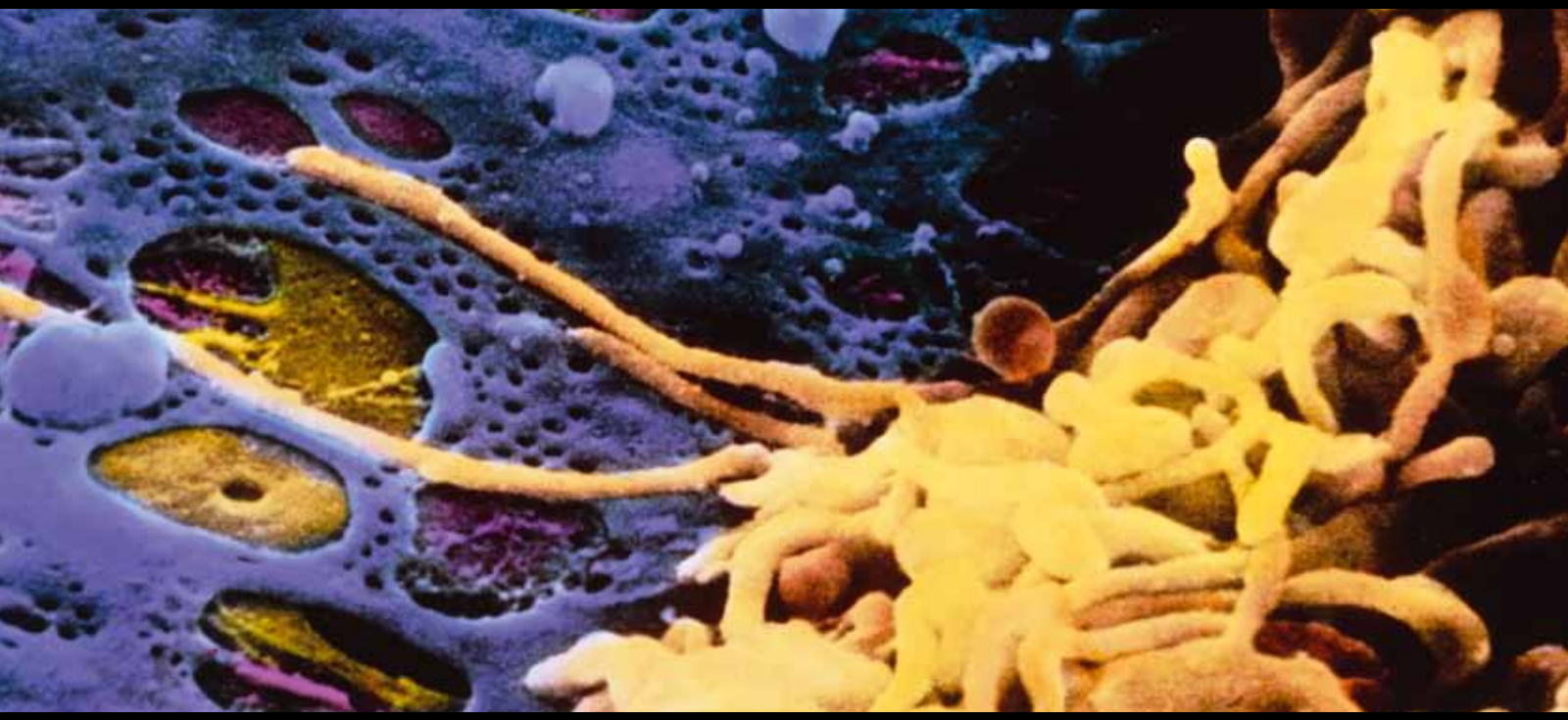
International Journal of Hepatology

Multiple Factors Involved in Nonalcoholic Hepatitis Pathogenesis

Guest Editors: Manuela Neuman, Nir Hilzenrat, Lawrence Cohen, and Robert E. Winkler



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Editorial Board

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Editorial

Multiple Factors Involved in Nonalcoholic Hepatitis Pathogenesis

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Nonalcoholic fatty liver disease (NAFLD) is the most common type of liver disease, with etiologies as varied as its presentations. This special issue of the International Journal of Hepatology examines some of the multiple factors involved in the pathogenesis of this chronic liver condition, ranging from inflammation, aberrant lipid metabolism, drug-induced liver injury, and babesiosis.

NAFLD is marked by a high degree of inflammation. K. Tajiri and Y. Shimizu (2012) describe the role of natural killer T (NKT) cells in NAFLD pathogenesis. The role of these inflammatory cells changes throughout the course of the disease, progressing from being initially protective during steatosis, to acting as progression factors during fibrosis. The interaction between NKT cells and the glycolipid antigen-presenting CD1d molecule is believed to play a key role in this process. NKT cells are lipid antigen-specific lymphocytes that produce high levels of T helper 1 response proinflammatory cytokines. NKT cells also have anti-inflammatory properties through T helper 2 polarization, thus playing a key role in modulating NAFLD. CD1d was shown to lead to hepatic inflammation through antigen presentation to NKT cells. In turn, this process is associated with insulin resistance and altered lipid metabolism. Thus, manipulating NKT cells may provide a therapeutic avenue in NAFLD (K. Tajiri and Y. Shimizu, 2012).

In the "two-hit theory" model, fatty acids and triglycerides accumulate in the liver, leading to inflammation, oxidative stress, and mitochondrial dysfunction, ultimately causing liver damage. M. Enjoji et al. (2012) review this mechanism from the point of view of cholesterol metabolism, while discussing its management as a potential treatment for NAFLD. NAFLD is marked by dysfunctional cholesterol metabolism, with cholesterol accumulation taking place as a result of *de novo* synthesis and plateauing excretion observed even if dietary intake is high. Based on findings from a Japanese population, the highest cholesterol intake was observed in nonobese NAFLD patients, while obese NAFLD patients had higher cholesterol intake than healthy volunteers. Excess liver cholesterol and its metabolite oxysterol are associated with steatosis and activation of the liver X receptor-α-sterol regulatory element-binding protein-1c pathway. Inhibiting cholesterol absorption or reducing dietary cholesterol intake may help reduce hepatocytic cholesterol accumulation, thus presenting valid cholesterol management techniques that may aid in the prevention of cholesterol overload-associated NAFLD (M. Enjoji et al., 2012).

Diets of obese patients are generally high in fat, particularly saturated fats and Ω-6 polyunsaturated fatty acids. Obesity is marked by a high volume of adipose

tissue, which is associated with increased production of monocyte chemoattractant protein (MCP-) 1. In turn, MCP-1 attracts macrophages, which leads to the development of an inflammatory cascade associated with high levels of proinflammatory cytokines, among which tumor necrosis factor-α is associated with insulin resistance and impaired glucose tolerance (P. Guturu and A. Duchini, 2012). Free fatty acids accumulate in the liver when a greater amount is delivered to the organ than that which is metabolized, particularly when coupled with deregulated *de novo* synthesis. P. Guturu and A. Duchini (2012) further argue that the type of free fatty acids is more important than their quantity with respect to the development and progression of NAFLD. Of particular concern were saturated fatty acids.

Paraoxonase (PON) is an esterase associated with the hydrolysis of various xenobiotics. Serum PON1 is synthesized in the liver. Serum PON1 levels are low in nonalcoholic steatohepatitis (NASH) patients. Furthermore, PON can be inactivated by oxidative stress, while decreased antioxidative potential may facilitate the evolution of NAFLD to NASH. *In vitro* experiments have shown that proinflammatory cytokines decrease PON1 mRNA levels, while decreased PON1 activity is related to the degree of liver injury in patients (O. Hussein et al., 2012). A methionine choline deficient diet (MCDD) was associated with increased liver weight in rats, characterized by substantial increases in hepatic triglycerides and cholesterol levels. Furthermore, MCDD is associated with decreased PON activity, as well as increased malondialdehyde levels (indicator of oxidation) in both serum and liver. Serum activity of PON was increased when animals were treated with metformin (M), rosiglitazone (R), ezetimibe (E), valsartan (V), M + R, R + M + V or R + M + V + E. In contrast, liver PON activity was only increased in animals treated with R, E, V, R + M + V and R + M + V + E. O. Hussein et al. (2012) show that insulin sensitizers decrease oxidative stress in both serum and liver.

The management of human immunodeficiency virus (HIV) infection is dependent upon the efficacy and safety of the different highly active antiretroviral therapy (HAART) regimens used. While hepatotoxicity is often present in HIV patients and generally improves upon HAART initiation, the intrinsic ability of certain antiretrovirals to cause hepatotoxicity is a factor that limits their usefulness. Hepatotoxicity is recognized as main type of adverse drug reaction (ADR) associated with HAART. Baseline hepatotoxicity, alcohol consumption, preexisting viral hepatitis, and old age are risk factors for developing drug-induced hepatotoxicity in HIV-positive patients (M. Neuman et al., 2012). Hepatotoxicity can manifest itself as hepatocellular injury, cholestasis, a mixed pattern of cytotoxic and cholestatic injury, or, less commonly, steatosis. Another common type of ADR affecting the liver is hypersensitivity reaction (HSR). Furthermore, gastrointestinal intolerance and pancreatitis are recognized as other treatment-limiting ADRs associated with HAART. The management of these reactions is paramount as they can lead to treatment non-adherence, which in turn can give rise to viral resistance. The authors conclude that therapeutic and drug monitoring of ART, using laboratory identification of phenotypic susceptibilities, drug interactions with other medications, drug interactions with herbal medicines, and alcohol intake might enable a safer use of this medication (M. Neuman et al., 2012).

J. C. Pritchett et al. (2012) describe in detail the association between drug-induced hypersensitivity reaction (DIHS), with a particular focus on both cutaneous and hepatic symptoms, and the reactivation of latent human herpes virus (HHV-) 6 infection. A "true" HSR is described by the triad of rash, fever, and organ involvement. The internal organ most often affected in the liver. Drug-induced liver injury presents itself as anomalies in liver function tests or hepatomegaly. Cutaneous reactions often present as maculopapular rash or generalized erythematous rash. HHV-6 is a lymphotropic DNA virus infecting close to 100% of the population in the first 2-3 years of life. Following the initial infection, HHV-6 remains dormant in the body and can become reactivated under conditions of immunosuppression (J. C. Pritchett et al., 2012). DIHS cases associated with HHV-6 reactivation show a more severe set of symptoms than cases without viral reactivation. Common symptoms of DIHS include fever, edema, lymphadenopathy, hypereosinophilia, and atypical lymphocytes, as well as elevated serum aminotransferase levels, cholestatic hepatitis, renal insufficiency, and multiorgan failure. Hypogammaglobulinemia is also widely observed, while elevations in serum IgG levels often indicate viral reactivation. Antiepileptics, sulfonamide antibiotics, allopurinol, and nonsteroidal anti-inflammatory drugs are some of the pharmaceutical agents associated with DIHS development. Symptoms of HSR generally improve following the discontinuation of the offending agent, but may relapse after 2-3 weeks, which coincides with the detection of viral reactivation (J. C. Pritchett et al., 2012).

Human babesiosis is transmitted through tick (*Idoxes* sp.) bites or through transfusion of infected blood. Symptoms of babesiosis include hepatitis, hydrothorax, pneumonia, myocarditis, splenomegaly, glomerulonephritis, hematuria, and hemolytic anemia. Individuals most at risk of developing this condition are neonates and the elderly, as well as splenectomised, immunocompromised, and AIDS patients. If not adequately treated, babesiosis can be fatal (H. S. Oz and K. H. Westlund, 2012). Hamsters are the preferred animal model in which to study babesiosis development and drug treatment. Treatments include the clindamycin/quinine combination, azithromycin/quinine, and atovaquone. Symptoms of babesiosis include low hemoglobin, high bilirubin, and elevated aspartate aminotransferase, creatinine, and hemoglobinuria levels. H. S. Oz and K. H. Westlund (2012) advocate for increased awareness, as well as improved diagnostic and therapeutic tools. An important preventive tool would be to screen blood donors for infection.

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Review Article

Etiopathogenesis of Nonalcoholic Steatohepatitis: Role of Obesity, Insulin Resistance and Mechanisms of Hepatotoxicity

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Incidence of nonalcoholic fatty liver disease is increasing with an estimated prevalence of 20–30% in developed nations. This is leading to increased incidence of chronic liver disease, cirrhosis, and hepatocellular cancer. It is critical to understand the etiology and pathogenesis of any disease to create therapeutic targets and develop new treatments. In this paper we discuss the etiology and pathogenesis of nonalcoholic steatohepatitis with special focus on obesity, role of insulin resistance, and molecular mechanisms of hepatotoxicity.

1. Introduction

The term non-alcoholic fatty liver disease (NAFLD) refers to the spectrum of diseases characterized by fatty infiltration of the liver ranging from steatosis, steatohepatitis, or cirrhosis. Hepatic steatosis with or without hepatitis, in the absence of alcohol use, was first described by Ludwig et al. and is referred to as non-alcoholic steatosis or non-alcoholic steatohepatitis (NASH) [1]. NAFLD is a common disease with an estimated prevalence in unselected population of developed nations around 20–30% [2]. The rapid rise in the incidence of the NAFLD might be explained by the recent epidemic of obesity and metabolic syndrome, which are manifested at hepatic level as NAFLD [3–5]. Most patients with NAFLD have simple hepatic steatosis without progression to steatohepatitis and fibrosis. However, in 2-3% of patients, NAFLD can progress to NASH that can eventually cause progressive fibrosis and lead to cirrhosis and related complications including hepatocellular carcinoma [3, 6, 7]. Once patients with simple steatosis develop NASH, up to 50% of them could develop advanced fibrosis [8].

A "two-hit hypothesis" was then proposed to explain the pathogenesis and progression of NAFLD, where the first hit causes accumulation of excess triglycerides in the liver leading to simple steatosis and the second hit causes the steatosis to progress to inflammation and fibrosis [9, 10]. The two-hit hypothesis was recently questioned as it was suggested that the hepatic accumulation of triglycerides in the liver might be instead protective towards further hepatic damage [11, 12].

Development of obesity or metabolic obesity, defined by isolated increase in visceral fat in people who are not obese, is often seen as the starting point for development of NAFLD [13], leading to the cascade of events ending in the formation of hepatic steatosis. How does increased visceral fat lead to increased fat accumulation in liver? What is the role of insulin resistance? What are the cellular and molecular mechanisms involved? What are the chemical mediators involved?

2. Factors Contributing to Development of Obesity

Development of obesity or metabolic obesity is seen as the initial step in the development of metabolic syndrome and non-alcoholic fatty liver disease. Obesity is likely due to contributions from multiple factors including but not limited to impaired central appetite regulation, genetic predisposition, and contribution from dietary factors and lack of physical activity [13].

Central nervous system plays a critical role in regulation of body weight via a negative feedback mechanism. Increase in body fat stores alert the appetite center in hypothalamus leading to appetite control and adipose tissue homeostasis [14]. Insulin and leptin are considered as prime mediators of this mechanism [15]. In overweight individuals, the amount of leptin in circulation is high but they develop resistance to leptin and so their appetite is not well controlled, leading to failure of the negative feedback mechanism [16, 17]. The molecular mechanisms leading to leptin resistance and its role in the development of obesity are discussed in detail elsewhere [18].

While increased caloric intake definitely has a critical role in the development of obesity, there has been considerable interest about various dietary components and their relative contribution to the development of obesity. Increased fructose consumption has been shown as a risk factor for development of NASH and that increased fructose consumption correlates with the severity of fibrosis in patients with NAFLD [19, 20]. The explanation is that fructose consumption leads to obesity or metabolic syndrome and that NAFLD is the hepatic manifestation but it is interesting to note that increased fructose consumption is an independent risk factor for development of fatty liver irrespective of metabolic syndrome [21, 22].

Patients with NALFD are shown to have increased percentage of dietary fat content and also get much lower percentage of their calories from fruits [23, 24]; not only total fat content in the diet but also the composition of fat has seen considerable interest in recent times. Current literature supports the fact that diet of patients with NAFLD might be high in saturated fatty acids and n-6 polyunsaturated fatty acids and low in n-3 polyunsaturated fatty acids and monounsaturated fatty acids [25–27]. Though much of the focus has been on diets with high percentage of fats, diet, rich in synthetic disaccharides have also been shown to induce hepatic fibrosis in rats [28].

Gut microbiota and its interaction with the consumed nutrients have also been the focus of research and microbiota could have a possible role in obesity by their influence on amount of nutrients absorbed. Several mechanisms were proposed including altered gut permeability and digesting the ingested polysaccharides thereby increasing the amount of energy absorbed [29].

While low physical activity might not directly contribute to NAFLD in otherwise healthy patients, increase in physical activity coupled with weight loss has been shown to improve liver profile in overweight patients with chronic liver disease [30].

3. Obesity Is a Proinflammatory State: Results in Insulin Resistance

Two types of adipose tissue are recognized in humans: brown adipose tissue and white adipose tissue. brown adipose tissue, mainly found in neonates, helps with heat production and has a protective effect against hypothermia. White adipose tissue, present in adults, consists of adipocytes, endothelial cells, fibroblasts, leukocytes, and bone marrow derived macrophages. The only function of white adipose tissue was initially thought to be energy store. Instead, new research is pointing towards adipose tissue having a more complex endocrine function mediated by the production of numerous proinflammatory cytokines called adipocytokines [31, 32]. It should also be noted that not all white adipose tissue might be the same; increasing volume of visceral adipose tissue and their production of pro-inflammatory cytokines seems to play an important role in development of insulin resistance compared to subcutaneous adipose tissue [33].

Adipocytokines produced by the adipose tissue include adiponectin, leptin, resistin, visfatin, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1; also known as CCL2 or CC-chemokine ligand 2), plasminogen activator inhibitor-1, angiotensinogen, retinol-binding protein-4, and serum amyloid A [34–38]. Adipocytokines are not exclusively produced by adipocytes but some, like TNF-α, are mainly produced by the macrophages in the adipose tissue. MCP-1 produced by adipocytes is a major factor contributing to macrophage recruitment to the adipose tissue [39]. Adipose tissue in obese individuals is associated with increased macrophage activity, which is responsible for almost all of the TNF-α and major part of the IL-6 expressed by the adipose tissue [40]. Thus, obesity is state of chronic inflammation characterized by abnormal cytokine (adipocytokines) production and activation of pro-inflammatory signaling pathways [41]. The following sequence of events has been proposed: development of obesity leads to increased volume of adipose tissue, followed by increased production of MCP-1 by the adipocytes, which attracts more macrophages to the adipose tissue itself. Once the macrophages in the adipose tissue are activated, a self-perpetuating inflammatory cascade is triggered by secretion of pro-inflammatory cytokines like TNF-α and IL-6 [31].

As noted above, the distribution of fat is also important in the pathogenesis of metabolic syndrome and visceral adipose tissue is considered a better indicator of insulin resistance and cardio vascular disease [42]. This could be due to either release of greater amounts of adipocytokines by visceral fat tissue compared to subcutaneous tissue in obese individuals [43] or could be due to the fact that visceral fat has direct access to portal circulation and thereby having stronger impact on liver [34].

Sodium salicylate an antiinflammatory medication has been used to decrease glycosuria associated with diabetes many years before the discovery of association between type-2 diabetes mellitus and increased inflammatory markers [44]. Since then more studies have shown increased levels of inflammatory mediators like C-reactive protein, interleukin-6, and plasminogen activator inhibitor-1 in patients with type-2 diabetes [45–48].

Obesity is a proinflammatory state with high levels of circulating pro-inflammatory cytokines and diabetes is also a state of chronic inflammation; how are these two conditions related? The answer to this question was provided by a study that has shown that TNF-α can induce insulin resistance in obese rodents and also that neutralization of TNF-α

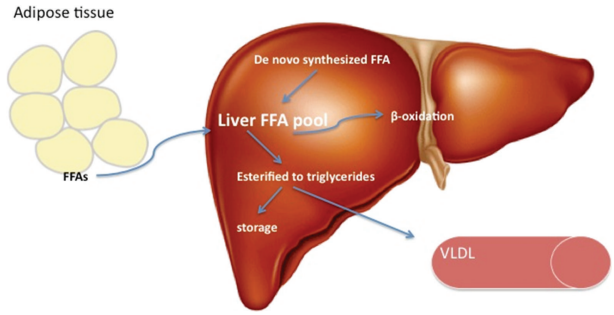

Figure 1: Lipid metabolism in liver. Liver FFA pool is derived from uptake of circulating FFAs and de novo synthesis. The FFAs are then either oxidized or esterified into triglycerides. Triglycerides are then released into circulation as VLDL or stored as vacuoles leading to hepatic steatosis.

can decrease the insulin resistance with resulting increased peripheral uptake of glucose [49]. Since then similar findings of elevated TNF-α were also found in humans with increased insulin resistance and impaired glucose tolerance [50–52].

High TNF-α levels can induce insulin resistance in animal models through the activation of I-kappa-B-kinase-β (IKKβ)/nuclear-factor-kappa-B (NF-κB) and Jun N-terminal kinase (JNK) pathways [53]. JNK can cause insulin resistance through the phosphorylation of serine residues in insulin receptor substrate-1 (IRS-1) [54, 55]. IKKβ activation leads to activation of NF-κB via transcription and subsequent increased expression of markers and mediators of inflammation causing insulin resistance. Increasing obesity will lead to increased production of adipocytokines like TNF-α, IL-6 that lead to perpetuating cycle of JNK, and NF-κB activation leading to worsening insulin resistance. Detailed review of signaling pathways associated with insulin resistance due to inflammation was discussed elsewhere [56].

4. Mechanisms of Hepatic Fat Accumulation: Role of Insulin Resistance

The liver plays a key role in lipid metabolism; its role includes uptake and de novo synthesis of free fatty acids (FFAs) followed by conversion of FFAs into triglycerides by esterification. These triglycerides are then released into the circulation as very low-density lipoproteins (VLDL) or stored as triglyceride vacuoles in hepatocytes [57]. FFAs that are not esterified into triglycerides will be metabolized in the liver by β-oxidation [58] (Figure 1).

In NAFLD, there is disruption of this cascade of events since the amount of FFAs delivered/synthesized in the liver exceeds its oxidative capacity. This leads to increased triglyceride synthesis and as the triglyceride synthesis continues to rise and exceed the amount that can be released as VLDLs, triglycerides accumulate in hepatocytes causing hepatic steatosis [58, 59]. This step of development of hepatic steatosis is considered as "first hit" in the pathogenesis of NAFLD [60, 61].

This raises the questions: what causes increased availability of FFAs to liver, is it increased delivery or is it due to increased de novo synthesis of FFAs in liver? What is the role of insulin resistance? Other than increased FFA availability, does disruption of other mechanisms like β-oxidation or VLDL synthesis contribute to hepatic lipid accumulation?

As much as 59% of hepatic triglyceride content is derived from free fatty acids and only 26.1% of the hepatic triglyceride was due to de novo synthesis as shown in this study, where isotope tracers were used to track hepatic fat content [62]. This increased delivery of FFAs to liver is due to insulin resistance because insulin resistance increases the total serum FFAs levels due to increased lipolysis in peripheral adipose [63, 64]. Cluster differentiation 36 pathway activation leads to increased FFA uptake by liver [65]. Increased expression of this pathway is seen in patients with insulin resistance and is implicated in pathogenesis of NAFLD [66]. Defective oxidation of the FFAs and dysfunctional VLDL synthesis were also thought to be a key factor in pathogenesis of NAFLD [67]. Though delivery of increased amounts of FFAs beyond the capacity of liver metabolism seems to be the primary cause of hepatic fat accumulation, it should be noted that disruption of other pathways could have a role and more importantly that insulin resistance is implicated in most of these mechanisms [13, 68, 69].

As discussed earlier, increased visceral adipose tissue is a risk factor for development of metabolic syndrome and visceral adipose tissue is more prone to insulin resistance when compared to peripheral fat. Insulin resistance in visceral fat leads to increased lipolysis and subsequent delivery of FFAs to the liver increases in an exponential manner due to its direct drainage into portal circulation [70].

5. Molecular Mechanisms and Mediators of Hepatotoxicity from Excess Lipids

Hepatic steatosis [61] was considered as first hit in the pathogenesis of NAFLD but it later became clear that accumulation of triglycerides is actually protective and that free fatty acids are the toxic substances that lead to steatohepatitis and fibrosis [71, 72]. Diacylglycerol acyltransferase 2 (DGAT2) is an enzyme responsible for esterification of FFAs into triglycerides; inhibition of triglyceride synthesis by genetically deleting this enzyme has reduced hepatic steatosis in mouse model but made fibrosis worse due to FFA toxicity [11]. Interruption of triglyceride synthesis could be the initiating event for FFAs-mediated lipotoxicity (cellular toxicity due to accumulated fat) in liver cells [73]. As such, hepatic triglycerides are called the "good fat" and FFAs are called the "bad fat" [74].

This raises the question: are all free fatty acids the same? Studies that looked at the composition of hepatic and circulating free fatty acids have revealed that patients with NAFLD have elevated levels of oleic acid (a monounsaturated fatty acid, MUFA) and palmitic acid (a saturated fatty acid, SFA) [75, 76]. On the other hand polyunsaturated fatty acids are not shown to be toxic to hepatocytes and could be protective in patients with NAFLD [13, 77]. Further information on this topic was provided by experimental studies that looked at the role of stearoyl-CoA desaturase-1

(SCD1), the enzyme that converts SFA to MUFA. Increased expression of SCD1 leads to more MUFA production, which was then incorporated into triglycerides and thus leading to well tolerated simple hepatic steatosis. But inhibition of SCD1 leads to accumulation of SFA and subsequent development of hepatocytes apoptosis and steatohepatitis [74, 78]. So for disease progression in NAFLD, the type of FFA accumulated is as important or may be more important than the quantity of FFAs accumulated in the hepatocytes [79].

Apoptosis is a process of programmed cell death and is considered an important mechanism in the progression of NAFLD [80–82]. Apoptosis is the key pathogenic mechanism noted in the biopsy specimens of the patients with NASH and in the spectrum of NAFLD presence of apoptosis distinguishes patients with simple steatosis from patients with NASH [83]. The extent and severity of the apoptosis correlates with the degree of inflammation and fibrosis, so patients with higher apoptosis rates will have advanced stage fibrosis [80]. Cytokeratin-18 fragments are markers for apoptotic hepatic cells and their circulating levels correlate with the severity of the fibrosis providing further evidence that apoptosis is an important feature of NASH [84]. Apoptosis mediated by FFAs is called lipoapoptosis [85] and the mediators of lipoapoptosis are further discussed here. Apoptotic pathways can be activated via extrinsic pathway mediated by receptors on cell surface or via intrinsic pathway mediated by intracellular organelles [86].

5.1. Toll-Like Receptors. Toll-like receptors (TLRs) are pattern recognition receptors that can identify pathogen-associated molecular patterns and in response, they activate the immune system via pro-inflammatory signaling pathways [87]. Saturated fatty acids like palmitic acid can activate TLR4-mediated upregulation of NF-κB with subsequent increased production of adipocytokines like TNF-α and IL-6 [88]. Decreased expression of TLR4 in mutant mouse model is shown to be protective against development of NASH [89].

In an experimental dextran sulfate sodium (DSS) colitis mouse model, mouse fed with high fat diet and DSS had increased levels of bacterial lipopolysaccharides in portal circulation, increased expression of TLR4, and severe hepatic inflammation when compared to controls [90]. TLR4 might be the crucial link in the gut microbiota-liver axis related to progression of NASH.

5.2. Death Receptors. Death receptors are cell surface receptors from the tumor necrosis factor family of receptors and play critical role in extrinsic apoptotic pathways [91]. The death receptors and their ligands expressed in liver include Fas, tumor necrosis factor receptor 1 (TNF-R1) and TNF-related apoptosis-inducing ligand receptor 1 and 2, TRAIL-R1 and TRAIL-R2, Fas ligand (FasL), TNF-α, and TRAIL [92]. In extrinsic pathway, death ligands activate their receptors forming adeath complex that in turn activates caspase-8 leading to apoptosis (caspases are death-inducing proteolytic enzymes). Overexpression of these death receptors and subsequent apoptosis is an important feature of NASH [80].

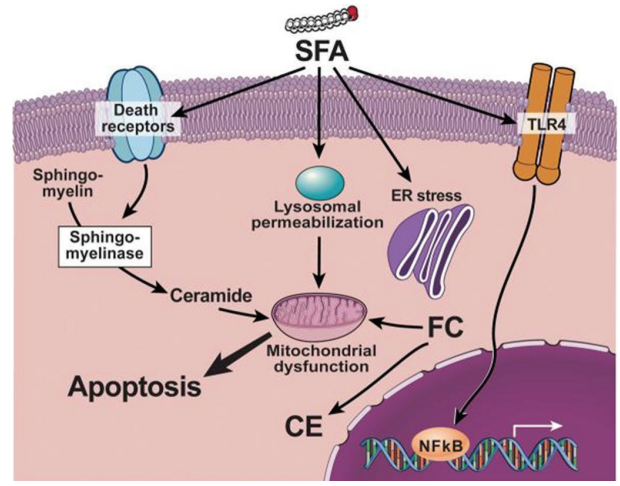

Figure 2: Reference [74]. FFA may activate several signaling pathways of apoptosis including upregulation and increased number of death receptors such as Fas and TRAIL receptor 5 (DR5), at the level of the plasma membrane, lysosomal permeabilization, and ER stress both coupled to mitochondrial dysfunction resulting in activation of the mitochondrial pathway of apoptosis. These toxic fatty acids may also activate TLR4 signaling resulting in up-regulation of several pro-inflammatory cytokines. Finally, other lipid types such as free cholesterol (FC) and ceramide may induce mitochondrial dysfunction and activate the mitochondrial pathway of apoptosis. Abbreviations: FFA: free fatty acids; SFA: saturated fatty acids. MUFA: monounsaturated fatty acids, FC: free cholesterol, CE. cholesteryl-ester; ER: endoplasmic reticulum.

5.3. Mitochondrial Dysfunction and Reactive Oxygen Species. Reactive oxygen species (ROS) are a group of free radicals derived from molecular oxygen, and oxidative stress refers to the cellular damage done by these free radicals [93]. ROS are formed via oxidative reactions in intracellular organelles and mitochondria are a principal source of ROS, but in a normal healthy cell, the levels of ROS are very low due to various anti-oxidant defense mechanisms [94, 95].

In normal healthy subjects, mitochondrial β-oxidation is the preferential way to dispose of the FFAs by liver [96]. But in NAFLD, there is an excess of FFAs, and increased β-oxidation by mitochondria leads to increased delivery of electrons to the electron transport chain causing overreduction of electron transport chain and formation of ROS [95]. Mitochondrial DNA is vulnerable to damage by ROS; increased generation of ROS leads to damage of mitochondrial DNA leading to mitochondrial dysfunction, which further potentiates ROS formation [97].

Intracellular stress caused by accumulation of ROS leads to mitochondrial dysfunction resulting in release of proapoptotic proteins like cytochrome c into the cytosol. Cytochrome c then combines with apoptotic-protein activation factor-1 (Apaf-1) and caspase 9 to form an activation complex called the apoptosome. Apoptosome activates the downstream caspases 3, 6, and 7 to complete the final steps of apoptosis [98].

5.4. Lysosomal Permeabilization. Mitochondrial dysfunction is considered the central pathophysiological process

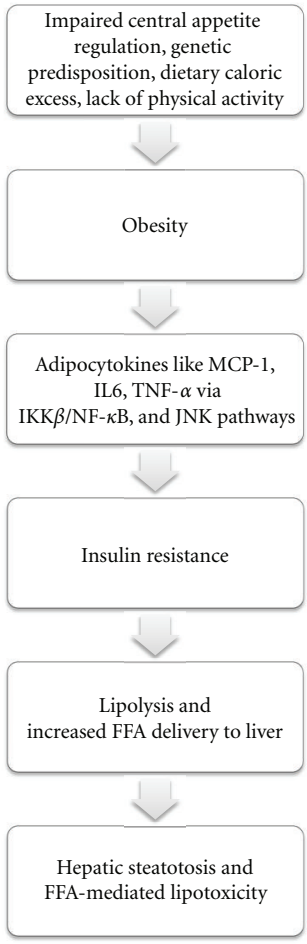

Figure 3: Development and progression of nonalcoholic fatty liver disease. Abbreviations: tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), I-kappa-B-kinase-β (IKKβ), nuclear-factor-kappa-B (NF-κB), Jun N-terminal kinase (JNK), and free fatty acids (FFAs).

contributing to progression of NALFD to NASH, and the quest to identify molecular mechanisms leads to the identification of lysosomal-mitochondrial axis in FFA-induced lipotoxicity and the potential role of lysosomal permeabilization in the progression of NASH [99]. In this study, liver cells were fed with high fat diet and observed in real time, lysosomal permeabilization and cathepsin B (a lysosomal protease) release in the cytoplasm occurred much earlier than mitochondrial dysfunction and cytochrome c release into the cytosol. Also inhibition of cathepsin B was protective against FFA-induced lipotoxicity [99]. Cathepsin B is also implicated in progression of liver fibrosis by its role in activation of hepatic stellate cells and aiding their differentiation into myofibroblasts [100].

5.5. Endoplasmic Reticulum Stress. Endoplasmic reticulum (ER) is an intracellular organelle with multiple important functions like protein synthesis, lipid synthesis, and so forth. When ER is put under stress (ER stress), it responds by a mechanism called unfolded protein response (UPR) [101]. UPR is designed to protect ER from the stress induced by various sources like viral infections, alcohol, or FFAs. But when the duration of ER stress is prolonged then UPR might not be able to cope and leads to apoptosis [102, 103]. Further information about the role of ER stress is addressed in this in vitro study where saturated fatty acid palmitic acid was able to induce ER stress and lead to apoptosis of hepatic cells [104].

Other mechanisms by which FFAs can lead to apoptosis include mitochondrial dysfunction via c-Jun N-terminal kinase (JNK) activation, pro-apoptotic protein Bax-induced mitochondrial permeabilization, free cholesterol-mediated ER stress, and ceramide-mediated apoptosis induced by death ligands like TNF/FAS [74, 105] (Figure 2).

Insummary, impaired central appetite regulation, genetic predisposition, dietary caloric excess, and lack of physical activity contribute to development of obesity. Obesity is a pro-inflammatory state and leads to insulin resistance via adipocytokines. Insulin resistance leads to increased lipolysis and exponentially high delivery of free fatty acids to liver. Accumulation of FFAs leads to hepatic steatosis and FFA-mediated lipotoxicity that eventually progresses to fibrosis/cirrhosis (Figure 3).

In conclusion, NAFLD is increasing in prevalence and could become the most common cause of chronic liver disease in the near future in the Western world. It is very important to understand the complex molecular mechanisms and the mediator involved to develop new therapeutic targets for this disease.

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Review Article

The Link between Hypersensitivity Syndrome Reaction Development and Human Herpes Virus-6 Reactivation

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Background. There are challenges in the clinical diagnosis of drug-induced injury and in obtaining information on the reactivation of human herpes viruses (HHV) during idiosyncratic adverse drug reactions. *Objectives*. (i) To develop a unified list of drugs incriminated in drug-induced hepatotoxicity and severe cutaneous reactions, in which drug hypersensitivity leads to HHV-6 reactivation and further complication of therapy and recovery and (ii) to supplement the already available data on reporting frequencies of liver- or skin-induced cases with knowledge of individual case reports, including HHV-6 reactivation and briefly introducing chromosomally integrated HHV-6. *Data Sources and Extraction*. Drugs identified as causes of (i) idiosyncratic reactions, (ii) drug-induced hypersensitivity, drug-induced hepatotoxicity, acute liver failure, and Stevens-Johnson syndrome, and (iii) human herpes virus reactivation in PubMed since 1997 have been collected and discussed. *Results*. Data presented in this paper show that HHV-6 reactivation is associated with more severe organ involvement and a prolonged course of disease. *Conclusion*. This analysis of HHV-6 reactivation associated with drug-induced severe cutaneous reactions and hepatotoxicity will aid in causality assessment and clinical diagnosis of possible life-threatening events and will provide a basis for further patient characterization and therapy.

1. Introduction

A hypersensitivity reaction (HSR) is a host-dependent idiosyncratic adverse drug reaction (ADR) that cannot be predicted by the dose, frequency, or length of the treatment. Furthermore, animal models cannot predict an HSR. A "true" HSR is defined by the triad of fever, rash, and internal organ involvement [1–6]. HSRs occur with an incidence of 1 in 1000 to 1 in 10000 drug exposures, with a mortality rate approaching 10% [5–8]. Symptoms of HSR can range from general manifestations, such as morbiliform rash, urticaria, angioedema, fever, malaise, anaphylaxis, bronchospasm, and erythema multiforme, to severe cutaneous adverse reactions (SCAR) such as drug-induced hypersensitivity syndrome (DIHS)/drug reaction with eosinophilia and systemic symptoms (DRESS), and Stevens-Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) [2, 4–7, 9–11]. Cutaneous eruptions are reported in the vast majority of DIHS/DRESS cases, often presenting as maculopapular rash or generalized erythematous rash [7, 8]. The internal organ most often affected is the liver, with drug-induced liver injury (DILI) presenting as anomalies in liver function tests or the presence of hepatomegaly. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were found to be increased an average of 9-10-fold above the upper normal limits in a literature review of 172 DRESS cases [7]. The presence of serum bilirubin raised >3 times over the upper limit of normal along with aminotransferase elevations is associated with a more severe set of symptoms than isolated aminotransferase abnormalities alone, an observation known as Hy's Law [12, 13]. Other internal organs, such as kidneys, are less often affected [4, 7]. Hypereosinophilia is the

third most common symptom of DIHS/DRESS [7]. Skin rash, liver involvement, high-grade fever, hypereosinophilia, lymphadenopathy, and the increased presence of atypical lymphocytes are symptoms associated with DIHS/DRESS cases classified as "probable/definite" based on the RegiSCAR scoring system [7].

Maculopapular rash, erythema multiforme, exfoliative dermatitis, acute generalized exanthematous pustular dermatosis-like eruptions, and erythroderma are types of cutaneous manifestations associated with DIHS, while mucosal involvement is rare. On the other hand, skin and mucosal involvement characterizes SJS/TEN [8]. A further distinction between DIHS/DRESS and SJS/TEN is the time to onset of symptoms, which is often delayed (4-5 weeks) in the former, while it occurs in the early stages of drug exposure (approximately 3 weeks) in the latter [8].

Carbamazepine was the drug most often associated with DRESS among 44 drugs linked to this reaction in 172 cases reported over a period of 12 years [7]. Anticonvulsants such as carbamazepine, phenytoin, phenobarbital, lamotrigine, zonisamide, and sodium valproate are the class of drugs most often related to DIHS/DRESS. Other pharmaceutical agents, including allopurinol, nonsteroidal anti-inflammatory drugs, chlormezanone, aminopenicillins, cephalosporins, quinolones cycline antibiotics, and antiretrovirals, as well as anti-infective sulfonamides, have also been implicated [6–8, 11, 14–16].

SCAR development results in patient hospitalization, and the culprit drug is interrupted on the first day of hospitalization. DILI is often diagnosed during the course of the disease. Corticosteroids are the treatment most often employed. The mean time to recovery was 6.4 weeks in 172 DRESS cases, while death was recorded in 9 (5.2%) patients. Death was connected with a more severe course of reaction, particularly observed in older patients, and often due to liver failure [7].

While the precise mechanism of HSR is unknown, various theories involve the interplay between metabolic and immunologic factors [2, 4, 6], genetic predisposition, and, more recently, infection with human herpes viruses (HHV), particularly HHV-6 [8].

Drug metabolites bind to cellular macromolecules such as proteins, creating covalent adducts that can serve as antigenic stimuli for the immune system [4, 6]. Several genetic factors are believed to predispose certain individuals to this type of adverse reaction. The subset of the population that is susceptible to HSRs carries defects in drug detoxifying pathways (e.g., epoxide hydrolase), such that a greater amount of a reactive drug metabolite is produced than that which can be detoxified [2, 4, 6]. Additional genetic predispositions that render certain individuals susceptible to HSRs include human leukocyte antigen (HLA) alleles, which are part of the major histocompatibility complex (discussed by Neuman et al. [17]).

Reactivation of latent viral infections has been linked to the development of more severe HSR symptoms, particularly in the context of DIHS/DRESS. For example, HHV-6 reactivation was associated with more severe organ involvement and a prolonged course of disease in 62 of 100 DIHS patients, compared with the remaining 38 patients who did not experience HHV-6 reactivation [18]. In the same study, all five deaths and ten cases of renal failure were observed among the 62 patients with HHV-6 DNA detected in the serum [18].

HHV-6 is a lymphotropic DNA virus belonging to the *Betaherpesviridae* subfamily [19, 20]. Two genetic variants of HHV-6 exist, HHV-6A and HHV-6B. While little is known about HHV-6A, HHV-6B infection often occurs during the first 2 years of life and is associated with acute febrile illness in young children and exanthem subitum in infants [19–26]. HHV-6 infection can be accompanied by convulsions and febrile status epilepticus. HHV-6 has been increasingly recognized as a cause of hepatitis and liver failure, as well as increased graft rejection and consequent decreased patient survival [20–26]. The rate of infection approaches 100% in individuals 2-3 years of age [21].

Following the initial infection, HHV-6B remains present in a latent phase, and infection can be reactivated during episodes of immunosuppression [20, 22, 24, 26]. Inherited forms of both HHV-6A and HHV-6B have been shown to integrate in chromosomes. This condition, known as chromosomally integrated HHV-6 (CIHHV-6), occurs in 0.8% of control populations but is found in approximately 2% of patient populations in Europe, United States, and the United Kingdom, with a lower rate in Japan. CIHHV-6 is characterized by the complete integration of the HHV-6 genome into the host germ line genome, such that CIHHV-6 DNA is found in every nucleated cell in the body [24, 27]. Persistently high viral copy numbers in whole blood or serum are observed in individuals with this condition [28, 29]. The viral genome of CIHHV-6 is transmitted vertically in the germline in a Mendelian manner [27]. Approximately 1% of newborns are diagnosed with congenital HHV-6, all of which are infected with CIHHV-6, directly or indirectly. In a sample of 43 infants with congenital infection, the majority (86.0%) were born with inherited CIHHV-6, while the remaining (14.0%) were not born with CIHHV-6 but became infected with HHV-6 transplacentally from their CIHHV-6-positive mothers [30].

The cellular DNA damage machinery responds to virus infection and the foreign genomes that accumulate in the nuclei of infected cells. Many DNA viruses have been shown to manipulate the cellular DNA damage response pathways in order to create environments conducive to their own replication. Some cellular factors are activated during infection while others are inactivated [31].

Arbuckle et al. showed that HHV-6 DNA integrates specifically and efficiently into the telomere sequences of chromosomes. The integrated virus can be chemically activated with trichostatin A, a stimulatory compound known to reactivate latent herpesviruses [32].

Active HHV-6 replication is determined by the presence of HHV-6 DNA in serum, plasma, or cerebrospinal fluid, or by reverse-transcriptase polymerase chain reaction (RT-PCR) in peripheral blood mononuclear cells (PBMC). RT-PCR is the sole technique that allows active HHV-6 replication to be differentiated from inactive CIHHV-6 [33]. It is important that active HHV-6 replication is differentiated from CIHHV-6 [34].

The present paper reviews recently published case reports of SCAR (DIHS/DRESS and SJS/TEN) and DILI developed secondary to drug exposure, with a particular focus on the role of HHV-6 reactivation and CIHHV-6 presence in the clinical outcome of the reaction. Our principal aim is to address the role of HHV-6 reactivation in the course of HSRs, particularly in DILI and DIHS/DRESS. In addition, we will focus on the complex cellular responses triggered by HHV-6 reactivation.

2. Materials and Methods

Studies discussed in this paper were selected based on a PubMed search of English language papers published in the last 15 years (1997–2011) using keywords such as "HHV-6 and hypersensitivity," "HHV-6 and DIHS," "HHV-6 and DRESS," "HHV-6 and SJS," "HHV-6 and TEN," "HHV-6 reactivation and drug," "HHV-6 and drug rash," and "chromosomally integrated HHV-6." Case reports presented deal largely with anticonvulsants and included HHV-6 reactivation. The selected case reports are part of a larger database of HSR cases with HHV-6 reactivation. In addition, our laboratory has studied HSRs and their mechanisms for the last 25 years, and we discuss the cases based on our experience related to the laboratory and clinical manifestations.

3. Results

3.1. HSR and HHV-6 Reactivation. Following initial infection in early childhood, HHV-6 continues to exist in a latent phase in the body. Consequently, HHV-6 DNA can be measured in different body compartments in HHV-6-positive individuals without active infection. Trace amounts of HHV-6 viral DNA were also detected by real-time quantitative PCR in 91 (26.8%) of 339 pediatric patients diagnosed and treated for acute lymphoblastic leukemia [34]. Low rates of HHV-6B DNA were detected in whole blood (8.0%), PBMCs (16.5%), and polymorphonuclear leukocytes (10.5%) belonging to 200 volunteers [54]. HHV-6A DNA was not observed in this sample. The mean observed HHV-6B viral loads were 81 copies/10^6 cells in whole blood, 62 copies/10^6 cells in PBMCs, and 34.5 copies/10^6 cells in polymorphonuclear leukocytes. Based on these findings, Géraudie et al. classify healthy individuals as having HHV-6B viral loads below 100 copies/10^6 cells [54]. HHV-6-DNA was positive in only 27 (39.1%) blood samples from 69 children undergoing elective tonsillectomy for moderate tonsillar hyperplasia or recurrent streptococcal infection without evidence of acute HHV-6 infection, while evidence of HHV-6 DNA was found in 100% of tonsil samples in the same population [55].

The common features of DIHS identified in a sample of 7 patients were high fever (≥39°C) in all patients, facial edema in all patients, diffuse lymphadenopathy in 5 (71.4%) patients, hypereosinophilia (>0.5 × 10^9/L) in 4 (57.1%) patients, atypical circulating lymphocytes in 4 (57.1%) patients, ALT elevated >3 times the reference level in 5 (71.4%) patients, and hypogammaglobulinemia in 4 (57.1%) patients [14]. The onset of DRESS took place after a mean period of 25 days (5 days–6 weeks) of therapy with the incriminated drug [14]. Similar findings are reported in other cohorts of DIHS patients [15, 56].

Several studies suggest that HHV-6 reactivation can only occur in susceptible individuals under conditions of transient immunosuppression, such as those transiently associated with the onset of DIHS [56, 57]. Lower serum IgG levels were observed in 10 adult anticonvulsant-induced HSR patients (mean 745 mg/dL) compared to 15 controls ($P < 0.001$). Serum IgG levels continued to decrease for several days after the drug was discontinued [57]. Similarly, circulating levels of B cells were also decreased in DIHS patients, compared to controls [15, 57]. In susceptible individuals, continuous therapy with anticonvulsants, which have been associated with DIHS, can lead to decreases in B cells and subsequent hypogammaglobulinemia. Drug-induced hypogammaglobulinemia could account for the delay between DIHS onset and HHV-6 [57]. Moreover, HHV-6 reactivation may occur at the same time, but it cannot always be detected in blood, since it takes 2–4 weeks for antibodies to appear after infection or reactivation. Because decreases in circulating IgG and B cell levels are not observed with other hypersensitivity conditions such as SJS/TEN, depressed levels of IgG and B cells can be used as biomarkers for the onset of DIHS [57].

HHV-6 reactivation may be partially explained by the immunosuppressive effects of DIHS-related drugs. The magnitude of inflammation is often proportional to the clinical manifestations, while hypogammaglobulinemia and HHV-6 reactivation worsen the clinical course of the disease [58]. Hypogammaglobulinemia is thus an early symptom of severe DIHS.

A more detailed description of disease progression is presented in case reports, some of which are summarized in Table 1 [35–52]. Fever was present in all patients described in these reports. Edema, predominantly on the face, was observed with a relatively high frequency [35, 37, 39–44, 47–49, 52], as were lymphadenopathy [35, 36, 39, 41–43, 45, 46, 49–51], hypereosinophilia [35–40, 42–52], and atypical lymphocytes [35, 36, 38–40, 42, 44, 46, 47, 52]. Internal organ involvement manifested itself largely as liver dysfunction, with elevated levels of liver enzymes [35–46, 48–50, 52] and cholestatic hepatitis with hepatocellular insufficiency [51]. Kidney failure was observed in a 75-year-old lamotrigine patient [51]. Multiple organ failure was observed in a couple of studies [47, 51], one of which resulted in death [47]. Hypogammaglobulinemia, another symptom of DIHS, was observed in a number of studies as well [35, 38, 39, 45, 49].

The offensive drug is immediately interrupted in DIHS patients, and treatment with prednisolone is often used to reverse the condition [15]. Symptoms begin to improve gradually, but both fever and skin manifestations often relapse approximately 2-3 weeks after the onset of DIHS. There is a delay until antibodies to the virus are generated. Therefore, there is a delay until the peak of the infection is detected. Symptoms relapse coincides with detection of HHV-6 reactivation, measured by circulating anti-HHV-6 antibodies and HHV-6 DNA [15, 56].

Table 1: Detailed analysis of select HSR cases with HHV-6 reactivation.

Diagnosed condition and patient characteristics	Preexisting medical conditions and previous drug exposure	Characteristics of HSR	HHV-6 reactivation characteristics	Status of other bacteria and viral reactivation	Treatment and symptoms resolution	Ref. no.
Carbamazepine DIHS 14-year-old Japanese male	Sodium valproate (600 mg/day) and carbamazepine (200 mg/day) for epilepsy Cefaclor (600 mg/day) for suspected bacterial infection	Fever after 17 days of carbamazepine Facial and body angioedema, generalized lymphadenopathy, mild hepatosplenomegaly, generalized erythema without erosion 11 days after first episode of fever WBC count 31.7×10^9/L (eosinophils 11%, atypical lymphocytes 12.5%), RBC count 4.46×10^{12}/L, hemoglobin 13.6 g/dL, platelet count 169×10^9/L, C-reactive protein 1.8 mg/dL, total protein 5.2 g/dL, albumin 3.1 g/dL, AST 137 IU/L, ALT 202 IU/L, LDH 714 IU/L, blood urea nitrogen 10 mg/dL, and creatinine 0.69 mg/dL Transient hypogammaglobulinemia (IgG 649 mg/dL upon admission and 1169 mg/dL on day 26, similar trends for IgM and IgA)	Anti-HHV-6 IgG titers increased from 1 : 10 upon admission to 1 : 10240 23 days later Anti-HHV-6 IgM titers unchanged HHV-6 DNA copy numbers decreased from 3.5×10^{12} copies/10^6 PBMCs on day 3 to 6.3×10^3 copies/10^6 PBMCs on day 46	Anti-HHV-7 IgG titers increased from 1 : 80 to 1 : 160 No changes in HHV-7 DNA copy numbers CMV, EBV, HSV, VZV, or parvovirus B19 serology negative	IV methylprednisolone (30 mg/kg) pulse therapy, followed by oral prednisolone (30 mg/day)	[35]
Carbamazepine DIHS 24-year-old Caucasian female	Carbamazepine (200 mg/day) for oligosymptomatic partial-complex seizures	Fever (38.6°C) with generalized lymphadenopathy and a maculopapular exanthema of the trunk and the perioral region after 6 weeks of carbamazepine WBC count 9.1×10^9/L (19.7% atypical lymphocytes and 8.1% eosinophils) and elevated ALT (50 IU/L) γ-GTP (160 IU/L) and alkaline phosphatase (114 IU/L)	Anti-HHV-6 IgM titers positive Anti-HHV-6 IgG titers increased from 1 : 320 to 1 : 1280 within 25 days of hospitalization HHV-6 DNA detected in serum	EBV, CMV, HBC, HCV, HIV, *Toxoplasma gondii* or *Borrelia burgdorferi* serology negative	n/a Symptoms resolved spontaneously	[36]
Carbamazepine DIHS 24-year-old Japanese female	Carbamazepine for 32 days for agitation and emotional instability Cefcapene pivoxil hydrochloride administered for supposed cervical lymphadenitis	Low-grade fever and cervical lymph node swelling within 2 weeks of carbamazepine Pleomorphic erythema of the trunk and lower extremities within another week Fever (39.0°C–39.9° C) and dark purplish pleomorphic erythema over the trunk and extremities within next 10 days WBC count 14.3×10^9/L (14.8% eosinophils), with severe liver dysfunction characterized by elevated AST (262 IU/L), ALT (423 IU/L), and LDH (827 IU/L) after 32 days of carbamazepine Inguinal lymph node swelling developed and edema in the lower limbs and face progressed Hepatic function worsened	Anti-HHV-6 IgG titer 1 : 80 and the IgM titer <1 : 10 at time of carbamazepine discontinuation Anti-HHV-6 IgG titers increased to 1 : 2560 and IgM titers became positive at 1 : 10	EBV, CMV, parvovirus B19 and antirubeola virus within normal ranges EBV and CMV serology negative	Not discussed	[37]

Table 1: Continued.

Diagnosed condition and patient characteristics	Preexisting medical conditions and previous drug exposure	Characteristics of HSR	HHV-6 reactivation characteristics	Status of other bacteria and viral reactivation	Treatment and symptoms resolution	Ref. no.
Carbamazepine DIHS 43-year-old Japanese female	Haloperidol, paroxetine hydrochloride, levomepromazine, amobarbital, bromovalerylurea chlorpromazine, and carbamazepine for anxiety and confusion	Generalized exudative erythema and fever (39.2°C) after 14 days of carbamazepine Increased WBC count, particularly atypical lymphocyte count Leukocytosis (27.5×10^9/L) with atypical lymphocytosis (36%, 9.9×10^9/L) and eosinophilia (4.5%, 0.88×10^9/L) Hepatic dysfunction with elevated AST (78 IU/L), ALT (106 IU/L), LDH (873 IU/L), and γ-GTP (556 IU/L) Transient hypogammaglobulinemia	Anti-HHV-6 IgG titers increased from 1 : 10 on day 6 to 1 : 5120 on day 11 Anti-HHV-7 IgG titers increased from 1 : 80 to 1 : 160 HHV-6 DNA detected on day 9, but not on day 11	EBV, CMV, HSV, VZV and parvovirus B19 serology negative	IV methylprednisolone (1000 mg/day) pulse therapy for 3 days, followed by oral prednisolone (60 mg/day)	[38]
Carbamazepine DIHS 54-year-old Turkish female	Carbamazepine for epilepsy	Fever after 20 days of carbamazepine Maculopapular rash, erythroderma, exfoliative dermatitis, fever, facial, and genital edema, lymphadenopathy, hypereosinophilia, hypogammaglobulinemia, atypical lymphocytosis, leukocytosis and abnormal liver function tests	Anti-HHV-6 IgG 1 : 60 on day 7 and 1 : 1920 on day 14 HHV-6 DNA detected by PCR on day 14	Not specified	Systemic steroids, IV immunoglobulin for 30 days	[39]
Carbamazepine DIHS 17-year-old Turkish male	Carbamazepine for brain surgery	Fever after 12 days of carbamazepine Maculopapular rash, erythroderma, bullous lesion, hypereosinophilia, anemia, atypical lymphocytosis, abnormal liver function tests	Anti-HHV-6 IgG 1 : 80 on day 7 and 1 : 2560 on day 14 HHV-6 DNA detected by PCR on day 14	Not specified	Systemic steroids for 21 days	[39]
Carbamazepine DIHS 23-year-old Turkish female	Carbamazepine for epilepsy	Fever after 24 days of carbamazepine Erythroderma, exfoliative dermatitis, vasculitis (purpura), genital ulcer, lymphadenopathy, leukocytosis, hypereosinophilia, atypical lymphocytosis, abnormal liver function tests	Anti-HHV-6 IgG 1 : 20 on day 7 and 1 : 1280 on day 14 HHV-6 DNA not specified detected by PCR on day 14	Not specified	Systemic steroids for 30 days	[39]
Carbamazepine DIHS 29-year-old Turkish male	Carbamazepine for brain surgery	Fever after 22 days of carbamazepine Maculopapular rash, vasculitis (purpura), facial and genital edema, hypereosinophilia, leukocytosis, abnormal liver function tests, splenomegaly	Anti-HHV-6 IgG 1 : 20 on day 7 and 1 : 1280 on day 14 HHV-6 DNA detected by PCR on day 14	Not specified	Systemic steroids for 20 days	[39]
Carbamazepine DIHS 15-year-old Turkish female	Carbamazepine for epilepsy	Fever after 20 days of carbamazepine Erythroderma, lymphadenopathy, leukocytosis, hypereosinophilia, atypical lymphocytosis, increased in serum amylase (pancreatitis), abnormal liver function tests, lung pneumonia	Anti-HHV-6 IgG 1 : 30 on day 7 and 1 : 1920 on day 14 HHV-6 DNA detected by PCR on day 14	Not specified	Systemic steroids for 18 days	[39]

Table 1: Continued.

Diagnosed condition and patient characteristics	Preexisting medical conditions and previous drug exposure	Characteristics of HSR	HHV-6 reactivation characteristics	Status of other bacteria and viral reactivation	Treatment and symptoms resolution	Ref. no.
Carbamazepine DIHS accompanied by dermatologic changes and HHV-6 reactivation 14-year-old Japanese male	Carbamazepine (200 mg/day) for localization-related epilepsy	Fever (37.8°C) after 3 weeks of carbamazepine Liver dysfunction with AST 403 IU/L, ALT 549 IU/L and LDH 637 IU/L upon admission Erythroderma with edematous changes spread over the entire body WBC count 34.5×10^9/L, with 19.5% atypical lymphocytes and 23.5% eosinophils prior to day 11 Liver dysfunction worsened with AST 1170 IU/L and ALT 700 IU/L on day 15 No cervical, axial, or inguinal lymphadenopathy detected	HHV-6 DNA (32000 copies/μg of DNA) detected by real-time PCR and anti-HHV-6 antibodies isolated from PBMCs on day 19 Anti-HHV-6 IgG increased 5120-fold on day 25	HAV, HBV, CMV, EBV serology negative	Corticosteroids (1 mg/kg/day)	[40]
Carbamazepine DIHS possibly triggered by HHV-6 reactivation 12-year-old Italian female	Amoxicillin Sodium valproate for generalized epileptic seizure, replaced with carbamazepine	Fever (>39°C), cutaneous rash with mild face edema and moderate laterocervical lymphadenopathy after 5 weeks of carbamazepine Normal hemochrome with moderate lymphopenia (0.8×10^9/L) and increased AST (200 IU/L), ALT (181 IU/L), and γ-GTP (116 IU/L)	Anti-HHV-6 IgG titers >1 : 128 HHV-6 and HHV-7 DNA detected	EBV, CMV, toxoplasma, *Bartonella* serologies, influenza, adenovirus, and respiratory syncytial virus serology negative	IV methylprednisolone (1 mg/kg) for 3 days, followed by oral methylprednisolone	[41]
Carbamazepine DIHS 48-year-old Japanese male	Carbamazepine (400 mg/day) for a psychiatric disease	High-grade fever and erythematous rash on his trunk after 43 days of carbamazepine Leukocytosis (9.3×10^9/L (0% eosinophilia/13% atypical lymphocytosis)) Lymphadenopathy Severe liver dysfunction with highest ALT level 1859 IU/L Worsening of rash and liver dysfunction at time of EBV reactivation Thyroid dysfunction at time of HHV-6 and HHV-7 reactivation	Increase in HHV-6 viral load Dramatic increase in anti-HHV-6 IgG titers	Increase in EBV viral load after 9 days of hospitalization HHV-7 reactivation	None	[42]
Phenobarbital DIHS 68-year-old Japanese male	Phenobarbital (120 mg/day) for epileptic fits	Erythematous rash on chest and trunk, fever and malaise after 43 days of phenobarbital Leukocytosis (17.4×10^9/L (6% eosinophilia/1% atypical lymphocytosis)) Lymphadenopathy Liver dysfunction with highest ALT level 323 IU/L Neurological symptoms developed in conjunction with increased HHV-6 viral load Neurological symptoms reappeared with rise in HHV-7 viral load	HHV-6 DNA detected 19 days after hospitalization Dramatic rise in anti-HHV-6 IgG titers but not IgM titers	EBV viral load increased as HHV-6 decreased HHV-7 viral load increased within a few months of HHV-6 CMV viral load increased with no clinical symptoms	Prednisolone (60 mg/day)	[42]

Table 1: Continued.

Diagnosed condition and patient characteristics	Preexisting medical conditions and previous drug exposure	Characteristics of HSR	HHV-6 reactivation characteristics	Status of other bacteria and viral reactivation	Treatment and symptoms resolution	Ref. no.
Salazosulfapyridine DIHS 68-year-old Japanese male	Salazosulfapyridine (2000 mg/day) for rheumatoid arthritis Previous exposure to prednisolone	High-grade fever, malaise, and rash extending to trunk and lower extremities after 28 days of salazosulfapyridine Leukocytosis (17.7×10^9/L (0% eosinophilia/2% atypical lymphocytosis)) Facial edema, lymphadenopathy and leucopenia upon admission Slight liver dysfunction with highest ALT level 44 IU/L Herpes labialis with herpetic stomatitis at time of HSV reactivation 13 days after admission Erythematous rash with transient high fever and dry cough developed at time of CMV antigens detection	HHV-6 DNA detected in blood upon admission Dramatic rise in anti-HHV-6 IgG titers	HSV reactivation HSV antigens EBV and CMV DNA detected	Prednisolone (40 mg/day)	[42]
Mexiletine DIHS 74-year-old Japanese male	Mexiletine for arrhythmia	High-grade fever and generalized erythematous rash on the chest and trunk after 31 days of mexiletine Leukocytosis (9.2×10^9/L (14% eosinophilia/2% atypical lymphocytosis)) Lymphadenopathy Liver dysfunction with highest ALT level 156 IU/L Cytomegalovirus antigenemia and massive internal bleeding following detection of CMV DNA Slight liver dysfunction following detection of EBV DNA	HHV-6 DNA detected Increased anti-HHV-6 IgG titers	Increased EBV and CMV viral loads	Prednisolone (50 mg/day) Ganciclovir (200 mg/day)	[42]
Anticonvulsant DIHS with HHV-6 reactivation developed after initial exanthema subitum complicated with febrile seizure 11-month-old Japanese female	IV diazepam for right tonic hemiconvulsions and upward deviation of the eyes Oral carbamazepine initiated in place of thiopental 10 days after hospitalization Carbamazepine replaced with phenobarbital IV phenytoin added on day 14 and continued for 11 days	Fever (39.1°C) without skin symptoms after 14 days of phenobarbital Erythematous rash appeared and spread over the entire body after 4 additional days Maculopapular erythematous rash appeared on face, extremities, and trunk after carbamazepine exposure Cervical lymphadenopathy Facial edema after phenobarbital discontinuation Elevated eosinophil count (1.9×10^9/L), AST (105 IU/L) and ALT (45 IU/L)	HHV-6 DNA first detected on day 5 (635 copies/mL in serum and 31.5 copies/mL in cerebrospinal fluid), coinciding with the eruptive stage of exanthema subitum HHV-6 DNA undetectable on day 16 HHV-6 DNA detected on day 28 (805 copies/mL in serum), coinciding with high fever and generalized erythema HHV-6 DNA increased on day 30 (4360 copies/mL in serum) when DIHS was suspected Anti-HHV-6 IgG titers negative upon admission Anti-HHV-6 IgG titers elevated on day 11 (256-fold) and day 34 (128-fold)	Not specified	Continuous infusion of thiopental and assisted mechanical ventilation, as well as IV methylprednisolone pulse therapy (30 m/kg/day for 3 days), γ-globulin, and acyclovir therapy for initial condition IV methylprednisolone for resolution of DIHS	[43]

Table 1: Continued.

Diagnosed condition and patient characteristics	Preexisting medical conditions and previous drug exposure	Characteristics of HSR	HHV-6 reactivation characteristics	Status of other bacteria and viral reactivation	Treatment and symptoms resolution	Ref. no.
Phenobarbital DIHS and fulminant hemophagocytic syndrome associated with HHV-6 reactivation 25-year-old Laotian female	Oral phenobarbital for epileptic seizures	Fever (40°C), diffuse pruritic exfoliative dermatitis with edema of the face and purpuric lesions on the extremities, enlarged cervical, axillary and inguinal lymph nodes, and hepatomegaly after 18 days of phenobarbital Atypical lymphocytes with leukocytosis (24.5×10^9/L), eosinophilia (5.14×10^9/L), and lymphocytosis (7.1×10^9/L) with predominant T phenotype (90% CD3, 1% CD19) Liver dysfunction with elevated ALT (465 IU/L), AST (165 IU/L), and LDH (19000 U/mL) Severe erythroderma complicated by methicillin-resistant *Staphylococcus aureus* septicemia on day 13 Acute hepatic failure with ALT 1415 IU/L and AST 2525 IU/L	Anti-HHV-6 IgG titers 1 : 80 on day 14 and >1 : 320 on day 29 HHV-6 DNA not detected in serum by PCR	EBV, CMV, HIV, human T-cell lymphotropic virus type 1, parvovirus B19, HCV, HBV, picornavirus, *Toxoplasma gondii* and *Treponema pallidum* serology negative	Oral corticosteroids and etoposide Corticosteroid therapy continued >1 year IV vancomycin (2 g/day)	[44]
Phenobarbital DIHS with multiple viral reactivations 43-year-old Japanese male	Phenobarbital (300 mg daily) for a brain aneurysm	High-grade fever, bilateral cervical and inguinal lymphadenopathy, and hepatomegaly after 44 days of phenobarbital Blood cell count 11.8×10^9/L, with eosinophil count 3.08×10^9/L Liver dysfunction with elevated AST 273 IU/L, ALT 770 IU/L, LDH 582 IU/L and C-reactive protein 12.7 mg/dL Hypogammaglobulinemia with serum IgG levels 769 mg/dL, IgA levels 47 mg/d/L and IgM levels 76 mg/dL Skin biopsy revealed dense infiltration consisting mainly of mononuclear cells and eosinophils in the upper dermis	Dramatic increases in anti-HHV-6 IgG on day 19	Dramatic increases in anti-CMV IgM on day 19 Increase in anti-HHV-7 IgG EBV serology negative	Initial HSR symptoms resolved spontaneously Treatment not administered	[45]
Phenobarbital DIHS 31-year-old Japanese female	Phenobarbital for depression	Fever, severe systemic erythematous eruptions followed by systemic lymphadenopathy and hepatosplenomegaly after 3 weeks of phenobarbital WBC count 105.4×10^9/L, with 25% eosinophils and 52% atypical lymphocytes, platelet count 161×10^9/L and hemoglobin concentration 9.4 g/dL Liver dysfunction with AST 70 IU/L, ALT 93 IU/L, alkaline phosphatase 824 IU/L, γ-GTP 592 IU/L, and LDH 364 IU/L	Anti-HHV-6 IgG 1 : 2560 and IgM 1 : 40 HHV-6 DNA 6.3×10^3 copies/mL in PBMCs	EBV, CMV, VZV, and HSV serology negative	Methylprednisolone (1000 mg/day) for 3 days, followed by prednisolone (60 mg/day)	[46]

Table 1: Continued.

Diagnosed condition and patient characteristics	Preexisting medical conditions and previous drug exposure	Characteristics of HSR	HHV-6 reactivation characteristics	Status of other bacteria and viral reactivation	Treatment and symptoms resolution	Ref. no.
Aromatic anticonvulsants DIHS developed concomitantly to HHV-6 seroconversion in 56-year-old Japanese male	Hypertension Sodium valproate (600 mg/day) for a 6-month history of generalized convulsion of unknown cause Phenytoin (134 mg/day) and phenobarbital (66 mg/day) for 3 weeks Acyclovir (20 mg/kg/day) for was believed to be HSV encephalitis	Generalized erythematous macules after 3 weeks of phenytoin and phenobarbital High-grade fever (39.8°C), generalized erythroderma and edema within 1 week WBC count 23.3 $\times$ 10^9/L (23% eosinophils and 9.5% atypical lymphocytes) Abnormal liver function Skin biopsy revealed a spongiotic epidermis with liquefactive degeneration of the epidermal basal layer Encephalopathy of nonmetabolic origins Death with multiple organ failure 18 days after falling into a coma and 5 weeks after rash appearance	Anti-HHV-6 IgG titers increased from 1 : 80 one month after appearance of macular rash to 1 : 640 within next 2 weeks Anti-HHV-6 IgM negative	HSV-1, HSV-2, VZV, CMV, EBV, measles, rubella, and mumps serology positive	Oral prednisone (30 mg/day) IV diazepam for frequent partial seizures developed during hospital stay	[47]
Anticonvulsant DIHS 6-year-old Tunisian child	Sodium valproate (600 mg/day) intermittently for 41 days Ethosuximide (500 mg/day) intermittently for 29 days for epileptic absences Ethosuximide interrupted prior to the hospitalization	High-fever (40°C), facial edema, diffuse pruritic morbilliform skin eruption with vesicular, and target lesions after 41 days of intermittent sodium valproate Leukocytosis of 17.9 $\times$ 10^9/L, eosinophilia of 2.0 $\times$ 10^9/L, lymphocytosis of 8.95 $\times$ 10^9/L and monocytosis of 1.43 $\times$ 10^9/L, with normal platelet count Elevated C-reactive protein (57 mg/mL), AST (70 IU/L), γ-GTP (50 IU/L), and LDH (1020 IU/L) Histologic analysis revealed a spongiotic epidermis with necrotic keratinocytes and a lymphocytic perivascular dermal infiltrate with a few hyperchromatic lymphocytes with irregular nuclei	Anti-HHV-6 IgG titers increased from 1 : 40 to 1 : 120 in the course of 120 days	EBV, HIV, HAV, HBV, HCV, CMV, parvovirus B19, herpes simplex virus and *Mycoplasma pneumoniae* serology negative	Oral prednisone (1 mg/kg/day)	[48]
Zonisamide DIHS 29-year-old Japanese male	Zonisamide (300 mg/day) for temporal epilepsy, interrupted 7 days prior to admission Cefcapene pivoxil hydrochloride hydrate, acetaminophen, tranexamic acid, and lansoprazole prescribed 7 days prior to admission	Acute kidney injury and diffuse skin rash with edema of the face after 2 months of zonisamide Fever, elevated AST (65 IU/L), ALT (132 IU/L), and γ-GTP (153 IU/L) levels 4 days prior to admission Facial edema and a generalized pruritic maculopapular rash with lichen upon admission Lymphadenopathy, eosinophilia, renal dysfunction, liver dysfunction, and hypogammaglobulinemia Splenomegaly and enlarged kidneys with serum creatinine level of 8.20 mg/dL Fever persisted and eruptions evolved to flaccid vesicles and bullae 4 days after admission Skin biopsy revealed multiple epidermal bullous formation with eosinophilic abscess Renal biopsy performed revealed glomeruli with minor abnormality, with infiltration of mononuclear cells and eosinophilic cells around each glomerulus and focal interstitium and interstitial edema with swelling and degeneration of tubular epithelial cells	HHV-6 DNA detected by PCR on day 6 but not on day 20 Anti-HHV-6 IgG levels increased from 1 : 16 on day 2 to 1 : 256 on day 27	Not specified	Hemodialysis Prednisolone (60 mg/day) started 4 days after admission	[49]

Table 1: Continued.

Diagnosed condition and patient characteristics	Preexisting medical conditions and previous drug exposure	Characteristics of HSR	HHV-6 reactivation characteristics	Status of other bacteria and viral reactivation	Treatment and symptoms resolution	Ref. no.
Lamotrigine DIHS 26 year-old Italian female	Additive mastoplasty for von Willembrandt disease in childhood Current escitalopram and lamotrigine for a bipolar disease Cefixime (400 mg twice/day) for 3 days, betamethasone, 1 mg and fexofenadine 120 mg for 1 day, and azithromycin 500 mg once/day for 3 days for fever ($\leq$39.5°C) developed 16 days prior to admittance	Fever after 30 days of lamotrigine Asthenia, nausea, myalgia, and arthralgia, as well as a maculopapular rash on the face, neck, trunk and superior and inferior limbs, hard and tender lymph nodes, and hepatomegaly upon admission Lymphadenopathy Eosinophilic leukocytosis (31.7×10^9/L, with 18% eosinophils) Increases in CD4 (3.7×10^9/L) and CD8 (5.8×10^9/L) T lymphocyte counts Severe liver dysfunction with 19-fold increased ALT and 14-fold increased AST High bilirubin level (2.84 mg/dL) and low prothrombin activity (39%)	Anti-HHV-6 IgG and IgM detected on day 11 HHV-6 DNA 8590 copies/mL in blood with real-time PCR	HAV, HBV, HCV, EBV, CMV, rubella, adenovirus, coxsackievirus, influenza virus A/B, parainfluenza virus serology negative Antibodies against *Borrelia burgdorferi*, *Rickettsia conori*, *Rickettsia typhi*, *Chlamydia trachomatis*, and *Leishmania infantum* negative	IV betamethasone (8 mg 3 times/day) and IV acyclovir (250 mg 3 times/day) for 8 days Fresh frozen plasma infusions, physiological solutions, and proton pomp inhibitors Prednisone (50 mg/day)	[50]
Lamotrigine DRESS 75-year-old French male	Lamotrigine (25 mg/day) for generalized tonic-clonic seizures	Diffuse exanthematous maculopapular rash affecting between 50% and 70% of the skin surface, fever, peripheral lymphadenopathies, and abdominal pain after 40 days of lamotrigine Hyperleukocytosis with hypereosinophilia Skin biopsy showed marked infiltration of the dermo-epidermal junction with lymphocytes and keratocyte necrosis Acute edematous pancreatitis diagnosed based on increased pancreas size Bilateral basal crackles on lung auscultation and hepatomegaly, as well as severe acute cytolytic and cholestatic hepatitis with hepatocellular insufficiency Oliguria and kidney failure Multiorgan failure by day 55	Positive anti-HHV-6 IgM serology HHV-6 DNA 11000 copies/mL	HIV, HAV, HBV, HCV, EBV, HSV-1 HSV-2, CMV serology negative	Prednisone (1 mg/kg/day) for 20 days	[51]
Carbamazepine DIHS 59-year-old Japanese male	Carbamazepine	Fever (>38.5°C) after 30 days of carbamazepine Liver dysfunction with ALT 119 IU/L and γ-GTP 79 IU/L WBC count 16.2×10^9/L and eosinophils count 0.45×10^9/L (16% atypical lymphocytes) Lymph node enlargement	Anti-HHV-6 IgG titers 1 : 160 during active phase and 1 : 1280 during recovery phase	Not specified	Prednisolone (40 mg/day)	[52]
Carbamazepine DIHS 56-year-old Japanese female	Carbamazepine	Fever (>38.5°C) after 22 days of carbamazepine Severe liver dysfunction with ALT 706 IU/L and γ-GTP 318 IU/L WBC count 11.5×10^9/L and eosinophils count 0.83×10^9/L (15% atypical lymphocytes) Lymph node enlargement	Anti-HHV-6 IgG titers 1 : 160 during active phase and 1 : 160 during recovery phase	Not specified	Prednisolone (40 mg/day)	[52]

Table 1: Continued.

Diagnosed condition and patient characteristics	Preexisting medical conditions and previous drug exposure	Characteristics of HSR	HHV-6 reactivation characteristics	Status of other bacteria and viral reactivation	Treatment and symptoms resolution	Ref. no.
Carbamazepine DIHS 62-year-old Japanese female	Carbamazepine	Fever (>38.5°C) after 51 days of carbamazepine No liver dysfunction with ALT 9 IU/L and γ-GTP 40 IU/L WBC count 7.8×10^9/L and eosinophils count 1.2×10^9/L (8% atypical lymphocytes) Lymph node enlargement	Anti-HHV-6 IgG titers 1 : 10 during active phase and 1 : 640 during recovery phase	Not specified	Betamethasone (2 mg/day)	[52]
Carbamazepine DIHS 53-year-old Japanese female	Carbamazepine	Fever (>38.5°C) after 26 days of carbamazepine Liver dysfunction with ALT 327 IU/L and γ-GTP 108 IU/L WBC count 6.4×10^9/L and eosinophils count 0.39×10^9/L Lymph node enlargement	Anti-HHV-6 IgG titers 1 : 640 during active phase and 1 : 10240 during recovery phase	Not specified	Corticosteroids not used	[52]
Carbamazepine DIHS 71-year-old Japanese male	Carbamazepine	Fever (>38.5°C) after 50 days of carbamazepine Liver dysfunction with ALT 108 IU/L and γ-GTP 140 IU/L WBC count 8.1×10^9/L and eosinophils count 0.50×10^9/L Lymph node enlargement	Anti-HHV-6 IgG titers 1 : 10 during active phase and 1 : 160 during recovery phase	Not specified	Prednisolone (30 mg/day)	[52]
Zonisamide DIHS 27-year-old Japanese female	Zonisamide	Fever (>38.5°C) after 26 days of zonisamide Severe liver dysfunction with ALT 401 IU/L and γ-GTP 220 IU/L WBC count 21.3×10^9/L and eosinophils count 0.66×10^9/L (49% atypical lymphocytes) Lymph node enlargement	Anti-HHV-6 IgG titers 1 : 40 during active phase and 1 : 1280 during recovery phase	Not specified	Prednisolone (30 mg/day)	[52]
Phenobarbital SJS 72-year-old Japanese female	Phenobarbital	Fever (>38.5°C) after 31 days of phenobarbital Mild liver dysfunction with ALT 72 IU/L and γ-GTP 28 IU/L WBC count 15.8×10^9/L and eosinophils count 0.42×10^9/L 5% detachment of the total body surface area	Anti-HHV-6 IgG titers 1 : 40 during active phase and 1 : 20 during recovery phase	Not specified	Prednisolone (40 mg/day)	[52]
Phenytoin SJS 19-year-old Japanese female	Phenytoin	Fever (>38.5°C) after 26 days of phenytoin Liver dysfunction with ALT 90 IU/L and γ-GTP 436 IU/L WBC count 12.8×10^9/L and eosinophils count 0.90×10^9/L Lymph node enlargement 10% detachment of the total body surface area	Anti-HHV-6 IgG titers 1 : 80 during active phase and 1 : 80 during recovery phase	Not specified	Prednisolone (60 mg/day)	[52]

Table 1: Continued.

Diagnosed condition and patient characteristics	Preexisting medical conditions and previous drug exposure	Characteristics of HSR	HHV-6 reactivation characteristics	Status of other bacteria and viral reactivation	Treatment and symptoms resolution	Ref. no.
Zonisamide TEN 71-year-old Japanese male	Zonisamide (300 mg/day) for symptomatic epilepsy History of long-term valproate sodium therapy	Fever (40.2°C) and rash after 23 days of zonisamide Erythematous macules resulting in diffuse areas of erythema with erosions and blisters located on the trunk and upper extremities 40% detachment of the total body surface area, with extensive hemorrhagic erosions on the lips, oral mucosa, pharynx, and larynx Elevated ALT (48 IU/L), LDH (379 IU/L), γ-GTP (111 IU/L), and C-reactive protein (11.3 mg/L) WBC count 12.5×10^9/L and eosinophils count 1.02×10^9/L Skin biopsy revealed prominent eosinophilic necrosis of the keratinocytes and subepidermal blister Moderate inflammatory infiltrate consisting of mononuclear cells in the upper dermis Skin eruptions and high fever returned 9 days after initial onset	Anti-HHV-6 IgG titers 1 : 10 during active phase (day 4) and 1 : 1280 during recovery phase (day 22) HHV-6 DNA levels increased from 2.0×10^1 copies/10^6 cells on day 4 to 1.3×10^2 copies/10^6 cells on day 22 in PBMCs	No change in HSV, CMV or EBV IgG titers	IV immunoglobulin therapy (5 g/day) for 3 days resulted in slowing of disease progress	[52]
Zonisamide TEN 66-year-old Japanese male	Zonisamide	Fever (>38.5°C) after 25 days of zonisamide Mild liver dysfunction with ALT 58 IU/L and γ-GTP 61 IU/L WBC count 10.1×10^9/L and eosinophils count 0.28×10^9/L 40% detachment of the total body surface area	Anti-HHV-6 IgG titers 1 : 40 during active phase and 1 : 40 during recovery phase	Not specified	Methylprednisolone (500 mg/day), followed by Prednisolone (30 mg/day)	[52]

Normal ranges: ALT 9–56 IU/L, AST 14–56 IU/L, γ-GTP 4–68 IU/L, LDH 116–250 IU/L, alkaline phosphatase 108 IU/L [35, 36, 38, 40, 44, 45, 52, 53], WBC 3.3×10^9/L–8.6×10^9/L, eosinophil count 0.07×10^9/L–0.45×10^9/L (eosinophils <8% of total circulating leukocytes), lymphocyte count 2.5×10^9/L–5.5×10^9/L, monocyte count $<1 \times 10^9$/L [48, 52, 53], IgG 778–1794 mg/dL, IgA 80–413 mg/dL, IgM normal 37–254 mg/dL [45].

Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; CMV: cytomegalovirus; EBV: Epstein-Barr virus; HAV: hepatitis A virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HHV: human herpes virus; HIV: human immunodeficiency virus; HSV: human herpes simplex virus; IV: intravenous; LDH: lactate dehydrogenase; PBMC: peripheral blood mononuclear cell; PCR: polymerase chain reaction; RBC: red blood cell; SJS: Stevens-Johnson syndrome; TEN: toxic epidermal necrolysis; VZV: varicella-zoster virus; WBC: white blood cell; γ-GTP: gamma-glutamyl transpeptidase.

Anti-HHV-6 IgG levels are elevated after 3 weeks following the onset of the reaction (time of drug withdrawal), but not after 2 weeks [58, 59]. Increases in HHV-6 DNA in PBMCs were found to precede the increases in anti-HHV-6 IgG levels in anticonvulsant HSR patients and were absent in controls [57]. The presence of HHV-6 DNA in PBMCs was found to correlate with the magnitude of anti-HHV-6 antibody titers ($P = 0.0002$) [60]. Intermittent detection of HHV-6 DNA in PBMCs was not indicative of illness, such that HHV-6 DNA can be found in the PBMCs at low levels in immunocompetent children, with and without symptoms [60].

Significant increases in anti-HHV-6 IgG titers were noted in all 6 patients included in a study. HHV-6 infection was confirmed in all 6 patients by PCR in PBMCs. HHV-6 reactivation was observed between 17 and 27 days after the onset of the reaction [56].

The presence of HHV-6 DNA in plasma or serum is evidence of active HHV-6 infection. In addition, the presence of serum anti-HHV-6 IgM and increases in the levels of anti-HHV-6 IgG levels are used to differentiate primary HHV-6 infection (i.e., seroconversion) from HHV-6 reactivation. Serum anti-HHV-6 IgG was detected in 7 DRESS patients analyzed, with serum anti-HHV-6 IgM detected in only 4 (57.1%) patients. Seroconversion was diagnosed in 2 patients, in which serum anti-HHV-6 IgM titers were initially undetectable and became detectable in subsequent serum samples, coinciding with increases in anti-HHV-6 IgG titers [14]. Seroconversion is described in detail in a 56-year-old male patient with aromatic anticonvulsant-induced HSR [47].

Aside from the presentation of fever and cutaneous symptoms, other features of HHV-6 reactivation can include lymphadenopathy, hepatosplenomegaly, encephalitis caused by the virus, and severe lymphocytopenia [56].

HHV-6 replication in hepatocytes led to severe hepatitis, associated with elevated enzyme levels, in an 18-year-old female [25]. Anti-HHV-6 antibody titers were elevated, as were HHV-6 DNA levels [25]. Higher odds of detecting HHV-6 DNA in PBMCs and liver biopsies were found in cases of fulminant hepatic failure or acute decompensation of chronic liver disease, compared to chronic liver disease ($P = 0.02$), in a sample of 23 children (median age 24 months) with acute liver failure. Similarly, higher HHV-6 DNA levels were found in cases of fulminant hepatic failure or acute decompensation of chronic liver disease, compared to chronic liver disease cases ($P = 0.0043$). These findings suggest that HHV-6 infection may cause liver failure in children [20].

Tumor necrosis factor- (TNF) α and interleukin- (IL) 6 levels were initially elevated prior to HHV-6 reactivation in 4 (66.7%) of 7 patients in a study. Although initially high, IL-6 levels decreased just prior to HHV-6 reactivation. IL-6 levels were once again elevated after HHV-6 infection in 5 (83.3%) of 6 patients [56]. IL-6 and TNF-α are produced primarily by monocytes and macrophages, the likely reservoir cells of latent HHV-6. Yoshikawa et al. propose that HHV-6 reactivation induces cytokine synthesis, which in turn modulate clinical manifestations [56]. These cytokines likely play an important role in viral reactivation, yet the low number of patients analyzed in this study makes a definite answer impossible [56]. Serum levels of IL-5, interferon (IFN)-γ, and eosinophil cationic protein were increased on day 29 and decreased on day 39 during an episode of carbamazepine DIHS. IL-6 was also increased at this time [58].

An interesting case of drug sensitization was observed in a patient who developed symptoms of DIHS within a few days of being exposed to the antibiotic cefaclor. This patient was previously exposed to cefaclor during an episode of carbamazepine-induced DIHS [58]. Slight increases in IL-5, IL-6, IL-10, IFN-γ, and eosinophil cationic protein were elevated in sera on day 6 of cefaclor DIHS [58]. A drug-induced lymphocyte stimulation test using PBMCs showed that the patient was sensitized to carbamazepine during the first episode, but not to cefaclor. Results of the test showed sensitivity to cefaclor during the second episode [58]. During the first episode, carbamazepine-induced HSR was diagnosed 17 days after the start of carbamazepine, consistent with DIHS. Also, symptoms persisted after the drug was interrupted, and the patient responded to pulsed intravenous (IV) methylprednisolone. In contrast, the patient developed the symptoms much quicker during the second episode, during which time the symptoms were milder and disappeared faster after interruption of cefaclor. The involvement of HHV-6 during the first episode was established with anti-HHV-6 IgG titers and HHV-6 DNA. Absence of HHV-6 reactivation could explain the milder symptoms during the second episode [58]. The magnitude of inflammatory cytokine increase was higher, and eosinophilia was more pronounced during the more severe carbamazepine-induced DIHS, prompting Aihara et al. to argue that HSR is mediated by both elevated inflammatory cytokine levels and activated eosinophils [58].

It is also possible that corticosteroids administered as initial treatment for drug toxicity are involved in the reactivation of HHV-6, Epstein-Barr virus (EBV), and cytomegalovirus (CMV), which became active in the later stage of the disease [15].

Anti-HHV-6 IgG titers were elevated in 7 DIHS patients 21–38 days after the onset of the reaction. Simultaneous elevation in anti-HHV-7 IgG was observed in 6 patients. An elevation in anti-CMV IgG was observed in all patients 35–54 days after the onset of the reaction. Only 2 patients experienced an elevation in anti-EBV IgG, 37 and 48 days after the onset of the reaction. Antibodies to herpes simplex virus, varicella-zoster virus, human parvovirus B19, rubella, and measles were not changed [15]. Viral DNA was detected in whole blood in all patients for whom anti-viral IgG was observed, and in serum for 6 of 7 HHV-6 patients, 5 of 6 HHV-7 patients, and all CMV and EBV patients. Rises in CMV viral loads followed rises in HHV-6 viral loads by 10–21 days [15]. Multiple viral reactivations in a single patient are reported in another study as well [45].

HHV-6 and/or HHV-7 often reactivate first, followed by CMV and/or EBV [15]. There appears to be a correlation between relapse of symptoms and detection of increased viral loads for the virus that gets reactivated first (i.e., HHV-6 or

HHV-7). Subsequent reactivation of CMV and EBV could be asymptomatic [15]. Further immune system perturbations brought about by the initial HHV reactivation are believed to be triggers for subsequent reactivation of other HHVs [15].

3.2. Chromosomally Integrated HHV-6. CIHHV-6 was described and quantified in healthy blood donors and various hospital populations (e.g., organ transplant recipients and immunosuppressed patients) [28, 61–67]. The mean viral loads in whole blood ranged from 10^6 copies/mL to 10^7 copies/mL [28, 62, 64].

Vertical transmission of inherited chromosomal integration is well documented [61, 68]. In addition, there are reports of CIHHV-6 acquisition through bone marrow transplant, followed by the presence of HHV-6 in every cell derived from hematopoietic stem cells [24, 69–71].

It is not known if CIHHV-6 can activate from its integrated state *in vivo*, but several lines of evidence suggest that this is a significant possibility. Integrated HHV-6 has been shown to be activated *in vitro* [32] using histone deacetylase inhibitors and other compounds known to reactivate herpesviruses from latency [27]. As previously discussed, transmission of CIHHV-6 from infected mothers to their non-CIHHV-6 infants, via the placenta, is another indicator that CIHHV-6 may be activated from its integrated state *in vivo* [30]. Children suspected of encephalitis in the United Kingdom had a four times greater rate of CIHHV-6 than the general population [72], and liver transplant patients with CIHHV-6 have higher rates of graft rejections and opportunistic infections [62]. Marek's Disease virus, a herpesvirus associated with tumors in chickens, can reactivate from its integrated state *in vitro* [27]. If specific drugs can cause integrated virus to activate, then individuals with CIHHV-6 may be at an increased risk for drug-induced HSR.

Only a few cases of HSR in CIHHV-6 patients have been reported. Watanabe et al. report the case of a 47-year-old Japanese male with fever (38.8°C) and generalized erythematous rash 78 days after carbamazepine exposure [73]. Mild hepatotoxicity (ALT 70 IU/L, AST 73 IU/L, γ-glutamyl transpeptidase 129 IU/L, and lactate dehydrogenase 686 U/L) and hypogammaglobulinemia (IgG 609 mg/dL, IgA 34 mg/dL and IgM 27 mg/dL) were measured. An abdominal skin biopsy revealed hydropic and vacuolar degeneration of epidermal basal cells, lymphocytic infiltration in the epidermis, and a dense upper dermal infiltrate consisting mainly of mononuclear cells [73]. Anti-HHV-6 IgG titers were increased 128-fold 6 weeks after the reaction onset, while anti-HHV-6 IgM titers remained unchanged throughout the course of the reaction, pointing towards HHV-6 reactivation. HHV-6 DNA was persistently high in serum (>10000 copies/mL) and whole blood. Fluorescent *in situ* hybridization revealed CIHHV-6 on chromosome 1q44 [73].

HHV-6 DNA levels in primary infection and acute reactivation are typically below 5.5×10^5 copies [27]. Of interest, the only cases reported of viral loads above this level (or in the same range as individuals with CIHHV-6) appear to be those with graft-versus-host disease or DIHS, two conditions that have very similar symptoms and courses [18, 27, 74].

4. Discussion

In the present paper, we review cases of HSR associated with HHV-6 reactivation. In the vast majority of patients, HHV-6 infection occurs during infancy or early childhood [60]. Following resolution of the primary infection, the virus remains latent in salivary glands, PBMCs, and the central nervous system. HHV-6 reactivates in immunosuppressed individuals and is associated with fever, rash, encephalitis, bone marrow suppression, and transplanted organ rejection. Primary HHV-6 infection can be identified by the presence of HHV-6 DNA in the serum, plasma, or cerebrospinal fluid, or reverse transcription PCR in the absence of elevated antibody titers [60]. Reactivation is marked by detection of both viral DNA and elevated anti-HHV-6 antibody titers [60]. As anti-HHV-6 IgG levels increase during the course of the reaction, anti-HHV-6 IgM levels remain constant around <1 : 20 in patients with HHV-6 reactivation [42, 75].

HSRs seem to occur with almost a thousandfold higher incidence in AIDS patients compared to immunocompetent individuals exposed to the same medication, as there is a much higher exposure to drugs in these patients, as well as a much higher incidence of viral infections [76, 77]. Aside from a higher incidence of HSR, HIV positivity can also lead to more severe symptoms, compared to HIV-negative patients [77]. In addition, an *in vitro* study showed increased DNA viral replication in PBMCs belonging to DIHS patients that were depleted for natural killer cells (NK) [78]. These findings point toward an extensive immune barrier aimed against DIHS development, while immune defects in components of these pathways can explain why only a relatively small amount of the population exposed to a drug will develop the reaction.

Interestingly, Kano et al. suggest that HHV-6 reactivation is not a consequence, but rather a prerequisite for anticonvulsant HSR [57]. The expansion of $CD4^+$ T cells and $CD8^+$ T cells in response to HHV-6 reactivation seems to be of extreme importance in the development of multiple organ failure during the course of anticonvulsant HSR. From their immunological findings, Kano et al. believe that continuous anticonvulsant therapy leads to transient immune dysfunction, marked by decreased IgG production [57]. While T cells protect against reactivation of viruses from a latent stage, antibodies prevent the dissemination of reactivating lytic virus. Therefore, DIHS may occur when transient drops in B cell counts and immunoglobulin production allow HHV-6 to be reactivated from latency, in conjunction with the presence of drug-specific T cells. The likely explanation for the lag between start of anticonvulsant therapy and onset of disease is that time is required for immunoglobulin levels to drop below a certain threshold [57].

An *in vitro* study using the human T cell line MT4 found that therapeutic doses of both sodium valproate and carbamazepine led to enhanced HHV-6B replication in a dose-independent manner, whereas phenytoin and sulfasalazine

had no effects on HHV-6B replication or cell proliferation [79]. *In vivo*, there have been reports of phenytoin and sulfonamide antibiotics leading to HHV-6 reactivation in DIHS patients [42, 43, 47, 52].

As such, while widespread, HHV-6 infection is not encountered in all DIHS cases. For example, HHV-6 DNA was detected by PCR in blood or serum in a 25-year-old female treated with phenobarbital, while it was absent in a 21-year-old female treated with phenytoin [16]. Matsuda et al. describe 2 cases of carbamazepine-induced DIHS in which no viral reactivation was observed [53]. Symptoms, including fever, skin eruptions, and liver involvement, occurred between 22 days and 4 weeks after carbamazepine initiation in the two patients, which is consistent with DIHS. Furthermore, eosinophilia and a drop in white blood cell count were observed in both patients [53].

HHV-6 reactivation typically occurs 2-3 weeks after the development of rash in DIHS patients [8, 76]. Evidence of this are elevated HHV-6 IgG titers and plasma HHV-6 DNA [76]. HHV-6 reactivation is often accompanied by relapse of fever and hepatitis [8]. A cascade of viral reactivation can then be started, with HHV-6, EBV, and/or HHV-7 at the top, followed, with some delay, by other herpes viruses, particularly CMV. Reactivation of these viruses is followed by development or exacerbation of clinical symptoms of DIHS, including various organ failures [8, 76, 80]. Of particular interest is CMV reactivation, which can be followed by transient fever, skin rash, myocarditis, pneumonia, or gastrointestinal bleeding [8].

Clinical symptoms (fever, hepatitis, and/or skin rash) correspond to the appearance of detectable HHV-6 DNA levels. Fever and/or hepatitis are the most common clinical features of HHV-6 reactivation in DIHS patients. Based on these findings, HHV-6 reactivation leads to a more severe course of disease in patients with DIHS [18]. HHV-6 reactivation is associated with more severe organ involvement and a prolonged course of the disease in patients with drug rash and systemic symptoms, compared with the remaining patients who did not experience HHV-6 reactivation [18]. Significant detection of HHV-6 DNA corresponds to the presence of anti-HHV-6 IgG titers, and there is a positive correlation between HHV-6 DNA detection and the development of fever and hepatitis [18].

While HHV-6 reactivation is a common feature of DIHS/DRESS, HHV-6 DNA was not detected in 15 patients with generalized morbilliform/maculopapular drug reactions [81]. HHV-7, CMV, or EBV DNA was not detected in any of the patients included in this study. *In situ* hybridization revealed HHV-6 mRNA in a small subset of infiltrating mononuclear cells around blood vessels and appendages in DIHS patients only [81]. Similarly, only 1 (25.0%) of 4 SJS/TEN patients for whom data was available showed an increase in anti-HHV-6 IgG titers from 1 : 10 to 1 : 1280 [52]. Aside from a lower likelihood of HHV-6 reactivation, SJS/TEN is marked by a quicker onset of reaction than DIHS/DRESS (2-3 weeks versus a mean 34 days) [52]. However, fever (>38.5°C), leukocytosis, eosinophilia, and atypical lymphocytosis were common features of both SJS/TEN and DIHS/DRESS, whereas liver dysfunction was observed predominantly in SJS/TEN patients and lymph node enlargement was observed predominantly in DIHS/DRESS patients. Treatments for SJS/TEN included systemic corticosteroids in 7 (87.5%) patients and IV immunoglobulin in 1 (12.5%) patient [52].

Furthermore, Teraki et al. warn that some of the clinical features of anticonvulsant-induced SJS/TEN may differ from SJS/TEN induced by other drugs, such as a more delayed onset of symptoms in the former category [52]. Additionally, the rate of hepatic dysfunction and hematological anomalies was also higher than that described with SJS/TEN induced by other drugs [52]. HHV-6 reactivation and atypical lymphocytosis were observed in DIHS patients predominantly, compared to SJS/TEN patients. The precise morphological nature of the skin reaction is another criterion used to differentiate DIHS from SJS/TEN. These findings should be interpreted with care due to the low sample size [52].

Carbamazepine was associated with 12 (52.2%) of 23 anticonvulsant HSR cases reported in a cohort study. All patients developed fever and pruritus. Maculopapular exanthematous eruptions were observed in 15 patients, erythroderma in 11 patients, exfoliative dermatitis in 3 patients, bullous eruption in 1 patients, urticaria in 1 patient, and purpura (vasculitis) in 2 patients [39]. There was no relationship between the drug used and the length or severity of the reaction. In addition to the skin, 20 (87.0%) patients had at least one other organ affected by the reaction, while 8 (34.8%) patients had at least two other organs affected by the reaction. DILI was observed in 12 (52.2%) patients, and kidney involvement was observed in 8 (34.8%) patients. Other affected organs include the spleen (splenomegaly), the lung, the thyroid (hypothyroidism), the heart, and the pancreas [39].

Drug-drug interactions are particularly problematic when several pharmaceutical agents capable of inducting HSR are administered simultaneously. For example, Conilleau et al. report the case of a child exposed intermittently to two anticonvulsants prior to developing DIHS [48]. Despite ethosuximide being interrupted prior to hospitalization and sodium valproate being associated more often with DIHS, it appears that the reaction was caused by the combination of these two structurally unrelated anticovulsants, as a patch test was positive for both ethosuximide and sodium valproate [48]. In a separate case, an infant that was exposed to carbamazepine, phenytoin, and phenobarbital presented with initial HHV-6 infection. Despite the patient being diagnosed with phenobarbital hypersensitivity [43], all three of these aromatic antiepileptic drugs have the potential to cause HSR [2]. Our group also describes patients susceptible to multiple antiepileptics [17].

Minocycline-induced DIHS with EBV reactivation was reported in a 24-year-old African American female. The presence of HHV-6 was not assessed in this study [82]. HHV-6 reactivation was observed in two patients with sulfasalazine severe hypersensitivity [83]. Sequential activation of HHV-6, HHV-7, herpes simplex virus, and CMV was observed in a 46-year-old male who developed DIHS after being exposed to cyanamide, a drug used to control alcoholism [84]. HHV-6 reactivation was observed in 2 elderly patients

with allopurinol DIHS [26, 79], and EBV reactivation was observed in a 40-year-old black male with allopurinol DIHS and pancreatitis [85]. The cause of the reaction was unknown in an 18-year-old female with SJS/TEN exposed to valacyclovir, promethazine, synthroid, belladonna, and orthotricycline [26]. Cacoub et al. provide a comprehensive list of 44 drugs associated with DIHS/DRESS [7].

In addition to anticonvulsants, patients can be exposed to additional medication for the treatment of comorbid diseases, opportunistic infections, or even for the management of HSR symptoms (e.g., antibiotics or nonsteroidal inflammatory drugs administered for the treatment of fever). Furthermore, epilepsy patients are often exposed to more than one anticonvulsant, most of which are capable of causing an HSR. While medication is interrupted at the time of HSR diagnosis and the patient is encouraged to avoid rechallenge with the drug that was deemed to have initiated the reaction, it is still important that all medication is considered and tested individually for its capacity to initiate an HSR. Investigators seldom check to see if there is an additive effect between the incriminated anticonvulsant and other medication, or if the anticonvulsant caused the reaction on its own. Also, it is unlikely that HHV-6 reactivation is measured upon admission. As a result, it is difficult to establish whether the drug or the virus initiates the reaction. Kano et al. argue that HHV-6 reactivation creates an environment that favors DIHS development [57]. The same investigators report the case of a 46-year-old woman who developed DIHS 4.5 months after initiating therapy with zonisamide and corticosteroids [80]. Since the reaction occurred well outside the time frame characteristic of DIHS and since reactivation of herpes viruses was not measured, it is possible that this phenomenon could have created the conditions necessary for DIHS to occur in this patient with long-term exposure to anticonvulsant therapy [80].

The presence of proinflammatory cytokines during DILI and SCAR is supported by findings that a significantly higher number of eosinophils was found both in the blood and tissue of patients with a drug-induced maculopapular exanthems, compared to control subjects with normal skin and skin from patients with psoriasis [86]. Furthermore, viral antigens can increase recruitment of eosinophils [87].

The presence of HLA-A*3101 allele and homozygosity for epoxide hydrolase 1 (EPHX1) single nucleotide polymorphism rs1051740 (T-C) in exon 3, associated with modifications in epoxide hydrolase activity, were found in a patient with anticonvulsant HSR, possibly triggered by HHV reactivation, suggesting a genetic predisposition to HSR upon HHV reactivation [41].

HHV-6 reactivation can be further observed in the absence of an HSR and is primarily associated with hepatotoxicity. Phillips et al. were able to show with the help of transmission electron microscopy that full and empty virus capsids accumulate in the nucleus of hepatocytes, while viral particles bud out into the nuclear envelope and the cytoplasm [19]. Over the years, several drugs were used to treat HHV reactivations *per se*, as well as opportunistic infections in HSR patients with HHV reactivation, including idoxuridine [88], cytarabine [89], and vidarabine [90]. More recently, ganciclovir was used in a patient with HHV-6 and CMV reactivations [42]. Vancomycin was used to treat an opportunistic infection with *Staphylococcus aureus* in a DIHS patient with HHV-6 reactivation [44]. However, treatment against HHV-6 reactivation is often withheld out of fear of worsening the condition due to an increasing number of drugs that the patient is exposed to [26].

Drug-induced adverse reactions represent a concern for patients, clinicians, the pharmaceutical industry, and health providers. Interestingly, very little mention of HHV-6 reactivation is made in a comprehensive article discussing DILI, including acute liver failure in major DILI registries from Europe and the United States [91].

In conclusion, this paper provides a multifaceted assessment of drugs implicated in HSR that have been reported to induce HHV-6 reactivation. This information may facilitate the possible link between the diagnosis of SCAR and DILI, and HHV-6 reactivation.

Abbreviations

ADR: Adverse drug reaction
ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
CIHHV-6: Chromosomally integrated human herpes virus-6
CMV: Cytomegalovirus
DIHS: Drug-induced hypersensitivity syndrome
DILI: Drug-induced liver injury
DRESS: Drug reaction with eosinophilia and systemic symptom
EBV: Epstein-Barr virus
HAV: Hepatitis A virus
HBV: Hepatitis B virus
HCV: Hepatitis C virus
HHV: Human herpes virus
HIV: Human immunodeficiency virus
HLA: Human leukocyte antigen
HSR: Hypersensitivity reaction
HSV: Human herpes simplex virus
IFN: Interferon
IL: Interleukin
IV: Intravenous
LDH: Lactate dehydrogenase
PBMC: Peripheral blood mononuclear cell
PCR: Polymerase chain reaction
SCAR: Severe cutaneous adverse reactions
SJS: Stevens-Johnson syndrome
TEN: Toxic epidermal necrolysis
TNF: Tumor necrosis factor
VZV: Varicella-zoster virus
γ-GTP: Gamma-glutamyl transpeptidase.

Authors' Contribution

J. C. Pritchett and R. M. Nanau contributed an equal amount of work.

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Review Article

Nutrition and Nonalcoholic Fatty Liver Disease: The Significance of Cholesterol

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Nonalcoholic fatty liver disease (NAFLD) is a common chronic liver disease that ranges in severity from simple steatosis to cirrhosis. NAFLD is considered to be associated with hepatic metabolic disorders, resulting in overaccumulation of fatty acids/triglycerides and cholesterol. The pathogenesis and progression of NAFLD are generally explained by the "two-hit theory." Most studies of lipid metabolism in the NAFLD liver have focused on the metabolism of fatty acids/triglycerides; therefore, the impact of cholesterol metabolism is still ambiguous. In this paper, we review recent studies on NAFLD from the viewpoint of hepatic lipid metabolism-associated factors and discuss the impact of disordered cholesterol metabolism in the etiology of NAFLD. The clinical significance of managing cholesterol metabolism, an option for the treatment of NAFLD, is also discussed.

1. Introduction

Histological features of nonalcoholic fatty liver disease (NAFLD) include steatosis, hepatocellular ballooning, the formation of Mallory bodies, apoptosis/necrosis, and inflammation [1]. Around 10–20% of patients with NAFLD have nonalcoholic steatohepatitis (NASH), which can develop into cirrhosis and hepatocellular carcinoma [2–5]. Because excess nutrition intake is one of the main causes, NAFLD is often accompanied by obesity, insulin resistance, hypertension, and/or dyslipidemia, which are manifestations of the metabolic syndrome [6]. Therefore, nutritional management and therapeutic exercise are fundamental steps to treat NAFLD.

The "two-hit theory" is increasingly being adopted to explain the pathogenesis of NAFLD and NASH [7]. In this theory, the first hit consists of the accumulation of fatty acids/triglycerides in the liver, while the second hit involves oxidative stress, mitochondrial dysfunction, and inflammation, which ultimately cause liver damage. It is also clear that inflammatory cytokines and insulin resistance are closely associated with fatty liver during the progression of NAFLD. In previous studies that examined lipid metabolism in the context of NAFLD, dysregulation of cholesterol metabolism has received much less attention than have fatty acids and triglycerides. In this paper, we focus on the role of cholesterol and its metabolites on the pathogenesis of NAFLD, and also the validity of cholesterol management as a method of treating this disease.

2. Fatty Acid Metabolism in the NAFLD Liver

Hepatic lipid homeostasis represents a balance between lipid uptake, synthesis, catabolism, and secretion. Therefore, steatosis, a typical characteristic of NAFLD, is expected to be caused by disordered lipid metabolism, particularly inhibition of fatty acid oxidation and enhanced lipogenesis. Many factors involved in hepatic lipid metabolism pathways have been identified, even though the precise cellular networks are not fully elucidated.

Adiponectin regulates hepatic fatty acid uptake and *de novo* lipogenesis. AMP-activated protein kinase (AMPK) works as a metabolic master switch, and its activity is regulated by adiponectin and tumor necrosis factor-α (TNFα).

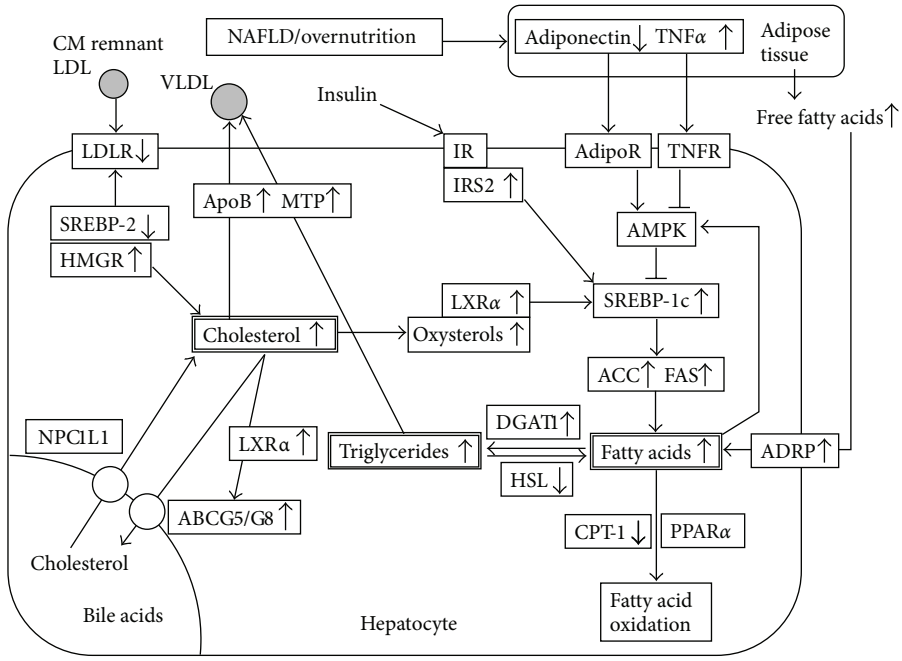

Figure 1: Expression profile of lipid metabolism-associated factors in nonalcoholic fatty liver disease (NAFLD). The established pathophysiological pathways in NAFLD involve increased delivery of fatty acids to the liver and increased SREBP-1c signaling because of cholesterol overload and insulin resistance. ABCG5/G8: ATP-binding cassette G5/G8; ACC: acetyl-CoA carboxylase; AdipoR: adiponectin receptor; ADRP: adipose differentiation-related protein; AMPK: AMP-activated protein kinase; ApoB: apolipoprotein B; CM: chylomicron; CPT-1: carnitine palmitoyltransferase-1; DGAT1: diacylglycerol acyltransferase 1; FAS: fatty acid synthase; HMGR: HMG-CoA reductase; HSL: hormone sensitive lipase; IR: insulin receptor; IRS2: insulin receptor substrate 2; LDLR: LDL receptor; LXRα: liver X receptor α; MTP: microsomal triglyceride transfer protein; NPC1L1: Niemann-Pick C1-like 1; PPARα: peroxisome proliferator-activated receptor α; SREBP: sterol regulatory element-binding protein; TNFα: tumor necrosis factor α; TNFR: TNF receptor.

Inhibition of AMPK results in the activation of sterol regulatory element-binding protein-1c (SREBP-1c), which upregulates fatty acid synthesis-associated enzymes, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). This leads to enhanced fatty acid synthesis and overproduction of triglycerides, ultimately resulting in liver steatosis [8]. Fatty acids are used for β-oxidation in mitochondria and peroxisomes under the regulation of peroxisome proliferator-activated receptor-α (PPARα). Fatty acids are ligands for PPARα, which transactivates the expression of genes involved in the transport, oxidation, and export of free fatty acids, including carnitine palmitoyltransferase-1 (CPT-1), the rate-limiting enzyme in fatty acid β-oxidation.

The relationship between NAFLD and lipid metabolism has been extensively investigated in studies that analyzed the hepatic gene expression profile in animals fed a high-fat diet [9] and in liver biopsy samples from NAFLD patients [10–14], and their expression profiles have been compared with those in normal individuals. Figure 1 summarizes the pathological changes in the NAFLD liver, which may lead to the accumulation of triglycerides, free fatty acids, and cholesterol. To our knowledge, the following events are known to occur in NAFLD. First, hepatic steatosis develops because of upregulated fatty acid synthesis, but it is questionable whether downregulation of fatty acid oxidation is also involved [15–18]. Second, adiponectin production is reduced because of increased visceral fat accumulation. Adiponectin levels are inversely proportional to insulin resistance and hepatic steatosis in NAFLD patients [19]. Third, insulin resistance, which is common in NAFLD, causes fatty liver, while increases in hepatocyte fatty acids levels cause hepatic insulin resistance [20]. Fourth, the severity of insulin resistance is correlated with the severity of NASH. Fifth, disturbed insulin signaling in hepatocytes leads to steatosis associated with the activation of SREBP-1c and the induction of fatty acid synthesis [21].

Recent findings suggest that the cannabinoid system is also involved in the development of fatty liver [22–24]. In animal studies, cannabinoid 1 (CB1) receptors were activated by a high-fat diet *via* induction of the synthesis of endocannabinoids, such as 2-arachidonoylglycerol and anandamide. CB1 receptor activation enhanced the expression of several lipogenic factors, including SREBP-1c, ACC and FAS, and downregulated CPT-1, resulting in increased *de novo* fatty acid synthesis and suppression of fatty acid oxidation. However, in the context of lipid metabolism, the signaling pathway downstream of the cannabinoid receptor has not been identified.

3. Cholesterol Metabolism in NAFLD

In humans, cholesterol is absorbed from the diet and synthesized by cells in various tissues. A healthy man weighing 60 kg contains approximately 140 g of cholesterol, but only 1% of

the total cholesterol is involved in a dynamic metabolic cycle [25]. In one study, the mean intake of dietary cholesterol was estimated to be 300–500 mg/day [14]. They also reported that the dietary cholesterol aggregates into micelles with biliary cholesterol (800–1300 mg/day) in the duodenum [14]. Physiologically, approximately 50% of the cholesterol is absorbed in the jejunum *via* a cholesterol transporter Niemann-Pick C1-like 1 (NPC1L1) expressed on the brush border membrane. The cholesterol is then transported to the liver in the form of chylomicrons and chylomicron remnants [26]. NPC1L1, which may facilitate the hepatic accumulation of cholesterol, is expressed on the canalicular membrane of hepatocytes in humans. Another transporter pump system involving ATP-binding cassette (ABC) G5/G8 excretes cholesterol into bile [27].

The main metabolic pathways of cholesterol in hepatocytes include (1) cholesterol *de novo* synthesis (acetyl-CoA-mevalonate-cholesterol pathway); (2) cholesterol uptake in the form of LDL and chylomicron remnants; (3) cholesterol excretion into the blood in the form of VLDL; (4) cholesterol excretion and uptake through bile *via* ABCG5/G8 and NPC1L1, respectively; (5) synthesis of bile acids and their excretion. Under normal conditions, these pathways interact with each other to maintain cholesterol levels within a specific range.

However, in NAFLD patients, these systems are highly disorganized. SREBPs act as regulators of hepatic cholesterol levels and activate genes involved in the synthesis of cholesterol and free fatty acids [28]. SREBP cleavage-activating protein (SCAP) has a cholesterol-sensing domain that senses intracellular cholesterol levels and directs the activity of SREBPs. Physiologically, when intracellular cholesterol levels are low, SREBPs are first translocated to the Golgi apparatus by SCAP and undergo proteolytic cleavage. Next, the cleaved activated form of SREBP is released to the nucleus. When intracellular cholesterol levels are high, SCAP activity and SREBP activation are suppressed. However, in the context of NAFLD, the regulatory loop of SREBP is disturbed, even if the intracellular levels of cholesterol and/or fatty acids are high [29]. In our study using liver biopsy samples from NAFLD patients, despite excess cholesterol accumulation in hepatocytes, *de novo* cholesterol synthesis remained greatly enhanced even though SREBP-2 expression was downregulated [30]. In the liver of these patients, as evidence of excess cholesterol accumulation, cholesterol uptake was reported to be suppressed because of markedly downregulated expression of LDL receptor (LDLR). Cholesterol excretion was enhanced *via* overexpression of ABCG5/G8, apolipoprotein B, and microsomal triglyceride transfer protein (MTP) [30], but it was considered that the secretion of cholesterol reaches a plateau in NAFLD patients. Even in this situation, cholesterol synthesis continued with upregulated expression of HMG-CoA reductase and synthase, farnesyl P-P synthase and squalene synthase [30–32]. This is because excess levels of cholesterol and its oxysterol metabolites, which are agonists for liver X receptor-α (LXRα) [32], cause excess fatty acid synthesis and steatosis by activating the LXRα-SREBP-1c pathway. LXRα expression was also upregulated in the liver of NAFLD patients [31, 32]. As shown in Figure 1, cholesterol uptake in the form of LDL is limited by the intracellular accumulation of fatty acid and cholesterol, while fatty acid and cholesterol synthesis are upregulated in the NAFLD liver. These findings suggest that the feedback system regulating intracellular lipids levels is disrupted in NAFLD.

4. Nutritional Analysis in NAFLD Patients

In some nutritional investigations, it has been suggested that high-fat, high-fat plus low-protein, high-carbohydrate, and/or high-cholesterol diets are the main causes of NAFLD [33–36]. Although many NAFLD patients show excess nutrition intake, obese, and/or insulin resistance, not all patients exhibit these features. In our nutritional analysis on Japanese population, nonobese NAFLD patients had some features that differed from those of obese patients [37]. Naturally, the dietary intake levels of total energy, fat, and carbohydrate were markedly higher in obese NAFLD patients with insulin resistance than those in nonobese NAFLD patients and healthy volunteers. The most interesting finding was that cholesterol intake was significantly higher in nonobese NAFLD patients than in obese NAFLD patients although cholesterol intake in obese patients was also significantly higher than that in healthy volunteers. In our hepatic expression analysis of lipid metabolism-associated genes, we found that LXRα expression levels were significantly higher in nonobese NAFLD patients than in obese NAFLD patients [13]. Of note, cholesterol overload can upregulate LXRα expression and activate fatty acid synthesis by increasing oxysterol levels, metabolites of cholesterol that act as agonists for LXRα and activate the LXRα-SREBP-1c pathway. These nutrition and gene expression profiles indicate that excess cholesterol intake (i.e., cholesterol supply) itself can be a strong stimulant for the development of steatosis, even though the total calorie intake may be within the normal range. Recent reports using model animals support our findings in nonobese NAFLD patients. Fatty liver without obesity can be established in animal models by feeding them with a hypercholesterolemic diet containing normal calorie level [38–40]. Although this animal model showed marked hypercholesterolemia, which was not observed in our patients, this may be because the diet for animals contains a very high cholesterol content (0.2–1.25%). Moreover, serum cholesterol levels may be preserved in NAFLD patients because dietary cholesterol is promptly taken up into the hepatocyte cholesterol pool.

5. Prospects for Cholesterol Management Therapy

As described above, it seems that cholesterol overload initiates the development of NAFLD. The progression from simple steatosis to steatohepatitis (NASH) usually involves the second hit, such as oxidative stress and inflammation. In some studies of nutritional animal models, the accumulation of cholesterol rather than fatty acids/triglycerides plays a critical role in this progression, possibly because of increased susceptibility to oxidative cell death [41]. It has

also been suggested that the regulation of cholesterol can control C-reactive protein levels and insulin sensitivity [41]. Conversely, in some reports, the progression of triglyceride accumulation and suppression of fatty acid oxidation were not hepatotoxic and actually protected against worsening liver damage [42]. Therefore, cholesterol management may be a promising treatment target for NAFLD.

Ezetimibe, a blood cholesterol lowering agent, is a NPC1L1-specific inhibitor and selectively blocks 54% of cholesterol absorption from the intestine in humans and in animals [43, 44]. Ezetimibe is quickly absorbed, enters the enterohepatic circulation, and has a half-life of 24 hours. From a nutritional point of view, it is important that ezetimibe does not inhibit the absorption of fat-soluble vitamins. In our clinical study, nonobese NAFLD patients showing excess intake of dietary cholesterol were treated with ezetimibe [45]. After starting the therapy, although significant changes were not seen in their body weight, their serum ALT levels decreased by 49.3 ± 16.1% and 45.3 ± 24.2% at 6 and 12 months, respectively. Moreover, steatotic findings on ultrasonography improved in some patients. Interestingly, NPC1L1 knockout mice with excess nutrition intake were resistant to fatty liver, while ezetimibe elicits therapeutically significant effects in animal models of NAFLD [46, 47]. These findings demonstrate that over intake and hepatic accumulation of cholesterol, leading to the activation of the LXRα-SREBP-1c pathway, are closely related to the development of NAFLD. Accordingly, inhibiting cholesterol absorption or reducing dietary cholesterol intake may offer a reliable therapeutic strategy for NAFLD. It has also been reported that HMG-CoA reductase inhibitors (statins) improve serum ALT levels in NAFLD patient [48–50].

Considering these findings, reducing hepatocytic accumulation of cholesterol may represent a fundamental treatment strategy for NAFLD [51]. To establish treatments focusing on cholesterol management, more clinical evidence is clearly needed. For example, cholesterol-restricted diet or lipid modulators (ezetimibe and statins) may be less effective in obese NAFLD patients with insulin resistance than in nonobese patients. This is because other factors associated with obesity and insulin resistance are involved in the development of fatty liver, and these factors may mask the effect of ezetimibe. There are other questions that also need to be answered. For example, does the therapeutic effect of statin in combination with ezetimibe surpass that of monotherapy? Can long-term cholesterol management with ezetimibe and/or statins really improve steatosis as well as ALT levels in NAFLD? It is also important to assess whether the clinical effects of cholesterol management are observed in patients with steatohepatitis (i.e., NASH) as well as patients with simple steatosis, and whether there is a synergistic/additive effect of cholesterol management in combination with antioxidant therapy or liver protection therapy.

6. Conclusions

The feedback system controlling intracellular lipids levels is greatly disrupted in NAFLD. Lifestyle modifications offer simple therapeutic targets for NAFLD. Cognitive nutritional support, which is aimed at reducing calorie-intake and avoiding overeating, should be developed alongside pharmaceutical treatments to prevent the disease progression to cirrhosis and HCC. Excess cholesterol intake, in particular, is a major stimulant for the development of fatty liver. The accumulation of cholesterol rather than triglycerides may play a critical role in the progression from simple steatosis to steatohepatitis. Accordingly, strategies targeting cholesterol accumulation offer basic therapeutic approaches for NAFLD patients, and cholesterol management therapy seems to represent a promising treatment for NAFLD. The potential clinical benefit of cholesterol management for treating NAFLD with respect to hepatic steatosis and injury should be estimated in appropriately designed trials.

Conflict of Interests

The authors have no conflict of interests to declare.

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Review Article

Role of NKT Cells in the Pathogenesis of NAFLD

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Nonalcoholic fatty liver disease (NAFLD) is the most frequent chronic liver disease and shows various inflammatory changes in the liver. Among those inflammatory cells, natural killer T (NKT) cells are found to have a critical role during the disease progression. NKT cells may have a protective role at the early stage with simple steatosis through modification of insulin resistance, whereas they act as a progression factor at the advanced stage with fibrosis. Those processes are thought to depend on interaction between NKT cells and CD1d molecule in the liver.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most frequent chronic liver disease in the world [1]. NAFLD shows various degrees of necroinflammatory changes and fibrosis in the liver and has been shown to lead to cirrhosis and hepatocellular carcinoma (HCC) [2]. As for the progression of NAFLD from simple fatty liver (FL) to nonalcoholic steatohepatitis (NASH), the "two-hit theory," which proposes the accumulation of fat as the first hit sensitizes the liver to a variety of second "hits" leading to hepatic injury, inflammation and fibrosis, has been generally accepted as an essential mechanism [3], although precise mechanism of the disease progression is still uncertain.

Because various degrees of inflammatory cell infiltration are seen in the livers with NAFLD, especially in NASH, immunological mechanisms are also thought to be profoundly associated with the pathogenesis and progression of NAFLD leading to fibrosis or HCC. However, the precise role of hepatic inflammation or contribution of immune responses in the pathogenesis of NAFLD has not been clarified yet. Recently, innate immune cells including natural killer T (NKT) cells have been shown to contribute to the pathogenesis. In this paper, we summarize and discuss the role of immune reactions in the pathogenesis of NAFLD, especially focusing on NKT cells.

2. Hepatic NKT Cells and Their Role in the Pathogenesis of Liver Diseases

2.1. NKT Cells in the Liver. The liver contains a unique population of resident mononuclear cells including innate immune cells such as Kupffer cells (KCs) or NKT cells, possibly because of a defense mechanism against constant exposure to a variety of toxins and antigens from intestinal bacteria through portal veins [4]. NKT cells are most abundant in the liver, and their regulatory roles in hepatic inflammation have been reported [5]. NKT cells are the unique subset of cells, which have both T-cell receptor (TCR) and specific surface molecules for natural killer cells [6] and are found with up to 30% of the intrahepatic lymphocytes in mice, and up to 10% of them in humans [7].

2.2. Immunoregulatory Role of NKT Cells. NKT cells are divided into type 1 and type 2 NKT cells according to the dependence on the interaction with CD1d, which is a nonpolymorphic glycolipid antigen-presenting molecule structurally related to the class I major histocompatibility complex (MHC). Type 1 NKT cells, which express an invariant TCR containing Vα14 in mice or Vα24 in human, recognize glycolipids in conjunction with CD1d, whereas the repertoire of TCR of Type 2 NKT cells are diverse although precise character is less well understood [6]. CD1d

is a molecule originally identified on thymocytes or antigen-presenting cells [8, 9]. In normal livers, CD1d is mainly expressed on KCs, but is also expressed on hepatocytes at a very low level. CD1d molecule is upregulated on both hepatocytes and bile duct epithelium in liver diseases including NAFLD [10, 11].

Invariant NKT cells, Type 1 NKT cells, are lipid antigen-specific lymphocytes and produce large amounts of T-helper (Th)1 (e.g., interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α)), Th2 (e.g., interleukin (IL)-4, IL-10), and Th17 (e.g., IL-17, IL-22) cytokines through recognition of glycolipid antigen presented on CD1d molecules [12]. Thus, invariant NKT cells may act as immune regulators in various liver diseases. Actually, alterations in the numbers of hepatic NKT cell have been found in various liver diseases such as autoimmune hepatitis, primary biliary cirrhosis, viral hepatitis, or alcoholic and nonalcoholic steatohepatitis. In human autoimmune liver diseases, NKT cells act as proinflammatory cells through hepatocyte apoptosis induced by the release of perforin or granzyme, or by the increased expression of FasL in addition to cytokine production such as IFN-γ or TNF-α [13]. The cells also play anti-inflammatory functions through Th2 polarization, Th17-dependent mechanisms or regulation of other types of immunoregulatory cells such as regulatory T cells [6, 13, 14]. In murine, a number of studies using concanavalin-A-(ConA-) induced hepatitis or alpha galactosylceramide (α-GalCer) induced hepatitis, both of which are murine models of autoimmune hepatitis, have shown the contribution of NKT cells in the disease progression [13, 15]. Collectively, hepatic NKT cells have both proinflammatory and anti-inflammatory functions and play an important regulatory role in the progression of liver diseases.

2.3. The Interaction between CD1d and NKT Cells in Lipid Metabolism. On the other hand, the liver has a central role in lipid metabolism with lipolyis, lipogenesis or fat storage. A recent study showed that the function of hepatic NKT cells is rapidly activated by lipids in a CD1d-dependent fashion [16]. Kotas et al. recently demonstrated that CD1d deficiency induces hepatic steatosis and glucose intolerance with high-fat or choline-deficient diet, and glucose intolerance was mainly induced by decreased hepatic insulin sensitivity [17]. Furthermore, CD1d deficiency also led to aggravation of metabolic parameters such as glucose homeostasis and hepatic lipid metabolism [17]. On the other hand, dietary fatty acids can modulate antigen presentation to hepatic NKT cells by a CD1d-dependent manner [18]. CD1d thus can also modulate insulin resistance and play an important role in lipid metabolism, leading to the formation of hepatic inflammation through antigen presentation to NKT cells.

3. NKT Cells and NAFLD

As described above, NKT cells modulate hepatic inflammation through CD1d recognition in conjunction with glycolipid antigen. Therefore, it is likely and reasonable to hypothesize that NKT cells contribute to the pathogenesis of NAFLD especially in the formation of hepatic inflammation and disease progression.

3.1. The Role of NKT Cells in Animal Models. In murine models, the association between NKT cells and NAFLD has been widely analyzed. Depletion of NKT cells has been reported in ob/ob mice, which are leptin deficient and regarded as a model of obesity-related fatty liver [19, 20]. In ob/ob mice, hepatic sensitization toward proinflammatory conditions is induced by endotoxin from gut, increased production of adipokines, or endoplasmic reticulum (ER) stress, as seen in human NAFLD [19, 21, 22]. Increase in adipokine production or ER stress activates cytokine production from hepatic KCs, especially IL-12, leading to selective depletion of hepatic NKT cells. Recently, Kremer et al. reported that hepatic NKT cells are decreased in hepatosteatosis in KCs- and IL-12-dependent manners [23]. They found that hepatic NKT cells are decreased as hepatosteatosis progresses that is developed by choline-deficient diet, but are preserved in IL-12 deficient mice. Moreover, administration of lipopolysaccharide leads to increase in hepatic IL-12 expression, and the depletion of KCs reduced hepatic IL-12 expression and restored NKT cells. In addition, administration of probiotics has been reported to improve high-fat diet-induced hepatic steatosis and insulin resistance by increasing hepatic NKT cells through reduction in the production of TNF-α and nuclear-factor-(NF)-κB-binding activity [24].

Adoptive transfer of NKT cells or treatment with glycolipid antigens has been shown to result in a reduction of hepatic steatosis and improvement of glucose intolerance in ob/ob mice [25, 26]. Moreover, adrenergic activation by norepinephrine injection has been reported to induce expansion of NKT cell population and improve hepatic steatosis [20]. In wild-type mice model fed by choline-deficient diet or high-fat diet, reduction in hepatic NKT cell numbers accompanied by increased Th1 cytokine production has been demonstrated [27, 28]. Those overall data show that hepatic NKT cells are involved in the process of hepatic steatosis through various metabolic factors or cytokines especially produced by KCs.

On the other hand, the role of hepatic NKT cells has not been elucidated during the progression of NAFLD, because neither ob/ob mice nor mice-fed high-fat diet develop significant liver fibrosis. Recent report by Syn et al. demonstrated that hepatic NKT cells are increased in the NAFLD liver partly due to hedgehog pathway activation, leading to promotion of liver fibrosis through activation of hepatic stellate cells (HSCs) [29]. Collectively, murine studies showed that NKT cells seem to act as important players in fat metabolic disorder and have an important role not only in hepatic steatosis (first hit) process but also in disease progression (second hit) in the pathogenesis of NAFLD.

3.2. The Role of NKT Cells in Humans. In humans, recent several studies including our report [30] also have provided important findings on the contribution of NKT cells to the pathogenesis of NAFLD. Xu et al. reported that peripheral

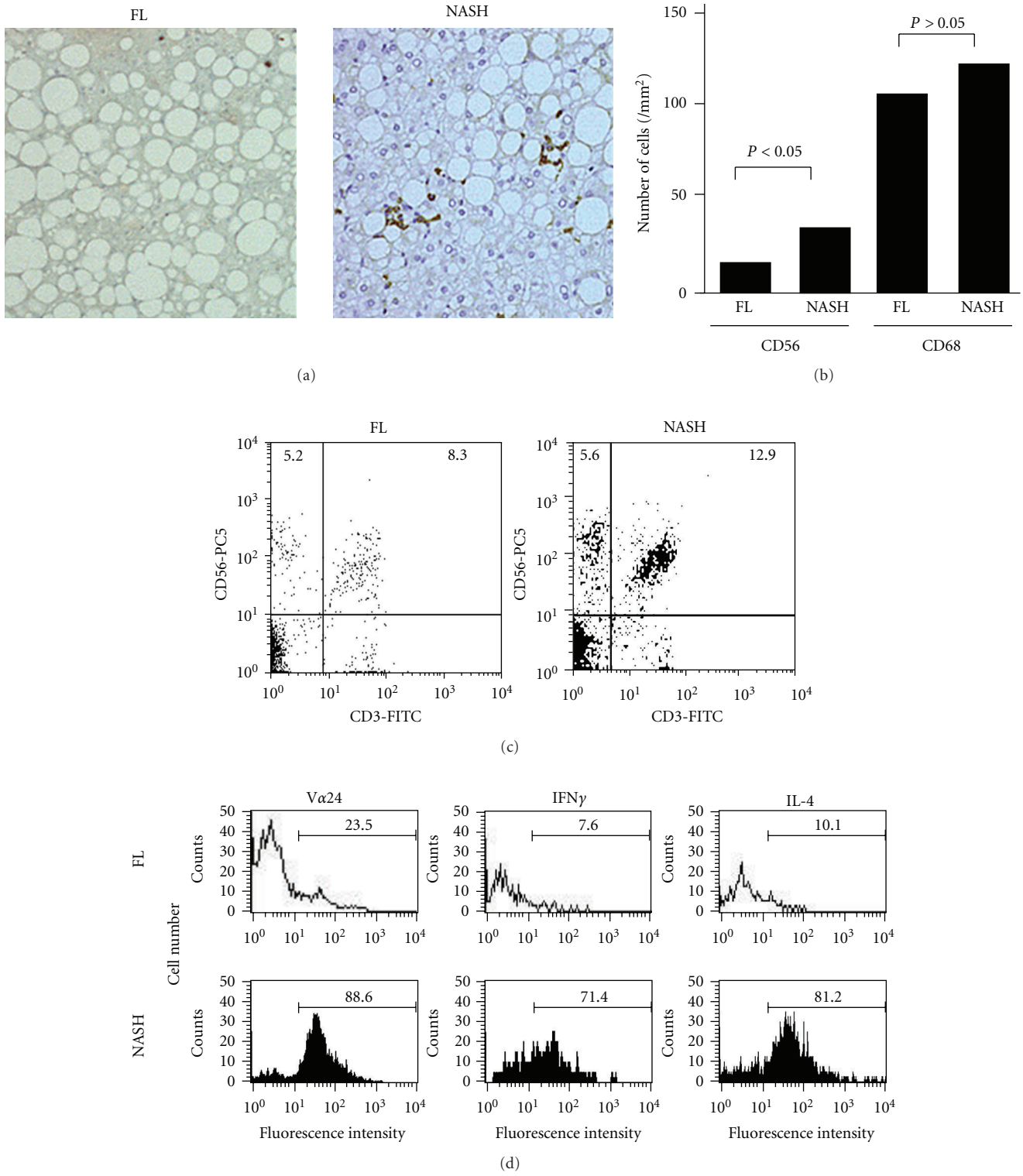

Figure 1: Accumulation of NKT cells in the NAFLD liver with high disease activity. (a) Immunohistochemical study using monoclonal antibody against for CD56 shows accumulation of $CD56^+$ cells in the liver of NAFLD with the disease progresses. (FL; fatty liver versus NASH; nonalcoholic steatohepatitis). (b) The number of $CD56^+$ or $CD68^+$ cells. $CD56^+$ cells are significantly increased as the disease progresses. (c) Flow cytometric analysis of isolated intrahepatic mononuclear cells with NAFLD. Numbers in the quadrant represent the percentage of positive cells. Right-upper quadrant represents NKT cells ($CD3^+CD56^+$ cells). (d) Flow cytometric analysis of Vα24 and intracytoplasmic cytokines of gated $CD3^+CD56^+$ cells among mononuclear cells isolated from livers with NAFLD. Numbers in each histogram represent the percentage of positive cells. These data have been previously presented in [30].

NKT cells are decreased in NAFLD patients as compared to healthy controls [31], may suggesting preferential recruitment of peripheral NKT cells to the liver. Our group recently analyzed the role of hepatic NKT cells in the pathogenesis of NAFLD using liver biopsy specimens of 54 patients with NAFLD [30]. First, we performed immunohistochemical staining for liver biopsy specimens with NAFLD using monoclonal antibodies against CD56 (NK marker) and CD68 (KCs marker). We found that $CD56^+$ cells are increased in the liver with NAFLD as the disease progresses (Figures 1(a) and 1(b)). Then, we analyzed the surface markers and intracytoplasmic cytokines of liver-infiltrating cells isolated from liver biopsy specimens by flow cytometry and found that the number of $CD3^+CD56^+$ NKT cells is increased in NASH as compared with simple FL (Figure 1(c)). Most of those NKT cells express Vα24, which is the phenotype of invariant NKT cells and produce both Th1 and Th2 cytokines in advanced NAFLD (Figure 1(d)). Furthermore, we demonstrated that antigen-presenting cells such as KCs are more activated and increased the expression of CD1d as the disease progresses [30]. Based on those data, we concluded that hepatic NKT cells could contribute to the disease progression in NAFLD through CD1d recognition. More recently, Syn et al. also reported the accumulation of NKT cells to the liver in progressive NASH accompanied with hedgehog pathway activation [29]. Furthermore, an increase in intrahepatic NKT cell has been shown in the livers with moderate-to-severe steatosis by Adler et al. [32]. On the other hand, Kremer et al. reported a different finding, in which NKT cells are decreased in the liver with NAFLD as steatosis developed. However, those results are from a small sample size with relatively mild NAFLD cases (little steatosis 5 cases, mild steatosis 4 cases, moderate steatosis 3 cases) [23]. Thus, because NKT cells are increased in the livers with human NAFLD at least in cases with advanced stages, they could contribute to the disease progression.

3.3. Comparisons of the Role of NKT Cells in the Pathogenesis of NAFLD between Animal Models and Humans. The results from human studies, in which NKT cells contribute to the progression of NAFLD, seem to be inconsistent with those from murine models. In murine models, NKT cells are decreased in the liver with steatosis [19–21, 23, 28] but NKT cells are increased according to the progression of NAFLD in humans [29, 30, 32]. One possible reason in the difference between human and mice is a distinct profile of adipokine production. It has been reported that serum leptin levels are increased in human NASH [33] but not in mice models of NAFLD, and administration of leptin to murine leptin deficiency models, ob/ob mice, actually increases the number of NKT cells [19]. Adipokines such as leptin may thus have a potential role in the regulation of the numbers of intrahepatic NKT cells. Alternatively, investigation on simple steatosis has not fully been done in humans as compared with murine studies, because patients with simple steatosis are usually healthy leading to lack of the opportunity to analyze the disease. Moreover, there has been no murine model for human NASH [34]. Those differences might make

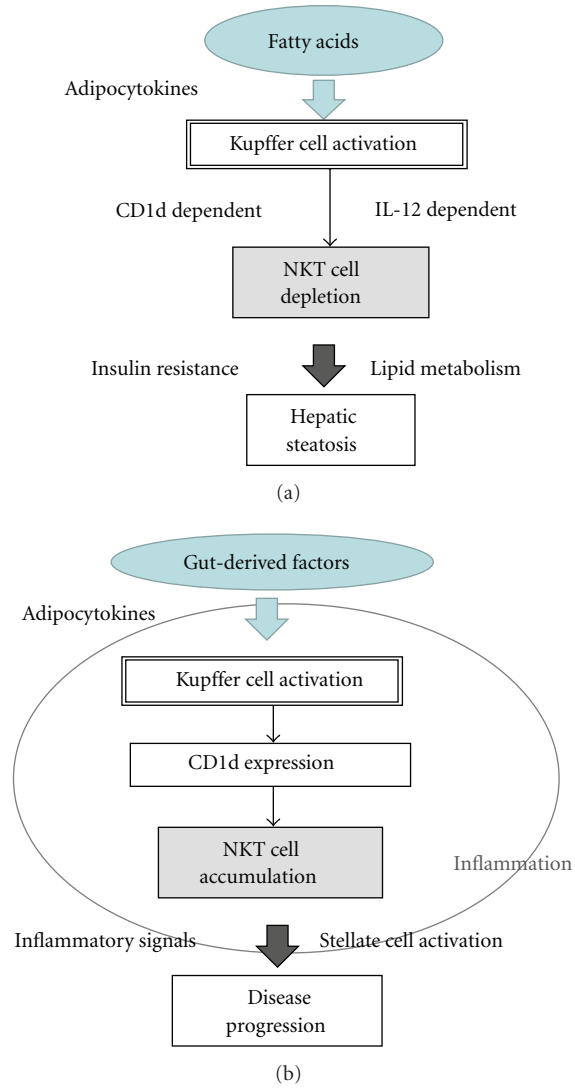

Figure 2: A hypothetical contribution of intrahepatic NKT cells in the progression of NAFLD at the early (a) and the late stage (b). Interaction between NKT cells and CD1d could play an important role in the pathogenesis during the entire phase of NAFLD.

the contribution of NKT cells in NAFLD in humans and murine inconsistent. Further investigation for the difference in the role of NKT cells between human and mice need to be done in the future.

3.4. Summary and Hypothetical Pathogenesis from Experimental Models (Figure 2). In summary, NKT cells seem to be decreased by activation of KCs through enhanced production of IL-12 at an early stage of NAFLD, whereas those are increased by upregulation of CD1d expression through increased production of adipokines or gut-derived microbiota at an advanced stage of NAFLD in humans. NKT cells may have a protective role at an early stage with simple steatosis by modification of insulin resistance (Figure 2(a)), whereas they act as a progressive factor at an advanced stage with fibrosis through increased proinflammatory

cytokine production, NF-κB activation, or HSCs activation (Figure 2(b)). These processes are mainly dependent on interaction of NKT cells with CD1d molecule in the liver. The change in degree or pattern of intrahepatic CD1d expression may thus influence the numbers or functions of NKT cells.

4. Conclusion

NKT cells have a regulatory role in the pathogenesis of lipid-metabolic disorder including NAFLD through interaction with CD1d on antigen-presenting cells. Manipulation of NKT cells might thus have a therapeutic potential.

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Review Article

"Human Babesiosis": An Emerging Transfusion Dilemma

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Babesiosis, a common disease of animals, can infect humans via vector "tick bite", particularly in endemic areas. The recent reports of fatal cases in Hepatitis C and postliver transplant patients resulting from transfusion of contaminated blood should alert the medical profession regarding this emerging dilemma in endemic as well as nonendemic areas and the need for accurate blood screening for transfusion. Here, we illustrate different stages of the parasite lifecycle, progression of babesiosis in animal model, some aspects of pathologic outcomes, ongoing therapeutic modalities, and a feasible Acridine Orange fluorescent methodology for the diagnostic evaluation of blood samples.

1. Introduction

Human babesiosis, is transmitted by the bite of species "*Ixodes*" tick (Figure 1) or by the blood transfusion from an infected individual in North America [1, 2]. Isolated reports have been documented of possible transplacental or congenital transmission of babesiosis to neonates [3]. In USA, clinical cases of babesiosis have been reported primarily in endemic areas, in the Northeast (New England and New York) to Midwest (Wisconsin and Minnesota), and California [4–7]. Recent cases from nonendemic area provide an alert to the medical professions including hepatology and gastroenterology community regarding emergence of the potential for transfusing contaminated blood and blood products with this infectious agent [7–9]. This includes fatal incident of babesiosis in two patients with the history of Hepatitis C, blood transfusion, and/or liver transplantation. Patient a, 43-year-old female from a nonendemic area (Delaware) was admitted after 3 days of fever, cough, and fatigue. The patient had been treated with pegylated interferon for 40 weeks and was blood-transfusion-dependent for the most of her life. She was identified with babesiosis allegedly resulting from an infection with *Babesia microti* (*B. microti*) after receiving a contaminated blood transfusion. She was treated with clindamycin/quinine, but patient expired 3 days after admission [7, 10]. The second case was a 73-year-old female from Indiana with the history of liver transplant, splenectomy, and blood transfusion. She was admitted for fever, dark urine, jaundice, and identified with 6% parasitemia. She was treated with a clindamycin/quinine combination and additional blood transfusion with no improvement, and increased parasitemia (50%). The patient expired 2 days later [8]. Additionally, 57-year-old female patient with a 26-years history of Crohn's disease and from endemic area (Cape Cod, MA) developed a severe fever (102°F) and syncope. She was on antitumor necrotic factor (anti-TNFα: Infliximab, Remicade) maintenance therapy (5 mg/kg every 8 weeks) for a period of 10 months and a history of tick bites. This patient was diagnosed with 15% parasitemia and was treated successfully with atovaquone/azithromycin combination that responded favorably and recovered [6].

2. *Babesia microti (B. microti)*

Babesia, is an intraerythrocytic protozoan parasite (Figures 2 and 3, blood from experimentally infected hamster with *B. microti*) which can cause a spectrum of flu-like symptoms, including benign headache and fever, in immunocompetent patients. However, in neonates, the elderly, splenectomised, immunocompromised, and AIDS patients [6–14] as well as animals [15–18], the disease can manifest with

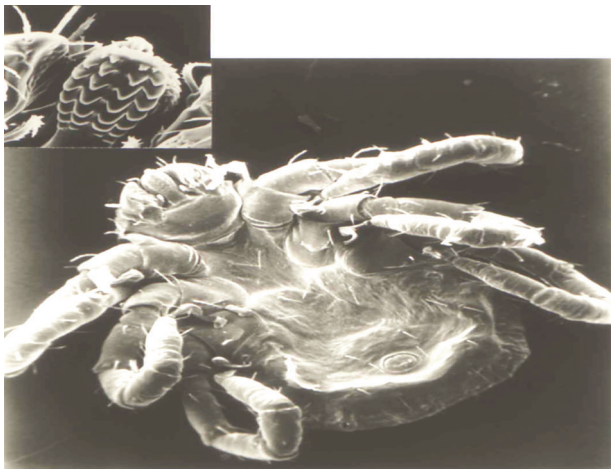

Figure 1: Scanning electron micrograph of an *Ixodes* tick larva with enlarged mouth part (mandibles and hypostome) demonstrating blade-shaped denticules. Tick inserts denticles into the flesh of the victim, opens them like an umbrella during blood meal, and infects the host with *babesia* (sporozoites).

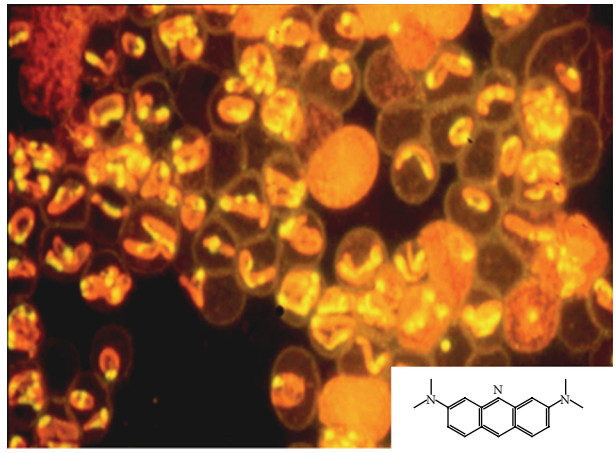

Figure 2: Blood smear, from hamster infected with *B. microti,* stained with acridine orange (molecular formula) and examined with fluorescent microscopy. Babesial organisms appear as single-ring forms (Trophozoites) or multinuclei (Merozoites) within the red blood cells (RBCs). Nuclei (DNA) stain yellow and the rest of the protein orange red inside the RBCs. RBCs appear as hollow ghost cells.

hepatitis (Figure 4(a), experimentally infected hamster), hydrothorax, pneumonia (Figure 4(b), infected hamster), myocarditis, splenomegaly, glomerulonephritis, hematuria, and hemolytic anemia resulting from fulminating babesiosis including fatal outcomes if not properly diagnosed and treated [6–8, 10, 15]. Babesiosis is named after Victor Babes, a Romanian microbiologist, who discovered the protozoan parasite in the cattle with febrile hemoglobinuria (1888).

3. Transmission

Babesia has a life cycle similar to malarial organisms with sexual stage in the vector *Ixodes* ticks (Figure 1) which transmit the organism. The sexual stage occurs in the tick salivary glands as extracellular sporozoites and sporoblasts. During a blood meal, the infested tick inserts its denticles into the flesh of the victim, opens them like an umbrella, and infects the host with sporozoites form. Rodents serve as reservoir host, and humans become accidental host when exposed to the infested tick in an endemic area. After an infected tick bite, babesial organisms enter the blood stream and invade the red blood cells (RBCs) and replicate asexual form as merozoites (Figures 2, 3(a), and 3(c), blood from infected hamster) and damage the organs. Parasites and infected RBCs harboring babesial organisms are picked up by the body immune cells, including neutrophils (Figures 3(b), 3(d), 3(e), and 3(f), blood from infected hamster), and macrophages and transferred to the splenic tissue which eventually become overwhelmed. Man can transmit the parasite by the blood transfusion, fetal congenital transmission, or infected organ transplantation [1–4, 15–18].

4. Animal Models

Hamster is a common animal model to study the disease and to explore drug therapy. Mice usually develop a low-grade parasitemia and not favored as a model. Hamsters are inoculated via *i.p* with an infected blood-harboring babesial organisms. The disease is usually subacute in hamsters, and peak parasitemia occurs in 2-3 weeks although infections may prove to be fatal in about l-2 months after inoculation [16]. Another hamster model was established by *B. microti* passaged into immunocompromised animals and then transitioned into immunocompetent (normal) golden hamsters [15]. Hamsters were immunosuppressed with one injection of Depo-Medrol followed by dexamethasone in daily drinking water. Then the hamsters were inoculated *i.p* with the infected blood. This model was found remarkably similar to the immunocompromised and severe cases of humans with babesiosis. Similar to acute cases in human, parasitemia raised rapidly to 70–90% in about 10 days following the inoculation. The tropism of the organism, histology, pathogenesis, and response to drugs in this fulminating hamster model was almost identical and mimicked those parameters in humans [15, 17].

5. Therapies

The clindamycin/quinine combination therapy has been described in the clearance of parasitemia and resolution of this disease, although, failures of this treatment approach have been reported [1, 2, 7–11, 13, 19]. In addition, blood exchange in severe cases has been shown with a variable success [7, 8, 10]. Previous studies described the efficacy of azithromycin/quinine against *B. microti* in a hamster model as a possible alternative for the clindamycin/quinine combination [16].

Furthermore, atovaquone was described to be more effective than the combination of clindamycin/quinine in prevention as well as therapeutic babesiosis in experimental studies [17, 18]. In contrast, proguanil (an antimalarial

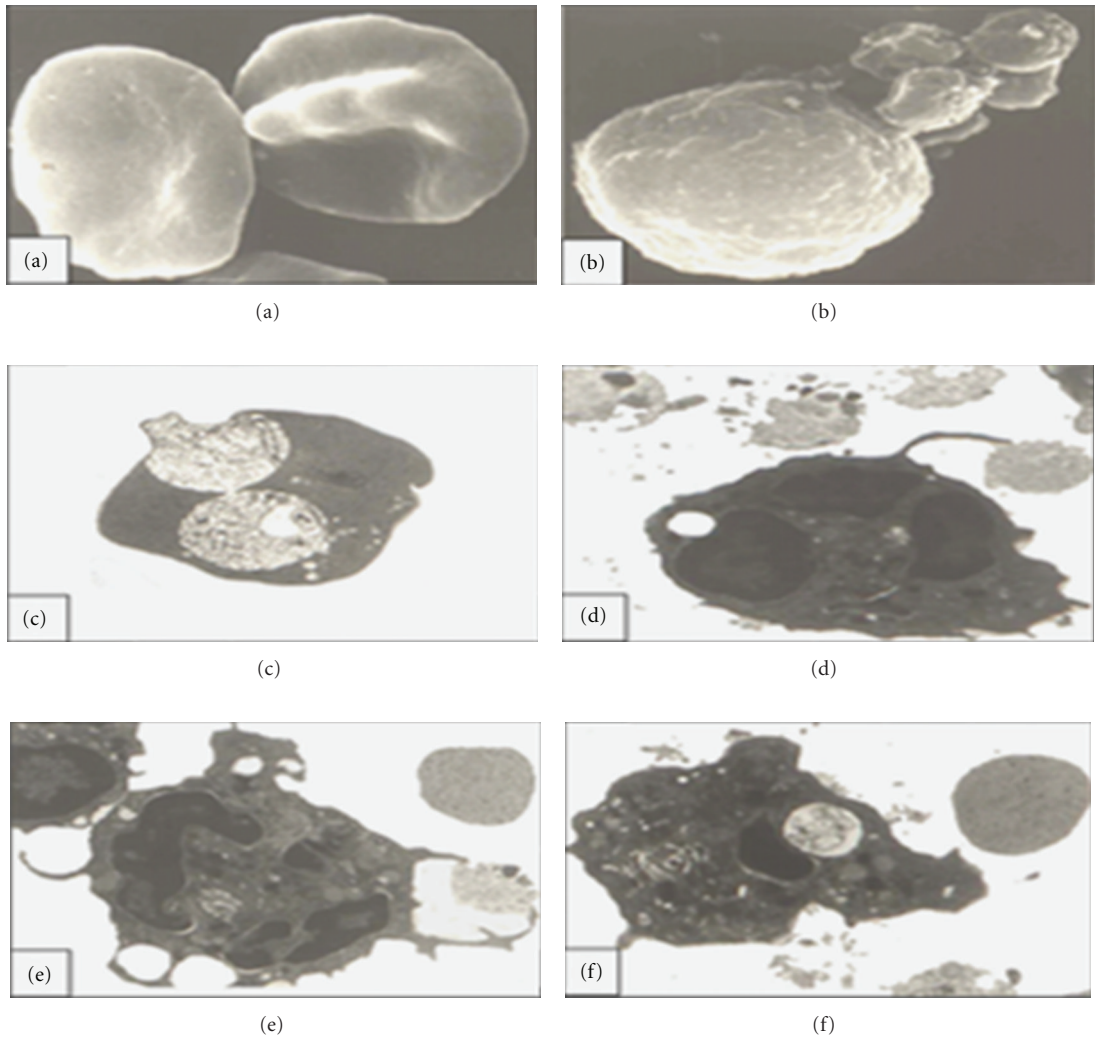

Figure 3: (a, b) are scanning electron micrographs and (c–f) transmission electron micrographs from the cross-sections. (a) infected RBCs from hamster harboring babesial organisms. (b) immune cell (neutrophil) attached to babesial organisms via extended pseudopods. (c) Cross section of the infected RBC from hamster harboring 2 distinct trophozoites. (d–f) cross-section of neutrophil extending pseudopods toward extracellular *babesia* during attachment (d), engulfment (e), and ingestion (f) of the organism (hamster blood).

agent) alone had no effect against the disease in the model [17, 18]. Currently, atovaquone and azithromycin combination have been reported effective in babesiosis patients from endemic area [6, 12] as well as those acquired infection via blood transfusion [1, 7, 20] including neonates [21].

Atovaquone is a 1,4-hydroxynaphthoquinone compound, an analog of ubiquinone, with potent antimalarial and antipneumocystic activity that has been effective in animals and humans for preventive and therapeutic management of *Pneumocystis carinii* pneumonitis [22–25], Toxoplasmosis [26], but not cryptosporidiosis [25]. Atovaquone has been reported, as generally, safe, well tolerated with minor side effects, and an effective alternative therapy compared to the clindamycin/quinine combination against babesiosis in cases [3, 6, 12]. More effective and better-tolerated therapeutic modalities are still needed to prevent possible development of drug resistance in the future.

6. Diagnosis

Patients are usually found to be asplenic, anemic with fever, and jaundice. Laboratory tests include low hemoglobin, high total Bilirubin, aspartate aminotransferase (200–7000 IU/L), creatinine levels, and hemoglobinuria [6–8, 10].

Currently Babesiosis is diagnosed using immunofluorescent assays, enzyme immunoassay EIA, ELISA, RT-PCR, and microarrays. *Babesial* immunoglobulin (IgG and IgM titers by immunofluorescent antibody (IFA) is somehow specific, and nested polymerase chain reaction (PCR) and real time to be more sensitive.

Then each case is confirmed using direct blood staining with Giemsa or Wright staining. For Giemsa staining, thin blood smears are fixed in methanol and stained for 20 min in 0.5% Giemsa stock solution in 4% PBS. The acridine orange technique (Figure 4) was found to be rapid and to provide

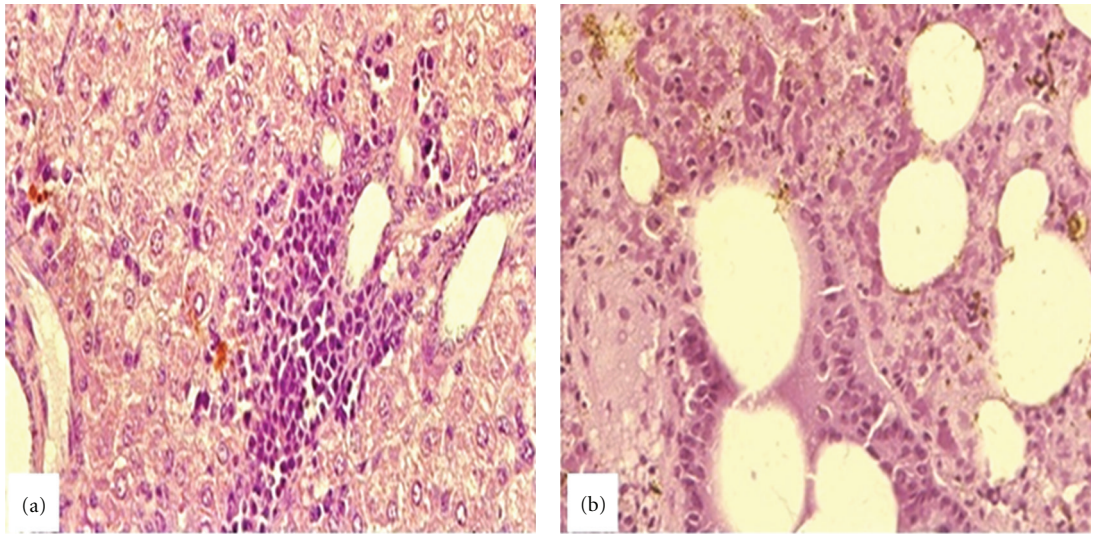

Figure 4: Histopathology sections from infected hamsters stained with hematoxylin and eosin. (a) Hepatitis: Hepatic section demonstrates degeneration of hepatocytes with diffuse periportal infiltration of inflammatory cells and bile stasis (brown spots) in parenchyma. (b) Pneumonitis: pulmonary section with severe edematous, diffuse infiltration of inflammatory cells, and hemosiderin deposits (brown spots) into parenchyma.

a feasible tool and better visualization of *B. microti* in blood smears than other direct staining methods. For Acridine orange staining, blood smears are fixed in methanol and stained in 0.1% of fluorescent dye diluted in Krebs Ringer-phosphate solution pH 7.4 and then examined 10 min later by fluorescent microscopy [15]. While Giemsa-stained slides are more stable and can be stored without degradation or bleaching over long periods, the fluorescent dyes do quench; however, slides stained with fluorescent dyes can be restained at a later time with minimal loss of discriminatory power [15]. Acridine orange is a cell-permeable dye, originally extracted from coal tar and creosote oil. It is a nucleic acid selective fluorescent cationic dye that interacts and binds to DNA of the parasite with a bright yellow-green light excitation. Acridine orange also sequesters protein compartments in the cell and becomes protonated to emit orange light under fluorescent light activation. Acridine orange technique was found to be a simple and effective diagnostic tool to confirm babesial infection compared to other traditionally used direct microscopic examination techniques, for example, Giemsa and Wright's stain [15, 18].

Additionally, differential diagnosis of babesiosis should be performed with lyme disease, malaria, and other blood-borne diseases using serology, PCR, and direct microscopic evaluations.

In conclusion, the awareness of the community for a possible babesial infection, implementation of additional diagnostic techniques, improved preventive measures, vector control to interfere with the parasite life cycle, blood and blood product sanitation, broader screening of the blood donors and/or blood for transfusion, and effective drug development should be combined to prevent increases in babesial infection in endemic as well as nonendemic areas.

Conflict of Interests

The authors declare that they have no conflict of Interests.

Acknowledgments

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Research Article

Paraoxonase Activity and Expression Is Modulated by Therapeutics in Experimental Rat Nonalcoholic Fatty Liver Disease

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Objective. The objective of the present study is to investigate the effect of rosiglitazone, metformin, ezetimibe, and valsartan (alone or in combinations) on paraoxonase (PON) activity and PON-mRNA expression in nonalcoholic fatty liver disease (NAFLD). *Methods*. 54 Male Sprague–Dawley rats were divided to 9 groups: chow diet group (15 weeks); methionine-choline-deficient diet (MCDD) group (15 weeks); MCDD-treated groups for the last 6 weeks with either metformin (M), rosiglitazone (R), metformin plus rosiglitazone (M+R), ezetimibe (E), valsartan (V), or a combination of R+M+V or of R+M+V+E for a total period of 15 weeks. *Results*. PON activities in serum and liver were decreased in MCDD rats. PON activity in serum increased significantly in all treatment groups. PON activity in liver was also increased significantly, except only in groups R, E, V, R+M+V, and R+M+V+E. Liver PON3 mRNA expression increased significantly in groups R+M, E, V, R+M+V, and R+M+V+E whereas liver PON2 mRNA expression increased significantly in MCDD, R+M, E, V, R+M+V, and R+M+V+E. *Conclusions*. PON activities in serum and liver were decreased in NAFLD. Treatment with insulin sensitizers, ezetimibe, and valsartan increased PON activity and reduced oxidative stress both in serum and liver.

1. Background

Paraoxonase (PON) aryldialkylphosphatase is an ester hydrolase that catalyzes the hydrolysis of some xenobiotics, such as organophosphates, unsaturated aliphatic esters, aromatic carboxylic esters and, possibly, carbamates [1]. The paraoxonase gene family contains at least three members, PON1, PON2, and PON3, which are located on chromosome 7q21.3–22.1 [2–4]. PON1 and PON3 mRNA are predominantly expressed in liver, whereas PON2 mRNA is found in different tissues [5] including human endothelial and aortic smooth muscle cells [6]. The enzymes PON1 and PON3 are circulating in serum and tightly bound with HDL in serum, and several studies suggest that it is this association that contributes to the protection conferred by HDL against LDL oxidation [7, 8]. PON2 is cell associated and is not circulating in the serum [6]. PON2 has been shown to reduce reactive oxygen species (ROS) in HeLa cells, reverse the oxidation of oxidized low-density lipoprotein, and inhibit the ability of oxidized low-density lipoprotein to induce monocyte chemotaxis [6], which can contribute to its antiatherogenic properties.

Serum PON1 is synthesized mainly in the liver. The gene expression has been observed only in the liver [9, 10]. Arylesterase and PON1 activities have been shown to be

functions of a single enzyme [11]. Lipid peroxidation products are increased [12, 13] and levels of endogenous antioxidants are decreased in patients with nonalcoholic liver disease (NAFLD) [14]. Chronic exposure to increased levels of oxidative stress may result in an excess of ROS within the hepatocytes which can contribute to the deterioration from NAFLD to non-alcoholic steatohepatitis (NASH). Liver antioxidant enzyme activities Cu/Zn-superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase were high in patients with NAFLD, but with normal thiobarbituric acid-reactive substance, which is a measure of the oxidative end product malonyl aldehyde (MDA) level [15]. Thus, in NAFLD, the increased antioxidant enzymes activities contained the oxidative stress and prevented the increment in MDA levels. Serum nitric oxide, SOD, GSHPx and PON1 activities [16], CuZn-SOD and catalase activities [17], and thiol levels [16, 18] were low in patients with NASH and incapable to compensate for oxidative stress. Increased lipid peroxidation and ROS in NAFLD consume antioxidant vitamins and can inactivate PON [19]. Decreased anti-oxidative defense may increase hepatocyte susceptibility to injury leading to aggravate NAFLD to NASH.

Decreased serum PON1 activity in NASH patients can be secondary to increased levels of proinflammatory cytokines such as interleukin-1 and TNF-α which downregulated mRNA expression of PON1 in HepG2 cells [20]. Decrease in liver microsomal PON1 activity is related to lipid peroxidation and liver injury in rats with CCl4-induced cirrhosis [21]. Decreased PON1 activity in sera of patients with chronic liver disease was suggested to be related to the degree of liver damage and not to allelic or genotypic differences [22]. On the contrary, in a recent study, there was no statistically significant correlation between degree of liver damage (grade and stage of NASH) from one side and between serum PON1 and MDA levels from the other side [23]. PON activity decreased significantly in the livers of Sprague-Dawley rats with experimental NAFLD induced by MCDD alone or enriched with olive oil, butter fat, or fish oil. The most prominent decrease in paraoxonase activity of 67.8% was observed in rats on MCDD enriched with olive oil [24]. The oxidative stress in the liver of this group was also higher than other groups on MCDD. As was previously reported by our group, MCDD rat group increased significantly the liver weight/rat weight ratio by 68%, hepatic triglyceride content by 1263%, and hepatic cholesterol content by 245% compared with the group on chow diet. In the group on chow diet (the control group), these parameters were 0.025 ± 0.003, 1.6 ± 0.3 mol/g, and 0.5 ± 0.0 mol/g, respectively. The MCDD group showed massive fatty infiltration, predominantly macrovesicular. There was mild ballooning degeneration and pericellular fibrosis [25]. Liver weight/body weight ratio in MCDD groups related to the ratio in chow group, liver, and serum triglyceride and cholesterol levels, serum alanine transaminase, fat infiltration in the liver and features of steatohepatitis were reported previously [25].

The key objectives of the present study are to investigate the effect of insulin sensitizers (rosiglitazone and metformin), ezetimibe, and valsartan (each alone or in combinations) on plasma PON activity and PON-mRNA expression in the liver and the potential protective role in NAFLD. To explain the paradox of increased oxidative stress with decreased PON activity in the MCDD rat group, we conducted this study.

2. Animal and Protocol

Male Sprague-Dawley rats (Harlan Laboratories Limited; Jerusalem, Israel) weighting 200–280 grams were studied. Rats were housed in regular cages situated in an animal room at 22°C. The rats before the beginning of the study were maintained on standard rat chow diet (Koffolk, Tel Aviv, Israel) and were given tap water to drink ad libitum. All animal studies were conducted according to the regulations for the use and care of experimental animals and treatment groups. The researchers were authorized to conduct the experiments after a formal training. The experiments were done in an authorized animal housing and laboratory systems.

Fatty liver was induced in rats fed by methionine-choline-deficient diet (MCDD TD.90262, Harlan Teklad, MadisonWI) for 9 weeks [26]. The constituents of MCDD as percent by weight were protein 14.9, carbohydrate 64.3, and fat 10.0. The rats were randomly divided to nine groups, 6 rats in each group. Group 1 served as control group and was maintained on standard chow diet for 15 weeks (control). Group 2 (MCDD group) was given only MCDD for 15 weeks. The following groups (3–9) were on MCDD but were treated during weeks 9–15 with various pharmaceutical interventions. Group 3 was fed MCDD with rosiglitazone (3 mg/kg), Group 4 was fed MCDD with metformin (200 mg/kg), Group 5 was fed MCDD with metformin + rosiglitazone, Group 6 was fed MCDD with ezetimibe (2 mg/kg), Group 7 was fed MCDD with valsartan (2 mg/kg), Group 8 was fed MCDD with metformin + rosiglitazone + valsartan, and Group 9 was fed MCDD with metformin + rosiglitazone + valsartan + ezetimibe. The dosage was selected for each intervention based on the results of previous studies using these agents in various liver diseases [27–30]. The dosage of the drugs in the combined treatments was the same as that in the groups treated with a single agent to maximize the combined effect. The standard rat chow diet contains 21.9% protein, 4.5% fat, 41% starch, 5% sugar, and 3.7% crude fibers. Diets were supplied in pellets form. The medications were given with food and in drinking water. All the drugs are water soluble and there was no need for additional solvents. The drugs were monitored daily and the formula was prepared and supplied by the local pharmacy of the Ziv Medical Center, Safed, Israel and not by pharmaceutical companies. The companies that produced the drugs were GlaxoSmithKline (rosiglitazone), Dexxon (metformin), MSD (ezetimibe), and Novartis (valsartan). The animals were kept at 37°C for 30 minutes before measurements, that were reported in previous publication, [25] were taken. Blood samples were taken by cardiac puncture. Rats were then sacrificed and liver and plasma were examined.

Malondialdehyde (MDA) levels were analyzed by the thiobarbituric acid reactive substances assay, which measures malondialdehyde equivalent [31].

Serum paraoxonase activity was determined by an adaptation of the spectrophotometric method of Furlong et al. [32]. Aliquots (10 μL) of diluted (1 : 5) serum were placed in microliter plate wells in triplicate; the reaction was initiated by adding 190 μL of the substrate (1.2 mmol/L paraoxon in 0.26 mmol/LTris-HCL, pH 8.5, 25 mmol/L CaCl2, and 0.5 mol/L Nacl). After mixing, the plate was read immediately at 450 nm to establish zero time values. Readings were repeated at 2 min interval for 10 min. Nonenzymatic hydrolysis of paraoxon was subtracted from the total rate of hydrolysis. The enzyme activity was calculated from the linear portion of the plot using the molar extinction for p-nitrophenol (17,100 mol/L). One unit of paraoxonase1 activity equals 1 μmol/L·min of released p-nitrophenol.

PON arylesterase activity was measured by using phenylacetate as substrate. Nonenzymatic hydrolysis of phenylacetate was not observed. The reaction took place in Tris buffer (50 mM Tris-HCl, pH 8, 1 mM $CaCl_2$) and was initiated by adding the substrate. Absorbance (λ = 270 nm) was measured at zero time and after 2 min. The enzymatic activity is expressed as micromoles of hydrolyzed phenylacetate.$min^{-1}\cdot mL^{-1}$ (U/mL) or per mg of liver tissue [33].

Liver PON2 and PON3 mRNA expression by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR): total RNA from rat livers was extracted with Trireagent (Sigma-Aldrich, Israel). cDNA was generated from 5 μg of total RNA using 0.5 μg random primers (Sigma-Genosys, Israel). Reverse transcriptase products were subjected to PCR amplification with Ready Mix PCR Master Mix (ABgene, Epsom, Surrey, UK). The amplification conditions were denaturation at 94°C for 15 s, annealing at 57°C for 20 s, and extension at 70°C for 15 s during 30 cycles for PON2 and PON3 [34]. The cDNA products were separated on 2% agarose gel with ethidium bromide. Densitometry was performed using Tina 2.0 g software (Fujifilm, Stockholm, Sweden).

3. Statistical Analysis

Results were expressed as mean ± standard deviation. ANOVA *Tukey's post hoc test* was used to examine differences between means. $P < 0.05$ was considered significant.

4. Results

Rats on MCDD for 9 weeks showed increased MDA levels in serum and liver by 264% ($P < 0.01$) and 124% ($P < 0.01$), respectively (Figure 1, Table 1). PON activities in serum and liver were decreased by 43% ($P < 0.05$) and 44% ($P < 0.01$), respectively (Figures 2(a) and 2(b)). In the liver, PON3 mRNA expression was not changed, while PON2 mRNA expression increased by 113% ($P < 0.05$) (Figures 2 and 3, Table 1).

Treatment with rosiglitazone in comparison with rats on MCDD did not affect serum MDA level, while liver MDA level was lowered by 36% ($P < 0.01$), which was not significantly different from control group (Figure 1). Serum PON activity increased under rosiglitazone therapy by 43% ($P < 0.01$) in comparison with control group and by 148% in comparison with MCDD group ($P < 0.01$). Liver PON activity decreased by 68% ($P < 0.01$) under rosiglitazone treatment in comparison with control group but was not different in comparison with MCDD group (Figure 2). PON2 mRNA expression decreased by 55% ($P < 0.01$) under treatment by rosiglitazone in comparison with MCDD group, back to the level of control group. PON3 mRNA expression was not significantly different in comparison with the control and MCDD groups (Figures 2 and 3).

In rats treated with metformin, MDA levels in serum and liver were decreased by 37% ($P < 0.01$) and 58.3% ($P < 0.01$), respectively, in comparison with MCDD group. Metformin normalized MDA level in the liver, while the level in plasma was still 2.25-fold higher than in control group ($P < 0.01$) (Figure 1). Metformin treatment increased serum PON activity by 134% ($P < 0.01$) in comparison to MCDD group to the normal activity in control group. Metformin did not affect the decreased liver PON activity in comparison with MCDD group. PON2 mRNA expression decreased by 55% ($P < 0.01$) under treatment by metformin in comparison with MCDD group, back to the level of control group. PON3 mRNA expression was not significantly different in comparison with the control and MCDD groups (Figures 2 and 3).

Combined treatment with rosiglitazone and metformin in comparison with rats on MCDD decreased serum and liver MDA levels by 39% ($P < 0.01$) and 52% ($P < 0.01$), respectively. Under this treatment, plasma MDA level was still higher than control group by 120% ($P < 0.01$) and liver MDA was not different from control group. Serum PON activity increased by the combination of rosiglitazone and metformin by 126% ($P < 0.01$) in comparison with MCDD group and was not different from the control group. Liver PON activity decreased by 47% ($P < 0.0001$) and by 71% ($P < 0.01$) in comparison with MCDD and control groups, respectively. PON2 mRNA expression in the liver was not different from that in MCDD group but 120% ($P < 0.01$) above that in the control group. PON3 mRNA expression increased in the liver by 118% ($P < 0.01$) and by 190% ($P < 0.01$) in comparison with the MCDD and control groups, respectively (Figures 2 and 3).

Treatments with ezetimibe, valsartan, combined treatment with rosiglitazone-metformin-valsartan, and combined treatment with rosiglitazone-metformin-valsartan-ezetimibe decreased serum MDA levels by 27% ($P < 0.01$), 31% ($P < 0.01$), 43% ($P < 0.01$), and 42% ($P < 0.01$), respectively. None of these treatments normalized plasma MDA levels in comparison with the control group. The same treatments decreased liver MDA levels in comparison with the MCDD group by 41% ($P < 0.01$), 35% ($P < 0.01$), 57% ($P < 0.01$), and 65% ($P < 0.01$), respectively. All these treatments normalized liver MDA levels in comparison with the control group. Plasma PON activity increased significantly ($P < 0.01$) by 100%, 119%, 91%, and 110% under treatments with ezetimibe, valsartan, combined treatment with rosiglitazone-metformin-valsartan, and combined treatment

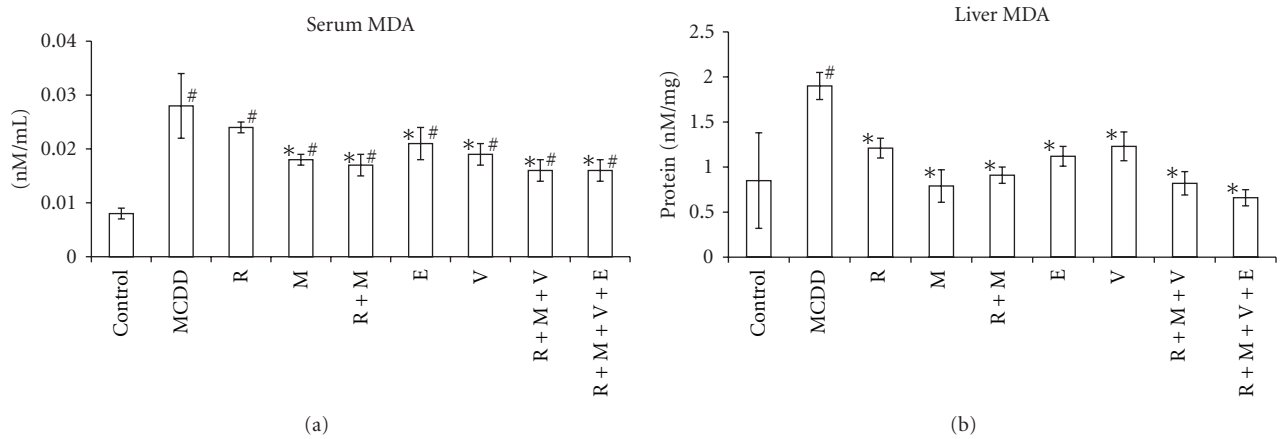

Figure 1: Malondialdehyde (MDA) in sera (a) and livers (b) in rat experimental fatty liver model. Fatty liver in Sprague-Dawley rats was induced by methionine-choline-deficient diet (MCDD) for 9 weeks. Rats were treated for other 6 weeks with rosiglitazone (R); metformin (M); rosiglitazone + metformin combination (R+M); ezetimibe (E); valsartan (V); rosiglitazone + metformin + valsartan in combination (R+M+V), or rosiglitazone + metformin + valsartan + ezetimibe (R+M+V+E). "#" indicates $P < 0.05$ versus control group (fed by standard rat chow). "*" indicates $P < 0.05$ versus MCDD group.

Table 1: Results of serum versus liver in rat experimental fatty liver model. Fatty liver in Sprague-Dawley rats was induced by methionine-choline-deficient diet (MCDD) for 9 weeks. Rats were treated for other 6 weeks with rosiglitazone (R); metformin (M); rosiglitazone + metformin combination (R+M); ezetimibe (E); valsartan (V); rosiglitazone + metformin + valsartan in combination (R+M+V) or rosiglitazone + metformin + valsartan + ezetimibe (R+M+V+E). Malondialdehyde (MDA), Paraoxonase (PON).

	Change by %	Serum MDA	Serum PON activity	Liver MDA	Liver PON activity	Liver PON2 mRNA	Liver PON3 mRNA
MCDD	Versus control	+264 $P < 0.01$	−43 $P < 0.05$	+124 $P < 0.01$	−44 $P < 0.01$	+113 $P < 0.05$	+33 P = NS
	Versus MCDD						
R	Versus control	+203 $P < 0.01$	+43 $P = 0.01$	+42 P = NS	−68 $P < 0.01$	−4 P = NS	−7 P = NS
	Versus MCDD	−15 P = NS	+148 $P < 0.01$	−37 $P < 0.01$	−40 P = NS	−55 $P < 0.01$	−30 P = NS
M	Versus control	+126 $P < 0.01$	+35 P = NS	−7 P = NS	−67 $P < 0.01$	+1 P = NS	+18 P = NS
	Versus MCDD	−37 $P < 0.01$	+134 $P < 0.01$	−58 $P < 0.01$	−39 P = NS	−53 $P < 0.01$	−11 P = NS
R+M	Versus control	+120 $P < 0.01$	+30 P = NS	+7 P = NS	−71 $P < 0.01$	+120 $P < 0.01$	+190 $P < 0.01$
	Versus MCDD	−39 $P < 0.01$	+126 $P < 0.01$	−52 $P < 0.01$	−47 $P < 0.01$	+3 P = NS	+118 $P < 0.01$
E	Versus control	+162 $P < 0.01$	+15 P = NS	+32 P = NS	−31 $P < 0.01$	+174 $P < 0.01$	+281 $P < 0.01$
	Versus MCDD	−27 $P < 0.01$	+100 $P < 0.01$	−41 $P < 0.01$	+28 P = NS	+29 $P = 0.048$	+188 $P < 0.01$
V	Versus control	+148 $P < 0.01$	+26 P = NS	+45 P = NS	−27 $P < 0.05$	+212 $P < 0.01$	+452 $P < 0.01$
	Versus MCDD	−31 $P < 0.05$	+119 $P < 0.01$	−35 $P < 0.01$	+35 P = NS	+47 $P < 0.05$	+316 $P < 0.01$
R+M+V	Versus control	+105 $P < 0.01$	+10 P = NS	−4 P = NS	−40 $P < 0.01$	+227 $P < 0.01$	+560 $P < 0.01$
	Versus MCDD	−43 $P < 0.01$	+91 $P < 0.01$	−57 $P < 0.01$	+12.5 P = NS	+54 $P < 0.01$	+397 $P < 0.01$
R+M+V+E	Versus control	+109 $P < 0.01$	+21 P = NS	−22 P = NS	−11 P = NS	+126 $P < 0.01$	+221 $P < 0.01$
	Versus MCDD	−42 $P < 0.01$	+110 $P < 0.01$	−65 $P < 0.01$	+66 $P < 0.01$	+6 P = NS	+142 $P < 0.01$

with rosiglitazone-metformin-valsartan-ezetimibe, respectively. All these treatments normalized plasma PON activity towards the control group. Liver PON activity did not change under treatments with ezetimibe, valsartan, or combined treatment with rosiglitazone-metformin-valsartan in comparison with MCDD group and was still significantly lower than the activity in control group. Combined treatment with rosiglitazone-metformin-valsartan-ezetimibe increased liver PON activity by 66% in comparison with MCDD group ($P < 0.05$) which was not significantly different from control group. PON2 mRNA expression in the liver under treatment with ezetimibe or combined treatment with rosiglitazone-metformin-valsartan-ezetimibe was not significantly different in comparison with MCDD group. When compared with the control group, it was increased by 174% ($P < 0.01$) in the ezetimibe group, by 212% ($P < 0.01$) in the valsartan group, by 227% ($P < 0.01$) in the combined treatment with rosiglitazone-metformin-valsartan, and by 126% ($P = 0.01$) in the rosiglitazone-metformin-valsartan-ezetimibe group.

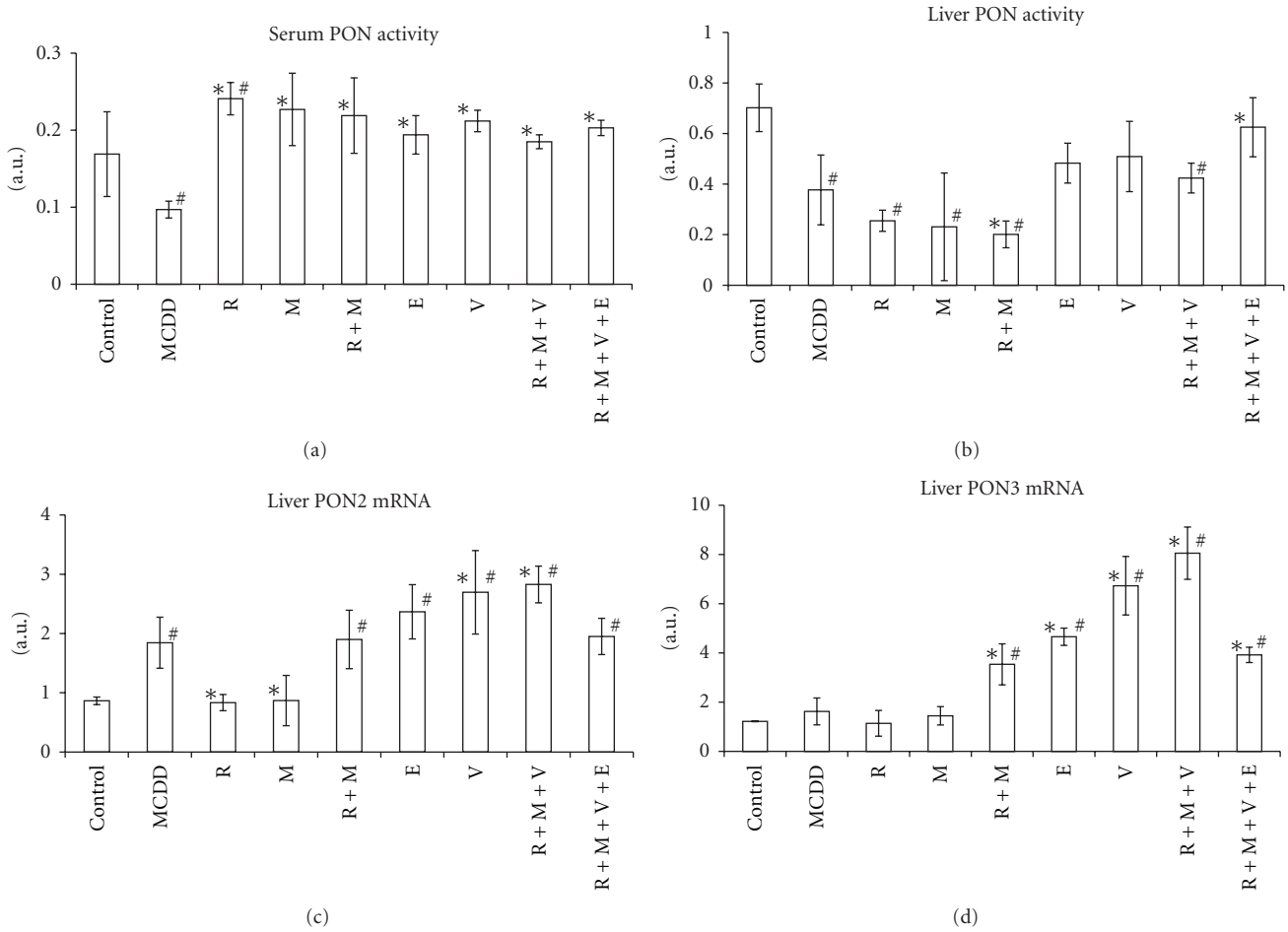

Figure 2: Paraoxonase (PON) activity in sera (a) and livers (b); PON2 (c) and PON3 (d) mRNA expression in rat experimental fatty liver model. Fatty liver in Sprague-Dawley rats was induced by methionine-choline deficient diet (MCDD) for 9 weeks. Rats were treated for other 6 weeks with rosiglitazone (R); metformin (M); rosiglitazone + metformin combination (R+M); ezetimibe (E); valsartan (V); rosiglitazone + metformin + valsartan in combination (R+M+V) or rosiglitazone + metformin + valsartan + ezetimibe (R+M+V+E). "#" indicates $P < 0.05$ versus control group (fed by standard rat chow). "$*$" indicates $P < 0.05$ versus MCDD group.

PON3 mRNA expression increased significantly ($P < 0.001$) in the groups ezetimibe, valsartan, rosiglitazone-metformin-valsartan, and rosiglitazone-metformin-valsartan-ezetimibe by 188%, 316%, 397%, and 142% in comparison with MCDD group and by 282%, 452%, 560%, and 221% in comparison with control group, respectively (Figures 2 and 3).

5. Discussion

As was shown in previous report, there was an increased oxidative stress in the serum and liver of MCDD rat group (experimental NAFLD) [25]. In the present study, it was demonstrated that this is accompanied by decreased PON activity in the serum and liver. In the liver of MCDD rat group, PON3mRNA expression did not increase, while PON2 mRNA expression increased without increased measured enzyme activity. This may be due to oxidative inactivation of the PON2 protein. For PON1 it was shown that oxidative stress in several systems can inactivate the protein [19]. Hydroxyl radicals may be active species primarily responsible for the oxidative inactivation of PON1 in *in vivo* system [19]. Oxidized lipids were shown to inactivate both serum and hepatic PON1 [35, 36]. Free sulfhydryl groups of PON1 interact with specific oxidized lipids and by that PON1 is inactivated. Xanthine oxidase (XO) produces superoxide anions which may decrease PON1 activity. Serum XO activity was negatively correlated with PON activity [35]. In parallel, in mouse peritoneal macrophages (MPMs), oxidative stress markedly increased PON2 mRNA levels but had no effect on PON3 mRNA levels. When MPM lipid peroxides content was increased, PON2 mRNA levels were significantly higher whereas PON3 mRNA levels were similar compared with control cells [34]. *Ex vivo* as well as *in vitro* studies, showed that under oxidative stress PON2 expression and enzymatic activity increased, whereas PON3 expression did not change, while its activity decreased [34]. In the present study, the increased hepatic oxidative stress was associated with increased hepatic PON2 mRNA expression, in accordance with an other study [34]. Despite that, the hepatic PON2

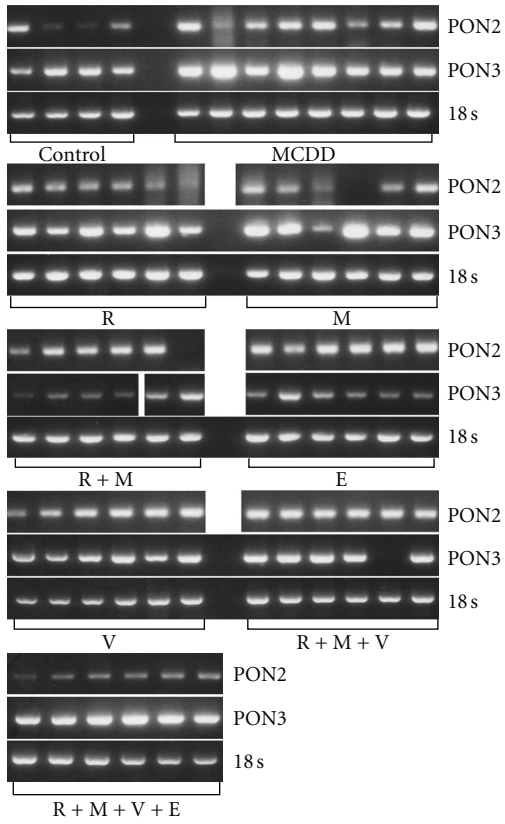

Figure 3: PON2 and PON3 mRNA expression in rat fatty liver experimental model. Fatty liver in Sprague-Dawley rats was induced by methionine-choline-deficient diet (MCDD) for 9 weeks. Rats were treated for other 6 weeks with rosiglitazone (R); metformin (M); rosiglitazone + metformin in combination (R+M); ezetimibe (E); valsartan (V); rosiglitazone + metformin + valsartan in combination (R+M+V) or rosiglitazone + metformin + valsartan + ezetimibe (R+M+V+E). 18 s ribosomal RNA was used for relative quantification. "#" indicates $P < 0.05$ versus control group (fed by standard rat chow). "*" indicates $P < 0.05$ versus MCDD group.

activity was decreased, possibly due to oxidative inactivation of the protein.

Oxidative stress is mediated by several oxidants which act by different mechanisms. So the next step was to examine the possibility whether polypharmacy therapy with different antioxidative mechanisms can have more potent effect to decrease the oxidative stress in NAFLD, and by that to increase the enzymatic activity of hepatic PON2. Treatments with rosiglitazone, metformin, rosiglitazone-metformin, ezetimibe, valsartan, rosiglitazone-metformin-valsartan, and rosiglitazone-metformin-valsartan-ezetimibe had all decreased oxidative stress in the liver to normal level. Liver tocopherol/MDA ratio [25], liver MDA level, and liver PON activity all were not different in the rat group which was treated by rosiglitazone-metformin-valsartan-ezetimibe in comparison with the control rat group. In the rosiglitazone-metformin-valsartan-ezetimibe rat group, serum PON activity was normalized, while serum MDA level improved significantly, but was still higher compared with the control rat group. It seems that oxidative stress sources in the serum are not limited to the liver, but can be from other sources such as skeletal muscles and adipose tissue. All these medications have direct or indirect antioxidant effect. In alloxan-induced type 1 diabetes in rats, metformin treatment led to a decrease in plasma lipid peroxidation levels compared to the non-treated group [37]. Valsartan have been shown to has antioxidative effect. Valsartan has phenolic moiety which may contribute to its free radical scavenging capacity [38]. Ezetimibe can have indirect antioxidative effect on serum LDL [28]. Moreover, in previous report, ezetimibe decreased significantly hepatic fat content, triglyceride content, and cholesterol content by 77%, 53%, and 25%, respectively [25]. Ezetimibe therapy for 2 years in patients with NAFLD improved significantly steatosis and necroinflammatory grades and ballooning and NAFLD activity scores [39]. Rosiglitazone exerted a significant vascular protective effect in hypercholesterolemic rabbits, most likely by attenuation of oxidative and nitrative stresses [40]. Rosiglitazone decreased O_2^- production when it was incubated together with cadmium or zinc [41]. Moreover, rosiglitazone upregulates the mRNA and protein expression of PON2 [42]. Rosiglitazone treatment in rats with metabolic syndrome decreased hepatic lipid peroxidation and increased hepatic paraoxonase activity [43].

In parallel with the decreased oxidative stress, PON activity in the serum was normalized in all treatment groups and increased in rosiglitazone group. Van Wijk et al. reported that rosiglitazone reduced fasting plasma peroxides and increased fasting PON-1 activity, without changing PON-1 mass in type 2 diabetic patients [44]. The only therapy that normalized the decreased activity of PON in the liver was rosiglitazone-metformin-valsartan-ezetimibe. Although PON2 and PON3 mRNA expressions were increased under treatments with rosiglitazone-metformin, ezetimibe, valsartan, rosiglitazone-metformin-valsartan, and rosiglitazone-metformin-valsartan-ezetimibe, only in the last group the PON activity was normalized in the liver. Increased PON2 activity can be due to stabilization of PON2 protein, increased translational state, or increased mRNA expression. These different mechanisms can explain the difference in PON2 activity under different treatments beyond the increased expression of mRNA PON2. The supplementation of E^0 mice with dietary antioxidants significantly increased macrophage PON3 activity, suggesting that oxidative stress was the cause for the reduced macrophage PON3 activity [34]. Moreover, PON3 lactonase activity decreases in parallel to the extent of the oxidative stress. Antioxidants have been shown to preserve PON1 activity [35] and pomegranate juice and vitamin E have similar effect on E^0 mice macrophage PON3 activity [34]. By pharmacologic intervention, serum and hepatic oxidative stress was decreased, protecting PON2 and PON3 protein from inactivation leading to increased serum and liver PON activities. On one side, PON activity can be increased by antioxidative therapy. On the other side, it is well documented that both PON2 and PON3 have antioxidant activity. Pretreatment of cultured aortic endothelial cells with supernatants from HeLa cells over expressing PON3 prevented the formation of mildly oxidized LDL [45]. PON2 overexpression lowers the intracellular

oxidative state of HeLa cells treated with hydrogen peroxide or with oxidized phospholipids [6]. PON2 and PON3 reduced oxidative stress in macrophages from E^0 mice [34]. PON1 reduces the content of macrophage lipid peroxides [46, 47]. It seems that PON2 and PON3 may act as potent cellular antioxidants [33, 45]. In EA.hy 926 cells, both confocal microscopy and biochemical cell fractionation demonstrated a prominent enrichment of PON2 in the nuclear envelope and the ER. Results were confirmed by microscopy of overexpressed PON2-iso1-GFP in several other cell types (SMCs, AoAFs, HeLa, HEK293T, and U2OS) [48]. Experiments performed in 3 major vascular cell types demonstrate that PON2 is capable of reducing oxidative stress [48]. In the present study, increased PON2 and PON3 activities by themselves due to the medications which were used can further decrease oxidative stress. PON3 mRNA expression was increased under treatments with rosiglitazone-metformin, ezetimibe, valsartan, rosiglitazone-metformin-valsartan, and rosiglitazone-metformin-valsartan-ezetimibe. With the potential antioxidative stress of these therapies, this can culminate in increased hepatic activity of PON3.

In the present study, combined therapy with rosiglitazone-metformin-valsartan-ezetimibe was the only treatment that normalized oxidative stress in the liver, decreased oxidative stress in the serum, normalized PON activity both in the serum and liver by increasing PON2 and PON3 mRNA expressions and/or due to higher resistance of PON2 and PON3 protein to inactivation. As previously reported, the combined therapy had greater effect to increase insulin sensitivity and to improve hepatic steatosis than monotherapy in the MCDD rat model of NAFLD [25].

So it can be deduced that combined therapy by its antioxidative effect and possibly by other direct effects increased PON2 and PON3 protein activity.

In this experimental model of NAFLD, the increase in PON2 mRNA levels may be the cell response to oxidative stress, as was shown for other cellular antioxidant enzymes [49].

To conclude, increased oxidative stress in experimental NAFLD decreased PON activity in serum and liver, despite the increased expression of PON2 mRNA in the liver. Treatment with insulin sensitizers, ezetimibe, and valsartan which have antioxidative properties by different mechanisms increased PON2 and PON3 activities which culminated in normalized oxidative stress both in serum and liver. This can be due to increased PON2 and PON3 mRNA translation and/or decreased PON inactivation due to lowered oxidative stress. Improved histological findings in these rats as was shown in previous report [25] can be due, in part, to the decreased oxidative stress.

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Review Article

HIV-Antiretroviral Therapy Induced Liver, Gastrointestinal, and Pancreatic Injury

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The present paper describes possible connections between antiretroviral therapies (ARTs) used to treat human immunodeficiency virus (HIV) infection and adverse drug reactions (ADRs) encountered predominantly in the liver, including hypersensitivity syndrome reactions, as well as throughout the gastrointestinal system, including the pancreas. Highly active antiretroviral therapy (HAART) has a positive influence on the quality of life and longevity in HIV patients, substantially reducing morbidity and mortality in this population. However, HAART produces a spectrum of ADRs. Alcohol consumption can interact with HAART as well as other pharmaceutical agents used for the prevention of opportunistic infections such as pneumonia and tuberculosis. Other coinfections that occur in HIV, such as hepatitis viruses B or C, cytomegalovirus, or herpes simplex virus, further complicate the etiology of HAART-induced ADRs. The aspect of liver pathology including liver structure and function has received little attention and deserves further evaluation. The materials used provide a data-supported approach. They are based on systematic review and analysis of recently published world literature (MedLine search) and the experience of the authors in the specified topic. We conclude that therapeutic and drug monitoring of ART, using laboratory identification of phenotypic susceptibilities, drug interactions with other medications, drug interactions with herbal medicines, and alcohol intake might enable a safer use of this medication.

1. Introduction

Knowledge about indications for antiretroviral therapy (ART) use in chronically human immunodeficiency virus (HIV-) infected patients, relative efficacy of different regimens, patient evaluation, and laboratory monitoring are essential in the success of viral eradication. There are different combination therapies presenting activity against both wild-type and multidrug resistant HIV.

Side effects of these therapeutic interventions include adverse drug reactions (ADRs) such as direct hepatocytotoxicity, hypersensitivity syndrome reactions (HSRs), nausea, headache, diarrhea, and pancreatic toxicity. An ADR represents any noxious, unintended, and undesired effect of a drug, which occurs at doses used in humans for prophylaxis, diagnosis, or therapy [1].

Pharmaceutical agents that can be combined to make up highly active antiretroviral therapy (HAART) can be divided into three categories, namely, nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs), based on their mechanism of action. Substrates of P-glycoprotein, an ATP-dependent efflux membrane multidrug resistance transporter, comprise one class of molecules that can limit the absorption of most PIs. For example, oral administration of saquinavir, indinavir, or nelfinavir in knockout mice lacking this transporter resulted in two- to fivefold increases in plasma drug concentrations [2]. Higher plasma drug

concentrations can therefore produce toxicities in human patients that might lack P-glycoprotein.

While drug interactions should be examined closely whenever prescribing medication in combination with PIs, this is a particularly important consideration with ritonavir, given its powerful inhibition of cytochrome p450 (CYP) 3A4 and its effects on several other mechanisms of drug interactions [3]. These can lead to increased levels of many coadministered medications, and consequently ADRs. Moreover, there is a potential for interaction with nutritional supplements [4].

Physicians should also be aware that patients with chronic viral hepatitis coinfection have additional impairment of CYP3A activity in the presence of ritonavir, compared to HIV patients without viral hepatitis, even at the low doses of 100 mg/day typically used for pharmacokinetic boosting [61].

The various ADRs associated with ART use encountered predominantly in the liver, including HSRs, as well as throughout the gastrointestinal (GI) system, including the pancreas, are presented hereinafter and summarized in Table 1.

2. Hepatotoxicity

Mendes-Corrêa et al. argue that liver damage exists in HIV patients independent of ART exposure [62].

In general, severe hepatic injury occurs in HAART patients, regardless of their treatment [63]. In his last published work, Zimmerman stated unequivocally that the necroinflammatory changes that can be seen in drug-related hepatotoxicity can overlap with those of chronic viral hepatitis [64].

The importance of histological changes in the diagnosis of drug-induced toxicity, its disease spectrum, and the fine structures of hepatocytotoxicity are considered in the discussion section. In the present section, we bring forth only evidence shown by investigators in their work, which is also summarized in Table 1.

Careful review of medication, both prescription and nonprescription, should be compiled in patients with new symptoms or signs of hepatitis, in order to address the possibility of drug toxicity.

Hepatic mitochondrial damage was found in ART-naive patients as well as patients exposed to the NRTIs zidovudine or didanosine [62]. The intensity of dense granules was higher in mitochondria from previously untreated patients, compared to current ART patients ($P < 0.05$). Qualitative analyses showed areas of mitochondrial hyperplasia, with changes in shape (elongation, baloonization, bizarre shapes) and size (megamitochondria) in both groups. There were also increases in the numbers of dense granules, matrix condensation, crista loss, lamellar distributed filamentous material, and crystalloid material [62].

The levels of ^{13}C-methionine exhaled, a measure of hepatic mitochondrial function, increased significantly in ART-naive patients after treatment initiation ($P < 0.001$) [5]. ^{13}C exhalation continued to decrease in ART-naive patients who continued to remain naive ($P = 0.04$), as well as patients who stopped treatment ($P = 0.043$). No changes in the ^{13}C-methionine breath test results were observed among ART-experienced patients who did not change their treatment ($P = 0.31$) or changed only the PI and NNRTI components of their treatments ($P = 0.34$), or among patients who remained on structured treatment interruption ($P = 0.068$). Reinitiation of ART led to significant improvements ($P = 0.008$) [5]. A switch from didanosine or stavudine to tenofovir or abacavir also led to a significant improvement in ^{13}C-methionine breath test performance ($P < 0.001$) [5].

Hepatotoxicity is a relatively common ADR leading to treatment interruptions in HIV patients, observed with different drug combinations (Table 1) [6–27]. Among these, nevirapine was often associated with the development of hepatotoxicity [8, 9, 12, 13, 15, 16, 18, 20, 22, 23, 25]. Nevirapine use was associated with a higher incidence of liver toxicity than efavirenz use [14]. The use of PIs in combination with either efavirenz or nevirapine was associated with an increased risk of hepatotoxicity compared to efavirenz or nevirapine alone (odds ratio (OR) 3.07, 95% confidence interval (CI) 1.01–9.32, $P = 0.04$) [65].

Increases in liver enzymes are also common ADRs characteristic of different ART regimens (Table 1) [20, 28–35, 66]. Increases in alanine aminotransferase (ALT) or/and aspartate aminotransferase (AST) are common symptoms of hepatotoxicity, while increases in alkaline phosphatase and γ-glutamyl transpeptidase were indicative of cholestasis in one study [66].

The median delay between HAART initiation and occurrence of hepatotoxicity was 2.5 months (interquartile range (IQR) 1 to 11 months) in one study [17] and 5 weeks (IQR 3 to 29 weeks) in another study [18]. While a similar number of patients discontinued nevirapine due to hepatotoxicity before month 3 and after a mean number of 9 months in another study [13], van Griensven et al. observed that only 27.6% of 29 cases of nevirapine hepatotoxicity occurred after 6 months of treatment [18].

One study found hepatitis to occur with a similar frequency among zidovudine/lamivudine, zidovudine/didanosine, or stavudine/lamivudine patients [27], whereas a separate study found a higher incidence of hepatotoxicity among stavudine/lamivudine patients [19].

Hepatic events were the most common drug ADRs associated with atazanavir/ritonavir [24]. Jaundice was also observed among atazanavir/ritonavir patients [24, 33, 36], but not among lopinavir/ritonavir patients [33]. Similarly, grade ≥ 3 increases in total bilirubin levels occurred more frequently in the atazanavir/ritonavir group than in the lopinavir/ritonavir group [33]. Acute liver failure, accompanied by jaundice, fever, vomiting, and hepatomegaly, was observed in a 10-year-old male [67]. The patient's condition started improving following liver transplantation and replacement of efavirenz with raltegravir [67]. Bilirubin levels did not affect the rate of hepatotoxicity in another study [35]. Grade 3 hyperbilirubinemia/liver toxicity was also observed with nevirapine [21].

Unconjugated bilirubin levels should be monitored in PI patients. The microsomal enzyme uridine diphosphate

Table 1

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
113 patients, 97 (85.8%) men	Germany	Stavudine/didanosine-based ($n = 73$ (64.6%))	Hepatic mitochondrial toxicity		Stavudine and didanosine	[5]
122 patients, 90 (73.8%) men	Germany	Zidovudine, lamivudine, abacavir, tenofovir	Hepatotoxicity	1 (0.8%) case	Tenofovir	[6]
600 adults, 430 (71.7%) women	Uganda	Zidovudine, lamivudine, abacavir ($n = 300$ (50.0%)) Zidovudine, lamivudine, nevirapine ($n = 300$ (50.0%))	Grade 4 liver function test abnormalities (ALT or/and AST raised >10 times over the upper limit of normal), including acute hepatitis/hepatic failure		Zidovudine Lamivudine Nevirapine	[7]
133 pregnant women	Brazil	Nevirapine-based	Grade ≤3 hepatotoxicity (ALT or/and AST raised ≤5 times over the upper limit of normal)	6 (4.5%) cases	Nevirapine	[8]
			Skin rash	21 (15.8%) cases	Nevirapine	
409 patients 244 (59.6%) pregnant women 87 (21.3%) nonpregnant women 78 (19.1%) men	Thailand	Zidovudine, lamivudine, nevirapine Stavudine, lamivudine, nevirapine	Hepatotoxicity, including asymptomatic hepatitis, symptomatic hepatitis or hepatitis occurring concomitant with rash	64 (15.6%) cases, including 19 (4.6%) symptomatic cases	Likely nevirapine	[9]
			Rash	66 (16.1%) cases, including 21 (5.1%) grade 4 cases	Nevirapine	
			SJS	6 (1.5%) cases	Nevirapine	
142 patients: 105 (73.9%) men, 124 (87.3%) white	Spain	Nevirapine-based Zidovudine, lamivudine ($n = 65$ (45.8%)) Stavudine, lamivudine ($n = 34$ (23.9%)) Stavudine, didanosine ($n = 33$ (23.2%)) Other combinations ($n = 10$ (7.0%))	Hepatotoxicity (ALT or/and AST raised >5 times over the upper limit of normal) Clinical hepatitis (ALT or/and AST raised >5 times over the upper limit of normal and/or nausea, asthenia, jaundice)	8 (5.63%) cases	Nevirapine	[10]
			Skin rash	16 (11.27%) cases	Nevirapine	
157 pregnant women	Brazil	Zidovudine, lamivudine, nevirapine	Grade ≤3 hepatotoxicity	7 (4.4%) cases	Nevirapine	[11]
			Skin rash	24 (15.3%) cases	Nevirapine	
540 patients	Niger	Stavudine, lamivudine, nevirapine	Grade ≤3 hepatotoxicity (ALT or/and AST raised ≤5 times over the upper limit of normal)	78 (15.7%) cases, including 6 (1.2%) grade 3 cases	Nevirapine	[12]
			Cutaneous ADRs	7 (1.3%) cases, including 5 (0.9%) rash cases and 2 (0.4%) pruritus cases	Nevirapine	

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
315 children median age 7.2 years: 158 (50.2%) girls	Rwanda	Stavudine, lamivudine, nevirapine ($n = 281$ (89.2%)). Stavudine, lamivudine, efavirenz ($n = 6$ (1.9%)) Zidovudine, lamivudine, nevirapine ($n = 19$ (6.0%)). Zidovudine, lamivudine, efavirenz ($n = 9$ (2.9%))	Severe hepatotoxicity, including asymptomatic hepatitis, symptomatic hepatitis, and clinical hepatitis	5 (1.7% of 300 nevirapine patients) cases before month 3, including 4 clinical hepatitis cases and 1 grade 3 asymptomatic case 3 (1.0% of 300 nevirapine patients) symptomatic hepatitis cases after a mean 9 months	Nevirapine	[13]
			Grade ≥3 skin manifestations, including SJS	16 (5.3% of 300 nevirapine patients) cases, including 2 (0.7%) SJS cases	Nevirapine	
8736 patient records	Tanzania	Various combinations	Liver toxicity	Higher in nevirapine patients than in efavirenz patients	Nevirapine Efavirenz	[14]
			Skin rash		Nevirapine Efavirenz	
253 women, 42 (16.6%) pregnant	United States	Nevirapine-based Zidovudine/lamivudine most common NRTI backbone	Hepatitis, including late-onset hepatitis	3 (1.2%) cases	Nevirapine	[15]
			Rash with concomitant hepatitis	2 (0.8%) cases	Nevirapine	
			Grade 1 rash	1 (0.4%) case	Nevirapine	
1110 patients, 631 (56.8%) men	Argentina	Nevirapine-based	Hepatotoxicity (ALT or/and AST raised >5 times over the upper limit of normal for patients with previously normal levels or >3.5 times over the baseline level for patients with abnormal basal levels)	35 (3.2%) cases	Nevirapine	[16]
			Severe rash	49 (4.4%) cases	Nevirapine	
290 women, 125 (43.1%) pregnant	Côte d'Ivoire	Zidovudine, lamivudine, nevirapine ($n = 265$ (91.4%)). Stavudine, lamivudine, nevirapine ($n = 25$ (8.6%))	Hepatotoxicity (ALT or/and AST raised >5 times over the upper limit of normal)	10 (3.4%) cases	Not specified	[17]
			Rash	15 (5.2%) cases	Likely nevirapine	

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
2190 adults, 1567 (71.5%) women	Rwanda	Stavudine, lamivudine, nevirapine	Hepatotoxicity (assessed based on ALT levels)	29 (1.3% of entire sample and 21.0% of 138 patients who stopped treatment due to nevirapine toxicity) cases	Nevirapine	[18]
			Skin rash	108 (4.9% of entire sample and 78.3% of 138 patients who stopped treatment due to nevirapine toxicity) cases	Nevirapine	
546 patients, 378 (69.2%) men	Peru	Lamivudine with either zidovudine (76% of cases), stavudine, or didanosine. Other drugs were nevirapine ($n = 314$ (57.5%)), efavirenz ($n = 210$ (38.5%)), lopinavir/ritonavir ($n = 19$ (3.5%)), atazanavir/ritonavir ($n = 2$ (0.4%)), or indinavir ($n = 1$ (0.2%))	Hepatotoxicity	7 (2.3%) cases	Not specified	[19]
			Rash	13 (2.4%) cases	Likely nevirapine or efavirenz	
765 patients: 614 (80.3%) men, 311 (40.6%) white, 265 (34.6%) black, 161 (21.0%) Hispanic	United States	Zidovudine, lamivudine, efavirenz ($n = 380$ (49.7%)). Zidovudine, lamivudine, abacavir, efavirenz ($n = 373$ (48.8%))	Grade 4 hepatotoxicity (ALT or/and AST, total bilirubin, direct bilirubin, alkaline phosphatase, γ-glutamyl transpeptidase raised >10 times over the upper limit of normal)	3 (4.3% of 70 patients who substituted efavirenz with nevirapine due to efavirenz toxicity) cases	Nevirapine	[20]
			Skin symptoms	18 (2.4%) cases 5 (33.3% of 15 patients who substituted efavirenz with nevirapine due to efavirenz toxicity) cases	Efavirenz Nevirapine	
103 pregnant women: 38 (36.9%) Caucasian, 24 (23.3%) Aboriginal	Canada	Nevirapine-based ($n = 56$ (54.4%))	Grade 4 hepatotoxicity (ALT or/and AST raised >10 times over the upper limit of normal)	1 (1.1% of 92 HAART-treated pregnancies) cases	Nevirapine	[21]
			Grade 3 hyperbilirubinemia (bilirubin raised 3–10 times over the upper limit of normal)	2 (2.2% of 92 HAART-treated pregnancies) cases	Likely nevirapine	
			Grade 2 rash and fever	2 (2.2% of 92 HAART-treated pregnancies) cases	Nevirapine	

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
			Grade 4 rash	1 (1.1% of 92 HAART-treated pregnancies) cases	Nevirapine	
137 patients, 103 (75.2%) men	Spain	Didanosine, stavudine, nelfinavir ($n = 67$ (48.9%)). Didanosine, stavudine, nevirapine ($n = 70$ (51.1%))	Hepatotoxicity		Nelfinavir Nevirapine	[22]
			Skin rash		Nelfinavir Nevirapine	
			GI ADRs (mainly diarrhea)	12 (14.9% of 67 nelfinavir patients and 2.8% of 70 nevirapine patients) cases	Nelfinavir Nevirapine	
573 patients, 366 (63.9%) men	Italy	Nevirapine-based 213 (37.2%) were taking zidovudine. 289 (50.4%) were taking stavudine. 71 (12.4%) were taking thymidine analogues. 97 (16.9%) were taking PIs	Hepatotoxicity (increases in serum liver function tests), including grade 3 hepatotoxicity (ALT or/and AST raised >5 times over the upper limit of normal if baseline levels were normal or >3 times over the baseline level if this was higher than the upper limit of normal)	44 (22.3% of 197 toxicity-related treatment interruptions) cases, including 5 cases of grade 3 AST elevations and 11 cases of grade 3 ALT elevations	Nevirapine and/or NRTIs	[23]
			Cutaneous reactions	84 (42.6% of 197 toxicity-related treatment interruptions) cases	Nevirapine	
			Unspecified GI ADRs	10 (5.1% of 197 toxicity-related treatment interruptions) cases	Nevirapine and/or NRTIs	
1318 patients, 967 (73.4%) men	Switzerland	Tenofovir, emtricitabine, atazanavir/ritonavir ($n = 144$ (10.9%)). Tenofovir, emtricitabine, efavirenz ($n = 374$ (28.4%)). Tenofovir, emtricitabine, lopinavir/ritonavir ($n = 216$ (16.4%)). Tenofovir, emtricitabine, nevirapine ($n = 50$ (3.8%)). Zidovudine, lamivudine, efavirenz ($n = 77$ (5.8%)). Zidovudine, lamivudine, lopinavir/ritonavir ($n = 204$ (15.5%)). Abacavir, lamivudine, efavirenz ($n = 77$ (5.8%))	Hepatic events	152 (11.5%) cases, including 42 (29.2%) of 144 atazanavir/ritonavir patients	Atazanavir/ritonavir (OR 2.55, 95% CI 1.01–6.42, $P = 0.047$) Other drugs	[24]

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
			HSR	38 (18.3% of 208 treatment interruptions due to drug toxicity) cases	Nevirapine (OR 3.33, 95% CI 1.43–7.77, $P = 0.005$) Atazanavir/ritonavir	
			GI tract intolerance	60 (28.9% of 208 treatment interruptions due to drug toxicity and 14.2% of 424 lopinavir/ritonavir patients) cases	Lopinavir/ritonavir (OR 5.50, 95% CI 2.67–11.3, $P < 0.001$)	
650 patients, 451 (69.4%) women	Botswana	Zidovudine/lamivudine, zidovudine/didanosine, or stavudine/lamivudine. 325 (50.0%) were taking nevirapine and 325 (50.0%) were taking efavirenz	Hepatotoxicity	11 (3.4% of 325 nevirapine patients) cases	Nevirapine	[25]
			Cutaneous HSR	19 (5.8% of 325 nevirapine patients) cases	Nevirapine	
			Pancreatitis	10 (3.3% of 325 nevirapine patients) cases	Likely nevirapine	
188 patients, 150 (79.8%) men	Spain	Efavirenz-based ($n = 117$ (62.2%)). Lopinavir/ritonavir-based ($n = 71$ (37.8%))	Hepatotoxicity	8 (5.1% of 117 efavirenz patients and 2.8% of 71 lopinavir/ritonavir patients) cases	Efavirenz Lopinavir/ritonavir	[26]
			Diarrhea	1 (0.9% of 117 efavirenz patients) case	Lopinavir/ritonavir Efavirenz	
2233 children	United States	Zidovudine, lamivudine ($n = 1336$ (59.8%)). Zidovudine, didanosine ($n = 1022$ (45.8%)). Stavudine, lamivudine ($n = 1154$ (51.7%)). Stavudine, didanosine ($n = 772$ (34.6%)). Didanosine, lamivudine ($n = 258$ (11.6%))	Hepatitis	56 (1.6% of 1336 zidovudine/lamivudine patients, 1.6% of 1022 zidovudine/didanosine patients, 1.6% of 1154 stavudine/lamivudine patients) cases	Zidovudine and lamivudine Zidovudine and didanosine Stavudine and lamivudine	[27]

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
			Bilirubin elevations	11 (0.5%) cases	Stavudine and lamivudine Stavudine and didanosine Lamivudine and didanosine Zidovudine and lamivudine Zidovudine and didanosine	
			Pancreatitis (assessed by routine monitoring of total amylase levels)	13 (1.7% of 772 stavudine/didanosine patients) cases	Stavudine and didanosine	
33 pregnant women: 20 (60.6%) Hispanic, 9 (27.3%) black, 4 (12.1%) white	United States	Zidovudine, lamivudine, nelfinavir	Grade ≤3 elevated AST and γ-glutamyl transpeptidase	2 (6.1%) cases	Treatment-related	[28]
289 patients: 204 (70.6%) men, 280 (96.9%) white	Spain	Nevirapine-based	Grade ≤3 elevated ALT/AST	6 (2.1%) cases	Nevirapine and hepatitis A virus coinfection	[29]
169 patients, 113 (66.9%) women	Cameroon	Zidovudine, lamivudine, nevirapine ($n = 85$ (50.3%)). Stavudine, lamivudine, nevirapine ($n = 84$ (49.7%))	Increases in ALT activity	1 (1.2% of 84 stavudine patients) case	Stavudine	[30]
			Rash	2 (1.2%) cases	Zidovudine and stavudine	
612 women: 443 (72.4%) black, 111 (18.1%) white, 53 (8.7) Hispanic	United States	Nevirapine-based ($n = 152$ (24.8%)). Nonnevirapine-based ($n = 460$ (75.2%))	Grade ≥2 liver enzyme elevations (graded according to the Toxicity Tables of the Division of Acquired Immunodeficiency Syndrome)	27 (4.4%) cases	Nevirapine and/or other drugs	[31]
			Grade ≥2 rash (graded according to the Toxicity Tables of the Division of Acquired Immunodeficiency Syndrome)	30 (5.7% of 526 patients included in the rash analysis and 26.3% of 114 patients who developed a new rash after therapy initiation) cases	Nevirapine and/or other drugs	
			SJS	1 (0.6% of 152 nevirapine patients)	Nevirapine	

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
88 children mean age 10.2 years: 51 (58.0%) girls, 38 (43.2%) white, 26 (29.5%) black	Switzerland	Lopinavir/ritonavir-based	AST elevation (185 IU/L)	1 (1.1%) case	Lopinavir/ritonavir	[32]
			HSR		Lopinavir/ritonavir	
			GI toxicity, including hepatic and pancreatic symptoms	5 (5.7%) cases	Lopinavir/ritonavir	
			Amylase elevation without serum lipase elevation (870 IU/mL)	1 (1.1%) case	Lopinavir/ritonavir	
883 patients, 606 (68.6%) men	Worldwide	Tenofovir, emtricitabine, atazanavir/ritonavir ($n = 440$ (49.8%)). Tenofovir, emtricitabine, lopinavir/ritonavir ($n = 443$ (50.2%))	Grade ≥3 increases in ALT/AST	25 (3.9% of 435 atazanavir/ritonavir patients and 1.8% of 431 lopinavir/ritonavir patients) cases	Atazanavir/ritonavir Lopinavir/ritonavir	[33]
			Grade ≥3 increases in total bilirubin levels	146 (33.6% of 435 atazanavir/ritonavir patients) cases	Mainly atazanavir/ritonavir	
			Jaundice No mention of Gilbert syndrome or hemolysis	3 (0.7% of 440 atazanavir/ritonavir patients) cases	Atazanavir/ritonavir	
			Diarrhea and grade ≥2 nausea	4 (0.9% of 443 lopinavir/ritonavir patients) cases	Lopinavir/ritonavir	
40 patients, 20 (50.0%) women	Uganda	Zidovudine, didanosine, lopinavir/ritonavir ($n = 36$ (90.0%)). Stavudine, didanosine, lopinavir/ritonavir ($n = 4$ (10.0%))	Elevated AST levels	2 (5.0%) cases	Not specified	[34]
			Nausea or vomiting	7 (17.5%) cases	Didanosine and unspecified drugs	
			Diarrhea	9 (22.5%) cases	Not specified	
49 children, 30 (61.2%) boys	Burkina Faso	Didanosine, lamivudine, efavirenz	Increases in liver enzyme levels	2 (4.1%) cases	Likely didanosine	[35]
			Increases in pancreatic enzyme levels without pancreatitis	1 (2.0%) case	Likely didanosine	
3333 patients	England	Not specified	Jaundice No mention of Gilbert syndrome or hemolysis	7 (3.4% of 203 treatment switches) cases	Atazanavir	[36]
			Suspected/actual HSR	5 (2.5% of 203 treatment switches and 62.5% of 8 treatment switches due to abacavir toxicity) cases	Abacavir	

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
			Unspecified GI side effects	9 (4.4% of 203 treatment switches and 100% of 9 treatment switches due to saquinavir toxicity) cases	Saquinavir	
			Diarrhea	7 (3.4% of 203 treatment switches) cases	Lopinavir/ritonavir	
158 patients, 104 (65.8%) men	Italy	Tenofovir, emtricitabine, efavirenz ($n = 41$ (25.9%)). Tenofovir, emtricitabine, various boosted PIs ($n = 46$ (29.1%)). Abacavir, lamivudine, efavirenz ($n = 12$ (7.6%)). Abacavir, lamivudine, various boosted PIs ($n = 41$ (25.9%)). Other combinations including boosted PIs ($n = 18$ (11.4%))	Early HSR	2 (3.8% among 53 abacavir patients) cases	Abacavir	[37]
56 patients: 49 (87.5%) men, 26 (46.4%) white, 18 (32.1%) black	United States	Tenofovir, another NRTI, lopinavir/ritonavir, fosamprenavir ($n = 28$ (50.0%)). Tenofovir, other NRTIs, lopinavir/ritonavir ($n = 14$ (25.0%)). Tenofovir, other NRTIs, fosamprenavir/ritonavir ($n = 14$ (25.0%))	HSR		Abacavir	[38]
600 patients, 430 (71.7%) women	Uganda	Zidovudine, lamivudine plus either abacavir or nevirapine	Suspected HSR (grade ≤ 3)	15 (3.0% of 300 nevirapine patients and 2.0% of 300 abacavir patients) cases	Nevirapine Abacavir	[39]
			Suspected HSR (grade 4)	4 (1.3% of 300 nevirapine patients) cases	Nevirapine	
357 HLA B*5701-negative adults: 348 (97.5%) men, 307 (86%) white	Australia	Tenofovir and emtricitabine, or abacavir and lamivudine. Other drugs included zidovudine, didanosine, stavudine, atazanavir, lopinavir, efavirenz or nevirapine	HSR		Abacavir	[40]
385 HLA-B*5701-negative adults 313 (81.3%), men 56 (14.5%) black	Europe	Abacavir, lamivudine, efavirenz. Tenofovir, emtricitabine, efavirenz	HSR		Abacavir	[41]

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
211 children mean age 5 years: 111 (52.6%) boys	Zambia	Stavudine, lamivudine, nevirapine	Grade ≤2 rash	15 (7.1% of 211 nevirapine patients and 37.5% of 40 ADRs judged to be definitely/probably related to nevirapine) cases	Nevirapine	[42]
57 patients, 39 (68.4%) men	China	Stavudine, didanosine, nevirapine ($n = 38$ (66.7%)). Stavudine, lamivudine, nevirapine ($n = 10$ (17.5%)). Zidovudine, lamivudine, nevirapine ($n = 9$ (15.8%))	Rash (including grade 3 rash)	3 (5.3%) cases	Likely nevirapine	[43]
173 adults, 107 (61.8%) men	Cambodia	Efavirenz was substituted with nevirapine	Cutaneous HSR	10 (5.8% of 173 patients substituting efavirenz with nevirapine and 52.6% of 19 patients who developed nevirapine-induced treatment-limiting HSRs) cases	Nevirapine	[44]
			Hepatic HSR	10 (5.2% of 173 patients substituting efavirenz with nevirapine and 47.4% of 19 patients who developed nevirapine-induced treatment-limiting HSRs) cases	Nevirapine	
394 patients, 263 (66.8%) men	Cambodia	Stavudine, lamivudine, nevirapine	Minor rash	17 (4.3% of 394 patients who switched efavirenz with full-dose nevirapine and 32.7% of 52 cases of nevirapine-induced ADRs) cases 49 (7.4% of 661 ART-naive patients commencing nevirapine-based HAART and 51.6% of 95 cases of nevirapine-induced ADRs) cases	Nevirapine	[45]

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
			Severe HSR, including severe rash, SJS, TEN and/or hepatitis	35 (8.9% of 394 patients who switched efavirenz with full-dose nevirapine and 67.3% of 52 cases of nevirapine-induced ADRs) cases, including 30 cases of severe rash, 2 cases of SJS, and 3 cases of grade ≥3 hepatitis. 44 (6.6% of 661 ART-naive patients commencing nevirapine-based HAART and 46.3% of 95 cases of nevirapine-induced ADRs) cases, including 36 cases of severe rash (one fatal), 2 cases of SJS (one fatal), one case of fatal TEN, and 5 cases of grade ≥3 hepatitis	Nevirapine	
			Grade ≤4 hepatitis		Nevirapine	
121 adolescents mean age 7 years: 70 (57.8%) boys	Jamaica	77 (63.6%) receiving HAART Zidovudine/lamivudine-based ($n = 72$ (93.5%)). Nevirapine-based ($n = 72$ (93.5%)). Zidovudine, lamivudine, nevirapine ($n = 67$ (87.0%))	HSR	3 (4.2% of 72 nevirapine patients and 3.9% of 77 HAART patients) cases	Nevirapine	[46]
			Nausea and vomiting		Either zidovudine, lamivudine and/or nevirapine	
10186 patients: 7395 (72.6%) men, 6227 (61.1%) Caucasian	Europe and Canada	Nevirapine-based ($n = 6547$) Zidovudine, lamivudine, nevirapine ($n = 4620$ (45.4%))	Hepatotoxicity without concomitant skin rash	124 (1.9% of 4620 nevirapine patients and 27.1% of 458 patients who interrupted nevirapine due to HSRs) cases	Nevirapine	[47]
			Skin rash	334 (5.1% of 4620 nevirapine patients and 72.9% of 458 patients who interrupted nevirapine due to HSRs) cases	Nevirapine	

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
			Unspecified GI symptoms	402 (6.14% of 4620 nevirapine patients) cases	Nevirapine and/or NRTIs	
			Unspecified pancreas-related toxicities	4 cases among nevirapine patients	Nevirapine and/or NRTIs	
217 patients, 122 (56.2%) men	Senegal	Didanosine, lamivudine, efavirenz ($n = 63$ (29.0%)). Stavudine, didanosine, efavirenz ($n = 44$ (20.3%)). Stavudine, lamivudine, efavirenz ($n = 11$ (5.7%)). Zidovudine, lamivudine, efavirenz ($n = 52$ (24.0%)). Lamivudine, zidovudine, nevirapine ($n = 28$ (12.9%)). Didanosine, lamivudine, nevirapine ($n = 8$ (3.7%)). Stavudine, didanosine, nevirapine ($n = 8$ (3.7%)). Stavudine, lamivudine, nevirapine ($n = 3$ (1.4%))	Hepatitis, including hepatitis with concurrent skin rash	3 (6.4% of 47 nevirapine patients) cases, including 2 (4.2%) cases with concurrent skin rash	Nevirapine	[48]
			Skin rash, including SJS and TEN	3 (6.4% of 47 nevirapine patients) cases, including 2 (4.2%) cases of SJS or TEN	Nevirapine	
			Hyperamylasemia	10 (4.6%) cases	Likely efavirenz or nevirapine	
230 adults, 172 (74.8%) men	India	Stavudine, lamivudine, nevirapine ($n = 157$ (68.3%)). Stavudine, lamivudine, efavirenz ($n = 18$ (7.8%)). Zidovudine, lamivudine, nevirapine ($n = 41$ (17.8%)). Zidovudine, lamivudine, efavirenz ($n = 14$ (6.1%))	Severe rash (SJS or TEN)	9 (3.9%) cases, including 1 (0.4%) case of fatal TEN	Likely nevirapine or efavirenz	[49]
126 patients, 109 (86.5%) men	Spain and Italy	Lamivudine, abacavir, efavirenz ($n = 63$ (50.0%)). Lamivudine, abacavir, lopinavir/ritonavir ($n = 63$ (50.0%))	HSR/rash	8 (6.3%) cases	Efavirenz Lopinavir/ritonavir	[50]
21 Caucasian patients, 16 (76.2%) men	France	Efavirenz-based ($n = 7$ (33.3%)) Nevirapine-based ($n = 14$ (66.7%))	HSR	6 (28.6%) cases	Efavirenz Nevirapine	[51]
650 adults, 451 (69.4%) women	Botswana	Either zidovudine and lamivudine, zidovudine and didanosine, or stavudine and lamivudine, plus either nevirapine or efavirenz	SJS	16 (2.5%) cases	Likely nevirapine or efavirenz	[52]

Table 1: Continued.

Study population	Study settings	Treatment	ADRs	Incidence of ADRs	Drugs associated with ADRs	Ref. no.
66 patients, 56 (84.8%) men	Spain	Lopinavir/ritonavir-based ($n = 33$ (50.0%)). Nevirapine-based ($n = 33$ (50.0%)). NRTIs were didanosine, stavudine, and/or zidovudine	Diarrhea	10 (15.2%) cases	Likely nevirapine or lopinavir/ritonavir	[53]
70 patients, 50 (71.4%) men	Spain	Lopinavir/ritonavir-based	Unspecified GI symptoms assessed using the Gastrointestinal Symptom Rating Scale	1 (1.4%) case	Lopinavir/ritonavir	[54]
23 patients, 18 (78.3%) men	Spain	Zidovudine, lamivudine, abacavir, tenofovir	Unspecified GI symptoms		Lopinavir/ritonavir, tipranavir	[55]
115 patients, 70 (60.9%) men	France	Indinavir/ritonavir-based Lopinavir/ritonavir-based Nelfinavir-based	Unspecified GI ADRs Diarrhea	4 (12.5% among 32 lopinavir/ritonavir patients) cases 1 (3.2% among 32 nelfinavir patients) case	Lopinavir/Ritonavir Nelfinavir	[56]
1771 patients, 1204 (68.0%) men	South Africa	Zidovudine, didanosine, efavirenz ($n = 444$ (25.1%)). Stavudine, lamivudine, efavirenz ($n = 444$ (25.1%)). Zidovudine, didanosine, lopinavir/ritonavir ($n = 440$ (24.8%)). Stavudine, lamivudine, lopinavir/ritonavir ($n = 443$ (25.0%))	Nausea, constipation, fatigue		Zidovudine and didanosine. Stavudine and lamivudine	[57]
630 patients, 494 (78.4%) men	Worldwide	Saquinavir/ritonavir-based ($n = 309$ (49.0%)). Lopinavir/ritonavir-based ($n = 163$ (25.9%)). Indinavir/ritonavir-based ($n = 158$ (25.1%))	Unspecified GI toxicity, including grade ≥3 GI ADRs		Saquinavir	[58]
12 patients: 11 (91.7%) men, 9 (75.0%) Caucasian, 3 (25.0%) black	United Kingdom	Saquinavir/ritonavir-based	Mild nausea and diarrhea	5 (41.7%) cases	Likely treatment-related	[59]
1081 patients, 708 (65.5%) men	Italy	Not specified	Pancreatic toxicity (at least 3-fold increases in serum pancreatic enzymes)	166 (38.2% of 435 patients with confirmed laboratory pancreatic abnormalities) cases	Concurrent use of didanosine, stavudine, lamivudine	[60]

glucuronosyltransferase (UGT) mediates conjugation of bilirubin.

Hyperbilirubinemia is an adverse effect that occurs in approximately 25% of indinavir patients, with total bilirubin rises to the 2.5 to 5 mg/dL range [68]. This represents largely indirect bilirubin and is insignificant except as a possible complication of pregnancy [68, 69]. Atazanavir also appears to impair UGT activity [70], such that the PIs atazanavir and indinavir are associated with hyperbilirubinemia. The relationship between underlying genetic risk factors and the risk of developing hyperbilirubinemia remains unclear. UGT1A1*28 allele was associated with jaundice in a study in which bilirubin levels were not measured [24]. In a study of patients who underwent genotypic analysis for polymorphisms associated with increased unconjugated bilirubin, 64 (66.7%) of 96 patients were positive for the UGT1A1*28 allele. [70]. Ocama et al. found that 23 (29.9%) of 77 consecutive HIV-infected patients presenting with hepatotoxicity (jaundice, right upper quadrant pain with fever or malaise, ascites, and/or tender hepatomegaly) had increased transaminase levels as a result of nevirapine and/or isoniazid hepatotoxicity [71]. Of these 23 patients with drug-induced liver disease, 14 (60.9%) presented with jaundice and recovered after drug discontinuation. Hepatitis B surface antigen was positive in 11 (14.3%) patients while antihepatitis C antibody was reactive in only 2 (2.6%). Granulomatous hepatitis due to tuberculosis was diagnosed in 7 (9.1%) patients. Other diagnoses included alcoholic liver disease, AIDS cholangiopathy, hepatocellular carcinoma, schistosomiasis, hemangioma, and hepatic adenoma. Twelve (15.6%) patients died during follow-up, of which 7 (9.1%) died because of liver disease [71].

The overall incidence of severe hepatic injury was not significantly different between NRTIs, NNRTIs, and PIs in a sample of 222 patients, of which 84 (37.8%) were coinfected with hepatitis C virus (HCV) [63]. Coinfection with hepatitis viruses is often associated with a higher risk of hepatotoxicity [63, 65]; however this is not always the case [24]. Elevated baseline liver function tests and older age are additional risk factors for hepatotoxicity [18].

3. Hypersensitivity Syndrome Reaction

HSRs have been associated with the NRTI abacavir, the NNRTIs nevirapine and efavirenz, and the PI amprenavir [72–74]. The potential for HSR development symbolizes a treatment-limiting and potentially life-threatening ADR.

Abacavir HSR is the major treatment-limiting toxicity of HAART regimens containing this drug. This ADR usually occurs in the first 6 weeks of treatment [75]. An HSR characterized by some combination of flu-like symptoms, fever, rash, as well as GI symptoms, including hepatotoxicity, generally occurs in 3–5% of patients starting abacavir [76]. Other symptoms of HSR include malaise, lethargy, myalgia, myolysis, arthralgia, edema, pharyngitis, cough, dyspnea, headache, and paresthesia. Physical findings may include lymphadenopathy, mucous membrane lesions (i.e., conjunctivitis, mouth ulcerations), and rash, which usually appears as maculopapular or urticarial, but can also lead to Stevens-Johnson syndrome (SJS).

Differentiation between abacavir HSR and viral respiratory infections can be problematic. Rash (OR 13.1, $P = 0.02$), nausea (OR 30, $P < 0.001$), vomiting (OR 17.1, $P = 0.001$), and diarrhea (OR 22, $P < 0.001$) were associated with HSR in 15 cases of abacavir HSR matched with 30 controls with culture-proven influenza A with no abacavir exposure [77]. The number of GI symptoms was also predictive of HSR ($P < 0.001$). Multivariate analysis confirmed that the number of GI symptoms (OR 8.6, $P = 0.0032$) and rash (OR 16.9, $P = 0.07$) was associated with abacavir HSR. Abacavir HSR-associated rash was typically mild to moderate in this study, occurring after an average of 9–11 days since treatment initiation [77]. Abacavir HSR was found to resolve itself rapidly following treatment modifications [37]. This reaction was observed in other studies as well [36–41].

Abacavir HSR is strongly associated with GI symptoms [77]. Laboratory abnormalities include elevated liver function tests, increased creatine phosphokinase or creatinine, and lymphopenia. Liver failure and death have occurred in association with HSR. Symptoms associated with HSR worsen with continued therapy but often resolve upon discontinuation of the drug [78, 79].

It is highly recommended that HSR patients avoid rechallenge with full-dose abacavir, as extremely severe symptoms and even death may result [80]. Reports describe the mechanisms of action, efficacy, and ADRs of abacavir in HIV-1-infected patients and illustrate the danger of serially rechallenging patients with this agent even if the patient was previously desensitized [78, 81–83].

A strong statistical association was identified between the human leukocyte antigen (HLA)-B*5701 allele, part of the major histocompatibility complex, and clinically diagnosed abacavir HSR [84]. While abacavir HSR occurs in approximately 5% of HIV patients treated with this drug, the HLA-B*5701 allele was discovered in 6.3% of 11000 genetic screens performed in a Canadian population [75]. As a consequence, prospective HLA-B*5701 screening is performed to identify patients at high risk for abacavir HSR before they are treated [85]. Genetic screening of potential abacavir patients can greatly help prevent HSRs and it can lead to individualizing of HAART in order to prevent toxicity and to improve adherence [75]. Carriers of HLA-B*5701 should avoid abacavir-based HAART [84, 86–91]. Despite prior HLA genotyping, the incidence of abacavir HSR was higher in an abacavir/lamivudine-based regimen compared to a tenofovir/emtricitabine-based regimen [40, 41]. This phenomenon indicates that an additional metabolic or immune mechanism might contribute to the ADR.

Approximately 17% of patients starting nevirapine and 10% of patients starting efavirenz will develop rash of varying severity with or without systemic features, typically between 1 and 3 weeks after starting the drug [92]. As HLA genotyping has the potential to reduce the incidence of abacavir HSR, nevirapine is the pharmaceutical agent most often associated with cutaneous HSRs (Table 1) [9–14, 16–18, 20–25, 31, 39, 42–48]. Moreover, Warren et al. [93] report that nevirapine can be associated with SJS. Albeit

infrequent, SJS and toxic epidermal necrolysis (TEN) are sometimes observed in conjunction with nevirapine and can be fatal [9, 13, 31, 45, 48, 49]. The median time for skin rash occurrence was 1.0 month (IQR 3 weeks to 3 months) [17, 18, 45]. The majority of patients who discontinued nevirapine due to HSR did so within 18 weeks (for both skin rash and hepatotoxicity without concomitant skin rash) [47].

Hepatitis is observed with relative frequency in HSR patients [15, 45, 48]. No patient suffered from both skin rash and liver abnormality in other studies [8, 11, 44, 47]. Hepatic involvement in HSR can also be observed without concomitant cutaneous reactions [44, 47].

Nevirapine-associated HSRs are usually moderate to severe and often require treatment change for the elimination of the agent that caused the reaction [8, 9, 13, 15, 16, 18, 20, 23, 31, 42, 44–46]. Nevirapine is usually substituted with efavirenz in HSR cases [42]. Dermal lesions were observed only in combinations that contained nevirapine and lamivudine in one study [66]. There was a higher incidence of severe rashes (grade ≥3) among nevirapine patients, compared to nonnevirapine patients ($P = 0.002$) in another study. While the same trend was observed overall for grade ≥2 rash, this association was no longer significant ($P = 0.099$) [31]. Efavirenz itself has been associated with skin symptoms [20, 50]. In such cases, a switch to nevirapine often results in the development of similar reactions on nevirapine as well, showing cross-reactivity between the two NNRTIs [20]. Efavirenz treatment did not lead to the development of cutaneous HSRs in a separate study [25].

The HLA-DRB1*01 allele was significantly associated with isolated rash alone in patients exposed to nevirapine or efavirenz ($P = 0.04$), whereas immunologic and genetic factors are associated with hepatotoxicity and systemic ADRs [51].

Lopinavir/ritonavir [50] and atazanavir/ritonavir [24] were also associated with HSR. Among other NRTIs, the development of rash led to zidovudine and stavudine substitution [30] and the development of pruritis led to didanosine substitution [35] in other studies.

There are also studies in which the drugs responsible for the HSR are not specified, but certain hypotheses can be made based on the medication regimen. In such instances, patients are often exposed to either nevirapine or efavirenz [19, 52].

Older age ($P < 0.003$) and a higher $CD4^+$ cell count ($P < 0.03$) were predictors of rash development [42]. No significant differences in plasma nevirapine concentrations were observed between patients who experienced skin rash and patients who did not [14]; however significantly more cases of grade ≤2 rash were identified in a group receiving a full dose of nevirapine, compared to a half-dose of the drug ($P = 0.003$) [42]. A strong association between grade ≥2 rash and nevirapine-based treatment was observed when only subjects with $CD4^+$ >250 cells/mm^3 were considered ($P = 0.001$), suggesting an interaction between the treatment and the $CD4^+$ count [42].

In addition, a trend of increasing risk of developing grade ≥2 rash was observed in pregnant subjects ($P = 0.054$). Pregnant subjects with baseline $CD4^+$ >250 cells/mm^3 were significantly at risk of developing grade ≥2 rash ($P = 0.042$). However, pregnancy alone is not a predictor of ADR development for women initiating nevirapine therapy. This is an important finding, as pregnant women were both more likely to start nevirapine-based treatment ($P < 0.001$) and to have higher baseline $CD4^+$ counts ($P < 0.001$) [31]. No independent risk factors for skin rash were identified in a separate study [18].

4. Gastrointestinal Intolerance

GI complaints, mainly diarrhea, vomiting, and abdominal disturbances, were the most frequently observed ADRs in several studies [24, 53]. These types of ADRs appeared mainly during the first 12 weeks of therapy and were mild (grade ≤2) and transient in most patients [53]. Gastroenterological intolerance (dyspepsia, nausea, vomiting, and diarrhea) is common effects of different drug combinations [66]. GI intolerance was the main cause of lopinavir/ritonavir therapy modification or interruption (Table 1) [24, 32, 33, 36, 54]. GI symptoms associated with lopinavir/ritonavir and tipranavir were the most common type of ADRs in patients exposed to these pharmaceutical agents [55]. Cases of GI toxicity associated with lopinavir/ritonavir discontinuation occurred between day 3 and week 15 [56]. While diarrhea was the most common ADR that led to lopinavir/ritonavir treatment changes, this ADR was less commonly associated with efavirenz discontinuations [26]. Compared with patients assigned to efavirenz, patients assigned to lopinavir/ritonavir had higher rates of nausea, diarrhea, and vomiting ($P < 0.01$) [57].

Nevirapine is another drug associated with a high rate of treatment discontinuations as result of GI intolerance [22, 23, 47]. Nevirapine discontinuations caused by GI symptoms often occur within the first 18 weeks of treatment [47].

The incidence of GI ADRs (mainly diarrhea) was higher in patients treated with nelfinavir compared to patients treated with nevirapine ($P = 0.01$) [22]. Vomiting and diarrhea were observed in other samples of patients treated with nelfinavir [28, 56].

GI intolerance was the main cause of saquinavir therapy modification or interruption as well [36]. A higher saquinavir C_{min} was associated with a higher incidence of serious GI ADRs [58]. In addition, higher saquinavir C_{min} was more prevalent in individuals with grade ≥3 GI side effects, compared with individuals with grade ≤2 GI side effects ($P = 0.028$) [58]. Mild nausea and diarrhea were also observed among saquinavir patients [59].

No patient on atazanavir/ritonavir discontinued treatment due to GI intolerance. More patients receiving lopinavir/ritonavir experienced grade ≥2 nausea, compared to patients receiving atazanavir/ritonavir [33].

GI symptoms were associated with treatment modifications in patients receiving treatment with dual-boosted PIs [94]. The drugs responsible for the observed ADRs are not specified [94]. GI toxicity was also reported in relation to didanosine [34]. Patients assigned to zidovudine and didanosine had higher rates of nausea, constipation, and

fatigue when compared to patients assigned to stavudine and lamivudine ($P < 0.05$) [57]. Drug-related GI toxicity leads to poor medication adherence and ultimate virological failure [34]. Mild GI intolerance that did not require treatment modifications was observed in a couple of other studies [46, 95].

5. Pancreatic Toxicity

Acute pancreatitis is an inflammatory condition of the pancreas characterized clinically by abdominal pain and elevated levels of pancreatic enzymes (serum amylase, isoamylase, and/or lipase). Abnormal exocrine and endocrine function can also occur during an acute attack.

Banks and Freeman argue that acute pancreatitis is characterized by two of either abdominal pain characteristic of acute pancreatitis, serum amylase, and/or lipase raised ≥3 times over the upper limit of normal, and characteristic findings of acute pancreatitis on computed tomography (CT) scan [96]. Based on this characterization, Manfredi and Calza found 46 (3.7%) patients who presented with serum amylase and/or lipase raised ≥3 times over the upper limit of normal and acute pancreatitis on CT scan [60]. A further 120 (11.1%) patients presented only with serum amylase and/or lipase raised ≥3 times over the upper limit of normal and were thus classified as asymptomatic. Only 31 (2.9%) patients had mild-to-moderate symptoms of abdominal pain, with only 9 cases of clinically assessed pancreatits, none of which required surgery or developed complications [60].

A relatively high incidence of at least one confirmed laboratory pancreatic abnormality, relating to at least two serum pancreatic enzymes over a mean follow-up period of 33.6 consecutive months, was observed in this large study [60]. The use of NRTIs like didanosine, stavudine, and lamivudine and coadministration of other medications such as pentamidine, cotrimoxazole, antituberculosis therapy, cytotoxic chemotherapy, or their combination for at least 6 months were significant risk factors for at least 3-fold increases in serum pancreatic enzymes ($P < 0.05$), as was drug or alcohol abuse for at least 6 months ($P = 0.04$). Opportunistic infections with potential pancreatic involvement (cytomegalovirus, cryptosporidiosis, mycobacteriosis, or disseminated tuberculosis) ($P = 0.03$), chronic liver, and/or biliary disease ($P = 0.01$), current administration of HAART regimen containing PIs ($P = 0.05$), hypertriglyceridemia for at least 6 months ($P = 0.02$), or a combination of the above risk factors ($P = 0.003$) were also associated with pancreatic toxicity [60]. The combination of hyperamylasemia with either elevated isoamylasemia or lipasemia was selected for evaluating laboratory abnormalities, and the authors do not separate hyperamylasemia from hyperlipasemia. Serum isoamylase and serum lipase measurements are more specific when compared with serum amylase alone for the diagnosis of pancreatitis [60]. Even so, Van Dyke et al. chose to diagnose pancreatitis based on total serum amylase rather than the more specific pancreatic serum amylase, as the former is routinely monitored [27]. The PI lopinavir/ritonavir was associated with amylase elevations in another study [32].

Pancreatitis likely attributable to didanosine was observed in a couple of studies (Table 1) [27, 35]. Grade ≥3 serum amylase elevations were similar in patients receiving either didanosine/lamivudine/efavirenz or lamivudine/zidovudine/efavirenz [97]. Hyperamylasemia and hyperuricemia were eventual findings without clinical relevance in another study [66]. Among NNRTIs, nevirapine was associated with pancreas-related toxicities [25, 47, 48], whereas efavirenz was not [25].

Recurrent episodes of acute pancreatitis may also suggest a misuse of alcohol or use of concomitant medication. There is no mention of how many drinkers were in a large study, yet the incidence of symptomatic and asymptomatic cases was similar between alcohol drinkers and abstainers [60].

6. HAART Interaction with Alcohol Consumption

Hepatic injury is often more common in individuals with alcohol abuse and in those with HCV coinfection. HAART-induced hepatic injury has the potential to limit the usefulness of this medication in HIV treatment [63]. Twelve (5.4%) patients were found to abuse alcohol in a sample of 222 patients, of which 84 (37.8%) were coinfected with HCV. Alcohol abuse was identified as a risk factor for developing hepatic injury of any grade (OR 3.42, 95% CI 1.04–11.19, $P < 0.05$), especially severe hepatic injury (OR 8.66, 95% CI 2.47–30.40, $P < 0.05$), measured by elevations in transaminase levels [63]. Alcohol intake greater than 40 g per day (OR 3.09, 95% CI 1.27–7.54, $P = 0.01$) was associated with a greater risk of severe hepatotoxicity in a sample of 108 patients [65].

Fourteen (10.6%) patients were alcohol abusers in a small sample of 132 HIV patients coinfected with HCV. Due to the low number of alcohol abusers in this sample, no association between alcohol abuse and hepatotoxicity was observed [98]. Alcohol consumption, both at baseline and during follow-up, was not linked to progression of fibrosis by ≥1 stages among 135 patients coinfected with HCV, of which 31 (23.0%) patients had an alcohol intake of >50 g/day [99].

Excessive alcohol consumption had no effect on the development of mild-to-moderate rash. However, severe rash plus/or hepatotoxicity was observed among 741 patients, of which 163 (22.0%) abused alcohol (≥168 g of alcohol per week for women and ≥252 g of alcohol per week for men) [16].

Since the primary metabolic pathways of abacavir are mediated by microsomal UDP glucuronyl transferase and cytosolic alcohol dehydrogenase, use and misuse of alcohol can lead to hepatotoxicity. A significant pharmacokinetic interaction was found following the coadministration of abacavir and ethanol. Twenty-four HIV-positive men received either a single 600 mg dose of abacavir, 0.7 g/kg ethanol (the equivalent of 5 alcoholic drinks), or a 600 mg dose of abacavir plus 0.7 g/kg ethanol on separate occasions. With coadministration, there was a 41% increase in abacavir area under the curve and a 26% increase in abacavir $t_{1/2}$, with no change in the pharmacokinetic profile of ethanol [100].

While not all studies found an association between alcohol consumption and a greater risk of HAART toxicity, studies where such parameters are investigated often use small population sizes, with a low proportion of alcohol abusers, making it difficult to uncover interactions.

7. Discussion

The present paper discusses hepatic, GI, and pancreatic ADRs related to various ART drugs and drug combinations. We also introduce a section on HSR, since HSRs encompass many of the clinical entities of hepatic and GI representations. We further describe some of the interactions between ART and other drugs and alcohol. Moreover, we briefly explore the influence of certain comorbidities, such as viral hepatitis, on ART-induced hepatotoxicity.

As with all ART medications, many clinically significant interactions are possible with PIs. For example, atazanavir cross-reacts with nevirapine. Atazanavir exposure is significantly lower when combined with this drug, and the risk of nevirapine toxicity may increase due to increased nevirapine exposure. In addition, atazanavir in combination with efavirenz is not recommended in treatment-experienced patients, since efavirenz significantly lowers atazanavir exposure. Concomitant didanosine/lamivudine exposure is not recommended in ART-naive patients receiving unboosted atazanavir due to potential toxicities. In addition, the virological response to abacavir may be diminished significantly by multiple NRTI-associated mutations and/or by reductions in phenotypic susceptibility to abacavir. However, many subjects showing evidence of baseline resistance to NRTIs respond to abacavir.

Of particular relevance to the HIV-infected population is coinfection with HCV. Hepatitis with aminotransferase elevations was reported, and it should be appropriately monitored [101].

Hepatocytotoxicity, or drug-induced liver injury, can be classified based upon clinical presentation and laboratory features, the mechanism of toxicity, and/or histological findings. The presence of serum bilirubin raised >3 times over the upper limit of normal along with aminotransferase elevations is associated with a more drastic prognosis than isolated aminotransferase abnormalities [102], an observation known as Hy's Law [103].

In addition to these acute hepatic presentations, some drugs are associated with chronic histological inflammatory changes and a clinical syndrome resembling autoimmune hepatitis, while others cause endothelial damage or thrombosis, leading to vascular complications such as venoocclusive disease [104]. Withdrawal of the offending drug usually leads to reversal of the injury. The patterns of acute injury may present as hepatocellular (cytotoxic) damage, cholestasis, a mixed pattern of cytotoxic and cholestatic injury, or, less commonly, steatosis [102]. Discontinuation of the offending agent usually results in complete recovery, although the prognosis is generally worse in patients with hepatocellular injury presenting with jaundice when compared to cytotoxic injury alone.

HIV *per se* may influence the ultrastructural architecture of the liver. In the liver of a patient living with AIDS, Phillips et al. found tubular structures mainly in the cytoplasm of endothelial cells and less frequently in Kupffer cells, macrophages, fibroblasts, and biliary cells. These changes were associated with the endoplasmic reticulum, representing a cellular response to virus-induced injury [105].

Drug-induced steatohepatitis may also resemble alcoholic liver disease [106, 107]. In addition, ethnicity plays a role in antiretroviral-induced toxicity [108].

Some of the subjects living with HIV that were included in these studies have a history or past or present alcohol consumption. From a histological point of view, alcoholic hepatitis presents enlargement of hepatocytes that may increase the vascular pressure in the acinus [109]. Mitochondrial changes, including megamitochodria or irregular mitochondria, as well as Mallory bodies, are also encountered. Mallory bodies (alcoholic hyalin) correspond to cytokeratine conglomerations of proteins that form filaments.

The predominant cell in the liver is the hepatocyte, which contains abundant cytoplasm. There are little amounts of carbohydrates and phospholipids in filaments seen in hepatocytes. These cytokeratine filaments represent an abnormal expression of the cytoskeleton. Ultrastructurally, irregular inclusions, which range from small conglomerates of filaments to large inclusions, occupy most of the cytoplasm [105]. A wide range of other ultrastructural changes in alcoholic liver disease can be seen in conjunction with HAART, such as cell necrosis, increased peroxisome numbers, and crystalloid inclusions. Also, there are infrequent bile duct proliferation and ground glass cytoplasmic inclusions that can be resolved after alcohol abstinence.

The genetic value of UGT in PI-induced hyperbilirubinemia is further discussed [110]. Rotger et al. showed that individuals homozygous for the $A(TA)_7TAA$ allele of UGT1A1*28 enzyme receiving atazanavir or indinavir were at increased risk of experiencing hyperbilirubinemia in the jaundice ranges. They studied in parallel a group of patients that have not been genotyped for UGT1A1 allele before prescribing atazanavir or indinavir as first-line agents versus patients that have been genotyped for UGT1A1*28. The "genotype-guided ART" narrowed the use of atazanavir or indinavir to individuals without the UGT1A1*28 allele. The authors conclude that genetic screening would lead to a theoretical 75% reduction in the incidence of hyperbilirubinemia in the jaundice range. The high incidence of the UGT1A1*28 allele might lead to high risk of developing jaundice in the setting of Gilbert syndrome when exposed to specific PIs [110]. UGT1A1 promoter $A(TA)_7TAA$ variant was most common among African Americans and least common among subjects of Asian origin [111]. Therefore the use of genetic screening for the $A(TA)_7TAA$ allele before initiation of antiretroviral therapy is controversial [112].

Ideally, genetic testing for this allele, in conjunction with testing for markers of immunotoxicity such as lymphocyte toxicity assay, may be used in the future in the clinical setting to prevent, diagnose, or monitor drug-induced ADRs in people living with HIV [113].

We conclude that antiviral pharmacodynamics is affected by a broad array of factors ranging from individual pharmacokinetic and pharmacogenetic parameters, to medication adherence and drug-drug interactions. Therefore, therapeutic and drug monitoring of HAART plays an important role. Using laboratory techniques to identify phenotypic susceptibilities, as well as knowing the interactions between ART and other drugs or herbal medicines, might enable a safer use of this beneficial type of medication in HIV patients. Adding to the complexity, many HIV-infected patients are unable to keep therapeutic medication safe due to their behavior patterns, such as alcohol misuse. Lack of pharmacovigilance is associated with HIV disease progression as well as toxicities.

The major objective of this article is to increase awareness on the possible toxicity of therapeutics prescribed in HIV. Moreover, there are other health products including traditional small molecule drugs, natural health products, biologics, and biotechnology products that are prescribed in HIV. These products may cause not only significant liver direct toxicity but also unpredictable, idiosyncratic hepatotoxicity. Therefore, in the process of achieving pharmacovigilance objectives, the investigational approach used for a particular therapeutic may have to be individualized based on the safety characteristics of the product as well as its proposed clinical application.

Abbreviations

ADR: Adverse drug reaction
ALT: Alanine aminotransferase
ART: Antiretroviral therapy
AST: Aspartate aminotransferase
CI: Confidence interval
CYP: Cytochrome p450
GI: Gastrointestinal
HAART: Highly active antiretroviral therapy
HCV: Hepatitis C virus
HIV: Human immunodeficiency virus
HLA: Human leukocyte antigen
HSR: Hypersensitivity syndrome reaction
IQR: Interquartile range
NRTI: Nucleoside reverse transcriptase inhibitor
NNRTI: Non-nucleoside reverse transcriptase inhibitor
OR: Odds ratio
PI: Protease inhibitor
SJS: Stevens-Johnson syndrome
UGT: Uridine diphosphate glucuronosyltransferase.

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