Redox Biology 42 (2021) 101937

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Impact of vitamin E on redox biomarkers in non-alcoholic fatty liver disease

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ARTICLE INFO

Keywords:
Hydroxyoctadecadienoic acid (HODE)
Lipid peroxidation biomarkers
Non-alcoholic fatty liver disease (NAFLD)
Tocopherols
Vitamin E
4-Hydroxynonenal

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disorder in Western nations and characterized by excessive accumulation of lipids in the liver. In this narrative review, we summarize the evidence from human trials that free radical-induced oxidation of macromolecules, in particular of lipids, is a characteristic feature of NAFLD and non-alcoholic steatohepatitis (NASH). We further synthesize the data in the scientific literature describing the impact of vitamin E (mainly α-tocopherol) on concentrations of redox biomarkers in liver biopsies from patients with NAFLD as well as animal experiments. In summary, the available evidence from clinical trials suggests that reactive species-mediated damage to macromolecules, predominantly lipids, occurs in NAFLD and NASH and that daily supplementation with at least 200 I.U. α-tocopherol may alleviate oxidative stress in the liver of NAFLD patients. We propose α-tocopherol as a useful model substance to identify and validate suitable redox biomarkers that may be employed in future clinical trials of new therapeutics for NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), a disease characterized by excessive accumulation of hepatic lipids, is becoming the most common liver disorder in Western nations [1]. NAFLD is a range of different liver diseases varying in severity from steatosis to non-alcoholic steatohepatitis (NASH), with or without fibrosis, to cirrhosis and in some cases hepatocellular carcinoma (Fig. 1). Liver biopsy remains the gold standard of diagnosis for NAFLD. Histologically, NAFLD is defined as the presence of steatosis, the excessive accumulation of lipids, in more than 5% of hepatocytes, while NASH is defined as steatosis in more than 5% of hepatocytes plus evidence of inflammation and ballooning, with or without fibrosis present [2]. NAFLD is highly prevalent, affecting almost a quarter of the global population [3] and these numbers are predicted to increase to 30% by 2030 [4]. The prevalence is even higher, namely 70%, in patients with obesity and type 2 diabetes mellitus [3]. Due to the rise in childhood obesity, NAFLD is becoming a significant pediatric problem as well, with an estimated 36% of obese children affected [5]. The actual prevalence may be even higher, as NAFLD is considered a silent disease with few symptoms until the late stages.

NAFLD is a multifactorial disease with contributions from the environment, gut microbiota, insulin resistance and genetics [6]. The disease is strongly associated with obesity [7] although lean NAFLD has been described. Around 40% of NAFLD patients progress to NASH [8,9] and this process is incompletely understood [6]. Steatosis alone is mostly viewed as benign, but in NASH there is evidence of hepatic cell death, inflammation, fibrogenesis and increased genesis of reactive species as well as an increased risk for hepatocellular carcinoma [6,10,11]. The liver injury in NASH can make a liver transplantation necessary and it has been estimated that NASH will become the leading cause for liver transplantations within the next decade [12].

Vitamin E was discovered in 1922 by Katherine J. Bishop and Herbert M. Evans as a dietary factor preventing fetal resorption in rats [13]. The term vitamin E describes a family of eight lipophilic compounds, namely α-, β-, γ-, and δ-tocopherol and α-, β-, γ-, and δ-tocotrienol, that feature a chromanol ring attached to either a saturated alkyl side chain, in the case of the tocopherols, or an unsaturated isoprenoid side chain, for the tocotrienols. The Greek letters are assigned based on the number and positions of up to three methyl groups attached at the chromanol ring. The saturated sidechain contains three chiral centers, which can be in either R- or S-configuration, and thus give rise to eight different stereoisomers (RRR, RRS, RSR, SRR, RSS, SRS, SSR, and SSS). All eight congeners are present in the diet and absorbed into the organism, but only α-tocopherol (αT) in its RRR conformation is selectively retained and incorporated into VLDL [14], while the non-αT congeners are preferentially metabolized and excreted [15–17]. αT is a potent chain-breaking antioxidant protecting membrane lipids from oxidation [18], but it is still debated whether or not this is the only function of αT

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https://doi.org/10.1016/j.redox.2021.101937
Received 30 January 2021; Received in revised form 26 February 2021; Accepted 3 March 2021
Available online 6 March 2021

in vivo [19].

The evidence for therapeutic effects of αT in the treatment of NAFLD have been extensively reviewed [20–22]. In brief, clinical trials have shown benefits of αT supplementation in NAFLD patients without diabetes mellitus [23], with diabetes mellitus [24] and in children [25]. The latest meta-analysis, including 768 NAFLD patients and seven clinical trials, found and overall improvement of histological parameters in patients treated with αT [26]. Until now, αT supplementation is the only intervention that improves transplant-free survival and reduces mortality in NASH patients [27]. The current evidence has prompted the American Association of Liver Diseases (AASLD) to recommend αT supplementation as a treatment of biopsy-proven NASH in non-diabetic patients [2].

In this review we do not wish to reiterate the beneficial effects of vitamin E on histological parameters of NAFLD, but rather focus on the influence of αT on redox biomarkers in the context of NAFLD. Because an overshooting generation of reactive species and in particular lipid peroxidation play a crucial role in NAFLD, we aim to investigate if αT can act as a model substance to validate clinically useful redox biomarkers that may be employed in future clinical trials of new therapeutics for NAFLD.

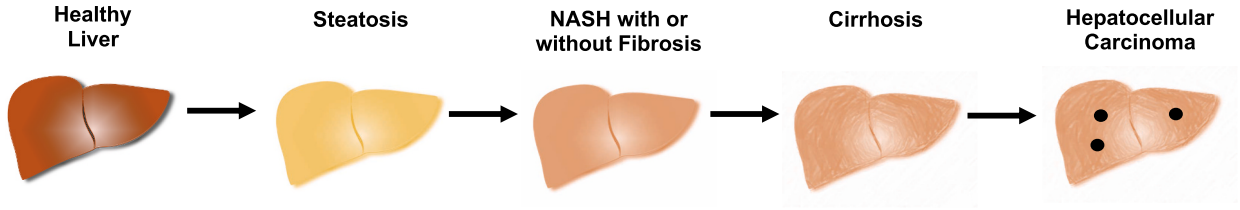
2. The role of reactive species in NAFLD

Oxidative stress has been defined as an imbalance between oxidants and antioxidants in a (biological) system in favor of the oxidants [28]. Under normal (healthy) conditions, the oxidants are maintained within a certain physiological range and carry out redox signaling functions. NAFLD is considered a disease of "multiple hits" [29] of which one is an imbalance of oxidants and antioxidants, leading to accumulation of oxidatively damaged molecules and ultimately oxidative stress. Pathways leading to an increase in reactive species within the liver include mitochondrial dysfunction, endoplasmic reticulum stress, iron overload as well as overexpression of enzymes, for example the phase 1 enzyme cytochrome P_{450} 2E1 (CYP2E1) [30].

Increased redox biomarkers, especially derived from lipid peroxidation, have been reported in blood samples and biopsies of NAFLD patients (Table 1, Fig. 1). Lipid peroxidation products can arise through distinct pathways through either free radical-mediated oxidation, enzymatic oxidation or non-enzymatic, radical independent oxidation [31]. Both, free radical and enzymatic oxidation of linoleic acid leads to oxidized fatty acids. Non-enzymatic oxidation predominantly generates 9- and 13- hydroxyoctadecadienoic acid (HODE). While enzymatic oxidation stereo-selectively generates either R- or S-stereoisomers and generally favors the production of 13-HODE, free radical-induced oxidation results in the formation of racemic mixtures of R- and S-stereoisomers and of both 9-HODE and 13-HODE equally [11]. Consistent with an increase in oxidative stress, both 9- and 13-HODE are increased in the plasma of NASH patients, but not of patients with steatosis. Furthermore similar amounts of the R- and S-HODE are present in NASH providing further evidence of free radical involvement [11].

Multiple alkenals are formed during lipid peroxidation of polyunsaturated fatty acids, including 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) [32]. 4-HNE can modify proteins at cysteine, lysine or histidine residues, forming stable protein adducts [33,34], whereas MDA triggers DNA damage [35]. In the liver of NAFLD patients, 4-HNE protein adducts [10,36,37] and conjugated dienes [38], another marker of lipid peroxidation are elevated. Several studies reported increased MDA concentrations in the plasma of NAFLD patients [38–41] and, although considered a rather unspecific marker for MDA [42], thiobarbituric acid reactive substances (TBARS) were also increased in liver biopsies obtained from patients with steatosis or NASH [43]. Interestingly, the same study also measured circulating TBARS and found no difference between controls and patients with either steatosis or NASH. This highlights the limited ability of circulating biomarkers to appropriately capture the organ specific redox state.

Carbonyl groups are introduced into proteins either by direct oxidation of amino acids, oxidative protein cleavage or through



Hepatic biomarker		
Protein carbonyls	↑↑	↑
8-Hydroxydeoxyguanosine	-	↑
4-Hydroxynonenal protein adducts	↑	↑
Thiobarbituric acid reactive substance	↑	↑
Cholesteryl ester hydroperoxides	↑	
Total glutathione	↓	↓
Superoxide dismutase activity	↓	↓
Catalase activity	↑	↓↓

Circulating biomarkers		
9- and 13-Hydroxyoctadecadienoic acid	-	↑
Malondialdehyde	↑	↑
Protein carbonyls	↑	↑
Advanced glycation end products	↑	↑↑
Conjugated dienes	↑	
Reduced glutathione	↑	↑
Superoxide dismutase activity	↑↓	↑
Catalase activity	↓	↓
Glutathione peroxides activity	↑	↑
Glutathione reductase activity	↑	↑
α-Tocopheryl quinone	↑	
8-Hydroxydeoxyguanosine	↑	↑

Fig. 1. Non-alcoholic fatty liver disease (NAFLD) comprises a spectrum of liver diseases ranging from steatosis to steatohepatitis (NASH) with and without fibrosis, cirrhosis and eventually hepatocellular carcinoma. Although redox biomarkers have not been evaluated in all stages, the current literature supports an increase of reactive species in steatosis and NASH in the circulation as well as in liver biopsies of patients.

Table 1
Biomarkers of oxidative stress in patients with NAFLD compared to controls.

Redox biomarker	Effect	Fold change of mean compared to controls	Number of patients	Reference
Plasma/Serum				
Malondialdehyde	↑	1.7	50	[38]
	↑	NAFL:1.9 NASH: 2.0[a]	175	[39]
	↑	2.4	34	[40]
	↑	1.4	81	[41]
4-Hydroxynonenal	↑	2.4[a]	23	[55]
Thiobarbituric acid reactive substance	–	–	53	[43]
Conjugated dienes	↑	1.5	50	[38]
Advanced glycation end products	↑	NAFL: 1.7 NASH: 2.5	175	[39]
Protein carbonyls	↑	3.8[a]	58	[45]
9- and 13- Hydroxyoctadecadienoic acid	↑	NAFL: 1.0; 0.9 NASH: 1.3; 2.0[a]	73	[11]
Superoxide dismutase activity	↑	NAFL: 1.6 NASH: 1.4[a]	174	[39]
	↑	1.2	50	[38]
	↓	0.8	34	[40]
	↓	0.9	81	[41]
Catalase activity	↓	NAFL: 0.4 NASH: 0.4[a]	174	[39]
	↓	0.8	50	[38]
	↓	0.7	81	[41]
Glutathione reductase	↑	NAFL: 1.1 NASH: 1.3[a]	174	[39]
	↑	1.1	50	[38]
	–	–	34	[40]
Glutathione peroxidase activity	↑	NAFL: 1.2 NASH: 1.1[a]	174	[39]
	↑	1.4	50	[38]
	–	–	34	[40]
Reduced glutathione	↑	NAFL: 1.7 NASH: 2[a]	174	[39]
	↓	0.5	50	[38]
	↑	1.2	34	[40]
α-Tocopheryl quinone	↑	2[a]	23	[55]
8-Hydroxydeoxyguanosine	↑	NAFL: 1.3 NASH: 1.5[a]	174	[39]
Liver				
Total glutathione	↓	NAFL: 0.4 NASH: 0.7[a]	43	[46]
Thiobarbituric acid reactive substance	↑	NAFL: 1.5 NASH: 1.8[a]	53	[43]
Superoxide dismutase activity	↓	NAFL: 0.8 NASH: 0.5[a]	43	[46]
Glutathione peroxidase	–	–	43	[46]
Catalase activity	↓	NAFL:0.5 NASH: 0.4[a]	43	[46]
	↓	NAFL: 0.8 NASH: 0.5[a]	53	[43]
Protein carbonyls	↑	NAFL: 3.3 NASH:1.3[a]	43	[46]
4-Hydroxynonenal protein adducts	↑	7.0	26	[36]
	↑	2.5[a]	22	[37]
	↑	NAFL: 18 NASH: 17	47	[10]
	↑	6.6	18	[69]
Cholesteryl ester hydroperoxides (CEOOH)	↑	NAFL: 1.4 NASH:1.8	22	[48]
8-Hydroxydeoxyguanosine	↑	NAFL: 2 NASH:11	47	[10]
	↑	NAFL:1.2 NASH: 1.5[a]	53	[43]

NAFL Non-alcoholic fatty liver (NAFL), Nonalcoholic Steatohepatitis (NASH).
[a] Graphically estimated.

secondary reaction with lipid peroxidation alkenals, such as 4-HNE [44]. Oxidative modification of proteins by reactive species can thus also be quantified as protein carbonyls. Protein carbonyls were reported to be increased in the plasma as well as in liver samples of patients with steatosis [45,46]. Compared to patients with steatosis, hepatic protein carbonyls are decreased in NASH [46]. Advanced glycation end products (AGE), modifications of proteins by sugar molecules, can also be formed as a consequence of oxidative stress [47]. Compared to healthy controls, patients with steatosis had increased AGE plasma concentrations and AGE levels were even higher in patients with NASH [39]. In addition to proteins, other macromolecules, such as cholesterol esters and DNA, can be modified by reactive species. Hepatic oxidation products of cholesteryl esters were increased in NAFLD patients [48]. Hepatic 8-hydroxydeoxyguanosine, a marker of oxidatively modified DNA, was primarily found as a consequence of NASH and to a lesser extent of steatosis [10,43]. In the plasma, on the other hand, increases in 8-hydroxydeoxyguanosine were already observed in patients with steatosis [39].

The antioxidant defense system in humans evolved to counteract the potentially harmful effects of reactive species. The enzyme superoxide dismutase (SOD) prevents oxidative damage from the superoxide radical (O_2^-) by converting it into hydrogen peroxide (H_2O_2), which then is detoxified by either catalase (CAT) or glutathione peroxidase (GPx). The glutathione peroxidase reaction requires the antioxidant tripeptide glutathione (GSH) as a cofactor, which is oxidized to glutathione disulfide (GSSG) in the process. GSSG can then be regenerated to GSH by glutathione reductase (GR).

The direction and extent of changes in the activities of antioxidant enzymes in human NAFLD patients is largely inconclusive. Two clinical trials reported an increase in SOD activity in plasma from 224 NAFLD patients [38,39], and decreased SOD activity has been reported in one smaller trial with 34 NAFLD patients [40] (Table 1). In the liver, reduced SOD activity was observed in one trial with 43 subjects with NAFLD [46].

The activity of catalase, on the other hand, was consistently reduced in plasma [38,39] and in the liver [43,46] in all reports of human trials with NAFLD patients. The activities of GPx, the enzyme converting H_2O_2 to H_2O, and GR, the enzyme regenerating the cofactor GSH from its oxidized form GSSG, are either increased [38,39] or unchanged in plasma [39] of NAFLD patients. The antioxidant peptide GSH has been reported to be increased [39,40] or decreased [38] in plasma of patients with NAFLD (Table 1). The concentration of total glutathione, the sum of GSH and GSSG, is decreased in the liver of NAFLD patients compared to healthy controls [46].

Although plasma concentrations of αT are lower in patients than in

healthy subjects [49], NAFLD does not appear to be a disease of hepatic deficiency of the antioxidant vitamin E, but rather one of sequestration of vitamin E in lipid droplets [50,51]. At least in animal models, this sequestration also decreases the formation of αT long-chain metabolites [50]. The consequence of the inhibition of metabolites, especially αT-13-COOH with its potent anti-inflammatory function [52–54], remains to be elucidated. The radical-mediated formation of the αT metabolite α-tocopheryl quinone is increased in the serum of NAFLD patients [55], giving further evidence for the antioxidant function of αT [50].

In summary, the above changes in the concentrations of markers of lipid, protein, and DNA oxidation and in the activities of antioxidant enzymes and endogenous antioxidants point towards increased oxidative stress in patients presenting with NAFLD. The plasma concentrations of these biomarkers may be influenced by other organs and may thus be representative of the systemic redox status, rather than that of the liver alone, and may explain the inconsistent and sometimes conflicting results in this compartment (Table 1). The available data from liver biopsies, on the other hand, paint a clearer picture, with all lipid, protein, and DNA oxidation biomarkers being increased and all antioxidant enzyme activities and total glutathione concentrations being decreased in NAFLD.

3. Vitamin E and hepatic redox biomarkers in animal models of NAFLD

The limited availability of liver biopsy samples from NAFLD patients and the fact that ethical considerations often prevent the sampling of liver biopsies from healthy (control) subjects result in the small number of human trials reporting redox biomarker data from humans. Hence, animal models of NAFLD and NASH are essential tools in understanding the molecular details of the redox balance in the etiology of these diseases.

Animal models for NAFLD and NASH use either genetic models, high-fat diet-induced lipid accumulation or the induction of liver injury by a methionine- and choline-deficient (MCD) diet (Table 2). In mice with MCD-induced liver injury, αT supplementation attenuated the MCD-induced increase in the formation of hepatic TBARS [56,57], the reduction in hepatic total glutathione [56] and SOD activity [58]. A similar decrease in diet-induced TBARS by αT was also observed in mice fed a high-fat and high-cholesterol diet [59].

Phosphatidylethanolamine N-methyltransferase (PEMT) is a hepatic enzyme that converts phosphatidylethanolamine to phosphatidylcholine and is important for the maintenance of VLDL secretion. In mice lacking PEMT, steatosis develops due to impaired VLDL secretion [60]. Feeding PEMT-deficient mice a high-fat diet also significantly increased TBARS and the ratio of reduced to oxidized glutathione (GSH/GSSG), both indicating increased oxidative insult. αT supplementation in this model attenuated both the rise in TBARS as well as the increase in the GSH/GSSG ratio [61] (Table 2).

The more specific measurement of hepatic concentrations of MDA by high-performance liquid chromatography largely confirms the findings reported for the less specific lipid peroxidation biomarker TBARS. Rats fed a high-fat diet for 4 weeks had lower concentrations of MDA in the liver when simultaneously given αT [62]. Pretreatment of ob/ob mice, a genetic model of obesity, with either α- or γ-tocopherol (500 mg/kg diet) for 5 weeks protected the livers from LPS-induced increases in MDA concentrations [63]. There are, however, two studies that did not find a reduction of hepatic MDA upon αT treatment. One study used the MCD diet-fed mouse model [58] and another trial used guinea pigs fed a high-fat diet [64]. While hepatic MDA was increased in the MCD model [58], and could thus theoretically have been reduced by αT, MDA was not increased in the control group of the high-fat diet guinea pig trial, thus preventing the detection of any potential effects of αT [64]. The lack of increase in MDA in the high-fat diet control compared to the normal-fat control group in the latter study might be explained by the high concentrations of vitamin C present in the diet [64].

4. Vitamin E and hepatic redox biomarkers in patients with NAFLD

To the best of our knowledge, there are currently only two published studies that investigated the effects of αT supplementation on redox biomarkers in humans. One study investigated the combination therapy with daily doses of 7.5 mg hydroxytyrosol and 10 mg all-rac-αT or placebo for 4 months in pediatric patients with biopsy-confirmed NAFLD (n = 80) [65]. Both the placebo as well as the treatment group showed an increase in reduced and oxidized glutathione in the serum, which is

Table 2
The effects of vitamin E supplementation in animal models on hepatic redox biomarkers in NAFLD and in serum and hepatic samples in clinical trials.

Redox biomarker	Effect	Model	Dose and duration	Reference
Animal Experiments				
Thiobarbituric acid reactive substances	↓	Mice, MCD diet[a]	250 mg/kg diet all-rac-α-tocopherol acetate, 5 weeks	[56]
	↓	Mice, MCD diet + partial hepatectomy	5000 mg/kg diet all-rac-α tocopherol, 1 week	[57]
	↓	PEMT$^{-/-}$ mice, high-fat diet	500 mg/kg diet vitamin E[b], 3 weeks	[61]
	↓	Mice, high-fat, cholesterol, and cholate diet	0.02% vitamin E[b] in the diet, 12 weeks	[59]
Total glutathione	↑	Mice, MCD diet	250 mg/kg diet all-rac-α-tocopherol acetate, 5 weeks	[56]
Malondialdehyde	–	Guinea pigs, high-fat diet	250 mg/kg diet RRR-α-tocopherol, 6 weeks	[64]
	–	Mice, MCD diet	100 mg/kg diet vitamin E[b], 3 weeks	[58]
	↓	ob/ob mice * LPS injection	500 mg/kg diet α-tocopherol or γ-tocopherol, 5 weeks	[63]
	↓	Rats, high-fat diet	30 IU/d vitamin E[b], gavage, 4 weeks	[62]
Superoxide dismutase activity	↑	Mice, MCD diet	100 mg/kg diet vitamin E[b], 3 weeks	[58]
GSH/GSSG	–	Guinea pigs, high-fat diet	250 mg/kg diet RRR-α-tocopherol, 6 weeks	[64]
	↑	PEMT−/− mice, high-fat diet	500 mg/kg diet vitamin E[b], 3 weeks	[61]
Clinical Trials				
Protein carbonyls[c]	↓	80 pediatric patients with biopsy proven NAFLD	7.5 mg hydroxytyrosol and 10 mg all-rac α-tocopherol acetate per day, 4 months	[65]
Advanced glycation endproducts[c]	↓			
Advanced oxidation protein products[c]	–			
4-HNE protein adducts[d]	↓	21 patients with biopsy proven NAFLD	200, 400 and 800 IU/d RRR-α-tocopherol per day, 4 weeks	[36]

[a] MCD diet, methionine- and choline-deficient diet.
[b] no further details provided.
[c] Serum.
[d] Hepatic samples.

likely the consequence of effective lifestyle and dietary advice. However, the serum redox markers AGE and protein carbonyls were only significantly reduced in the treatment group. Advanced oxidation protein products, are another measure of oxidized proteins [66], decreased but did not reach statistical significance. Although αT was investigated in combination with hydroxytyrosol, which also has antioxidant properties [67], the data overall points to a decrease in circulating redox biomarkers, although whether changes also occurred in the liver remains unknown. In the only study, that investigated hepatic redox biomarkers, patients with biopsy-proven NAFLD (n = 21) were randomly assigned to receive either 200, 400 or 800 I.U. RRR-αT per day for four weeks [36]. Prior to supplementation and after the four-week period, liver biopsies were obtained and the amounts of 4-hydroxynonenal protein adducts quantified immunohistochemically. When investigating all three doses combined, αT supplementation for four weeks significantly reduced concentrations of 4-HNE protein adducts. Even though there were no significant differences in the observed effects between the three doses tested (200, 400, 800 I.U. per day), the two higher doses reduced 4-HNE formation by ca. 60%, whereas the 200 I.U./d dose only reduced 4-HNE by ca. 20% relative to the baseline values [36], thus providing preliminary evidence that the highest benefit is seen with doses of at least 400 I.U./d. These preliminary findings warrant further investigation and confirmation by larger clinical trials.

5. Conclusion

The available evidence supports that oxidative stress, an imbalance between the generation of reactive species and antioxidant defense, plays an important role in the pathological process of NAFLD. Novel experimental approaches and methods are needed to evaluate the success of treatments that target reactive species and redox imbalances. The immunohistochemical detection of 4-HNE protein adducts has been validated in patients with NAFLD [36] and was successfully employed to demonstrate the antioxidant activity of αT in the livers of these patients. Using 4-HNE protein adducts, however, requires multiple biopsies, which may not be feasible in most human studies.

While concentrations of redox biomarkers in blood are easy to obtain, have been shown to be elevated in NAFLD (Table 1), and respond to antioxidant supplementation (Table 2), circulating redox biomarkers do not necessarily reflect the redox status of the liver, but may be better biomarkers of systemic redox balance. The use of redox-sensitive contrast agents coupled with magnetic resonance imaging is a minimally invasive diagnostic method and could be a promising novel tool to investigate the redox status of the liver [68]. The current evidence, both from animal models and NAFLD patients, suggests that αT supplementation is capable of decreasing the hepatic concentrations of biomarkers of lipid peroxidation and increasing the activity and concentrations of endogenous antioxidants in the liver (Table 2). Vitamin E supplementation is thus a valuable tool for the development and validation of novel methods for the detection and quantification of hepatic oxidative stress in general and lipid peroxidation biomarkers in particular.

Declaration of competing interest

The authors have no known conflict of interest.

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