The effect of folate deficiency and different doses of folic acid supplementation on liver diseases

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Abstract

Liver has multiple functions such as detoxification, metabolism, synthesis and storage. Folate is a water-soluble vitamin B9, which participates in one-carbon transfer reactions, maintains methylation capacity, and improves oxidative stress. Folic acid is a synthetic form commonly used as a dietary supplement. The liver is the main organ for storing and metabolizing folate/folic acid, and the role of folate/folic acid in liver diseases has been widely studied. Deficiency of folate results in methylation capacity dysfunction and can induce liver disorders. However, adverse effects of excessive use of folic acid on the liver have also been reported. This review aims to explore the mechanism of folate/folic acid in different liver diseases, promote further research on folate/folic acid, and contribute to its rational clinical application.

Keywords： folate; folic acid; NAFLD; ALD; HCC

Abbreviations: THF, tetrahydrofolate; MTHF, methyltetrahydrofolate; RDA, recommended dietary allowance; DFEs, dietary folate equivalents; DHFR, dihydrofolate reductase; PCFT, proton-coupled folate transporter; RFC, reduced folate carrier; DHF, dihydrofolate; 5-MTHF, 5-methyltetrahydrofolate; MRP3, multi-drug resistance associated protein 3; SHMT, serine hydroxymethyltransferase; MTHFD1L, methylenetetrahydrofolate dehydrogenase 1 like; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; dUMP, deoxyuridylate; dTMP, thymidylate; TS, thymidylate synthase; CCl4, carbon tetrachloride; SREBP-2, sterol regulatory element-binding protein-2; CREB, cAMP response element-binding protein; NF-Y, nuclear factor Y; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ROS, reactive oxygen species; NAFLD, Non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor-κB; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1β; HFD, high-fat diet; PPARα, peroxisome proliferator-activated receptor alpha; SIRT1, silence information regulation factor 1; AMPK, AMP-activated protein kinase; AMP, adenosine monophosphate; LKB1, liver kinase B1; ACC, acetyl coenzyme A carboxylase; NADPH, nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase; GSH, glutathione; GSSG, oxidized glutathione; Pparg, peroxisome proliferator activated receptor gamma; Srebf, sterol regulatory element

binding transcription factor; Nr1h, nuclear receptor subfamily 1 group H member; PPARγ, peroxisome proliferator-activated receptor γ-2; MCP-1, monocyte chemoattractant protein-1; NOX1, NADPH oxidase 1; BiP, binding immunoglobulin protein; MTHFR, methylenetetrahydrofolate reductase; Cidec, DFF45-like effector c; TG, triglyceride; ALD, alcoholic liver disease; CYP2E1, cytochrome P-450 2E1; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; SREBP-1c, sterol regulatory element binding protein-1c; ALT, alanine transaminase; AST, aspartate transaminase; TC, total cholesterol; LDL, low-density lipoprotein; DNMT3a, DNA methyltransferase 3 alpha; CPG, carboxypeptidase G; Foxp3, forkhead box P3; PINK1, putative kinase 1; Drp1, dynamin-related protein 1; DILI, drug-induced liver injury; DNMT1, DNA methyltransferase 1; EZH2, enhancer of zeste homolog 2; H3K27me3, lysine 27 on histone H3; CFTR, cystic fibrosis transmembrane conductance regulator; Fas, programmed cell death-receptor; Akt1, protein kinase B; IFN-γ, interferon gamma; TB, tuberculosis; INH, isoniazid; RIF, rifampicin; NPSH, nonprotein-soluble thiol; TBARS, thiobarbituric acid reactive substances; CDs, conjugated dienes; VEGF, vascular endothelial growth factor; p-eNOS, phosphorylated-endothelial nitric oxide synthase; IAP2 or Birc2, inhibitor of apoptosis 2; Bcl-2, B-cell leukemia/lymphoma 2; Col1α2, procollagen type I α2; Mmp7, matrix metalloproteinase 7; ALA, α- Linolenic acid; TGF- β1, transforming growth factor β1; DHA, docosahexaenoic acid; HCC, hepatocellular carcinoma; EMT, epithelial-to-mesenchymal transition; ZEB2, zinc finger E-box binding homeobox 2; Oct4, octamer-binding transcription factor 4; PRRX1, paired related homeobox 1; PERK, protein kinase R-like endoplasmic reticulum kinase; ATF4, activing transcription factor 4; LAMP3, lysosome-associated membrane glycoprotein 3; MTHFD, formyltetrahydrofolate synthetase; LCN2, lipocalin 2; H3K9Me2, histone H3 lysine 9 di-methylation; DEN, diethylnitrosamine; HEV, viral hepatitis E.

1.Introduction

Folate is a water-soluble B9 vitamin synthesized in plants from 6-hydroxymethyldihydropterin, p-aminobenzoate and glutamate, and is a co-enzyme substrate involved in one-carbon transfer reactions [1,2]. Natural folate exists physiologically in the form of tetrahydrofolate (THF, active form) as well as methyltetrahydrofolate (MTHF, primary form found in blood) [3]. Folic acid is a synthetic (stable) form commonly used in dietary supplement and food fortification [1,3]. At present, studies have found that folate deficiency is associated with many diseases, including macrocytic anemia, mucositis, infertility, muscle weakness, cardiovascular diseases, nervous system diseases, liver diseases, cancer, etc [4]. Moreover, folate supplementation can reduce the occurrence of birth defects including fetal neural tube diseases, anemia and neurological disorders in newborns [5-7]. Because of these benefits, fortification of flour and grain products with folic acid has been applied in many countries [8].

Although folate has an indispensable and important role in our body, there is concern for adverse effects with excessive folic acid supplementation. Currently, the recommended dietary allowance (RDA) for folate by the U.S. Food and Nutrition Board varies by age and sex [9]. The RDA for folate in children aged 1 to 13 years old is 150-300 mcg of dietary folate equivalents (DFEs). The RDA for folate in people aged 14 and above is 400 mcg DFEs, while the RDA of folate in pregnant and lactating women is 600 mcg DFEs and 500 mcg DFEs, respectively [9]. Studies have found that daily intake of 400 μg of folic acid can lead to unmetabolized folic acid in plasma [10]. However, most folic acid supplements contain more than 400 μg of folic acid [11], and some population groups have total folic acid intake that exceeds the tolerable upper limit, due to the use of fortified foods and dietary supplements [12,13]. Kalmbach et al. also found that folic acid fortification is related to increased exposure to circulating folic acid [14]. The impact of folic acid on pregnant women and fetuses has always been a concern. In mice models, the recommended dose of folic acid for rodents is 2 mg/kg. The study found that a high-folic acid diet (20mg/kg) in pregnant mice is associated with embryo loss, delayed embryo growth, a higher incidence of ventricular septal defect, and

thinning of the left and right ventricular walls during embryonic development [15]. However, in human studies, the use of high doses of folic acid during pregnancy is neither harmful to fetal growth, nor associated with the occurrence of oral clefts or and congenital heart disease [16]. It's worth noting that another observational study in humans showed that higher plasma folate was associated with higher 2-h glucose and higher odds of gestational diabetes mellitus [17]. It is well known that gestational diabetes can lead to fetal growth restriction and an increased risk of maternal diabetes. Therefore, we believe that further clinical studies are needed to address the impact of excessive folic acid supplementation on both pregnant women and fetuses.

Moreover, high folic acid intake has been linked to an increased risk of adenomatous lesion and prostate cancer [18,19]. It has also been found that increased levels of unmetabolized folic acid in plasma can reduce cognitive test scores in seniors who aged ≥ 60 years, and also can dampen cytotoxicity of natural killer cells in postmenopausal women [20,21]. In rats fed with high fat diet, it has been reported that excessive folic acid can exacerbate weight gain, fat accumulation, impaired glucose tolerance, and white adipose tissue inflammation [22].

The liver is an important organ of the human body, which has the functions of detoxification, metabolism, synthesis and storage [23], and participates in the metabolism of carbohydrates, proteins and lipids, the clearance of drugs, toxins and pathogens in the blood, and the regulation of immune responses [24]. However, many drugs, environmental toxins and dietary ingredients can induce liver injury, including dysregulation of lipid metabolism, degeneration and necrosis of hepatocyte and activation of immune responses [25].

Folate plays an important role in maintaining methylation ability, and many methylation reactions occur in the liver [2]. The liver is also the main organ for folate/folic acid storage and metabolism [1], and the reduction of folic acid depends on the role of dihydrofolate reductase (DHFR) in the liver [3]. Therefore, there is an inseparable relationship between liver and the biological effects of folate/folic acid. In order to increase people's understanding and awareness of folate and folic acid intake, especially for people with pre-existing liver diseases, this article systematically summarizes the effects and mechanisms of folate/folic acid in the

liver by referring to published literature, and provides theoretical support for clinical doctors and nutritionists to use folate and folic acid-related drugs and supplements reasonably.

2.Molecular structure, pharmacokinetics and biological function of folate/folic acid

2.1 Molecular structure and source of folate/folic acid

The chemical formula of folic acid is $C_{19}H_{19}N_7O_6$ (Figure 1), and its molecular core is composed of a heterocyclic pterin structure. The methyl group at the sixth position is combined with para-aminobenzoic and glutamic acid, thus presenting pteroylglutamic acid [4,26]. Pterin is composed of pyrimidine and pyrazine rings with substituting keto- and amino groups in the second and fourth positions [4].

Folate and folic acid are different forms of vitamin B9, and the main difference between these two is their sources. Folate is a natural form and can be present in dark green leaves, mushrooms, animal liver, yeast and so on [4,27]. Mammals lack the enzymatic capacity to synthesize folates, so we need to intake the folate with dietary source [27]. Folic acid is a synthetic and stable form of folate which is normally used for extra oral supplementation and fortification [27-29]. Therefore, in some countries, such as the United States, Canada, etc, cereals, grains and breads fortified with folic acid are an important source of folate/folic acid [27]. In addition, studies have found that folate-producing bacteria in the cecum, colon (mainly includes Lactobacillus and Bifidobacterium), and proximal small intestine can also serve as a source of folate [30,31,32].

2.2 Absorption, bioavailability and pharmacokinetics of folate/folic acid

Dietary folate is mainly absorbed by the duodenum and proximal jejunum [32,33], while folate synthesized by gut bacteria may be adsorbed from the colon [4]. The main mechanism of folate absorption is that folate (polyglutamate) is hydrolyzed to monoglutamate by glutamate carboxypeptidase II (folate hydrolase) in the brush-border membrane of small intestine. Then, monoglutamate transported into cells by proton-coupled folate transporter (PCFT) and reduced folate carrier (RFC). The pH of the proximal small intestine is 5.8-6.0, while RFC performs its optimal transport function at pH 7.4 and the activity of RFC also

decreases as the pH decreases. Therefore, the contribution of RFC to folate absorption is much less than that of PCFT [4,33,34]. After absorption by enterocytes, folic acid undergoes reduction to dihydrofolate (DHF), THF, and 5,10-methylene-THF, ultimately converting to biologically active 5-methyltetrahydrofolate (5-MTHF) which is the dominant physiological form in the blood [3,4,35]. 5-MTHF is then transported to the blood through the basolateral membrane under the action of multi-drug resistance associated protein 3 (MRP3), thus achieving rapid and efficient transepithelial transport [33,34]. In terms of bioavailability, folic acid is generally believed to be more bioavailable than natural folate in food [28]. Because folic acid is a monoglutamate, it can be directly absorbed in the intestine without hydrolysis step, and the bioavailability is very high which can be approximately up to 100% [9,28]. Afterwards, 5-MTHF enters the hepatic portal system through the bloodstream and is transported to the hepatic sinuses. There are three main metabolic routes for 5-MTHF after entering the liver. First, it can be converted to polyglutamate for storage. Second, it can be secreted into the bile, returned to the duodenum and jejunum and subsequently reabsorbed, thus completing the enterohepatic circulation. Third, it can enter the hepatic vein and eventually reach the systemic circulation, where it is taken up by peripheral tissues, converted to THF , and participates in one-carbon transfer reactions [1,27,35]. 5-MTHF that does not bind to serum protein is reabsorbed by the proximal tubules after glomerular filtration [27,35]. According to previous studies, 5-MTHF can be detected in the liver, kidneys, bile acid, etc., while the liver is the main organ for storing and metabolizing folate/folic acid [27,29]. The liver plays a central role in the homeostasis of folate/folic acid [35]. Therefore, studying the role of folate/folic acid in the liver is crucial for exploring the systemic effects of folate/folic acid [36].

2.3 Functional mechanism and signaling pathways

Folate plays an important role in one-carbon transfer reactions involved in nucleic acid biosynthesis, methylation reactions and sulfur-containing amino acid metabolism [1]. The

one-carbon metabolism of folate is mediated by THF and is a metabolic network of interdependent biosynthetic pathways, which mainly occurs

in mitochondria, cytoplasm and nucleus [37]. In the mitochondria, it is mainly the interconversion of activated one-carbon units carried by THF [38]. First, serine, glycine, dimethylglycine, and sarcosine undergo catabolism under the action of mitochondrial serine hydroxymethyltransferase (SHMT), aminomethyltransferase, dimethylglycine dehydrogenase, and sarcosine dehydrogenase, respectively [26,37]. This process depends on THF and produces 5, 10-methylene-THF [26]. Then, 5, 10-methenylTHF is formed under the action of 5,10-methylenetetrahydrofolate dehydrogenase, and further oxidized to 10-formyl-THF by 5, 10-methyltetrahydrofolate cyclohydrolase [37]. Finally, formate and free THF are formed under the action of methylenetetrahydrofolate dehydrogenase 1 like (MTHFD1L) [26,37,39]. In addition, Met-tRNA forms fMet-tRNA under the cofactor 10-formyl-THF to initiate mitochondrial protein synthesis [26]. Serine, glycine and formate are transported to the cytoplasm through the mitochondrial membrane and become carbon donors in the cytoplasm [38,40]. In addition, one-carbon can also be directly produced in the cytoplasm through the catabolism of histidine, purine, and serine, but formate from mitochondrial one-carbon metabolism is the main source [26,37]. In the cytoplasm, folate-mediated one-carbon metabolism involves three interdependent biosynthetic pathways, including de novo synthesis of purine nucleotides, thymidylate, and remethylation of homocysteine to methionine [26]. The pathway involved is that serine transfers a carbon unit to THF under the action of SHMT to form 5,10-methylene-THF and glycine [38]. 5,10-methylene-THF can synthesize thymidylate directly under thymidylate synthase [37,38,40]. While 5,10-methylene-THF can be oxidized to 10-formyl-THF for purine synthesis, formate can also produce 10-formyl-THF [37,38,40]. Conversely, 5,10-methylene-THF can be reduced to 5-MTHF, which is a carbon donor for the remethylation of homocysteine. Methionine is synthesized by homocysteine under the action of methionine synthase, and then enters the methyl cycle by S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) [26,37,38,40]. In the nucleus, 5,10-methylene-THF, as a carbon donor, catalyzes the methylation of deoxyuridylate (dUMP) to thymidylate

(dTMP) under thymidylate synthase (TS). Moreover, the nucleus also contains the mutual conversion of THF and DHF and the process of serine acting as a carbon donor through the SHMT reaction, which involves three enzymes (SHMT, TS and DHFR) constituting the dTMP synthesis cycle [37,38].

Both dTMP synthesis and homocysteine remethylation are sensitive to folate deficiency [7]. Folate deficiency reduces dTMP levels, which increases errant incorporation of uracil into DNA, leading to abnormalities in DNA and chromosome structure [41]. Previous study has found that folate deficiency can induce DNA deletion, cytochrome c oxidase dysfunction, membrane depolarization and superoxide overproduction in rat liver, thereby promoting mitochondrial oxidative decay. Folic acid supplementation can ameliorate this defect [42]. In a carbon tetrachloride (CCl4)-induced rat model of liver injury, reduced folate levels interfered with DNA synthesis and prevented or delayed liver regeneration [43]. Alcoholics have lower levels of folate [44], and folate deficiency may contribute to alcoholic liver disease through epigenetic effects that reduce methyl supply for silencing gene expression and/or DNA stability [45]. Folate deficiency also can promote alcoholic liver injury by disrupting liver methionine metabolism and DNA stand break in a micropig experiment [46]. In addition, there is a strong negative correlation between folate and plasma homocysteine levels [47,48]. Increased homocysteine leads to increased serum and cellular SAH levels which is a potent inhibitor of SAM-dependent methylation reactions including protein and DNA methylases [7]. Hyperhomocysteinemia can cause the activation of multiple transcription factors including SREBP-2 (sterol regulatory element-binding protein-2), CREB (cAMP response element-binding protein), and NF-Y (nuclear factor Y) in the liver and lead to an increase in HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase and cholesterol biosynthesis, which resulting in hepatic lipid accumulation and hypercholesterolemia [49]. Combined treatment with folic acid and vitamin B12 can normalize plasma homocysteine levels and reduce oxidative stress to attenuate alcohol-induced liver injury [48]. Moreover, folate deficiency is associated with impaired antioxidant enzyme activity, increased production of reactive oxygen species (ROS) and lipid peroxidation [42,50,51]. A study has shown that folate

may play a direct effect on free-radical-induced oxidation of low-density lipoprotein [52]. In conclusion, folate-mediated one carbon metabolism plays a crucial role in liver diseases. Studying the role of folate in liver disease and optimal folic acid dose has potential to guide clinical treatment. The next section addresses the role and mechanism of folate/folic acid in different liver diseases.

3.The role of folate/folic acid in liver diseases

3.1 Folate/folic acid and NAFLD/NASH

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease characterized by steatosis, inflammation, fibrosis and liver injury. There is currently no effective treatment for this disease, apart from strengthening exercise and controlling diet [1]. NAFLD can develop into non-alcoholic steatohepatitis (NASH), a more severe inflammatory and hepatocyte damage process typically accompanied by pericellular fibrosis, which may develop into cirrhosis [53,54]. A study has shown that low serum folate level is an independent risk factor for NAFLD in Chinese population and is associated with the severity of hepatic steatosis [55]. The hepatic steatosis is often observed in disorders of one-carbon metabolism [2]. It has been reported that patients with NAFLD have lower circulating folate levels, but it is unclear whether folate deficiency is a cause or a consequence of NAFLD. In addition,although serum folate levels may be associated with the development of NAFLD [1], there is evidence that moderate amount of folic acid can prevent NAFLD, while excessive folic acid may be counterproductive.

Studies have found that folic acid supplementation can inhibit the nuclear factor-κB (NF-κB) pathway by reducing the concentration of ROS and homocysteine. At the same time, folic acid can also inhibit the expression of pro-inflammatory cytokines (interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β)), thereby improving liver inflammation in mice fed high-fat diet (HFD) and HepG2 cells treated with palmitic acid or homocysteine [56,57]. Folic acid can improve HFD induced steatohepatitis in rats, partly because it can increase peroxisome proliferator-activated receptor alpha (PPARα) levels

through a silence information regulation factor 1 (SIRT1)-dependent manner, thereby improving liver lipid metabolism. Another reason is that folic acid administration can also restore depleted liver one-carbon metabolism and gut microbiota diversity [58]. During HFD feeding, folic acid supplementation restores AMP-activated protein kinase (AMPK) activation by increasing adenosine monophosphate (AMP) levels and liver kinase B1 (LKB1) phosphorylation in the liver, which contribute to ameliorate glucose and cholesterol metabolism impaired by high-fat dietary intake [59]. Further research in high-fructose-fed rats revealed that folic acid enhanced the levels of phosphorylated AMPK and LKB1 and increased phosphorylation (inactivation) of acetyl coenzyme A carboxylase (ACC) in the liver, thereby inhibiting hepatic lipogenesis and ameliorating hepatic steatosis [53]. The activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the liver of HFD fed mice significantly increased. However, supplementation with folic acid can downregulate the gene expression of NADPH oxidase subunits (including $gp91^{phox}$, $p22^{phox}$ and $p47^{phox}$) and inhibit the activation of NADPH oxidase by inhibiting NF-κB pathway, thus improving liver oxidative stress [60]. Moreover, folic acid can also increase the activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase, and correct the equilibrium between reduced (GSH) and oxidized (GSSG) glutathione, suggesting a protective role of folic acid against HFD-induced oxidative damage in the liver [60]. These results illustrate the mechanism and therapeutic role of folic acid in NAFLD/NASH, suggesting that folic acid may become a therapeutic drug for NAFLD/NASH in the future. However, in a randomized controlled trial of the effects of folic acid supplementation on liver enzymes, lipids, and insulin resistance in patients with NAFLD, folic acid supplementation (1 mg/d) for 8 weeks was able to prevent an increase in homocysteine, but did not significantly alter serum liver enzyme levels, degree of liver steatosis, insulin resistance, and lipid levels. Therefore, the course and dosage of folic acid supplementation in NAFLD patients in the future are also the focus of exploration [61]. The trial included only 66 patients and needs to be confirmed in larger trials (Figure 2).

Although most studies have shown the protective effect of folic acid on NAFLD/NASH, detrimental roles due to its excessive dosage have also been indicated. When detecting the cell viability of HepG2 cells exposed to FA at different gradient concentrations, treatment with 5-75 μg/mL of FA had no statistics effect on HepG2 cell viability,whereas high concentration of FA (higher than 100 μg/mL) appeared to be toxic and reduced the cell viability [(57)]. In addition, excessive intake of folic acid can affect the metabolic processes of glucose and lipids, exacerbating metabolic syndrome. In HFD rats, feeding excess folic acid (7.5 mg/kg,12 weeks) exacerbates weight gain, fat mass and glucose intolerance [(22)]. The increased adipose size and mass has been proven to be induced by increased key transcriptional regulators of lipid metabolism (peroxisome proliferator activated receptor gamma (Pparg), sterol regulatory element binding transcription factor 1 (Srebf1), Srebf2, nuclear receptor subfamily 1 group H member 2 (Nr1h2), Nr1h3), and lipogenic genes. Excess folic acid can increase TG accumulation by upregulating the expression of peroxisome proliferator-activated receptor γ-2(PPARγ) in 3T3-L1 cells. Moreover, excessive folic acid increases the inflammation of white adipose tissue in rats by increasing the levels of monocyte chemoattractant protein-1 (MCP-1), TNF-α, NADPH oxidase 1 (NOX1) and binding immunoglobulin protein (BiP) [(22)]. High dose of folic acid intake (20 mg/kg folic acid, 6 months) reduces the level of methylenetetrahydrofolate reductase (MTHFR) protein and activity, resulting in MTHFR deficiency, as well as the reduction of MTHF and methylation capacity. MTHFR-deficient hepatocyte cannot alleviate the effects of phospholipid and lipid disorders, thereby leading to hepatocyte damage and liver injury [(11)]. It has also been found that excessive folic acid supplementation (40 mg/kg diet) in pregnant mice will lead to reduced islet β-cell mass and insulin synthesis in their offspring. At the same time, excessive folic acid also can result in increased expression of fat metabolism related genes *Pparγ2* and cell death-inducing DFF45-like effector c (*Cidec*), and higher liver triglyceride (TG) content in the offspring [(62)]. These suggest that excessive folic acid may lead to glucose intolerance, fat accumulation, and adipose tissue inflammation, thereby exacerbating the development of metabolic syndrome. Excessive folic acid supplementation may interfere with lipid

metabolism, promote changes in one-carbon metabolic pathways and gene expression patterns, thereby leading to liver injury and harmful effects (Figure 3).

In conclusion, the decrease of folate level can aggravate the steatosis of the liver. Moderate supplementation of folic acid may alleviate NAFLD/NASH by improving the oxidative stress and lipid metabolism of the liver and reducing the inflammation level of the liver, but excessive supplementation of folic acid may aggravate NAFLD/NASH. Unfortunately, how to determine the "moderate" and "excessive" level of folic acid is currently unknown in human experimental studies, because most of the research is based on cells and animals. So it is necessary to explore the appropriate clinical dose for NAFLD/NASH in the future study.

3.2 Folate/folic acid and alcoholic liver disease (ALD)

The report states that about 69% to 80% of alcoholics have low serum folate levels [(63)], which may be due to multiple reasons. People who chronically dependent on excessive alcohol may develop folate deficiency due to poor diet, reduced folate absorption and liver uptake, and increased renal excretion. Moreover, folate deficiency may promote the development of ALD by exacerbating abnormal methionine metabolism.[(64)]. Ethanol can change liver methionine metabolism, resulting in the interference of SAM-dependent transmethylation [(65)]. Folate deficiency increases plasma homocysteine levels, decreases SAM/SAH ratio and GSH levels, and increases cell apoptosis and DNA strand breaks. Therefore, folate deficiency promotes alcohol induced liver damage by exacerbating liver methionine metabolism disorders and DNA damage [(46,64)]. In addition, cytochrome P-450 2E1 (CYP2E1) and endoplasmic reticulum (ER) stress signals including glucose-regulated protein 78 (GRP78), caspase 12, and sterol regulatory element binding protein-1c (SREBP-1c) are activated in response to folate deficiency. Their expression levels are positively correlated with SAH and/or homocysteine levels in the liver, and negatively correlated with SAM/SAH ratio. These results indicate that abnormal hepatic methionine metabolism induced by combination of ethanol and insufficient folate is closely associated with CYP2E1 activation and enhanced ER stress pathway which aggravated steatosis and apoptosis [(66)].

Therefore, folic acid supplementation may become a key method for the treatment of ALD. Folic acid supplementation can reduce alanine transaminase (ALT) and aspartate transaminase (AST) activities, decrease lipid and DNA oxidation, increase GSH levels, decrease homocysteine and ameliorate oxidative stress to reduce hepatotoxicity caused by chronic alcohol intake [67,68]. Folic acid inhibits the elevation of TG, total cholesterol (TC), and low-density lipoprotein (LDL), and liver fat deposition caused by ethanol. The potential mechanism is that folic acid supplementation ameliorates hepatic Th17/Treg imbalance in mice with long-term alcohol exposure by reducing DNA methyltransferase 3 alpha (DNMT3a) levels and then downregulating the methylation levels of carboxypeptidase G2 (CPG2) and CPG3 in forkhead box P3(Foxp3) promoter region, thereby improving ethanol-induced inflammatory damage [69]. In addition, folic acid can improve mitochondrial function and inhibit mitophagy and mitochondrial fission by reducing the expression of PTEN induced putative kinase 1(PINK1)-parkin and dynamin-related protein 1 (Drp1), thereby preventing hepatocyte apoptosis [63]. In summary, folic acid can improve ALD by regulating oxidative stress, lipid metabolism and mitophagy. Considering the harm of folate deficiency in ALD patients and the benefits of supplementing folic acid, taking folic acid is recommended for ALD patients (Figure 4).

3.3 Folic acid and drug-induced liver injury (DILI)

Most studies have shown that folic acid has a protective effect on DILI induced by different etiologies. In a randomized clinical trial, folic acid can better reduce ALT and AST levels in response to antiepileptic drug induced liver injury compared with silymarin, suggesting an advantage of folic acid in the treatment of DILI [70]. Folic acid can significantly increase the activities of SOD and catalase, as well as GSH levels, thereby alleviating oxidative stress caused by acetaminophen and reducing the area of liver necrosis [71]. In liver injury induced by homocysteine, on the one hand, folic acid supplementation can effectively inhibit the generation of superoxide anion mediated by NADPH oxidase, prevent oxidative stress, and thus reduce liver lipid peroxidation [72]. On the other hand, folic acid can inhibit

the expression of DNA methyltransferase 1 (DNMT1) and enhancer of zeste homolog 2 (EZH2), decrease levels of SAM and SAH and increase the intracellular ratio of SAM/SAH. These result in inhibition of homocysteine-induced DNA methylation and trimethylation of lysine 27 on histone H3 (H3K27me3) of cystic fibrosis transmembrane conductance regulator (CFTR) promoter, thereby upregulating CFTR expression, accelerating homocysteine metabolism and alleviating ER stress and hepatocyte apoptosis [73]. In CCL4-induced liver injury, folic acid can restore the activities of catalase and GSH, downregulate the mRNA expression levels of programmed cell death-receptor (Fas) and TNF-α, and upregulate the concentration of cell survival signals protein kinase B (Akt1), interferon gamma (IFN-γ), so as to prevent lipid peroxidation, reduce inflammation and improve liver injury. While the combination of folic acid and melatonin has a better effect in protecting liver function [74]. In a rat model of liver injury induced by antituberculosis (TB) drugs (isoniazid (INH) and rifampicin (RIF)), folic acid supplementation can alleviate liver injury caused by TB drugs. The potential mechanism may be associated with regulating n-acylethanolamine metabolism, enhancing the detoxification and clearance of INH and RIF, promoting liver regeneration, downregulating inflammation, and so on [75]. Folic acid combined with vitamin B12 can increase the activities of SOD, catalase and the level of nonprotein-soluble thiol (NPSH) in liver, inhibit the increase of thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CDs), and thus relieve liver tissue degeneration and DNA damage and prevent liver injury caused by arsenic exposure [76]. However, there is no study on the improvement of arsenic-induced liver injury by folic acid supplementation alone, which still needs deeper exploration. Therefore, the efficacy, side effects and optimal dose of folic acid in the treatment of DILI need to be further studied (Figure 5).

3.4 Folic acid and liver fibrosis/cirrhosis

Plasma folate concentrations are low in patients with cirrhosis, and low folate levels are significantly associated with the severity of fibrosis [77,78]. The role of folic acid in liver fibrosis or cirrhosis is still controversial. Ho et al. found that in cirrhotic rats (with or without

hyperhomocysteinemia), folic acid (100 mg/kg/day) could significantly reduce the shunting degree and mesenteric vascular density, thereby decreasing splanchnic blood flow and mitigating the stress to form portosystemic collaterals. In addition, the pathological angiogenesis was reduced by downregulating the protein expression of mesenteric vascular endothelial growth factor (VEGF) and phosphorylated-endothelial nitric oxide synthase (p-eNOS) [77]. In addition, a cross-sectional study of 5,417 participants showed that individuals with higher serum folate levels had a lower chance of developing advanced liver fibrosis [78]. However, another study confirmed that moderately high folic acid (25 mg/kg) administration aggravated the development of CCL4-induced liver fibrosis in rats. The underlying mechanism was proven that folic acid significantly decreased the expression of the inhibitor of apoptosis 2 (IAP2 or Birc2) and the B-cell leukemia/lymphoma 2 (Bcl-2) genes, increased the expression of procollagen type I α2 (Col1α2) and matrix metalloproteinase 7 (Mmp7). In addition, a higher hepatic collagen content, serum AST, bilirubin and a lower serum albumin concentration was detected in rats receiving folic acid supplements(25 mg/kg) [79]. A recent study also showed that dietary restriction of folate can promote the regression of liver fibrosis in NASH mice. Folate can shift to mitochondrial metabolism during HSC activation and result in depletion α- Linolenic acid (ALA), which maintaining transforming growth factor β1 (TGF- β1) signaling and lead to fibrosis. However, the restriction of folate can block SHMT2/MTHFD2 mediated mitochondrial folate metabolism, prevent depletion of ALA, increase the biological transformation of ALA to docosahexaenoic acid (DHA), and thus inhibit TGF-β1 signaling by downregulating TGFBR1 mRNA expression. Therefore, blocking mitochondrial folate metabolism is expected to be an important step in improving liver fibrosis in NASH mice [80]. In view of this, further studies are needed to confirm whether folate/folic acid improves or worsens liver fibrosis and cirrhosis, and to explore the therapeutic or pathogenic dosage of folic acid.

3.5 Folate/folic acid and hepatocellular carcinoma (HCC)

Studies have shown a negative correlation between folate level and the development of HCC [81,82], and low blood folate status can promote the progression of HCC [83]. This may be

due to several mechanisms. First, folate deficiency effects epithelial-to-mesenchymal transition (EMT). Su et al. found that folate deficiency could significantly upregulate mesenchymal markers such as Snail, zinc finger E-box binding homeobox 2 (ZEB2) and Vimentin in HCC cells, and downregulate E-cadherin to promote EMT. In addition, cancer stem-like cell markers including octamer-binding transcription factor 4 (Oct4), β-catenin and CD133 increased, and paired related homeobox 1 (PRRX1) decreased in folate deficiency group which indicating promoted tumor stem-like phenotype and metastatic potential [(84)]. However, another study suggested that epithelial markers (Syndecan-1, e-cadherin) did not change significantly under folate deficiency, and mesenchymal marker (vimentin) significantly decreased. Therefore, folate deficiency was shown to reduce the transition of epithelial cells to mesenchymal cells, and inhibit the invasion and migration of cancer cells, but this study was not confirmed in vivo [(85)]. Second, folate deficiency can induce apoptosis [(85)]. It was found that folate deficiency in HepG2 cells could lead to S phase cell accumulation and G2/M phase block, thus inducing apoptosis [(86)]. This may due to the increased accumulation of homocysteine caused by folate deficiency, which leads to the overproduction of hydrogen peroxide and the overactivation of the redox-sensitive transcription factor NF-κB [(87)]. Folic acid administration during cell culture can save apoptotic culture and restore the cell cycle to normal [(86)]. Third, folate deficiency can lead to genomic instability. In a folate/methyl-deficient rodent model of hepatocarcinogenesis, DNA methyltransferase activity was increased and p53 gene expression was decreased in HCC group compared with control and preneoplastic group. These changes can induce genomic instability and clonal neoplastic phenotype expansion in the liver [(88)]. Moreover, previous study also proved DNA damage, DNA hypomethylation, and tumor progression in folate/methyl-deficient rodent model [(89)]. Fourth, folate deficiency can induce ER stress. Goyal et al. found that folate deficiency could induce ER stress by activating protein kinase R-like endoplasmic reticulum kinase（PERK)/activing transcription factor 4 (ATF4)/lysosome-associated membrane glycoprotein 3 (LAMP3) pathway, and PERK inhibitors could inhibit the migration and invasion of HepG2 cells and further lead to the

reduction of EMT and apoptosis [85]. In addition, there is overexpression of folate-related enzymes in HCC, including methylenetetrahydrofolate dehydrogenase, cyclohydrolase, and formyltetrahydrofolate synthetase 1 (MTHFD1), SHMT2, methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), and MTHFD1L, which is associated with low survival rate, short recurrence time, poor prognosis, and metastasis in HCC patients [90]. Among them, MTHFD1L contributes to the production and accumulation of NADPH, and MTHFD1L knockdown can increase oxidative stress and make cancer cells sensitive to sorafenib [91].

Folic acid plays a certain therapeutic role in HCC. Folic acid supplementation can depress an important oncogene-lipocalin 2 (LCN2) by promoting the level of histone H3 lysine 9 di-methylation (H3K9Me2) in LCN2 promoter, thereby inhibiting cell proliferation and cell cycle, and playing a chemopreventative role in the tumorigenesis of HCC [92]. Folate deficiency in some poorly-differentiated and invasive subclone variant, such as SK-Hep-1 cells, can promote rodex adaptation and upregulate GRP78 and survivin which acts as ROS inhibitors to induce multi-drug resistance and conducive to the progression of HCC. So, it is a potent clue to supply folic acid to prevent its deficiency in order to achieve more satisfactory chemotherapy effect in the treatment of HCC [93]. Moreover, the treatment of folic acid combined with chemotherapy has been widely studied presently. Folic acid is used as a tumor targeting part because of its high affinity with folic acid receptors, which can overexpress in many tumors including liver cancer [94]. Folate/folic acid can combine with polyethylene glycol cyclodextrin nanoparticles, paclitaxel, doxorubicin, selenium nanoparticles and other substances to improve the effectiveness of targeted nanomedicine therapy and play an important role in the treatment of HCC [95-97]. However, Sharma et al. have found that excessive dietary folic acid supplementation can promote the early progression of HCC. In the rat model of establishing HCC with diethylnitrosamine (DEN), an excessive diet of folate (20 mg/kg) resulted in an increase case of HCC and a decrease case of cirrhosis when compared with folate normal group (2 mg/kg) [98]. Therefore, when using treatment regimens containing folic acid, it is necessary to pay attention to the dosage of folic acid. In summary, folate plays an important role in the occurrence and development of HCC. The development

of targeted drugs containing folic acid has clinical value in the treatment of HCC, but the dietary or drug supplementation dosage of folic acid needs further exploration (Figure 6).

3.6 Folate/folic acid and viral hepatitis

Folic acid may play an active role in viral hepatitis. In the treatment of viral hepatitis, folic acid and corsal treatment can significantly increase the clearance and elimination rate of antipyrine and reduce the area under the pharmacokinetic curve, which may be due to the involvement of its derivatives in de novo synthesis of nucleotides [(99)]. In pregnant patients with viral hepatitis E (HEV), genetic alterations in folate pathway genes, folate receptor alpha deficiency, and decreased vitamin B12 level may cause elevated homocysteine which promote oxidative stress, therefore increasing the risk of preterm delivery, disease severity, and negative pregnancy outcomes [(100)]. Supplementation with vitamin B12 and folic acid may be an effective treatment options to combat elevated homocysteine in HEV infected pregnancy cases and diminish associated fetal and maternal morbidity and mortality [(100)]. At present, there are still few studies on folic acid and viral hepatitis, so further exploration of its therapeutic effect is needed to provide more clinical reference.

Conclusion

Folate, as a B-group vitamin, participates in various biological reactions in the body, such as lipid metabolism, oxidative stress and methionine metabolism. Therefore, it plays a crucial role in the human body. This article provides a detailed overview of the role of folate/folic acid in NAFLD/NASH, ALD, DILI, liver fibrosis/cirrhosis, HCC, and viral hepatitis. By describing the association and potential pathogenesis between folate deficiency or folic acid excess and liver diseases, clinical doctors can have a more comprehensive understanding on the role of folate/folic acid. When studying the published literature, we found that the dosage of folic acid as a dietary or drug supplement still needs further exploration, especially for people who have already presented with liver diseases. We look forward to more animal and clinical studies on folic acid dosage to guide the rational clinical application. In addition,

there is relatively little research on the role of folic acid in liver fibrosis/cirrhosis and viral hepatitis, despite conflicting views in current research. Therefore, further research is needed in this field. In summary, this review can help people systematically understand folate/folic acid in the context of liver diseases, which is beneficial for the clinical application and development of targeted drugs containing folic acid.

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Author Contributions

CMJ and MH conceived and designed the article. HM and HL authored drafts of the paper. CMJ ,MH and HL corrected the content and language. HM and YTY prepared pictures and references. All authors have read and approved the manuscript and agree to be accountable for all aspects of the article.

Declaration of Interests

The authors declare that they have no competing interest.

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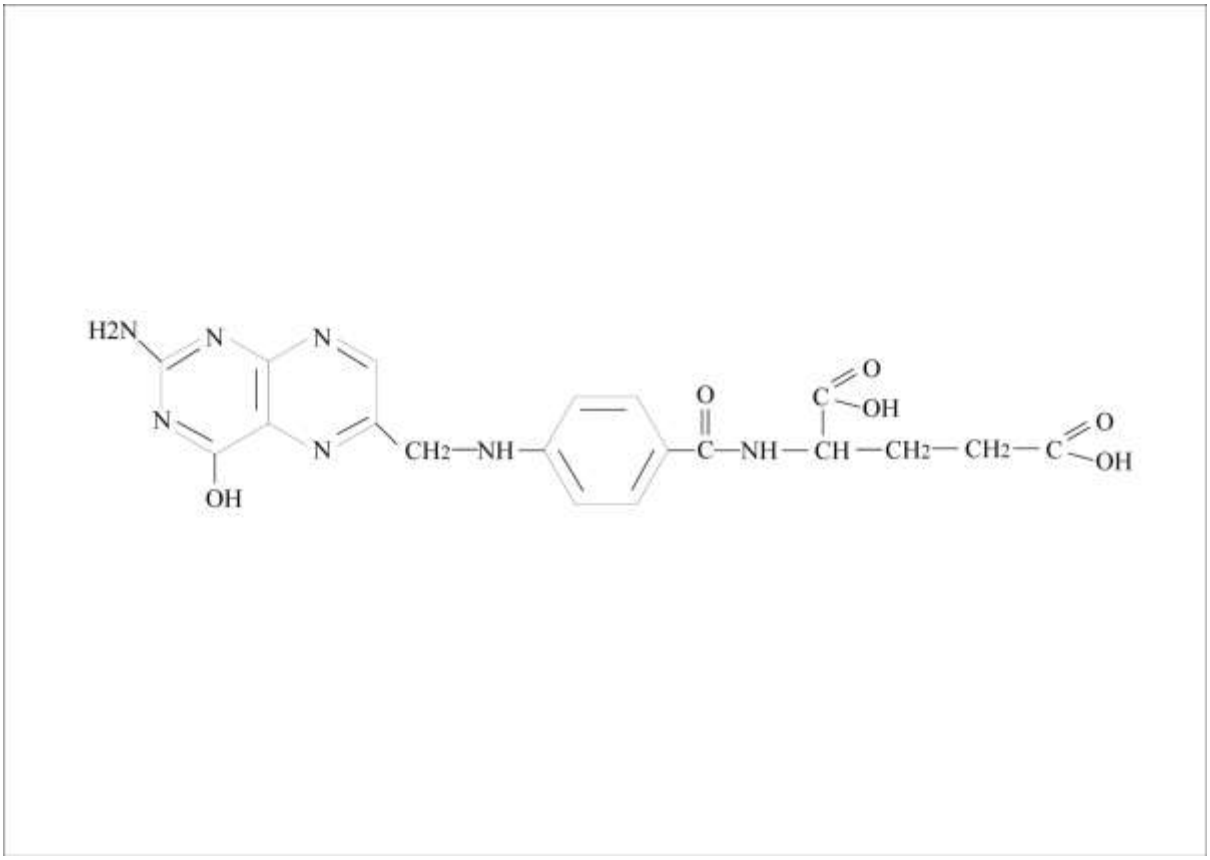

Figure 1 Chemical structure of folic acid (4).

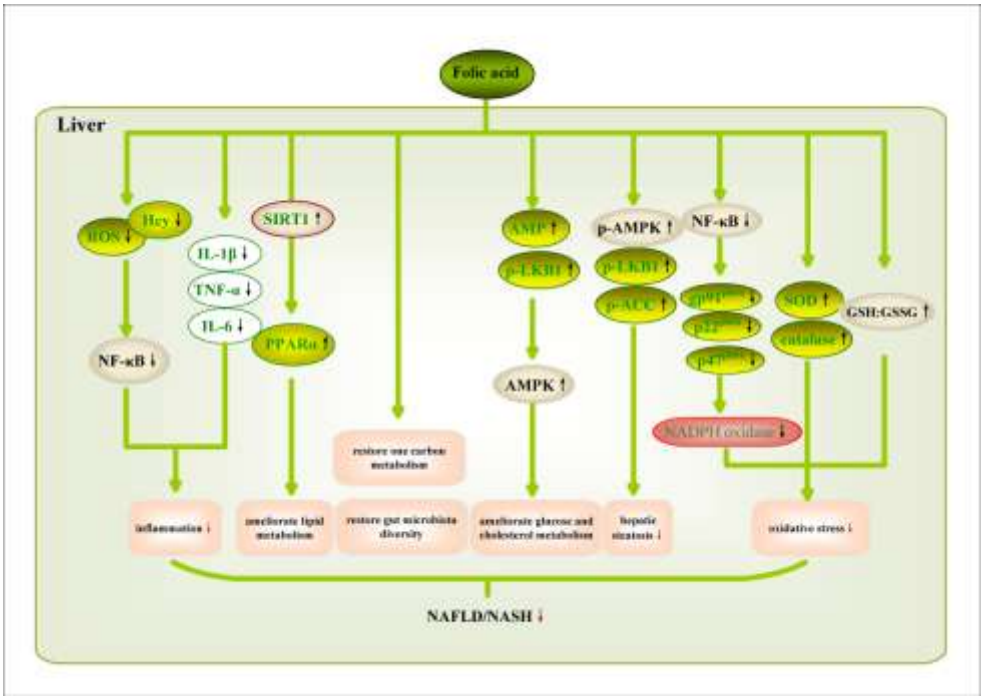

Figure 2 Folic acid supplementation and NAFLD/NASH. Folic acid inhibits the NF-κB pathway by decreasing ROS and Hcy concentrations and inhibits IL-6, TNF-α, and IL-1β to improve liver inflammation. Folic acid improves hepatic lipid metabolism by increasing PPARα levels in a SIRT1-dependent manner and restores hepatic single carbon metabolism and gut microbiota diversity. Folic acid restores AMPK activation by increasing AMP and LKB1 phosphorylation levels, thus ameliorating glucose and cholesterol metabolism. Folic acid inhibits hepatic steatosis by increasing phosphorylation of AMPK and LKB1 and ACC. Folic acid improves liver oxidative stress by inhibiting the activation of NADPH oxidase, increasing the activities of SOD and catalase, and correcting the equilibrium between reduced (GSH) and oxidized (GSSG) glutathione. ROS, reactive oxygen species; Hcy, homocysteine; NF-κB, nuclear factor-κB; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin-1β; SIRT1, silence information regulation factor 1; PPARα, peroxisome proliferator-activated receptor alpha; NASH, non-alcoholic steatohepatitis; AMP, adenosine monophosphate; p-, phosphorylation; LKB1, liver kinase B; AMPK, AMP-activated protein kinase; ACC, acetyl coenzyme A carboxylase; NADPH, nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase; GSH, glutathione; GSSG, oxidized glutathione; NAFLD, Non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.↑increase; ↓decrease; ↓alleviate

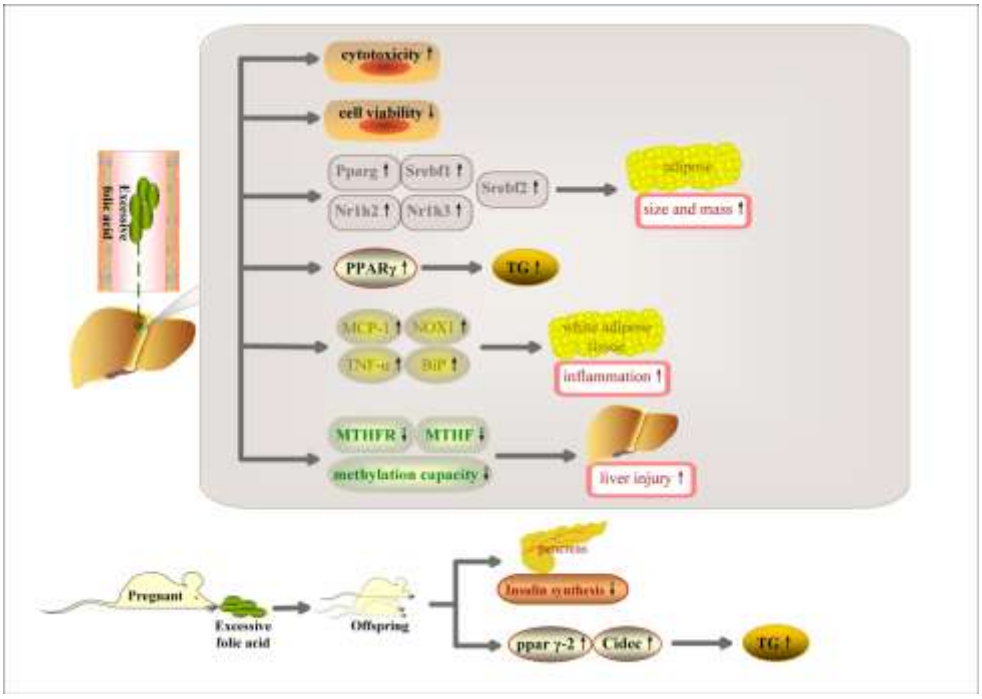

Figure 3 Excessive folic acid and NAFLD/NASH. High doses of folic acid can cause cytotoxicity and decrease cell viability. Excessive folic acid promotes the increase of Pparg, Srebf1, Srebf2, Nr1h2, Nr1h3, and induces the increase of fat size and mass. Excess folic acid upregulates PPARγ to increase TG accumulation. Excessive folic acid increases the levels of MCP-1, TNF-α, NOX1, and BiP to promote inflammation in white adipose tissue. High dose folic acid intake causes MTHFR deficiency and reduces MTHF and methylation capacity, which leading to liver damage. Excessive supplementation of folic acid during pregnancy can reduce insulin synthesis and increase TG content by raising the expression of Pparγ2 and Cidec in the offspring. Pparg, peroxisome proliferator activated receptor gamma; Srebf, sterol regulatory element binding transcription factor; Nr1h, nuclear receptor subfamily 1 group H member; PPARγ, peroxisome proliferator-activated receptor γ; TG, triglyceride; MCP-1, monocyte chemoattractant protein-1; TNF-α,tumor necrosis factor-alpha; NOX1, nicotinamide adenine dinucleotide phosphate oxidase; BiP, binding immunoglobulin protein; MTHFR, methylenetetrahydrofolate reductase; MTHF, methyltetrahydrofolate; Cidec, cell death-inducing DFF45-like effector c. ↑increase; ↓decrease; ↑aggravate

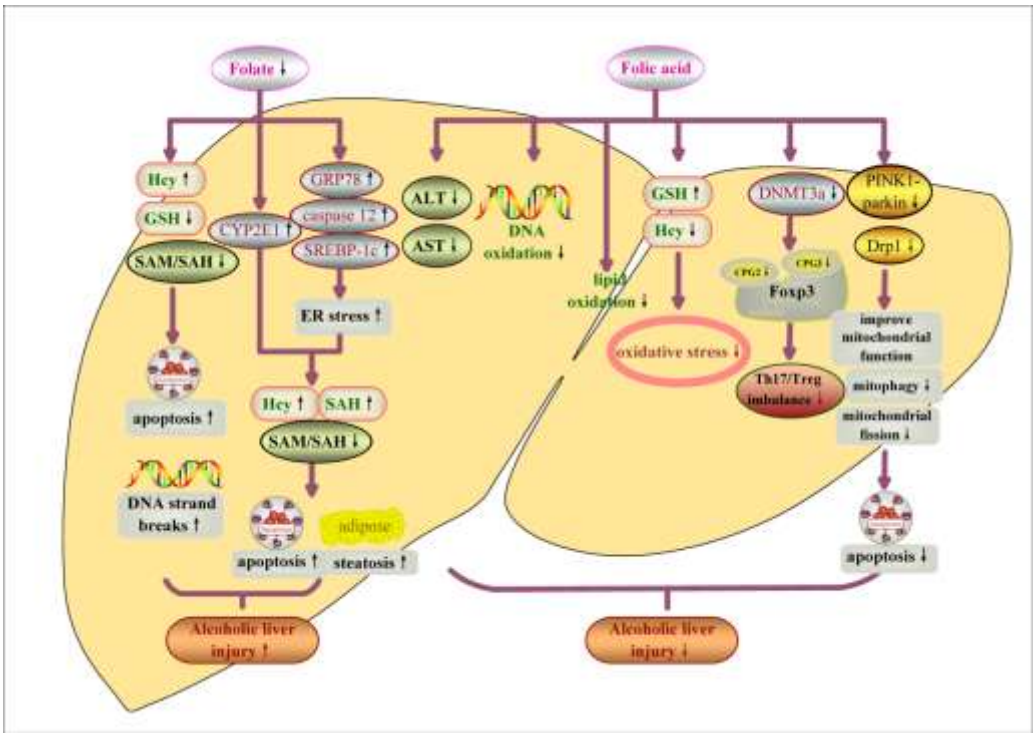

Figure 4 The mechanism of folate deficiency and folic acid supplementation in ALD. Folate deficiency increases Hcy levels, decreases SAM/SAH ratio and GSH levels, and increases apoptosis and DNA strand breakage. Folate deficiency leads to activation of CYP2E1 and ER stress signals including GRP78, caspase 12, and SREBP-1c, thereby increasing levels of SAH and homocysteine, reducing the SAM/SAH ratio, and exacerbating steatosis and apoptosis. Folic acid supplementation can lower ALT and AST, reduce lipid and DNA oxidation, and improve oxidative stress by increasing GSH and decreasing Hcy levels. Folic acid can improve Th17/Treg imbalance by decreasing DNMT3a level, and downregulating CPG2 and CPG3 methylation levels in Foxp3 promoter region. Folic acid can reduce the expression of PINK1-parkin and Drp1, improve mitochondrial function, inhibit mitochondrial autophagy and mitochondrial division, and thus prevent hepatocyte apoptosis. Hcy, homocysteine; GSH, glutathione; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DNA, deoxyribonucleic acid; GRP78, glucose-regulated protein 78; SREBP-1c, sterol regulatory element binding protein-1c; ER, endoplasmic reticulum; ALT, alanine transaminase; AST, aspartate transaminase; DNMT3a, DNA methyltransferase 3 alpha; Foxp3, forkhead box P3; CPG, carboxypeptidase G; PINK1, PTEN induced putative kinase 1; Drp1, dynamin-related protein 1. ↑increase; ↓decrease; ↑aggravate; ↓alleviate

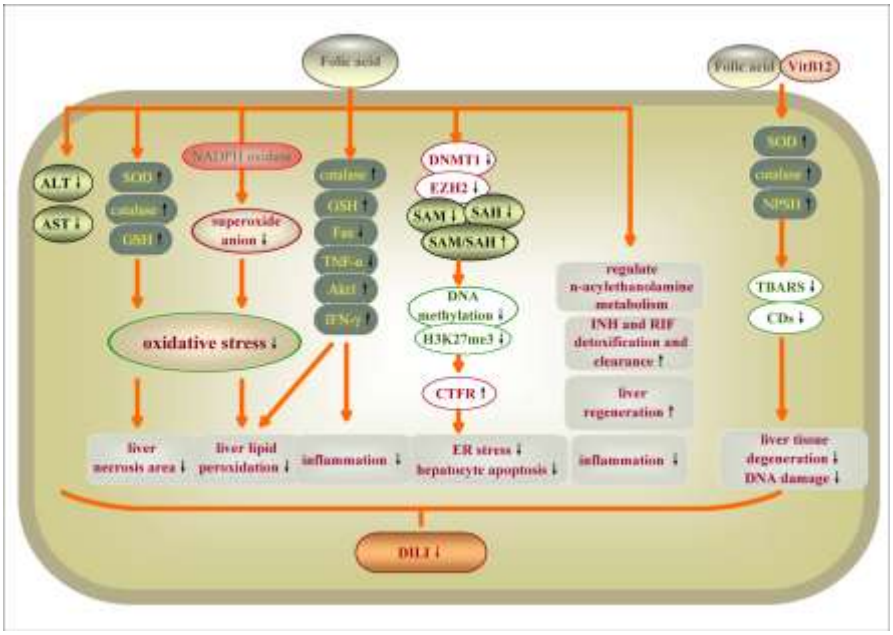

Figure 5 Folic acid and DILI. Folic acid supplementation can lower ALT and AST levels. Folic acid can increase the activities of SOD and catalase and the level of GSH, thereby alleviating oxidative stress and reducing the area of liver necrosis. Folic acid supplementation can inhibit the generation of superoxide anions mediated by NADPH oxidase and prevent oxidative stress, thereby reducing hepatic lipid peroxidation. Folic acid can restore the activities of catalase and GSH, downregulate the levels of Fas and TNF-α, and upregulate the concentrations of Akt1 and IFN-γ, so as to prevent lipid peroxidation, reduce liver inflammation. Folic acid can inhibit DNMT1 and EZH2, decrease SAM and SAH levels, increase SAM/SAH ratio, inhibit DNA methylation and H3K27me3, thereby upregulating CFTR expression, alleviating ER stress and hepatocyte. Folic acid can regulate n-acylethanolamine metabolism, enhance the detoxification and clearance of INH and RIF, promote liver regeneration, downregulate inflammatory response, and thus improve liver injury. Folic acid combined with vitamin B12 can increase SOD, catalase and NPSH levels, and reduce TBARS and CDs, thus alleviating liver tissue degeneration and DNA damage. ALT, alanine transaminase; AST, aspartate transaminase; SOD, superoxide dismutase; GSH, glutathione; NADPH, nicotinamide adenine dinucleotide phosphate; Fas, programmed cell death-receptor; TNF-α,tumor necrosis factor-alpha; Akt1, protein kinase B; IFN-γ, interferon gamma; DNMT1, DNA methyltransferase 1; EZH2, enhancer of zeste homolog 2; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DNA, deoxyribonucleic acid; H3K27me3, trimethylation of lysine 27 on histone H3; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; INH, isoniazid; RIF, rifampicin; NPSH, nonprotein-soluble thiol; TBARS, thiobarbituric acid reactive substances; CDs, conjugated dienes; DILI, drug-induced liver injury; VitB12, vitamin B12. ↑increase; ↓decrease; ↓alleviate

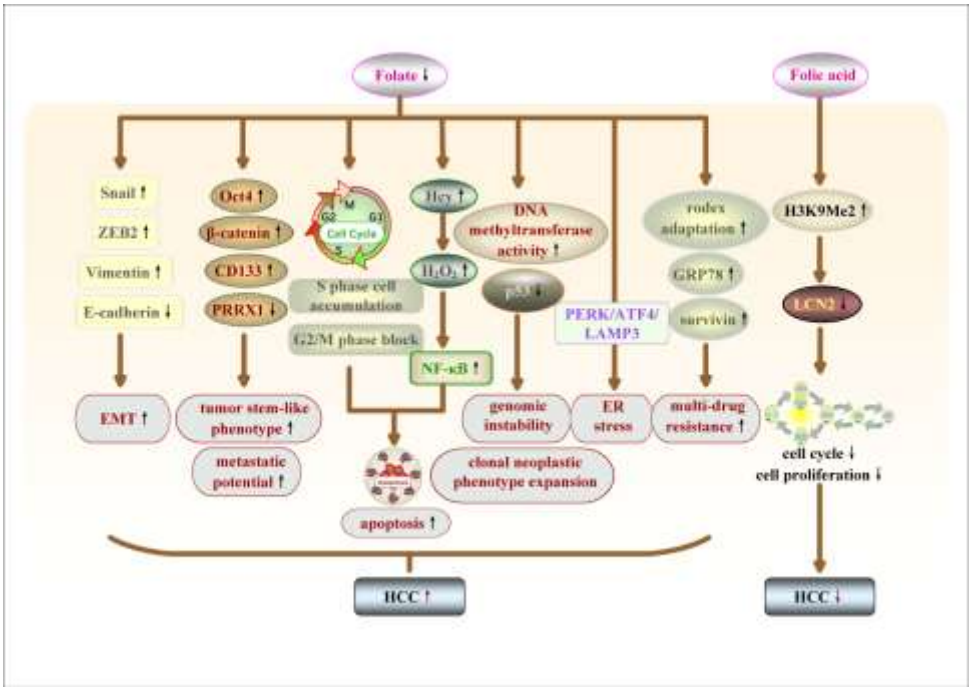

Figure 6 The mechanism of folate deficiency and folic acid supplementation in HCC. Folate deficiency upregulates Snail, ZEB2 and Vimentin, downregulates E-cadherin to promote EMT. Folate deficiency increases Oct4, β-catenin and CD133, and decreases PRRX1, which indicating promoted tumor stem-like phenotype and metastatic potential. Folate deficiency leads to S phase cell accumulation and G2/M phase arrest in the cell cycle and can promote increased Hcy accumulation, resulting in excess H2O2 production and NF-κB activation, thereby inducing apoptosis. Folate deficiency increases DNA methyltransferase activity and decreases p53 gene expression, which induce liver genomic instability and clonal neoplastic phenotype expansion. Folate deficiency can induce ER stress by activating the PERK/ATF4/LAMP3 pathway. Folate deficiency can promote rodex adaptation and upregulate GRP78 and survivin to induce multi-drug resistance, which is not conducive to the treatment of HCC. Folic acid supplementation inhibit LCN2 by promoting the level of H3K9Me2, thereby inhibiting cell proliferation and cell cycle. ZEB2, zinc finger E-box binding homeobox 2; EMT, epithelial-to-mesenchymal transition; Oct4, octamer-binding transcription factor 4; PRRX1, paired related homeobox 1; Hcy, homocysteine; H2O2, hydrogen peroxide; NF-κB, nuclear factor-κB; DNA, deoxyribonucleic acid; PERK, protein kinase R-like endoplasmic reticulum kinase; ATF4, activing transcription factor 4; LAMP3, lysosome-associated membrane glycoprotein 3; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; H3K9Me2, histone H3 lysine 9 di-methylation; LCN2, lipocalin 2; HCC, hepatocellular carcinoma. ↑increase; ↓decrease; ↑aggravate; ↓alleviate