Squalene epoxidase as a novel therapeutic target in non-alcoholic fatty liver disease: abridged secondary publication

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KEY MESSAGES

1. Squalene epoxidase (SQLE), a key enzyme in cholesterol biosynthesis, is upregulated in non-alcoholic fatty liver disease (NAFLD).
2. Liver-specific SQLE overexpression in mice induces spontaneous onset of steatosis and accelerates NAFLD progression in dietary models.
3. The SQLE inhibitor terbinafine suppresses NALFD development in both dietary and genetic mouse models.
4. Serum SQLE is a potential diagnostic biomarker for NAFLD.

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a leading cause of liver disease and a substantial healthcare burden worldwide. NAFLD encompasses a spectrum of liver pathologies, ranging from steatosis to non-alcoholic steatohepatitis (NASH).[1] The latter is an aggressive form of NAFLD associated with hepatocellular injury and inflammation; it can progress to fibrosis, cirrhosis, and eventually hepatocellular carcinoma.[2] Although steatosis can be reversed by lifestyle modifications, NASH often causes irreversible damage such as cirrhosis. Dysregulated hepatic metabolism can cause steatosis and progression to NASH. Thus, hepatic metabolic pathways have been targeted to suppress NAFLD progression or promote NASH resolution. Cholesterol, a major lipotoxic molecule, plays a central role in NASH pathogenesis.[3] Squalene epoxidase (SQLE) is a rate-limiting enzyme in the endogenous cholesterol biosynthesis pathway and an aetiological factor in both NAFLD and hepatocellular carcinoma.[4] We therefore hypothesise that SQLE serves important roles in the development of NAFLD and NASH.

In this study, we demonstrated that SQLE is upregulated in patients with NAFLD. Liver-specific *Sqle* overexpression in mice led to spontaneous steatosis and exacerbated high-fat, high-cholesterol (HFHC) diet–induced NASH in mice. In contrast, Sqle knockout (ko) mice exhibited less severe NAFLD and NASH. Pharmacological inhibition of SQLE via terbinafine ameliorated NASH in multiple mouse models. Moreover, serum SQLE could serve as a novel biomarker for diagnoses of NAFLD and NASH.

Methods

The role of SQLE in NAFLD development was examined using hepatocyte-specific Sqle transgenic (tg) mice. Mice were fed various diets to induce NAFLD including an HFHC diet and a methionine- and choline-deficient diet. To target SQLE, we repurposed terbinafine, a Food and Drug Administration–approved SQLE inhibitor, for NAFLD treatment in multiple mouse models. Serum SQLE levels were compared between a cohort of patients with NAFLD and a cohort of healthy controls to assess its diagnostic performance on NALFD and NASH.

Results

Hepatocyte-specific *Sqle* overexpression in mice accelerates diet-induced NASH

Sqle tg mice and wildtype mice were fed an HFHC diet for 28 weeks to induce NASH. The HFHC diet increased both body and liver weight in wildtype mice; these effects were exacerbated in *Sqle* tg mice (Fig 1a). Liver and serum cholesterol and triglyceride levels were significantly increased in HFHC diet–fed *Sqle* tg mice (Fig 1b and 1c). Insulin and glucose tolerance tests revealed marked insulin resistance in HFHC diet–fed *Sqle* tg mice compared with wildtype mice (Fig 1c). Serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) (Fig 1d) were significantly increased, along with levels of liver thiobarbituric acid reactive substances (TBARS), indicating liver damage and oxidative stress in HFHC diet–fed *Sqle* tg mice. Haematoxylin and eosin staining showed that HFHC diet–fed *Sqle* tg mice developed steatohepatitis with increased

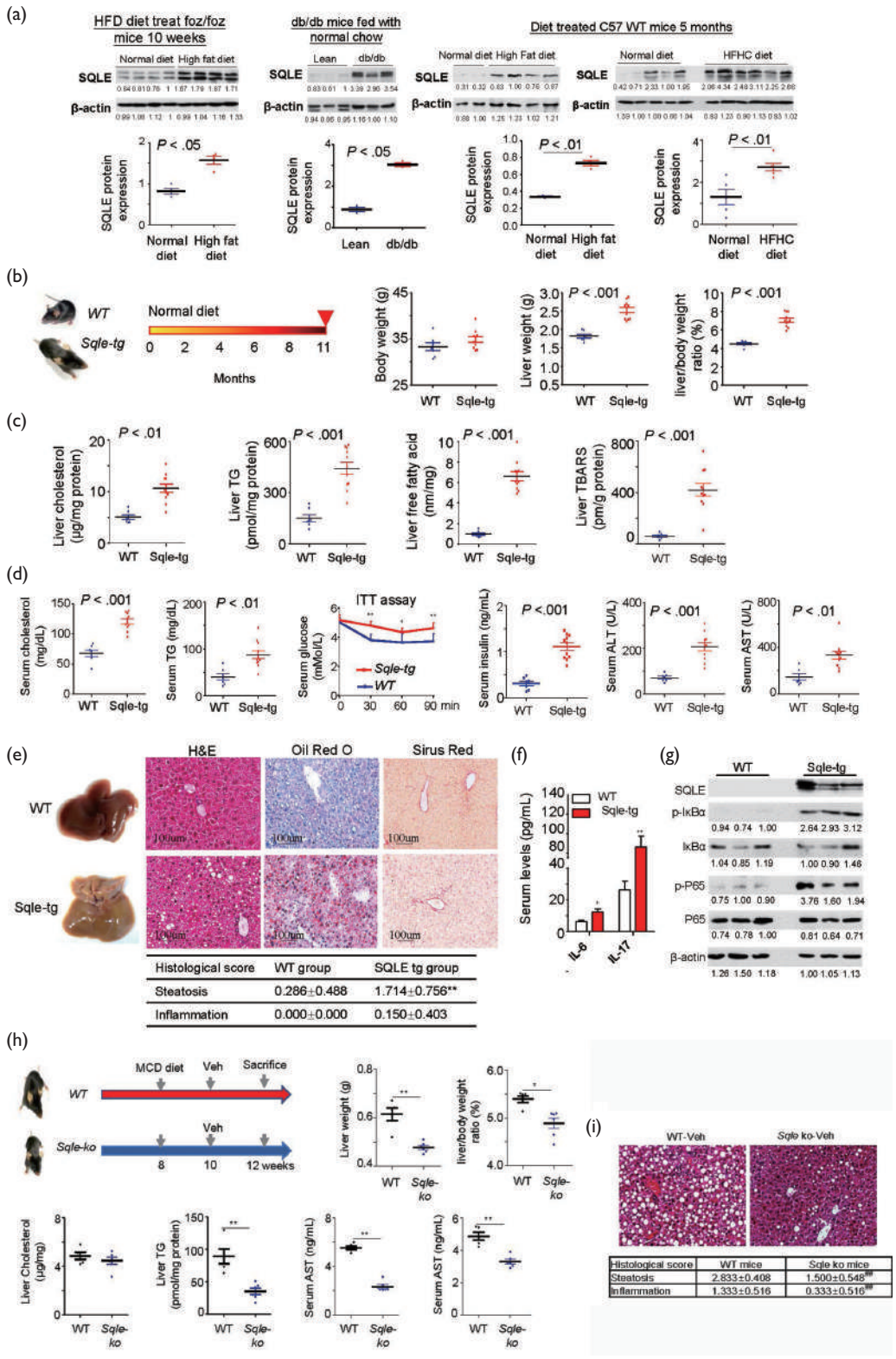


FIG 1. Diet-induced overexpression of liver squalene epoxidase (Sqle) in mice triggers spontaneous non-alcoholic fatty liver disease (NAFLD).
(a) Liver SQLE expression is upregulated in both dietary and genetic mouse models of non-alcoholic steatohepatitis (NASH). Hepatocyte-specific *Sqle* overexpression in mice induces spontaneous liver steatosis. Comparing standard chow–fed hepatocyte-specific *Sqle* tg and wildtype mice, (b) *Sqle* overexpression increases liver weight and liver/body weight ratio, without affecting body weight. (c) *Sqle* tg mice exhibit higher levels of liver cholesterol, triglycerides, free fatty acids, and thiobarbituric acid reactive substances (TBARS). (d) *Sqle* tg mice exhibit significantly elevated serum levels of cholesterol, triglycerides, alanine transaminase (ALT), and aspartate transaminase (AST), as well as enhanced insulin resistance. (e) Histological analyses, including haematoxylin and eosin (H&E) staining, Oil Red O staining, and Sirius red staining, reveal significantly increased steatosis and inflammation scores in livers of *Sqle* tg mice, compared with wildtype mice. (f) *Sqle* tg mice exhibit significantly elevated serum levels of IL-6 and IL-17, compared with wildtype mice. (g) Western blot analysis shows increased hepatic expression of p-P65 and p-IκBα in *Sqle* tg mice. In hepatocyte-specific *Sqle* ko mice fed a methionine- and choline-deficient diet, (h) *Sqle* ko mice show decreases in liver weight, liver/body weight ratio, liver cholesterol and triglyceride levels, and serum levels of ALT and AST compared with wildtype mice. (i) Haematoxylin and eosin staining demonstrates lower steatosis and inflammation scores in *Sqle* ko mice.

steatosis (P<0.01) and inflammation (P<0.01) scores, compared with HFHC diet–fed wildtype mice. This observation was confirmed by hepatic lipid accumulation and the presence of fibrosis according to Sirius red staining (Fig 1e). Consistent with a role for Sqle in NASH induction, serum cytokine and chemokine assays revealed elevated levels of pro-inflammatory cytokines (eg, IL-1β, IL-6, MCP-1, MIP-1β, and TNF-α) in *Sqle* tg mice (Fig 1f). NF-κB pathway activation was observed in *Sqle* tg mice, as evidenced by increased levels of p-Iκbα and p-p65 (Fig 1g). These findings suggest that Sqle accelerates NASH progression in the context of an HFHC diet.

Hepatocyte-specific *Sqle* knockout in mice inhibits diet-induced NASH

We constructed hepatocyte-specific *Sqle* ko mice and then fed both *Sqle* ko mice and their wildtype littermates a methionine- and choline-deficient diet for 8 weeks. *Sqle* ko mice displayed decreases in body weight, liver/body weight ratio, liver cholesterol and triglyceride levels, and serum levels of ALT and AST (Fig 1h). *Sqle* ko mice fed a methionine- and choline-deficient diet also developed less severe steatohepatitis, as evidenced by decreased steatosis (P<0.01) and inflammation (P<0.01) scores (Fig 1i). Taken together, these results suggest that the absence of Sqle attenuates diet-induced NASH.

Pharmacological inhibition of Sqle ameliorated NASH in Sqle tg mice

Terbinafine is an SQLE inhibitor for treatment of fungal infections. To evaluate the utility of SQLE as a therapeutic target for NAFLD/NASH, we administered terbinafine (80 mg/kg/d) to HFHC diet–fed *Sqle* tg mice at 26 weeks after HFHC diet initiation until week 34. Terbinafine reversed the *Sqle* tg-associated increase in liver/body weight ratio (Fig 2a). Terbinafine also abolished *Sqle* tg-induced accumulation of liver cholesterol, triglycerides, and free fatty acids (Fig 2b), as well as serum levels of cholesterol and triglycerides (Fig 2c). Furthermore, terbinafine normalised insulin sensitivity, as evidenced by insulin tolerance test results and serum insulin levels. In addition to the alleviation of steatosis, terbinafine lowered serum levels of ALT and AST (Fig 2c), as well as levels of liver TBARS (Fig 2b), indicating the reversal of Sqle-induced liver damage and oxidative stress. Histological evaluation confirmed that terbinafine significantly attenuated the severities of steatohepatitis, lipid accumulation, and fibrosis (Fig 2d). Consistent with these mitigating effects on steatohepatitis, terbinafine-treated *Sqle* tg mice showed reduced levels of serum pro-inflammatory cytokines including IL-1β, IL-6, MCP-1, and TNF-α (Fig 2e), as well as suppressed NF-κB activation (p-Iκbα and p-p65) [Fig 2f]. No obvious toxicity was observed during terbinafine treatment in mice. These results collectively suggest that SQLE targeting can be a treatment modality for NASH.

Pharmacological inhibition of SQLE ameliorated NASH development in wildtype mice

We evaluated the efficacy of terbinafine (80 mg/kg) in mitigating HFHC diet–induced NASH in wildtype mice. Treatment was initiated at 26 weeks after HFHC diet initiation and continued until week 32. Terbinafine significantly reduced liver weight and liver/body weight ratio (Fig 3a). It also abolished HFHC diet–induced accumulation of liver triglycerides and free fatty acids (Fig 3b) and lowered serum triglyceride levels (Fig 3c), while improving insulin and glucose tolerance. Moreover, better control of liver damage was confirmed by decreases in liver TBARS levels (Fig 3b) and levels of serum ALT and AST (Fig 3c). Histological evaluation confirmed that terbinafine attenuated steatohepatitis, lipid accumulation, and liver fibrosis, as evidenced by lower steatosis and inflammation scores (Fig 3d). Consistent with these findings, terbinafine suppressed serum pro-inflammatory cytokines (Fig 3e), indicating its effectiveness in reducing HFHC diet–induced liver inflammation. Overall, SQLE targeting confers therapeutic benefits in HFHC diet–induced NASH.

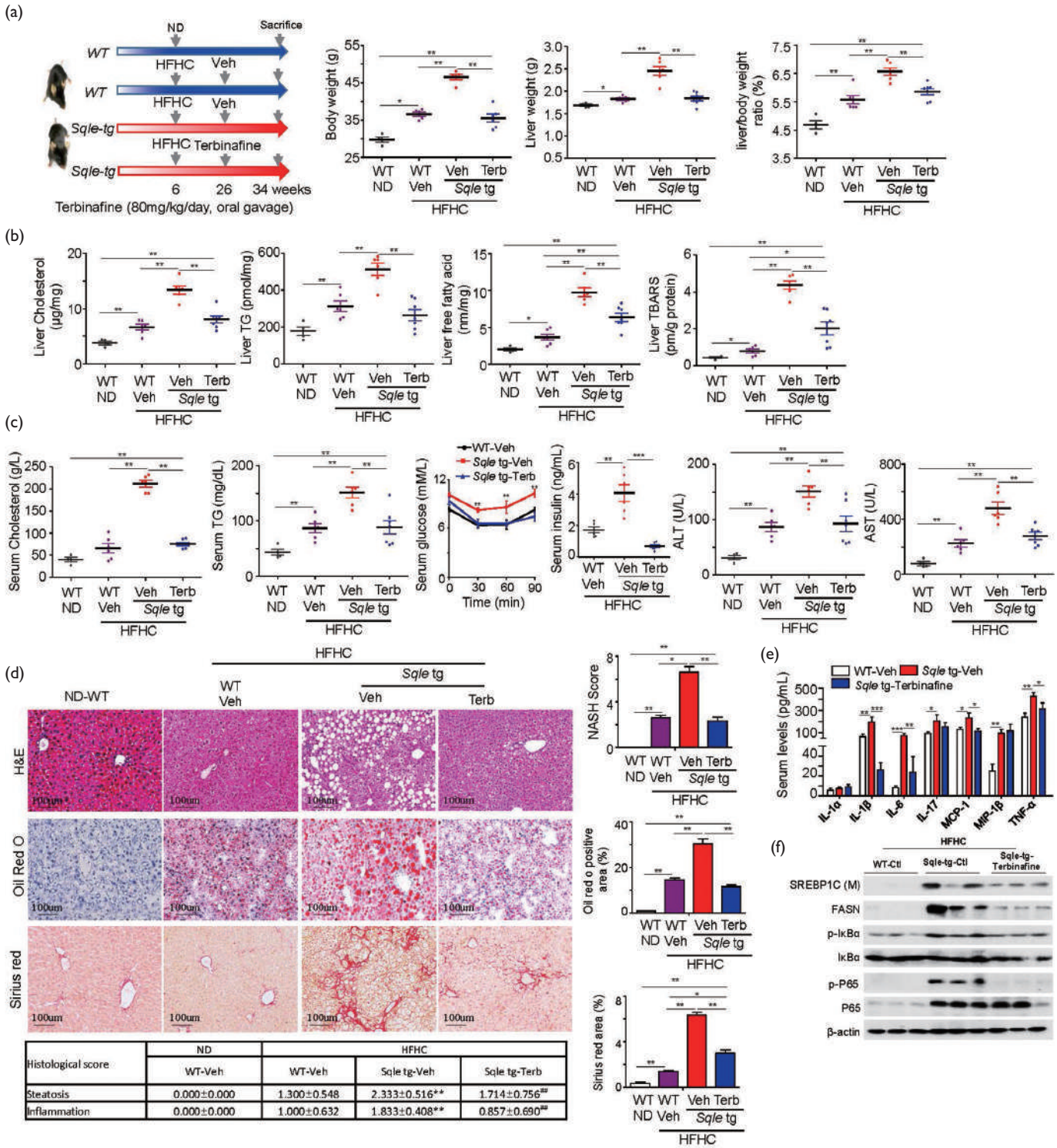
SQLE is a potential diagnostic biomarker for NAFLD and NASH

We measured serum SQLE levels in 829 individuals across four patient cohorts. The area under the receiver operating characteristic curve was 0.602 for distinguishing patients with NAFLD from healthy controls, 0.628 for distinguishing patients with NASH from healthy controls, and 0.611 for distinguishing patients with NASH from patients with steatosis (Fig 3f). Thus, serum SQLE can be a diagnostic biomarker for NAFLD and NASH.

Discussion

Our findings indicate that SQLE plays a pivotal role in NAFLD development. Hepatocyte-specific *Sqle* overexpression in mice led to spontaneous steatosis and exacerbated diet-induced NASH. SQLE increases cholesterol and triglyceride levels in hepatocytes, while activating pro-inflammatory signalling. Pharmacological inhibition of SQLE ameliorated NASH in experimental mouse models, suggesting that SQLE can be a therapeutic target for NASH.

High dietary cholesterol can induce NASH in mouse models,[5] although the role of the cholesterol biosynthetic pathway in NASH pathogenesis remains unclear. In the present study, we demonstrated a causative role for SQLE in NASH using hepatocyte-specific *Sqle*-overexpressing transgenic mice (*Sqle* tg).



Histological score	ND	HFHC		
	WT-Veh	WT-Veh	Sqle tg-Veh	Sqle tg-Terb
Steatosis	0.000±0.000	1.300±0.548	2.333±0.516**	1.714±0.756##
Inflammation	0.000±0.000	1.000±0.632	1.833±0.408**	0.857±0.690##

FIG 2. Pharmacological inhibition of Sqle ameliorated NASH in mice: in HFHC-fed *Sqle* tg mice treated with terbinafine, (a) terbinafine reverses *Sqle* tg–associated increases in body weight, liver weight, and liver/body weight ratio. (b) Terbinafine abolishes hepatic *Sqle* expression–induced levels of liver cholesterol, triglycerides, free fatty acids, and thiobarbituric acid reactive substances (TBARS), as well as (c) serum levels of cholesterol, triglycerides, alanine transaminase (ALT), and aspartate transaminase (AST), along with insulin resistance. Histological analyses using haematoxylin and eosin staining, Oil Red O staining, and Sirius red staining of wildtype and *Sqle* tg mouse livers demonstrate that terbinafine significantly attenuated (d) the severities of steatohepatitis, lipid accumulation, and fibrosis, as well as (e) serum levels of IL-1βIL-6, MCP-1, and TNF-α in vehicle and terbinafine-treated livers. (f) Western blot analysis shows that terbinafine suppressed SQLE-induced protein expression of Srebp1c, Fasn, p-P65, and p-IκBα.

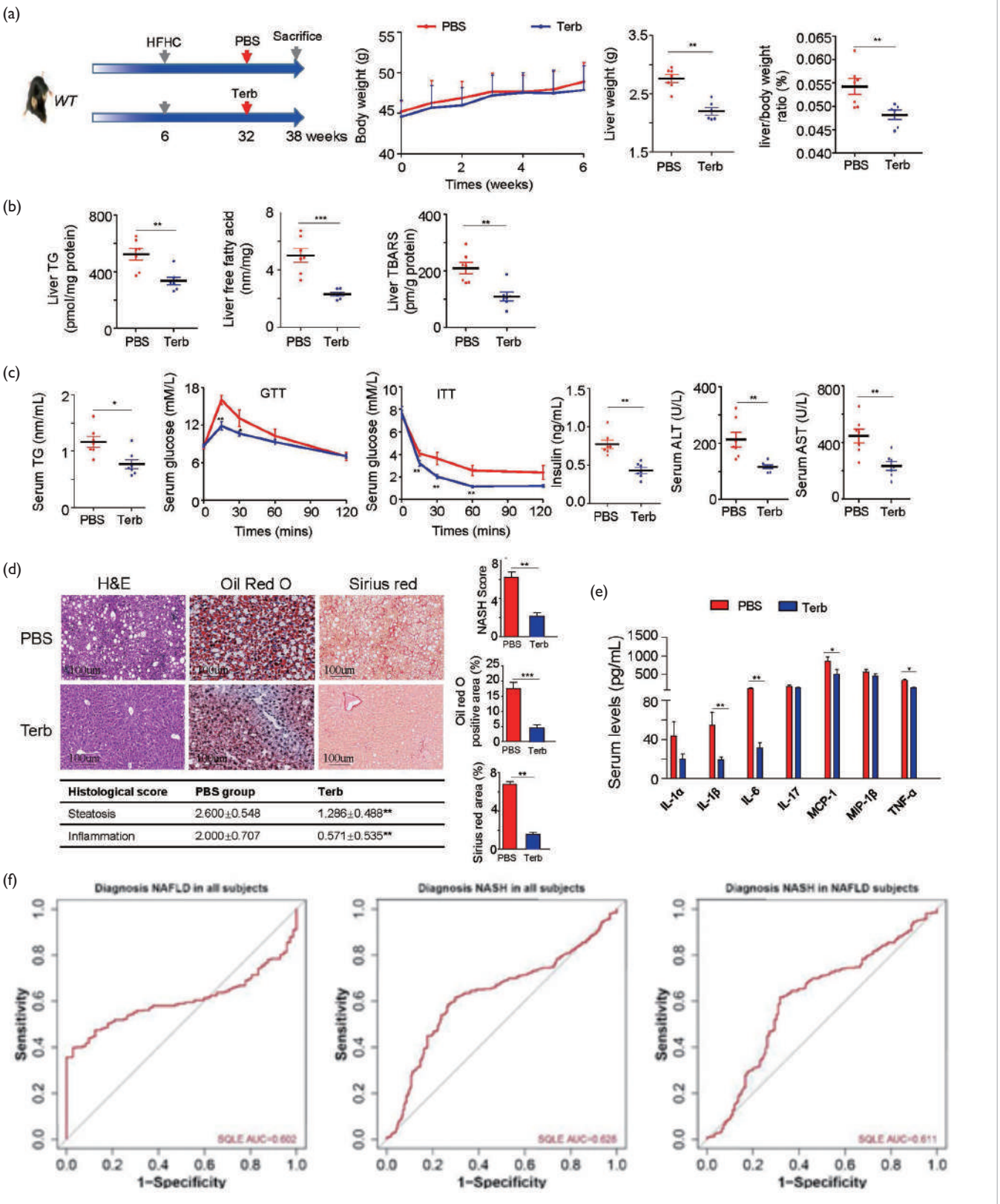


FIG 3. Pharmacological inhibition of SQLE ameliorated NASH in wild type mice. In mice with HFHC diet–induced NASH treated with terbinafine, (a) terbinafine significantly reduces liver weight and liver-to-body weight ratio. (b) Terbinafine abolishes HFHC diet–induced accumulation of liver triglycerides, free fatty acids, and thiobarbituric acid reactive substances (TBARS), as well as (c) serum triglycerides, alanine transaminase (ALT), and aspartate transaminase (AST), while improving insulin and glucose tolerance. (d) Liver histology analyses indicate that terbinafine attenuated steatohepatitis, lipid accumulation, and liver fibrosis, as well as (e) serum levels of IL-1α, IL-1β, IL-6, IL-17, MCP-1, MIP-1β, and TNF-α. (f) Diagnostic performance of serum SQLE in distinguishing patients with NAFLD from healthy controls, patients with NASH from healthy controls, and patients with NASH from patients with NAFLD.

On a standard diet, *Sqle* tg mice developed hypercholesterolaemia, hyperlipidaemia, spontaneous hepatic steatosis, and liver damage. When fed an HFHC diet, *Sqle* tg mice exhibited rapid development of NASH with increased inflammation and fibrosis, compared with wildtype mice. In contrast, *Sqle* ko mice exhibited alleviation of NAFLD and NASH development. Collectively, our data suggest that SQLE plays a functional role in the initiation of hepatic steatosis and its progression to NASH.

No drug has been approved for the treatment of NASH. In our study, we found that terbinafine ameliorated NASH in multiple mouse models. This effect is likely due to the inhibition of cholesterol and lipid biosynthesis, thereby suppressing the major causative metabolic pathways involved in NASH. No apparent toxicity was observed during terbinafine treatment. Targeting SQLE is a promising approach for the prevention and treatment of NASH.

Serum SQLE showed good diagnostic performance in distinguishing patients with NALFD or NASH from healthy controls, and patients with NASH from patients with steatosis. Thus, SQLE is a promising diagnostic biomarker for NALFD and NASH.

Conclusion

SQLE is a pivotal gene in the initiation and progression of NASH. It is involved in cholesterol biosynthesis, de novo lipogenesis, and inflammation. Targeting SQLE confers a therapeutic benefit in animal models. Serum SQLE is a diagnostic biomarker for NASH.

Funding

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Disclosure

The results of this research have been previously published in:

1. Liu D, Wong CC, Zhou Y, et al. Squalene epoxidase induces nonalcoholic steatohepatitis via binding to carbonic anhydrase III and is a therapeutic target. Gastroenterology 2021;160:2467-82.e3.
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3. Li C, Wang Y, Liu D, et al. Squalene epoxidase drives cancer cell proliferation and promotes gut dysbiosis to accelerate colorectal carcinogenesis. Gut 2022;71:2253-65.

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