# Role of folate in non-alcoholic fatty liver disease (NAFLD)

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Role of folate in non-alcoholic fatty liver disease (NAFLD)

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Running head: Hepatic protection of folate supplementation

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of chronic liver conditions that are characterized by steatosis, inflammation, fibrosis and liver injury. The global prevalence of NAFLD is rapidly increasing in proportion to the rising incidence of obesity and type 2 diabetes. Since NAFLD is a multifaceted disorder with many underlying metabolic abnormalities, currently, there is no pharmacological agent that is therapeutically approved for the treatment of this disease. Folate is a water-soluble B vitamin that plays an essential role in one-carbon transfer reactions involved in nucleic acid biosynthesis, methylation reactions and sulfur-containing amino acid metabolism. The liver is the primary organ responsible for storage and metabolism of folates. Low serum folate levels have been observed in patients with obesity and diabetes. It has been reported that a low level of endogenous folates in rodents perturbs folate-dependent one-carbon metabolism, and may be associated with development of metabolic diseases such as NAFLD. This review highlights the biological role of folate in the progression of NAFLD and its associated metabolic complications including obesity and type 2 diabetes. Understanding the role of folate in metabolic disease may position this vitamin as a potential therapeutic for NAFLD.

Key words: folate, non-alcoholic fatty liver disease, steatosis, oxidative stress, inflammation
Introduction

Non-alcoholic fatty liver disease (NAFLD) is a broad spectrum of chronic liver disorders that ranges from steatosis to steatohepatitis, cirrhosis, and hepatocellular carcinoma. The prevalence of NAFLD is 15 to 30% in the general population, and 70 to 90% in patients with type 2 diabetes and obesity (Ahmed 2015). Obesity is defined as abnormal or excessive fat accumulation that presents a risk to health. Obese patients typically exhibit a body mass index (BMI) greater than or equal to 30kg/m$^2$ and have an increased susceptibility towards NAFLD development (WHO 2016). NAFLD is strongly associated with the metabolic syndrome, a cluster of the most dangerous heart attack risk factors such as diabetes and prediabetes, abdominal obesity, high cholesterol and high blood pressure (IDF 2005). Patients with NAFLD often develop characteristics of the metabolic syndrome such as hyperglycemia, dyslipidemia, insulin resistance, and hypertension (Farrell and Larter 2006; Marchesini et al. 2001). The pathophysiology of NAFLD is complex and incompletely understood, therefore a number of genetic and dietary animal models have been developed to investigate NAFLD pathogenesis. These animals display histopathological features of NAFLD (Larter and Yeh 2008; Takahashi et al. 2012). Hepatic steatosis or fatty liver occurs when hepatic lipid content is greater than 5% of liver weight (Fabbrini et al. 2010). In contrast to steatosis, non-alcoholic steatohepatitis (NASH) is characterized by hepatic lipid accumulation, inflammation, liver injury, and fibrosis (Brunt and Tiniakos 2010; Kleiner et al. 2005). Although the progression of steatosis to NASH is reversible, NASH can irreversibly advance to cirrhosis in which hepatic tissue is replaced with collagenous fibrotic lesions (Brunt and Tiniakos 2010; Cohen et al. 2011). Cirrhosis may further progress to hepatocellular carcinoma (Argo et al. 2009; Cohen et al. 2011). However, the primary cause of mortality in NAFLD patients is cardiovascular disease. One long term follow-up study revealed
that coronary artery disease was the most common cause of death among NAFLD patients (Rafiq et al. 2009). Patients with NAFLD had a high cardiovascular risk profile. Compared with control subjects matched for age and gender, and with similar prevalence of features of the metabolic syndrome, NAFLD patients also exhibited higher degree of insulin resistance associated with endothelial dysfunction (Villanova et al. 2005), which may contribute to the increased cardiovascular risk in these patients. There is currently no pharmacological agent approved for the treatment of this multifaceted disorder (Schuppan and Schattenberg 2013). Although insulin sensitizing agents (eg. metformin, thiazolidinediones) and statins have been investigated for NAFLD management, these agents have not been approved for the treatment of NAFLD due to lack of the information on their long term safety and efficacy (Chalasani et al. 2012). The world is in search of a safe and effective treatment avenue for NAFLD. Folate, a water-soluble B vitamin, participates in one-carbon transfer reactions that are essential for cell metabolism (Stover 2004; Tibbetts and Appling 2010). Dysregulation of folate-dependent one-carbon metabolism has been implicated in NAFLD-related comorbidities such as obesity, type 2 diabetes, and the metabolic syndrome (da Silva et al. 2014; Hirsch et al. 2005; Mahabir et al. 2008; Nilsson et al. 2015). In this article, we provide an overview regarding the role of folate in NAFLD and its associated metabolic disorders. This review aims to summarize the current understanding on the impact of folate metabolism in the development and progression of NAFLD.

**Folate and folic acid**

Folate is a water-soluble B9 vitamin that serves as a co-enzymatic substrate for one-carbon transfer reactions. Folate-dependent one-carbon transfer reactions are important for nucleic acid
biosynthesis, methylation reactions, and sulfur-containing amino acid metabolism (Stover and Field 2011; Tibbetts and Appling 2010). Mammals lack the enzymatic capacity to synthesize folates, and therefore the intake of dietary folates is essential to meet their physiological requirements (Lucock 2000; Zhao et al. 2009a). The dietary reference intake (DRI) for folate in healthy individuals is 400ug of dietary folate equivalence (DFE) per day. In pregnant women, the DRI increases to 600ug DFE per day to satisfy fetus requirements (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate 1998). Although folate is widely distributed in a variety of foods, animal liver and dark green leafy vegetables are the most abundant sources of naturally occurring folates (Lucock 2000; Zhao et al. 2009a). Dietary folates are often in the reduced and polyglutamated forms (Wright et al. 2007; Zhao et al. 2009a). In contrast, folic acid is an oxidized, monoglutamate with higher bioavailability than its natural counterpart (Iyer and Tomar 2009) and is the synthetic (stable) form of folate that is used for dietary supplementation and fortification (Wright et al. 2007; Zhao et al. 2009a). In addition to dietary folate/folic acid, folate-producing bacteria in the colon and the proximal small intestine may serve as an endogenous source of folates (Camilo et al. 1996; Rong et al. 1991). However, the contribution of intestinal bacteria to whole body folate homeostasis in mammals is significantly less than the dietary source of folates (Visentin et al. 2014).

**Folate absorption**

Dietary folate absorption primarily occurs in the proximal intestine within an acidic microenvironment (Figure 1). The reduced folate carrier (RFC/SLC19A1) and the proton-coupled folate transporter (PCFT/SLC46A1) are highly expressed along the apical membrane of
enterocytes (Visentin et al. 2014; Zhao et al. 2011). Folate uptake is mainly mediated by PCFT, which optimally functions at an acidic pH in the small intestine (pH 4 to 6) (Qiu et al. 2006; Visentin et al. 2014). Natural dietary folates (polyglutamate form) are hydrolyzed to monoglutamates by glutamate carboxypeptidase II (in human intestine) or γ-glutamyl hydrolases (in rodent intestine) prior to absorption at the intestinal brush border membrane (Shafizadeh and Halsted 2007). Compared to folic acid, dietary folates tend to be more reduced and are commonly found as formyl and methyl polyglutamates (Perry 1971; Wright et al. 2007). Folic acid is a monoglutamate, therefore hydrolysis is not necessary for intestinal absorption (Hu et al. 2016). Folic acid approaches 100% bioavailability and at least 85% of folic acid is bioavailable when consumed in the diet. Compared to folic acid, only 50% of natural dietary folates are bioavailable (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate 1998; Iyer and Tomar 2009). Upon absorption by enterocytes, folic acid is reduced to dihydrofolate (DHF). Subsequently, DHF is further reduced to tetrahydrofolate (THF) that is the biological active form of folate (Bailey and Ayling 2009; Pietrzik et al. 2010; Wright et al. 2007). Although both of these reactions are catalyzed by DHF reductase, the initial enzymatic reduction of folic acid to DHF is the rate-limiting step (Bailey and Ayling 2009). The addition of one-carbon moiety to THF by serine hydroxymethyltransferase, generates 5,10-methylene-THF. The 5,10-methylene-THF is further reduced to 5-methyl-THF (5-MTHF) by 5,10-methylene-THF reductase (Stover and Field 2011; Tibbetts and Appling 2010). The 5-MTHF is the main form of folate that is transported across the basolateral membrane of enterocytes, and enters the portal circulation (Pietrzik et al. 2010; Wright et al. 2007). Since DHF reductase has a low capacity for reduction of folic acid in enterocytes, unmetabolized folic acid may be present in the portal circulation (Hu et al. 2016).
High intakes of folic acid may also lead to accumulation of its unmetabolized form in the portal vein (Pietrzik et al. 2010; Raghunathan et al. 1997). The 5-MTHF and folic acid derived from the gut is subsequently delivered to the liver via the portal vein (Hu et al. 2016; Pietrzik et al. 2010; Wright et al. 2007). The RFC and PCFT are widely expressed on the basolateral membrane of hepatocytes (Zhao et al. 2011; Zhao et al. 2009a). However, previous studies suggest that the PCFT plays a major role in mediating folic acid and 5-MTHF transport to the liver (Horne et al. 1993; Hu et al. 2016). Once inside the cell, folic acid is metabolized to 5-MTHF, which undergoes polyglutamation by folylpolyglutamate synthase (Visentin et al. 2014; Zhao et al. 2009a). Folate polyglutamates are efficiently retained by hepatocytes relative to their monoglutamate forms, and are preferential substrates for folate-dependent enzymatic reactions (Blom et al. 2006; Zhao et al. 2009a). While up to 20% of the 5-MTHF is retained by the liver, the remainder is delivered to extra-hepatic tissues by the systemic circulation or secreted into bile via the bile duct (Steinberg et al. 1979). Bile folates may be reabsorbed by the intestine and are subsequently distributed to the liver and other tissues (Steinberg et al. 1979; Zhao et al. 2009a). Since liver is a major organ for folate storage and metabolism (Wright et al. 2007), it plays an important role in maintaining whole body folate homeostasis (Steinberg et al. 1979).

**Folate metabolism and its biological function**

Folate mediated one-carbon transfer reactions are differentially distributed within the cytoplasm, nucleus, and mitochondria in mammalian cells (Shin et al. 1976; Stover and Field 2011; Tibbetts and Appling 2010) (Figure 2). Intracellular one-carbon transfers are mediated by co-enzymatic forms of THF, which carries one-carbon moieties (derived from histidine, serine, glycine and formate) for amino acid, nucleotide biosynthesis, and methylation reactions (Blom et al. 2006;
In the cytoplasm and nucleus, 5,10-methylene-THF serves as a substrate for biosynthesis of deoxymethylvalylate (dTMP) from deoxyuridylate (dUMP), as well as for interconversion of glycine and serine (Herbig et al. 2002; Tibbetts and Appling 2010). Serine and glycine biosynthesis may also occur in the mitochondria. In contrast, *de novo* purine nucleotide biosynthesis and remethylation of homocysteine to methionine are folate-dependent reactions that take place in the cytoplasm (Stover and Field 2011; Tibbetts and Appling 2010). The 5,10-methylene-THF is reversibly converted to 5,10-methenyl-THF by methylenetetrahydrofolate dehydrogenase (Herbig et al. 2002). The 5,10-methenyl-THF is a precursor for 10-formyl-THF synthesis. The 10-formyl-THF provides one-carbon moieties for purine nucleotide synthesis, while 5-MTHF donates carbon units for methionine synthesis (Tibbetts and Appling 2010). Nucleotide biosynthesis is required for RNA and DNA synthesis, and plays a key role in cell growth and proliferation. Remethylation of homocysteine to methionine also prevents homocysteine accumulation. Methionine is an essential precursor for the synthesis of S-adenosylmethionine (SAM), a principle methyl donor in cells. This cofactor regulates a number of fundamental cellular processes involved in cell signaling, protein localization, degradation of molecules, as well as gene transcription and translation (Miranda and Jones 2007). Folate deficiency attenuates methionine synthesis from homocysteine, inhibits generation of SAM from methionine, and promotes accumulation of S-adenosylhomocysteine (SAH), a potent inhibitor of SAM-dependent methylation reactions (Stover 2004). Folate deficiency compromises one-carbon metabolism and is implicated in diseases such as hyperhomocysteinemia, alcoholic fatty liver disease (ALD) and NAFLD (Christensen et al. 2010; Medici and Halsted 2013; Stover 2004). One-carbon metabolism in the mitochondria is important for the synthesis of formate, which is a major source of carbon units.
for one-carbon metabolism that occurs in the cytoplasm (Barlowe and Appling 1988; Tibbetts and Appling 2010). The 10-formyl-THF serves as a carbon donor for formate synthesis and donates a formyl group to the methionine-tRNA (formylmethionine-tRNA), which is required for initiation of mitochondrial protein synthesis (Pike et al. 2010; Tibbetts and Appling 2010). Taken together, folate-dependent one-carbon transfer reactions are vital for regulation of various intracellular metabolic processes (Stover and Field 2011; Zhao et al. 2009a).

**Implication of folate status in disease**

Mandatory folic acid fortification of flour and grain was established in 1998 for prevention of birth defects in Canada and the United States (Centers for Disease and Prevention 2010). General populations in these countries have achieved adequate folate intakes since implementation of the folic acid fortification policy (Bailey et al. 2010). However, folate deficiency may occur in countries without mandatory folic acid fortification policies (Dhonukshe-Rutten et al. 2009; Garcia-Casal et al. 2005). Moreover, folate absorption is impaired in individuals with chronic alcoholism and malabsorptive disorders (Lucock 2000; Medici and Halsted 2013). Hereditary folate malabsorption due to mutations in the PCFT gene abolishes folate intestinal absorption and transport into the central nervous system, leading to folate deficiency (Qiu et al. 2006). Folate deficiency leads to anemia and the development of neurological disorders in newborns (Cario et al. 2011; De Wals et al. 2007; Stover 2004). Dihydrofolate reductase deficiency can compromise folate status in red blood cells and cause megaloblastic anemia, a condition of large, abnormal red blood cells (Cario et al. 2011; Lucock 2000).

A low level of serum folates is frequently observed in patients with alcoholic fatty liver disease (ALD) (Medici and Halsted 2013) and hyperhomocysteinemia. Hyperhomocysteinemia is an
independent predictor of cardiovascular disease (Clarke 2000; Homocysteine Studies 2002; Wald et al. 2002). Methylenetetrahydrofolate reductase deficiency is a common hereditary disorder of folate metabolism that causes severe hyperhomocysteinemia (Brattstrom et al. 1998; Burda et al. 2015). Although folic acid supplementation could effectively reduce homocysteine levels in the circulation (Boushey et al. 1995; Lonn et al. 2006), cardiovascular risk was not improved in patients with vascular disease (Lonn et al. 2006). Since folate is a crucial player in one-carbon metabolism, its deficiency has been associated with dysregulation of intracellular metabolic processes (Christensen et al. 2010; da Silva et al. 2014). Emerging evidence suggests that low circulating folate levels may be associated with the development of metabolic disorders including obesity and NAFLD (Hirsch et al. 2005; Mahabir et al. 2008).

**Non-alcoholic fatty liver disease (NAFLD)**

NAFLD is a broad spectrum of liver disorders that develop independently of alcohol consumption (Ahmed 2015; Brunt and Tiniakos 2010). The two-hit hypothesis was initially proposed to describe the pathogenesis of NAFLD. This theory recognizes that perturbations in lipid metabolism (first hit) may sensitize the liver to secondary hits such as inflammation and oxidative stress (Day and James 1998). However, subsequent studies suggest that multiple parallel factors may contribute to fatty liver disease. The multiple-hit theory suggests that insulin resistance, lipotoxicity, oxidative stress, inflammation, gut-derived endotoxins, adipokines, or genetic factors may simultaneously contribute to the development of NAFLD (Buzzetti et al. 2016; Tilg and Moschen 2010). NAFLD is highly prevalent in patients with obesity and type 2 diabetes (Ahmed 2015). A number of clinical studies identified that serum folate levels were

Obese and overweight patients had significantly lower serum folate levels compared to normal weight individuals. Serum folate levels were decreased by 1.7% in correspondence to each unit increment of BMI in obese patients (Mahabir et al. 2008). A study based on the national health and nutrition examination survey (NHANES; 2003-2006) reported that serum folate levels were significantly reduced in obese patients (12.4ug/L; \( n=1141 \)) compared to normal weight individuals (13.1ug/L; \( n=1236 \)). The regression analysis indicated an inverse relationship between serum folate and BMI regardless of adjustment for vitamin intakes and demographic variables such as gender, age, ethnicity, smoking status, and alcohol use. Low levels of serum folates were directly associated with decreased intakes of the vitamin in obese patients (Bird et al. 2015). Another study performed on childbearing age women before and after the USA folic acid fortification program (data obtained from NHANES III 1988-1994; \( n=5018 \) and from NHANES 1999-2000; \( n=1351 \)) revealed that even after controlling for folate intake in food and supplements, a high BMI was associated with low serum folate levels. It was estimated that women with a BMI greater or equal to 30kg/m\(^2\) had to take an additional 350ug/day of folate to achieve similar serum folate levels as women the lowest BMI category (less than 20kg/m\(^2\)). The significant decrease of serum folates suggests that obese patients may be at risk for folate deficiency despite dietary folic acid fortification (Mojtabai 2004). In contrast, folate status was significantly improved in morbidly obese patients after gastric bypass surgery (Updegraff and Neufeld 1981). Since treatment of obesity was able to restore folate levels in patients, this suggests that obesity may be the underlying cause for the imbalance of endogenous folate levels. Low folate levels in obese patients were associated with increased NAFLD severity. It was
reported that serum folate levels were significantly reduced in obese female patients with severe NAFLD (21nmol/L; n=17) compared to obese women with normal liver morphology or minimal liver damage (27nmol/L; n=26) (Hirsch et al. 2005). One limitation of this study is a small sample size (Hirsch et al. 2005). Circulating folate levels were also markedly lower in patients with type 2 diabetes. Such a reduction in blood folate levels was correlated with high levels of fasting blood glucose, and increased expression of hepatic genes involved in the development of diabetes (Nilsson et al. 2015). Folate depletion may also lead to epigenetic and transcriptional alterations in the liver, and contribute to the pathogenesis of type 2 diabetes (Nilsson et al. 2015).

While the sum of clinical evidence suggests that low folate status may have an important implication in metabolic disease, a causal relationship cannot be established. In addition to the findings from human studies, animal based studies also support the notion that folate homeostasis is impaired in obesity and diabetes. In the study by Lam et al. experiments were conducted in diabetic/obese (db/db) and non-diabetic/lean (m/db) C57BL/KsJ mice. It was demonstrated that plasma folate concentrations in the db/db mice (29.62ug/L) were profoundly reduced compared to m/db mice (45.22ug/L). A limitation of this study was that it was unclear whether blood was collected under fasting or fed conditions, which might affect the interpretation of the plasma folate readings (Lam et al. 2009). Overall, the reduction in serum folate levels observed in obese patients and in animal models suggests a potential interrelationship between perturbation of folate status and the development of NAFLD.

**Role of folate in lipid and carbohydrate metabolism**

Steatosis is a benign condition that develops when lipogenesis and fatty acid uptake exceed the rate of lipid export in the liver (Cohen et al. 2011; Fabbrini et al. 2010). Hepatic expression of
genes involved in the de novo lipid synthesis was significantly elevated in NAFLD patients (Kohjima et al. 2007). In general, lipogenesis contributes to less than 5% of hepatic triglyceride content in healthy individuals. However, 26% of triglycerides stored in the liver derive from lipogenesis in patients with NAFLD. Such an increase in lipid biosynthesis may contribute to hepatic lipid accumulation (Donnelly et al. 2005). In addition, adipose tissue lipolysis is also increased in NAFLD patients, which accelerates the rate of fatty acid uptake by the liver (Fabbrini et al. 2008). An elevated supply of fatty acids to the liver derives from hepatic lipogenesis and adipose tissue lipolysis, which, in turn, contributes to increased hepatic triglyceride synthesis in NAFLD (Fabbrini et al. 2008; Musso et al. 2009). Although hepatic oxidation of fatty acids and triglyceride export by very low density lipoproteins (VLDL) were upregulated in patients with NAFLD, these processes could not restore metabolic homeostasis in the liver (Adiels et al. 2006; Fabbrini et al. 2008; Sanyal et al. 2001).

A number of studies have suggested that folate deficiency may also contribute to the development of steatosis in rodents (Christensen et al. 2010; da Silva et al. 2014). Depletion of dietary folates in rodents was associated with high expression of lipid biosynthetic genes, which perturbs lipid metabolism in the liver (Champier et al. 2012). Moreover, hepatic lipid transport by VLDL was impaired in folate-deficient mice (Christensen et al. 2010; da Silva et al. 2014; Kim et al. 1994). Folate is essential for the synthesis of S-adenosylmethionine (SAM) from methionine. Phosphatidylethanolamine N-methyltransferase (PEMT) utilizes SAM as a methyl donor to catalyze the methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC), which is necessary for VLDL assembly in hepatocytes (Fast and Vance 1995; Zhao et al. 2009b). The synthesis of PC from PE by PEMT was reduced during folate deficiency (Christensen et al. 2010; Li and Vance 2008). A reduction in the PC to PE ratio may compromise
lipid export by VLDL, and promote hepatic lipid accumulation (Christensen et al. 2010). Moreover, a low PC to PE ratio may also enhance cell membrane permeability and lead to leakage of cellular components, activation of immune cells and cytokine secretion (Noureddin et al. 2015). In addition to lipoprotein assembly, PC is a major phospholipid required for bile secretion by the liver (Noga and Vance 2003). Bile production, which is essential for absorption and digestion of dietary fats, was also decreased in folate-deficient rodents. Inhibition of bile production may have adverse effects on lipid metabolism (McNeil et al. 2008; Sehayek et al. 2003). On the other hand, hepatic insulin resistance can perturb the regulation of lipid metabolism (Birkenfeld and Shulman 2014; Fabbrini et al. 2010), and is a common feature observed in human and rodent models of NAFLD (Larter and Yeh 2008; Utzschneider and Kahn 2006). The regulation of glucose and lipid production is disrupted when insulin action is compromised in the liver (Birkenfeld and Shulman 2014; Saltiel and Kahn 2001). Abnormal lipid and carbohydrate metabolism in the liver is often associated with dysregulation of AMP-activated protein kinase (AMPK). The AMPK is a key regulator of metabolism in correspondence with energy balance (Hardie et al. 2012). Inactivation of AMPK has been associated with hepatic lipid accumulation, hyperglycemia, and hyperinsulinemia in animal models with high-fat diet induced NAFLD (Pu et al. 2012; Sid et al. 2015). Our recent study demonstrated that folic acid supplementation was able to restore AMPK activation in high-fat diet fed mice, leading to improvements in hyperinsulinemia as well as in lipid and glucose metabolism (Sid et al. 2015). Therefore, the hepatoprotective effect of folate may be attributed to its important role in metabolic regulation.
Role of folate in oxidative stress and inflammation

Oxidative stress is a complex, biological phenomenon that reflects an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms (Halliwell and Whiteman 2004; Kaludercic et al. 2014). This imbalance is clearly demonstrated in patients with NAFLD, who exhibited elevated levels of ROS and hepatic lipid peroxides (Seki et al. 2002; Videla et al. 2004), as well as compromised hepatic and systemic antioxidant defenses (Hardwick et al. 2010; Loguercio et al. 2004). Hepatic oxidative stress is a key mediator of hepatocellular injury (Rolo et al. 2012; Seki et al. 2002). Folic acid, as well as naturally occurring folates, were suggested to have antioxidant functions due to their ability to directly scavenge ROS (Gliszczynska-Swiglo and Muzolf 2007; Joshi et al. 2001). Dietary folate depletion in rodents was shown to significantly increase hepatic lipid peroxidation, and impair the activities of various antioxidant enzymes (Henning et al. 1997; Huang et al. 2001). Low folate status may disrupt mitochondrial redox homeostasis, and stimulate oxidative damage in rodent liver (Chou et al. 2007; Sawyer and Van Houten 1999). Our previous studies demonstrated that folic acid could confer protective effects against oxidative stress in the liver (Sarna et al. 2012; Woo et al. 2006) and kidney in rodents (Hwang et al. 2011). Folic acid supplementation effectively inhibited NADPH oxidase-mediated superoxide production, and restored the antioxidant response in hyperhomocysteinemic rats, and in high-fat diet induced obese mice (Hwang et al. 2011; Sarna et al. 2012; Woo et al. 2006). Moreover, intracellular folate metabolism significantly contributes to the generation of NADPH, a source of reducing power for defense against oxidative stress. Depletion in folate metabolic enzymes was associated with low levels of NADPH and a reduction in the ratio of reduced glutathione to oxidized glutathione, which is an indicator of oxidative stress (Fan et al. 2014). Hepatic inflammation is
another important pathological mediator of NAFLD (Day 2006). Oxidative stress may contribute to the progression of NAFLD by triggering the hepatic immune response. The by-products of lipid peroxidation (i.e. malonyldialdehyde and 4-hydroxynonenal) may induce activation of liver immune cells and promote inflammation (Sutti et al. 2014). In NAFLD, chronic stimulation of the hepatic inflammatory response may enhance liver susceptibility to tissue injury, fibrosis, and cirrhosis (Elsharkawy and Mann 2007; Sutti et al. 2014). The degree of inflammation in the liver is exacerbated with increased severity of NAFLD (Kleiner et al. 2005). Patients with NAFLD have high levels of pro-inflammatory cytokines, which are secreted by hepatocytes and immune cells (Day 2006). Folic acid supplementation in overweight and hyperhomocysteinemic patients was associated with reduction in pro-inflammatory cytokine levels (Solini et al. 2006; Wang et al. 2005). In contrast, macrophages grown in a folate-depleted medium had high expression of pro-inflammatory mediators such as IL-1β, MCP-1, IL-6 and TNF-α (Kolb and Petrie 2013). Macrophage-mediated release of inflammatory cytokines may contribute to the development of vascular disease (Ito and Ikeda 2003; McLaren et al. 2011). We have observed that treatment of macrophages with folic acid attenuated homocysteine-induced pro-inflammatory cytokine expression (Au-Yeung et al. 2006). The ability of folic acid to reduce inflammatory cytokine levels further suggests that it may attenuate the inflammatory response that is associated with development of cardiovascular disease (Au-Yeung et al. 2006; Kolb and Petrie 2013). Taken together, the sum of available evidence suggests that folic acid may exhibit both antioxidant and anti-inflammatory functions.
Conclusions and future considerations

Folate plays a fundamental role in one-carbon transfer reactions that are involved in cell growth, gene regulation, and metabolism. Low levels of folates are observed in patients with chronic alcoholism, dietary folate deficiency, malabsorption disorders and hyperhomocysteinemia. Emerging studies suggest that low serum folate levels may be also associated with the development of NAFLD and obesity. In this review, we have summarized the current research evidence on the role of folate in NAFLD development (Figure 3). Folate plays an essential role in lipid metabolism and folic acid supplementation can attenuate steatosis in rodent liver. In addition to its lipid lowering effects, folate can scavenge ROS, regulate the activity of ROS-generating enzymes, and restore the activity of antioxidant enzymes. Folate can also blunt the increase of inflammatory cytokines secreted by immune cells. Supplementation with folic acid has been shown to improve lipid metabolism and oxidative stress in rodent models of NAFLD. Despite the known beneficial effects of folic acid supplementation, there are concerns regarding folic acid oversupplementation in masking vitamin B12 deficiency and impairing cognitive function. Proper clinical trials are warranted to determine the optimal dose of folic acid supplementation in specific populations such as individuals with fatty liver or metabolic disease to minimize adverse effects. Although low circulating folate levels have been reported in NAFLD patients, it is unclear whether depletion of folates may be cause or consequence of NAFLD. Future studies are necessary to determine whether low folate status may be a risk factor for NAFLD. Overall, folate plays an important role in regulating hepatic metabolism, as well as reducing oxidative stress and inflammation. The multifaceted role of folate suggests that supplementation of this vitamin may potentially have therapeutic implications for the management of NAFLD.
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Figure 1. Folate absorption and metabolism in the intestine and liver.

The major routes of folate absorption and metabolism in the intestine and liver are illustrated. The key forms of folate that enters these organs are shown in bold. All enzymes are italicized. DHF, dihydrofolate; THF, tetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; DHFR, dihydrofolate reductase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFC, methylenetetrahydrofolate cyclohydrolase; MS, methionine synthase; GCPII, glutamate carboxypeptidase II; FPG synthase, folylpolyglutamate synthase; PCFT, proton-coupled folate transporter; RFC, reduced folate transporter.

Figure 2. Compartmentalization of folate-dependent one-carbon metabolism.

One-carbon metabolism for nucleotide biosynthesis, amino acid metabolism, and methylation reactions are distributed within intracellular compartments such as the cytoplasm, nucleus and mitochondria. The box with solid lines denotes reactions that only occur in the cytoplasm, the box with dotted lines denotes reactions that mainly occur in the mitochondria, and the box with double dotted lines denotes reactions that occur in both the cytoplasm and nucleus. The other one-carbon metabolism reactions may occur in all three cellular compartments. DHF, dihydrofolate; THF, tetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; DHFR, dihydrofolate reductase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase; MTHFD, methylenetetrahydrofolate dehydrogenase; MTHFC, methylenetetrahydrofolate cyclohydrolase; FTHFS, formyltetrahydrofolate synthetase; TS, thymidylate synthase; dTMP, deoxythymidylate; dUMP, deoxyuridylate; MS, methionine synthase; MAT, methionine adenosyltransferase; CBS, cystathionine-β-synthase; CSE,
cystathionine-\(\gamma\)-lyase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; \(SAHH\), adenosylhomocysteine hydrolase; fmet-tRNA, formylmethionine-tRNA; \(FT\), formyltransferase.

**Figure 3. Potential role of folate in NAFLD**

The relationship between folate and NAFLD is illustrated. NAFLD is characterized by steatosis, inflammation, oxidative stress, as well as insulin resistance and hyperglycemia. Folate supplementation in rodents reduces metabolic abnormalities associated with NAFLD.
Figure 1
Figure 2

- **Folic acid**
  - THF
  - dTMP
  - dUMP
  - 10-formyl-THF
  - 5,10-methylene-THF
  - 5-MTHF
  - Methionine
  - Methionine Cycle
  - Homocysteine
  - CBS/CSE
  - Glutathione
  - Transulfuration pathway
  - SAM
  - Methylation reactions

- **Cytoplasm**
  - Purines
  - THF
  - 10-formyl-THF (MTTHC)

- **Cytoplasm and Nucleus**
  - dTMP
  - dUMP
  - TS
  - 10-formyl-THF
  - 5,10-methenyl-THF
  - 5,10-methylene-THF
  - Methionine (MAT)

- **Mitochondria**
  - THF
  - Formate
  - fmet-tRNA
  - MTHFC

- **Cytoplasm, Nucleus or Mitochondria**
  - DHFR
  - 10-formyl-THF
  - FTHFS
  - FT

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Figure 3

NAFLD

- Steatosis
- Inflammation
- Oxidative stress
- Insulin resistance
- Hyperglycemia

Folate