, due to the high prevalence of chronic diseases; this is a potential confounder, since hepatotoxicity may be related to multiple drug exposure rather than to obesity.

Nevertheless, it is believed that patients with NAFLD could be more prone to develop DISH. The reason for this increased susceptibility is multifactorial, but usually reflects the triggering by the drug of similar steatogenic, inflammatory, and/or fibrotic pathomechanisms that are in operation in NAFLD, or changes in drug detoxification systems. Although these potential mechanisms are plausible, there are still very little high quality clinical data to support them.

Robust evidences to confirm a higher incidence of hepatotoxicity in obese and NAFLD patients are lacking. However, physicians should be aware that many drugs, herbals compounds, and dietary supplements are consumed by obese patients in order to manage different associated disorders or to prompt weight loss. Therefore, obese individuals are more likely to develop DILI due to the higher exposure to drugs, irrespective of whether they have or not an intrinsic higher susceptibility to DILI due to their diseased livers.

Considering the growing epidemic of obesity and the high prevalence of NAFLD in the general population, the study of the impact of DILI warrants extensive investigation. Particularly, efforts should be made to further characterise specific mechanisms of DILI in patients with underlying NAFLD, and to identify risk factors for this condition to prevent its development and aggravation. From the everyday clinical practice standpoint, it seems a good practice to limit (when possible) the number of prescribed medications in obese/NAFLD patients, to advise against over-the-counter self-medication, and to introduce drugs in a stepwise manner. Also, a closer follow-up and biochemical monitoring should be advised when drugs with known hepatotoxic potential are to be administered in patients with NAFLD or whatever risk factors for this condition, including diabetes and obesity.

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