**Hepatocyte-secreted Autotaxin Exacerbates Nonalcoholic Fatty Liver Disease Through Autocrine Inhibition of the PPARalpha/FGF21 axis**

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**Short title:** Inhibition of hepatic autotaxin alleviates NAFLD

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**Abbreviations**: AAV, Adeno-associated viruses; Acox-1, acyl-CoA oxidase 1; Acta2, actin alpha 2; ALT, alanine transaminase; AST, aspartate transaminase; ATX, autotaxin; BAT, brown adipose tissue; BMI, body mass index; CDAHF60, choline-deficient, L-amino acid-defined, high-fat diet (60%kcal); Colla1, alpha-1 type I collagen; Cpt1α, carnitine palmitoyltransferase 1A; ENPP2, ectonucleotide pyrophosphatase/phosphodiesterase 2; FAO, fatty acid oxidation; Fasn, fatty acid synthase; FFA, free fatty acid; FGF21, fibroblast growth factor 21; Fn1, fibronectin 1; HFD, high-fat diet; IgG, Immunoglobulin G; Il-1β, Interleukin 1 Beta; INT, iodonitrotetrazolium; KD, knock-down; Ldlr, low density lipoprotein receptor; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LysoPLD, lysophospholipase D; MDM2, mouse double minute 2; NADH, reduced nicotinamide adenine dinucleotide; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PPARα, peroxisome proliferator-activated receptor; rhATX, recombinant human autotaxin; rmATX, recombinant mouse autotaxin; Scd-1, stearoyl-CoA desaturase-1; SEM, standard error of mean; shRNA, short hairpin RNA; STC, standard chow diet; TG, triglyceride; Timp-1, TIMP Metallopeptidase Inhibitor 1; Tnfa, tumor necrosis factor alpha; Vlcad/Lcad/Mcad/Scad, very-long/long/medium/short-chain acyl coenzyme-A dehydrogenase; WAT, white adipose tissue; WT, wild type.

**Conflicts of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.
Synopsis

Serum autotaxin levels are closely associated with histological severity of NAFLD in obese individuals. Hepatocyte-secreted autotaxin exacerbates NAFLD by autocrine inhibition of PPARα, thereby leading to suppression of hepatic FGF21 production. Genetic ablation or antibody-mediated neutralization of hepatic autotaxin ameliorates hepatic steatosis, inflammation and fibrosis in mice.

Abstract

Background & Aims: The prevalence of non-alcoholic fatty liver disease (NAFLD) has reached epidemic proportions globally due to the rapid rise in obesity. However, there is no FDA-approved pharmacotherapy available for NAFLD. This study aims to investigate the role of autotaxin (ATX), a secreted enzyme that hydrolyzes lysophosphatidylcholine to produce lysophosphatidic acid (LPA), in the pathogenesis of NAFLD and to explore whether genetic or pharmacological interventions targeting autotaxin ameliorate NAFLD.

Methods: Clinical association of autotaxin with the severity of NAFLD was analyzed in 125 liver biopsy-proven NAFLD patients. C57BL/6N mice or FGF21-null mice were fed with high-fat diet or choline-deficient diet to interrogate the roles of autotaxin-FGF21 axis in NAFLD development by hepatic knockdown and antibody neutralization. Huh7 cells were used to investigate the autocrine effects of autotaxin.

Results: Serum autotaxin levels were positively associated with histological scores and severity of NAFLD. Hepatocytes but not adipocytes were the major contributor to increased circulating autotaxin in both patients and mouse models with NAFLD. In mice, knocking-down hepatic autotaxin or treatment with a neutralizing antibody against autotaxin significantly reduced high-fat diet-induced NAFLD and high fat- and choline-deficient diet-induced non-alcoholic steatohepatitis (NASH) and fibrosis, accompanied by a marked elevation of serum FGF21. Mechanistically, autotaxin inhibited the
transcriptional activity of PPARα through LPA-induced activation of ERK, thereby leading to suppression of hepatic FGF21 production. The therapeutic benefit of anti-autotaxin neutralizing antibody against NAFLD was abrogated in FGF21-null mice.

**Conclusions:** Liver-secreted autotaxin acts in an autocrine manner to exacerbate NAFLD through LPA-induced suppression of the PPARα-FGF21 axis and is a promising therapeutic target for NAFLD.

**Keywords:** Hepatokines; Nonalcoholic steatohepatitis; Fibroblast growth factor; Autocrine actions; Antibody therapeutics.
Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease that has reached epidemiological proportions worldwide due to the rapid rise in the prevalence of obesity and type-2 diabetes [1, 2]. The patho-histological spectrum of NAFLD ranges from benign steatosis to non-alcoholic steatohepatitis (NASH), the latter of which is characterized by lobular inflammation and ballooning of hepatocytes with or without perisinusoidal fibrosis. Patients with NAFLD are more susceptible to end-stage liver diseases such as cirrhosis and hepatocellular carcinoma and are at increased risk for type-2 diabetes, cardiovascular disease, cancer and overall mortality [3]. NAFLD is expected to become a major indication for liver transplantation in the coming decade [2, 4, 5]. Despite the rapid rise in the healthcare burden of NAFLD, there are currently no approved pharmacotherapies for this chronic liver disease.

Liver-secreted hepatokines and adipose tissue-secreted adipokines play important roles in controlling metabolic homeostasis, insulin sensitivity and immunometabolism in the liver [6]. Aberrations in the production and/or function of a large number of hepatokines and adipokines have been implicated in the pathogenesis of NAFLD and its related liver complications [6, 7]. A number of hepatokines and adipokines, such as fibroblast growth factor 21 (FGF21) and adiponectin, have been developed as potential therapeutic agents or non-invasive diagnostic biomarkers for NAFLD [6, 8]. Autotaxin (ATX), also known as ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), is an adipokine abundantly secreted from mature adipocytes [9, 10], although it is also expressed in many other tissues including the liver. Autotaxin possesses lysophospholipase D (lysoPLD) activity, thereby converting lysophosphatidylcholine, LPC) to lysophosphatidic acid (LPA) [11], the latter of which is a bioactive lipid that activates multiple cell signaling pathways involved in cell aggregation, proliferation, chemotaxis and inflammation via binding to its G-protein-coupled receptors [12, 13].
A growing body of studies suggests the involvement of the autotaxin-LPA axis in obesity and its related metabolic complications [14-17]. In adipose tissue, the autotaxin-LPA axis acts in an autocrine manner to impair insulin actions in white adipocytes, inhibit thermogenesis in brown adipocytes, and in a paracrine manner to promote adipogenesis of preadipocytes [9]. Adipose deletion of autotaxin has recently been shown to protect against high-fat diet (HFD)-induced obesity, insulin resistance and glucose intolerance in mice [18, 19]. However, another earlier study reported a more obese phenotype of adipose-specific autotaxin knockout mice [20]. In older humans with obesity, serum autotaxin correlates positively with adiposity and is an independent predictor of insulin resistance [21]. Aberrant serum autotaxin levels have been observed in patients with severe obesity, viral hepatitis and fibrosis [16, 22-27], and inhibition of hepatic autotaxin or adipose-derived autotaxin has been reported to alleviate the development of hepatocellular carcinoma [16] or steatosis [17]. However, the pathophysiological role of the autotaxin-LPA axis in the onset and progression of NAFLD remains to be established.

In this study, we measured the dynamic changes in circulating levels of autotaxin and its hepatic expression in several different mouse models with NAFLD, and explored the relationship of serum autotaxin with histological severity and progression of NAFLD in liver biopsy-proven patients. Furthermore, we investigated the role of liver-secreted autotaxin in NAFLD development by both knockdown of hepatic autotaxin and treatment with a neutralizing antibody against autotaxin, and delineated the pathogenic mechanism whereby aberrant autotaxin-LPA axis exacerbates NAFLD via dysregulation of FGF21.

**Results**

Serum autotaxin levels are closely associated with abnormal liver function and NAFLD severity
in obese individuals.

To investigate the clinical association of autotaxin with liver function and the severity of NAFLD, we consecutively recruited 151 obese individuals receiving bariatric surgery. The clinical, biochemical, and histological characteristics of the 151 obese subjects included in this study are shown in Table 1. The mean±SD age (years) and body mass index (BMI) (kg/m$^2$) of the participants were 30.35±1.35 and 40.23±1.24, respectively. Histological examination of liver biopsies showed that the prevalence of hepatic steatosis (at least 5% of fat within hepatocytes), ballooning, inflammation and fibrosis was 132 (87.4%), 80 (53.0%), 70 (46.3%) and 38 (25.2%), respectively. 14 (9.3%) displayed normal liver histology whilst 31 (20.5%), 62 (41.1%), and 44 (29.1%) were classified into simple steatosis, borderline NASH, and NASH respectively according to the fatty liver inhibition of progression (FLIP) algorithm [28]. Consistent with previous reports [21], serum autotaxin levels in females (508.53±21.58 ng/mL, n=85) were significantly higher than in males (417.33±22.48 ng/mL, n=66) (P=0.004, Mann-Whitney U test). Notably, serum autotaxin levels exhibited significant, positive correlations with hepatic fat content (determined by MRI), serum levels of the liver injury markers AST and ALT (Figure1A-C), severity scores of hepatic steatosis, ballooning and inflammation grade as well as histological progression of NAFLD from normal liver, benign steatosis to NASH. These significances remained even after adjustment for sex, age and BMI (Table 2). Real-time PCR analysis showed a significantly higher protein level of autotaxin in the liver tissue than in subcutaneous and visceral adipose tissues of obese individuals (Figure 1D). Furthermore, there was a strong positive association between serum autotaxin levels and the autotaxin mRNA expression in the liver, but not in adipose tissues (Figure 1E). Taken together, these findings suggest that elevated serum autotaxin is closely associated with the development of liver dysfunction and NAFLD progression in obese individuals and the liver is perhaps an important contributor to increased circulating autotaxin.
Liver but not adipose tissue contributes to increased circulating autotaxin in mouse models with NAFLD.

To confirm our findings in the above clinical study, we monitored the dynamic changes of the autotaxin mRNA expression in major organs, serum autotaxin levels as well as its lysophospholipase D (lysoPLD) activity in HFD-induced obese mice with NAFLD and lean controls for a period of 20 weeks. Serum autotaxin levels and its lysoPLD activity exhibited progressive elevation with the progression of diet-induced obesity (Figure 2A and B) but remained little changed in STC-fed lean mice. At 20 weeks following HFD start, serum autotaxin levels and lysoPLD activity in obese mice were 1.65 and 2 folds higher than in lean mice, respectively (Figure 2A and B). In line with previous reports showing autotaxin as an adipokine [9, 18, 29], real-time PCR analysis detected highly enriched mRNA expression of autotaxin in several adipose depots, including epididymal white adipose tissue (eWAT) and interscapular brown adipose tissue (iBAT) (Figure 2C). However, the autotaxin mRNA expression in these two adipose depots were paradoxically decreased in HFD-induced obese mice compared to that in lean mice, although there was a modest elevation of autotaxin gene expression in subcutaneous WAT of obese mice. In contrast, the autotaxin mRNA expression level in the liver of HFD-induced obese mice was significantly increased by almost 2 folds, whereas its mRNA abundance in other tissues was comparable between obese and lean mice, except for lymph node where autotaxin mRNA expression in obese mice was lower than in lean mice. Further cellular fractionation analysis demonstrated that the autotaxin gene expression in hepatocytes was more than 5-fold higher than in non-parenchymal cells (NPCs), whilst obesity-induced upregulation of the autotaxin gene expression occurred predominantly in hepatocytes, but not in NPCs (Figure 2D).

To further dissect the contribution of adipose tissue and liver to circulating autotaxin in mice with
NAFLD, we next utilized adipocyte-specific murine double minute 2 knockout (Adipo-MDM2-KO) mice which displayed complete loss of adipose tissues, severe hyperglycemia, dyslipidemia, and massive fatty liver [30, 31]. Surprisingly, the autotaxin mRNA expression in the liver of the lipodystrophic mice was more than 3-fold higher than in wild-type littermates, accompanied by a significant elevation of serum autotaxin (Figure 2E and F). On the other hand, the autotaxin gene expression level in other tissues was comparable between these two genotypes of mice (Figure 2F). Taken together, these findings suggest that hepatocytes, but not adipose tissues, are the major production site for increased circulating autotaxin in both dietary obese mice and lipodystrophic mice with NAFLD.

**Knock-down of hepatic autotaxin expression alleviates NAFLD by increasing fatty acid oxidation in obese mice.**

Conventional autotaxin KO mice are embryonic lethal due to the pivotal role of autotaxin in angiogenesis and development [32]. To investigate the role of liver-secreted autotaxin in obesity-induced metabolic complications and fatty liver disease, we employed the adeno-associated virus 2/8 (AAV 2/8) delivery system to knock down hepatic autotaxin in adult mice by intravenous injection of AAV directly to the liver. Compared with the mice injected with AAV expressing scramble control (SC), the mRNA expressions of autotaxin in the liver of STC-fed lean mice and HFD-fed obese mice were significantly decreased by 40% and 45%, respectively, after injection of AAV bearing shRNA specific to the autotaxin (KD) gene for 15 weeks (Figure 3A), whereas autotaxin gene expression in adipose tissues remained unchanged (Figure 3B). Notably, HFD-induced elevations of serum autotaxin levels and lysoPLD activity were largely abrogated by AAV-mediated knockdown of hepatic autotaxin (Figure 3C and D), further confirming the liver as a major contributor to increased circulating autotaxin.
Knock-down of hepatic autotaxin did not exert obvious effect on body weight, total fat mass and food intake (Figure 3E-G) but significantly improved lipid profiles in dietary obese mice, including reductions in serum triglyceride, cholesterol, and free fatty acids (Figure 4A). Moreover, hepatic knockdown of autotaxin markedly alleviated HFD-induced elevations of liver weight (Figure 4B) and hepatic steatosis, as determined by H&E and Oil Red O staining (Figure 4C) and biochemical analysis for hepatic triglyceride contents (Figure 4D). Accordingly, elevated serum levels of ALT and AST, the two well-established markers of liver injury, were markedly decreased in obese mice with knockdown of hepatic autotaxin (Figure 4E).

Excessive hepatic lipid accumulation is often caused by an imbalance between fatty acid oxidation (FAO) and de novo lipogenesis [4]. We next examined the impact of knockdown of hepatic autotaxin on expression of genes involved in hepatic lipid metabolism and found that several FAO-related genes, including carnitine palmitoyltransferase 1A (Cpt1a), medium/long/short-chain acylcoenzyme-A dehydrogenase (Mcad/Lcad/Scad), and acyl-CoA oxidase 1 (Acox1) were greatly increased in both lean and obese mice with AAV-mediated knockdown of hepatic autotaxin (Figure 4F), whereas genes involved in fatty acid synthesis such as fatty acid synthase (Fasn) and stearoyl-CoA desaturase-1 (Scd-1) were not affected. Furthermore, increased hepatic FAO gene expression in the autotaxin knockdown mice was accompanied by significant elevation in FAO activity in the liver, as determined by NADH-dependent production of INT-formazan (Figure 4G). Taken together, these findings suggest that blocking augmented hepatic autotaxin expression is sufficient to counteract obesity related NAFLD, possibly by increasing FAO.

Treatment with anti-autotaxin neutralizing antibody potently ameliorates hepatic steatosis and
increases hepatic FGF21 in obese mice.

Given that autotaxin is a secreted enzyme with lysoPLD activity, we generated a neutralizing antibody (IgG) against autotaxin (anti-ATX) and evaluated whether neutralization of secreted autotaxin has therapeutic benefits on obesity-related NAFLD. Ex vivo incubation of serum from obese mice with anti-ATX IgG dose-dependently inhibited the endogenous lysoPLD activity with IC50 and maximum inhibitory effects at the concentrations of 0.069 uM and 0.251 uM, respectively, whereas nonimmune IgG had no such an inhibitory effect (Figure 5A). Furthermore, treatment with anti-ATX but not non-immune IgG blocked the lysoPLD activity of recombinant mouse autotaxin (rmATX) and suppressed the stimulatory effects of rmATX on phosphorylation of ERK1/2 in HepG2 hepatocytes in a concentration-dependent manner (Figure 5B and C). The circulating half-life of anti-ATX IgG in mice was approximately 5 days (Figure 5D).

Treatment of mice with anti-ATX IgG for a period of 7 weeks decreased circulating lysoPLD activity (Figure 6A) and significantly reduced HFD-induced elevations of serum triglycerides, cholesterol and FFAs (Figure 6B), although it had no significant effect on body weight, body composition and food intake (Figure 5E-G). In line with the findings in mice with knockdown of hepatic autotaxin, obese mice treated with anti-ATX IgG exhibited obvious improvements in NAFLD, as evidenced by significant reductions in liver weight (Figure 6C), hepatic steatosis (Figure 6D), triglyceride accumulation (Figure 6E) and serum levels of ALT and AST (Figure 6F). These improvements in anti-ATX IgG-treated mice were accompanied by upregulated expression of hepatic genes involved in FAO (Figure 6G) and increased FAO activity in the liver (Figure 6H).

Crosstalk between liver-secreted hepatokines and adipose-secreted adipokines play important roles in regulating hepatic lipid metabolism [6]. Therefore, we next examined the effects of anti-ATX IgG on serum levels of a panel of well-established hepatokines and adipokines involved in the
pathogenesis of NAFLD [6]. Treatment of mice with anti-ATX IgG had no obvious effects on mRNA expression levels of lipocalin-2, Fgf15, Angptl3/4/6, Fetuin-a, Fgl-1, Dpp4, Fabp4, Leptin, and Apelin. (Figure 7A and B), but markedly increased serum levels of FGF21 as well as the hepatic mRNA expression of FGF21 (Figure 7C and D), a well-known hepatokine with protective effects against NAFLD [8]. Accordingly, serum level of adiponectin, a downstream effector of FGF21 with anti-steatosis activity [33], was also elevated by anti-ATX IgG (Figure 7E). Likewise, significant elevations in hepatic FGF21 expression and serum levels of FGF21 and adiponectin were also observed in mice with AAV-mediated knockdown of hepatic autotaxin expression (Figure 7F-H).

**Autotaxin inhibits hepatic FGF21 production through LPA-induced ERK activation and PPARα inhibition.**

The above findings suggest autotaxin may exacerbate NAFLD via inhibition of hepatic FGF21 production. Therefore, we next investigated the direct effects of autotaxin and its enzyme product LPA on FGF21 expression in Huh7 hepatocytes. The Fgf21 mRNA abundance and FGF21 secretion into conditioned medium were significantly decreased by treatment with recombinant human autotaxin (rhATX), but were obviously increased by incubation with PF8380 (a chemical compound that selectively inhibits the lysoPLD activity of autotaxin to produce LPA [34]) and K16425 (a pharmacological LPA receptor antagonist [35]) (Figure 7I and J), suggesting autotaxin-LPA axis acts in an autocrine manner to suppress endogenous FGF21 production in hepatocytes. Likewise, treatment of Huh7 hepatocytes with rhATX and LPA dose-dependently inhibited the PPARα agonist fenofibrate-induced Fgf21 mRNA expression and its protein secretion, whereas such inhibitory effects of endogenous autotaxin were reversed by preincubation with PF8380 and K16465 (Figure 7K and L). Autotaxin-derived LPA exerts its pleiotropic effects by activating several intracellular signaling
cascades, especially PI3K/Akt and MAPK/ERK pathways [36]. Our results showed that treatment with the selective inhibitor of MEK/ERK (U0126) markedly reversed the inhibitory effect of LPA on fenofibrate-induced FGF21 production, whereas the PI3K inhibitor (wortmannin) had no obvious effect (Figure 7O). Given that PPARα is a key regulator for FGF21 gene expression in hepatocytes [37, 38], we next investigated whether LPA suppresses FGF21 production through ERK-mediated inhibition of PPARα. In Huh7 cells transfected with the PPRE (PPAR response element) X3-TK-luc promoter plasmid [39], LPA treatment significantly decreased fenofibrate-induced transcriptional activity of PPARα as determined by the luciferase reporter analysis, whereas such a suppressive effect of LPA was largely reversed by U0126, but not wortmannin (Figure 7P). Likewise, LPA significantly reduced the expression of another well-known PPARα target gene Vlcad through ERK activation (Figure 7O). In contrast, the PPARα mRNA abundance was not affected by either LPA or U0126 (Figure 7O).

PPARα activity can be modulated posttranslationally by ERK-mediated phosphorylation [40-42], and two ERK phosphorylation sites (S12 and S21) have been identified in mouse PPARα, which are highly conserved between species [41, 42]. We next explored the effect of LPA on PPARα phosphorylation at these two sites with Western blotting. As expected, LPA induced ERK phosphorylation at Ser 21, which was blocked by U0126 (Figure 7M and N), whereas neither LPA nor U0126 had any effect on phosphorylation at Ser 12. Taken together, these findings suggest that autotaxin inhibits hepatic FGF21 expression via LPA-induced ERK activation and subsequent PPARα suppression.

FGF21 is an obligatory mediator for the therapeutic benefits of anti-ATX antibody against steatosis, NASH and fibrosis.
FGF21 analogs and agonists have been shown to be highly effective in ameliorating NAFLD in both animal models and patients [8, 43]. We next investigated whether the therapeutic effect of autotaxin neutralization on fatty liver requires hepatic upregulation of FGF21 using FGF21 knockout mice [44, 45]. Treatment with anti-ATX IgG led to a comparable degree of suppression on lysoPLD activity in HFD-fed FGF21 knockout mice and wildtype (WT) littermates (Figure 8A). However, the effects of anti-ATX IgG on improvements of serum lipid profiles, reduction of liver weight and alleviation of hepatic steatosis and liver injury were observed only in WT mice, but not in FGF21 knockout mice (Figure 8B-F). Similarly, anti-ATX IgG-induced upregulation of FAO-related genes, increases in hepatic FAO activity and in serum adiponectin levels were markedly blunted in FGF21 knockout mice (Figure 8G-I), supporting the role of FGF21 as an indispensable downstream effector conferring the hepatoprotective effects of anti-ATX IgG in obese mice.

In light of the fact that recent clinical trials have shown promising therapeutic potential of FGF21 analogs for patients with NASH and fibrosis [8, 43, 46], we next explored whether neutralization of autotaxin with anti-ATX IgG ameliorates advanced stages of NAFLD in choline-deficient, L-amino acid-defined, 60% high-fat diet (CDAHF60)-fed mice, which develops all histological features of NAFLD, including severe steatosis, inflammation and fibrosis [47, 48]. Although CDAHF60 feeding did not increase body weight and fat mass, it caused a time-dependent elevation in serum level of autotaxin and its lysoPLD activity, as well as the autotaxin mRNA expression in the liver but not in adipose tissue, as compared to age-matched STC-fed mice (Figure 9A-C, E-G). Typical histopathology of NAFLD and liver injury were readily detected at 4 weeks after feeding CDAHF60 (Figure 9D). Treatment with anti-ATX IgG led to a comparable level of reduction in circulating lysoPLD activity between FGF21 knockout mice and WT littermates (Figure 10A), and markedly increased serum FGF21 levels in WT mice but not in FGF21 knockout mice (Figure 10B). Notably, the significant
effects of anti-ATX antibody on amelioration of serum triglycerides, cholesterol and FFAs and elevation of serum adiponectin were observed only in WT mice, but not in FGF21 knockout mice (Figure 10C and D). Furthermore, anti-ATX IgG significantly decreased liver weights, attenuated steatosis inflammation, fibrosis and NASH scores (NAS) as determined by histological examination (Figure 10E-G), obviously reduced triglyceride accumulation in the liver and serum levels of ALT and AST (Figure 10H and I), improved FAO rate (Figure 10J) and inhibited the expression of several fibrosis-related genes including Acta2, Coll1a1, Timp-1, Fn1 (Figure 10K). However, all these therapeutic benefits of anti-ATX IgG on NASH and fibrosis was largely abolished in FGF21 knockout mice, indicating that anti-ATX IgG-mediated neutralization of autotaxin is effective in treating CDAHF60-induced NASH and fibrosis by induction of FGF21.

Discussion

In the present study, we provide both clinical and experimental evidence that elevated autotaxin is an important contributor to the pathogenesis of NAFLD through LPA-induced inhibition of PPARα. Furthermore, siRNA-mediated blockage of hepatic autotaxin production or treatment with an antibody which neutralizes the lysoPLD activity of autotaxin potently ameliorates fatty liver, NASH and hepatic fibrosis in two different mouse models with NAFLD, by promoting FGF21 production (Figure 11). These findings identify the autotaxin-LPA axis as an endogenous inhibitor of hepatic FGF21 expression, and a promising therapeutic target for the treatment of NAFLD.

Autotaxin-LPA axis regulates a plethora of cellular functions including proliferation and growth, development, chemotaxis, vasoregulation, calcium dynamics and energy homeostasis, by the binding of LPA to its G protein-coupled receptors (LPA1-6) [36]. Augmented autotaxin expression has been
implicated in multiple types of cancers, chronic inflammatory diseases and cardiomyopathy [49-51]. Although autotaxin was previously thought to be an adipokine abundantly expressed in adipose tissue in rodents, our present study demonstrates that liver, but not adipose tissue, is a major contributor to elevated circulating autotaxin in both humans and several types of mouse models with NAFLD. In obese subjects, autotaxin expression in liver is much higher than in visceral and subcutaneous adipose tissues. Furthermore, autotaxin expression in liver, but not in adipose tissues, correlates strongly with serum autotaxin, which is closely associated with several histopathological features as well as staging of biopsy-proven NAFLD. In a lipodystrophic mouse model without adipose tissues, circulating autotaxin remains elevated due to its markedly increased hepatic expression. Furthermore, in NAFLD mouse models induced by both dietary obesity and CDAHF60 diet, autotaxin expression is markedly elevated in liver but remains little changed in other tissues, whilst selective knockdown of hepatic autotaxin expression alone is sufficient to counteract dietary obesity and CDAHF60-induced elevation of circulating autotaxin, accompanied with obvious amelioration of NAFLD and liver injury. Taken together, these findings support the causal role of liver-secreted autotaxin in the development of NAFLD in both animals and humans. In line with our finding, a previous study found that hepatocyte-secreted autotaxin contributes to lipid accumulation and carcinogenesis in the liver in a DEN/CCL4 induced HCC mouse model [16].

FGF21, a hormone predominantly secreted from hepatocytes, possesses pleiotropic protective activities against obesity and its related metabolic complications including NAFLD [8, 46, 52, 53]. In mice, FGF21 knockout exacerbates methionine and choline deficient diet-induced hepatic steatosis, inflammation and fibrosis [44, 54], whilst treatment with recombinant FGF21 or its analogs markedly ameliorates these pathological changes in the liver of both mice and rodents [44, 54]. FGF21 has been shown to exert its hepato-protective effects through its direct actions in the liver, by promoting fatty
acid oxidation, enhancing hepatic insulin sensitivity, attenuating endoplasmic reticulum and oxidative stresses [8, 52, 53], limiting endotoxin-induced liver inflammation and injury, blocking NF-Kb [55], and mitigating fibrogenic gene expression in hepatic stellate cells [56]. Furthermore, administration of FGF21 or its analogs in both mice and humans induces the production of adiponectin, an adipokine with insulin-sensitizing, anti-inflammatory and hepato-protective properties [33]. Notably, several recent clinical trials have shown highly promising therapeutic benefits of FGF21 analogs (such as PEGylated long-acting forms of FGF21) and FGF21 receptor agonists in the treatment of biopsy-confirmed NASH patients [8, 43, 46, 57, 58]. In another clinical study with biopsy-confirmed F1-F3 NASH patients, treatment with efruxifermin (a long-acting FGF21 fusion protein with Fc fragment) lead to more than 70% reduction in liver fat contents, accompanied with resolution of histological features of NASH and fibrosis, reduction of liver injury and elevation of adiponectin (NCT05039450) [59]. A remarkable reduction in liver fat content and markers of liver fibrosis and injury has also been observed in obese adults treated with another genetically engineered FGF21 analogue LLF580 [60].

In the present study, we observed a marked elevation in both circulating levels of FGF21 and its hepatic expression in both dietary obese mice and CDAHF60-fed mice with autotaxin knockdown or antibody neutralization, whilst the effects of knocking down or neutralizing autotaxin in ameliorating steatosis, NASH and fibrosis, and in promoting hepatic fatty acid oxidation are largely abrogated in FGF21 knockout mice, suggesting that elevation of endogenous FGF21 production by pharmacological or genetic inhibition of hepatic autotaxin may represent another therapeutic approach for treatment of NAFLD by targeting FGF21.

Another notable finding of our study is that the autotaxin-LPA axis acts in an autocrine manner to suppress PPARα activity, which is a master regulator of hepatic lipid metabolism and an obligatory transcription factor for hepatic FGF21 production [37, 38, 61]. In Huh7 hepatocytes, both autotaxin
and LPA dose-dependently inhibit fenofibrate-induced PPARα activation and FGF21 production possibly through ERK1/2-induced Ser21 phosphorylation of PPARα located within the A/B domain of PPARα, and such effects are reversed by pharmacological inhibition of autotaxin or ERK. In line with our findings, ERK-induced phosphorylation and deactivation of PPARα has been implicated in cardiac hypertrophic growth and myocardial lipid accumulation [42]. However, another study reported that PPARα can be activated by ERK1/2 in response to insulin or Ps. Aeruginosa stimulation in lung epithelial cells [40, 41], suggesting that ERK regulates PPARα activity in a cellular context-dependent manner. PPARα lowers triglycerides by transactivation of a cluster of genes involved in fatty acid metabolism and ketogenesis, in which FGF21 acts as an obligatory downstream effector [37, 61-64]. Mice with global and hepatocyte-specific PPARα are more susceptible to HFD-induced hepatic steatosis and methionine/choline-deficient diet-induced NASH [65], whilst treatment with treatment with PPARα agonists ameliorates hepatic steatosis and inflammation in several different types of NAFLD mouse models [66, 67]. However, clinical trials failed to demonstrate any therapeutic benefits of either PPARα agonists or PPARα/δ agonist on liver histology in NASH patients despite of significant improvements in circulating lipid profiles (NCT02704403)[68], suggesting that PPARα activation alone is insufficient for the treatment of NAFLD. In this connection, augmented autotaxin-LPA axis has been linked to several pathophysiological pathways involved in NAFLD development, including exacerbation of pro-inflammatory responses [69], induction of insulin resistance [18, 49], apoptosis [70] and fibrosis [15, 16, 71] in different disease models. Therefore, pharmacological interventions targeting autotaxin is expected to produce additional therapeutic benefits beyond PPARα activation and FGF21 production and may represent a more efficacious strategy for treatment of NAFLD.

A large number of chemical inhibitors of autotaxin and LPA receptor antagonists have recently been developed for the treatment of different chronic diseases, such as idiopathic pulmonary fibrosis,
systemic sclerosis, neurodegenerative diseases [36]. However, several clinical trials on the inhibitors of autotaxin (such as GLPG1690) and LPAR antagonist (such as BMS-986020) were terminated due to severe adverse effects of these chemical compounds (https://clinicaltrials.gov/) [72]. Therapeutic antibodies have several advantages over small molecule drugs with respect to target specificity, safety and stability. In this connection, our study provides proof-of-concept evidence that antibody-mediated neutralization of autotaxin activity is highly efficacious in ameliorating fatty liver and NASH in two different mouse models, suggesting that the neutralizing antibodies against autotaxin may represent a promising strategy for drug development.

Methods

Study approval

Human study was approved by the Institutional Review Board of Jinan University (2016-017) and the University of Hong Kong/ Hospital Authority Hong Kong West Cluster (UW 20-700) and conducted according to Declaration of Helsinki principles. Written informed consent was received from participants prior to their inclusion in the study. Animal studies were approved by the Committee on the Use of Live Animals in Teaching & Research at the University of Hong Kong.

Clinical studies

151 obese patients were consecutively recruited at the bariatric surgery clinic, the First Affiliated Hospital of Jinan University. Overweight and obesity class were classified by body mass index (BMI) according to the World Health Organization Asian Standard. Overweight, class I and class II obesity were defined as BMI 23.0-24.9 kg/m², BMI 25-29.9 kg/m² and BMI ≥30 kg/m² respectively. 26 individuals were excluded due to: 1) Age<18 years old; 2) With Syphilis or HIV infection; 3) heavy alcohol-drinking history (≥40 g/day for up to 2 weeks); 4) Lack of sufficient information. Demographic, clinical, and laboratory data (after an overnight fasting of at least 10 hours) for each patient were
collected within one week prior to their surgery and liver biopsies were obtained during the bariatric surgery. Liver biopsies were taken from the middle of the right lobe during bariatric surgery. Thin sections of liver biopsies were stained with Hematoxylin-eosin or Masson trichrome, and were evaluated by 3 independent experienced liver pathologists (Dr. Subrata Chakrabarti, Western University, Canada; Dr. Hema Mahajan, Western Sydney University, Australia; and Dr. Sen Yan, Dr. Everett Chalmers Hospital, New Brunswick, Canada). The scores of steatosis (0-3), hepatocellular ballooning (0-2), lobular inflammation (0-3), and fibrosis (0-4) were evaluated according to the NASH Clinical Research Network (NASH CRN) scoring system [73]. For measurement of autotaxin mRNA and protein levels, biopsies of intraabdominal (visceral) and subcutaneous adipose tissues and liver obtained during bariatric surgery were immediately frozen in liquid nitrogen and stored at −80°C. Serum was isolated by centrifugation, and immediately stored at −80°C for further analysis.

Animal experiments

C57BL/6N mice were obtained from the animal unit of the University of Hong Kong. Mice were housed at a controlled temperature (23±1°C) with a 12-hour light/12-hour dark cycle and free access to drinking water and different types of diet: standard chow (catalog #5053; LabDiet), rodent high-fat diet with 45 kcal% fat (Research Diets #D12451, USA), or L-Amino Acid rodent diets with 60 kcal% fat with low methionine and no added choline diet (Dyets #CDAHF60, USA). Fgf21 knockout (KO) mice and their wild type littermates in C57BL/6N were generated as previously described [33, 74]. Genotyping was conducted as previously described [45]. Adipocyte specific murine double minute 2 knockout (Adipo-MDM2-KO) mice were generated by crossing MDM2floxed/floxed mice with Adipoq-Cre mice (expressing Cre recombinase under the control of the adiponectin promoter; The Jackson Laboratory) as previously described [31]. Mice were subjected to AAV infection or antibody treatment
as indicated in each figure legend. Body composition was determined by the Bruker Minispec LF90 II Body Composition Analyzer (Bruker Biospin, USA). Food intake was measured every week and shown as calculated average daily calorie intake per mouse.

**Histological analysis and Oil Red O staining**

Liver specimen (around 5 mm x 5 mm) taken from the right lobe near the portal vein was fixed in 4% (w/v) paraformaldehyde, dehydrated and embedded in paraffin as previously described [75]. The paraffin-embedded liver sections (5 µm) were deparaffinized, rehydrated, and stained with hematoxylin-eosin (H&E), Sirius Red (SR, Picro Sirius Red Staining Kit, ab150681, Abcam, UK), or Trichrome Stain (Masson’s) (Sigma-Aldrich, #SDHT15, USA) according to the manufacturer’s instructions. For Oil Red O (ORO) staining, liver embedded in OCT compound was immediately frozen on dry ice. Frozen sections (7 µm) were obtained using Leica CM 1900 Cryostat (Leica Biosystems, Nussloch, Germany). Rehydrated sections were rinsed with 60% isopropanol and stained for 20 min with 0.3% Oil Red O solution, followed by three times of rinse in 60% isopropanol and twice in double distilled water. Slides were further counter-stained with hematoxylin and mounted.

**Immunoblot analysis and real-time PCR**

Proteins were isolated from cells or various mouse tissue using radioimmunoprecipitation assay buffer (RIPA buffer: 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecyl sulfate), 50 mM Tris, pH 8.0) containing protease inhibitors cocktail (Roche, #0469315900, USA), separated by SDS–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and incubated with various primary antibodies at 4°C for overnight, using HRP-linked anti-rabbit IgG (Cell Signaling Technology, #7074, USA) as a secondary antibody. The following primary antibodies were used for immunoblot analysis: HSP90 (#4874S), Erk1/2 (#9102s), phospho-Erk1/2 (#9110S) from Cell Signaling (MA, USA); PPAR alpha (phospho S12) (#ab3484) from Abcam.
(MA, USA); phospho-PPAR alpha (#AF8054) from Affinity Biosciences (OH, USA); PPAR alpha (sc-398394) from Santa Cruz (California, USA). The protein bands were visualized by enhanced chemiluminescence reagents (GE Healthcare) and quantified using the ImageJ software based on the intensities of bands. For the determination of serum levels of anti-ATX neutralizing IgG, a standard curve was generated by densitometry using a series of rabbit non-immune IgG standards with known quantity, and concentration of anti-ATX neutralizing IgG was calculated based on the equation generated from the standard curve.

Total RNA was extracted from cells or various mouse tissues using Trizol (Invitrogen, USA) following the manufacturer’s instruction and subsequently reverse transcribed into complementary DNA using reverse transcription kit (PrimeScript RT reagent kit, Takara, #RR037A, USA). Real-time PCR was performed using SYBR Green master mix (Takara, #RR420D, USA) on the Applied Bio-systems Prism 7000 sequence detection system (Thermo Fisher Scientific, USA), using primers listed in Table 3. All mouse genes are normalized against the mouse beta-actin gene and human genes are normalized against the human beta-actin gene.

Liver fractionation

Liver fractionation was performed as previously described [76]. In brief, liver was perfused with EGTA solution (Calcium-free Hank's Balanced Salt Solution with 25 mM HEPES and 0.2 g/L EGTA) and sequentially perfused with digestion solution (Iscove's Modified Dulbecco's Medium with GlutaMAX™ (ThermoFisher Scientific, #35050061, USA), 500 mg/L Collagenase type 4) under a warming infrared light. The liver was then carefully removed from the abdominal cavity and placed on a Petri dish filled with 5 mL of post-digestion solution (DMEM with GlutaMAX™, 25 mM HEPES and 10% FBS) and was mechanically disrupted by a scalpel blade. Minced liver was collected and
diluted with 45 mL of post-digestion solution into a 50 mL conical tube on ice. The suspension was passed through a 70 µm cell strainer and centrifuged at 68 g for 5 min. The pellet was then washed with PBS for 2 times at 68 g for 10 mins to obtain parenchymal cells. The top 40 mL was transferred into a new tube and centrifuged at 460 g for 10 mins. The pellet was resuspended in 37.5% Percoll (Sigma, #P1644, USA) containing 5 mM EDTA, followed by centrifugation at 850 g for 30 mins. The pellet was resuspended in the red cell lysis buffer (Invitrogen, #00-4333-57, USA) followed by centrifugation at 460 g for 10 mins to collect non-parenchymal cells (NPC) in the pellets.

**Biochemical and immunological analysis**

Mouse autotaxin, FGF21, and adiponectin were measured with ELISA kits (Immunodiagnostics Limited, #32770, 32180, 32010 respectively, Hong Kong, China). Serum levels of human FGF21 and human autotaxin were quantified with ELISA kits (Immunodiagnostics Limited, #31180, 31770 respectively, Hong Kong, China). Serum FFAs were determined by FFAs Half Micro Test kit (Roche, USA). Serum triglyceride, cholesterol, ALT and AST were measured using commercial kits from STANBIO Laboratory, USA. To measure liver TG content, liver tissues (~50mg) were homogenized in chloroform/methanol (2:1 v/v) using a tissue homogenizer. Lipid extracts were prepared by the Folch method, as previously described [77], and were then dried under nitrogen flow and further dissolved in ethanol. Fatty acid oxidation (FAO) rate was measured using a commercial kit (Biomedical Research Service of University at Buffalo, E-141, USA) following the manufacturer’s instructions [78, 79]. This FAO activity assay is based on the oxidation of octanoyl-CoA (an intermediate during beta-oxidation). Generation of NADH in this reaction is coupled to the reduction of the tetrazolium salt INT (iodonitrotetrazolium) to formazan. The intensity of the red-colored formazan is proportional to FAO activity. Briefly, 10 mg of liver tissue was lysed in 200 uL lysis buffer (provided in the kit) and incubated on ice for 5 min, followed by centrifugation at 11,000 g for 5 min.
at 4℃. The supernatant was transferred into a new tube, and lysis buffer was added to adjust protein concentration of the supernatant to 5 mg/mL. 10 uL of supernatant was then mixed with 50 uL blank solution or reaction solution (provided in the kit) in a 96-well plate and incubated at 37°C for 1 hour. 50 μL of 3% fresh-made Acetic acid solution was subsequently added into each well to stop the reaction, and the absorbance was measured at 492 nm for the calculation of formazan production (mol). FAO activity was presented as INT-formazan (mol) per gram protein.

**LysoPLD activity assay**

LysoPLD activity was measured as previously described [80]. Briefly, 10 μL mouse serum or rmATX (2 ug/ml in PBS) was incubated with 2 mM 1-myristoyl (14:0)–LPC (Avanti Polar Lipids Inc., USA) in 90 μL of assay buffer (100 mM Tris–HCl (pH 9.0), 500 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, and 0.05% Triton X-100) for 4 hours at 37°C to allow for autotaxin-mediated choline release. Subsequently, 100 μL of reaction buffer containing 4.5 mM 4-aminoantioyrine, 2.7mM TOOS reagent (N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline, a hydrogen donor), 20 Unit/ml Horseradish peroxidase, 3 Unit/ml choline oxidase, 4.5 mM MgCl₂, 100 mM Tris (pH 8.0) was added and incubated for another 10 min at room temperature to allow for color reaction. The absorbance was measured at 555nm for the calculation of choline production (nmol) per μL serum per hour (for serum), or choline production (μmol) per mg protein per minute (for rmATX).

**Production, purification, and validation of anti-autotaxin neutralizing antibody**

Recombinant mouse autotaxin (rmATX, #42771, Immunodiagnostics Ltd, Hong Kong) was used as antigen for four consecutive immunization of New Zealand white rabbits for two months. After the antibody titer in the serum reached more than 10⁶, blood samples were taken from marginal ear veins of sedated rabbits, followed by sequential affinity purification of anti-autotaxin IgG with protein G-agarose and rmATX-conjugated sepharose 4b resin. Non-immune rabbit IgG purified from pre-
immune rabbit serum was used as control. The neutralizing activity of anti-autotaxin IgG was validated by its ability to block the activity of rmATX in vitro and to inhibit rmATX-induced Erk activation in HepG2 cells (ATCC, Manassas, VA, USA) were maintained in DMEM (Gibco, USA).

**Cell culture and luciferase reporter assay**

Huh7 cell lines obtained from the Cell Bank of Type Culture Collection, Chinese Academy of Sciences were maintained in DMEM (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 100 IU/ml penicillin, and 100 μg/ml streptomycin. To evaluate the effects of autotaxin-LPA axis on FGF21 expression and secretion, cells grown in DMEM were treated with recombinant human autotaxin protein (rhATX, ImmunoDiagnostics Limited, #41771, Hong Kong), lysophosphatidic acid (Cayman, #62215, USA), Fenofibrate (Abbott Laboratories, IL, USA), U0126 (Cell Signaling Technology, #9903, USA); Wortmannin (Sigma, #W1628, USA); PF8380 (Cayman, #12018, USA), Ki16425 (Cayman, #10012659, USA) at the concentration specified in each figure legend. For luciferase reporter assay, Huh7 cells were seeded in 24-well plates and were transfected 0.5μg PPRE X3-TK-luc plasmid (Addgene plasmid #1015) and 0.5μg pRL-TK Renilla luciferase reporter plasmid per well with polyethylenimine (PEI). At 48 hours post transfection, cells were further treated with LPA (10μM), U0126 (10μM), wortmannin (2μM) in the presence or absence of fenofibrate (50μM) for 24 hours, and were then collected for analysis of firefly luciferase/Renilla luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, E1960, USA). See Supplemental table 5 for the chemical used in the study.

**Generation and titration of adenovirus associated viruses (AAV)**

The short hairpin RNA (shRNA) targeting autotaxin (target sequence: CCTGTACCAAATCTGACATAT) and control shRNA with scrambled sequence (CCTAAGGTTAAGTCGCCCTCG) in pAAV-EGFP-U6 vectors were purchased from VectorBuilder,
USA. The sequence targeting 3’UTR of autotaxin mRNA was chosen from the database provided by VectorBuilder, based on the predicted high knockdown efficiency. In vitro validation for the knockdown efficiency of shRNA was conducted by transfection of shRNA targeting autotaxin or control shRNA into Hepa1-6 cells (ATCC, Manassas, VA, USA) cultured in DMEM (supplemented with 10% FBS, 100IU/ml penicillin, and 100 ug/mL streptomycin) and examination of mRNA level of autotaxin at 4 days post transfection.

To generate AAV, 500 µL of DMEM was mixed with 120 µl of 1 mg/ml PEI to generate solution A, while another 500 µL of DMEM was mixed with 12 µg of shRNA plasmid, 10 µg of pXX6 and 10 µg of p5E18-VD2/8 to make solution B. After mixing solution A and B vigorously, the PEI-DNA complex was incubated at room temperature for 20 mins and added into the 150mm culture dish of HEK293T cells (ATCC, Manassas, VA, USA). 6 hours after transfection, the medium was replaced with fresh complete DMEM medium. The medium of HEK293T cells was harvested 3 days after transfection. Concentration and purification of AAV were conducted following the standard protocol of AAVanced™ Concentration Reagent (#AAV110A-1, System Biosciences Inc., USA). For the titration of AAV, 5 µL of concentrated AAV were added with Buffer AL (QIAGEN Inc., #19075, USA) and incubated at 55 °C for 30 mins. The mix was then proceeded to Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation, USA). Eluted DNA was used as the amplification template for the titration of AAV by real-time PCR, and serially diluted shRNA plasmids were used to generate a standard curve showing the correlation between CT value and copy number of vector genome (v.g.).

Statistics

For animal studies, data statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc. La Jolla, CA92037, USA). The results are expressed as mean ± standard error of mean (SEM). Animal sample size (N number) for each experiment was chosen according to the previous...
reports of similar type of experiments. For the comparison between two groups, the statistical significance was determined by 2-tailed Student’s t-test. For multiple comparisons within more than two groups, statistical calculations were performed by one-way ANOVAs followed by Tukey’s multiple comparisons using Prism 6. Values of P <0.05 were considered statistically significant. For the clinical study, statistical analyses were performed with SPSS software version 26.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6. Data were presented as mean ± standard error of mean. P values were assessed by Mann Whitney U test or Kruskal Wallis test with Dunn’s multiple comparison test among groups for continuous parameters and by χ² test for categorical parameters. Multiple regression was used to assess associations and adjust for conventional confounders such as age, sex, and BMI for correlation coefficient. Values of P <0.05 were considered statistically significant.

All authors had access to the study data and had reviewed and approved the final manuscript.

**Author Contributions:** H.Q. and A.X. completed conception and design of this study. H.Q., E.S., and A.X. drafted the manuscript and prepared the figures. H.Q., E.S., Y.H., T.L., K.M.K., L.Y.C., Q.W., and X.W. conducted the experiments. E.S., C.W., B.C., R.H., Y.W., and A.X. edited the manuscript. A.X. approved the final version of the manuscript.
References:


Figure legends

**Figure 1:** Association of serum autotaxin levels with liver fat contents, liver injury markers and hepatic autotaxin mRNA expression in obese human subjects. (A-C) Correlation of serum autotaxin levels with liver fat content determined by MRI (A, n=101), serum levels of AST (B, n=125), and ALT (C, n=125) (D) Protein levels of autotaxin in subcutaneous fat (scWAT), visceral fat (vWAT), and liver in male or female obese patients (n=10 each) measured by ELISA. The difference was analyzed with one-way ANOVAs followed by Tukey’s multiple comparisons. 

**Figure 2:** Liver is a major contributor to increased circulating autotaxin in mice with NAFLD. (A-D) 6-week-old C57BL/6N male mice (n=5) were fed with standard chow diet (STC) or high-fat diet (HFD) for 20 weeks and were then sacrificed to collect serum and various tissues for further analysis. (A-B) Time-dependent changes in serum level of autotaxin protein (A) and lysoPLD activity (B). (C-D) The relative mRNA abundance of mouse autotaxin in different tissues (C, shown as fold change relative to BAT of STC group), and in different fractions of the liver (D, shown as fold change relative to hepatocytes of STC group) determined by real-time PCR analysis. BAT: brown adipose tissue; msWAT: mesenteric adipose tissue; eWAT: epididymal adipose tissue; NPC: non-parenchymal cells. *p<0.05, **p<0.01, ***p<0.001 versus STC group. Data are presented as the mean ± SEM. (E-F) 12-week-old male adipocyte-specific MDM2 knockout (Adipo-MDM2-KO) mice and their wild type littermates (Adipo-MDM2-WT) were sacrificed to collect serum and various tissues for measurement of circulating autotaxin (E, n=8-12) and relative mRNA abundance of autotaxin (F, n=5, shown as fold change relative to lymph node of Adipo-MDM2-WT group) by ELISA and real-time PCR respectively. For (A)-(F), the statistical significance was determined by 2-tailed Student’s t-test. *p<0.05, **p<0.01, ***p<0.001. Data are presented as the mean ± SEM.

**Figure 3:** Knockdown of hepatic autotaxin expression decreases obesity-induced elevations of circulating autotaxin levels and lysoPLD activity but has no effect on body weight and food intake. 6-week-old C57BL/6N male mice) were intravenously injected with 1x10^{11} v.g. (vector genome) of adeno-associated virus (AAV) encoding shRNA specific to autotaxin (KD) or scramble control (SC), and were then fed with standard chow diet (STC) or high-fat diet (HFD) for 15 weeks. (A-B) Real-time PCR analysis for mRNA expression levels of autotaxin in liver (A) (shown as fold change against SC-STC group) and in different adipose depots (B) (shown as fold change against BAT of SC-STC group). BAT, brown adipose tissue; eWAT, epididymal adipose tissue; scWAT, subcutaneous adipose tissue. (C) Serum autotaxin levels measured at different time points after AAV injection. (D) Serum lysoPLD activity measured at 15^{th} week after AAV injection. (E-G) Body weight (E) and fat mass (F) measured at indicated time points; average daily food intake shown as kcal per day (kcal/day) (G). n=6-8. For (A)-(G), statistical calculations were performed by one-way ANOVAs followed by Tukey’s multiple comparisons using Prism 6. *p<0.05, **p<0.01, ***p<0.001. Data are presented as the mean ± SEM.

**Figure 4:** The effect of knock-down of hepatic autotaxin expression on obesity-related NAFLD. 6-week-old C57BL/6N male mice were fed with standard chow diet (STC) or high-fat diet (HFD) for 15 weeks after intravenous injected with 1x10^{11} v.g. (vector genome) of adeno-associated virus (AAV) encoding shRNA specific to autotaxin (KD) or scramble control (SC). (A) Serum lipid profiles including levels of triglyceride, cholesterol, and free fatty acid. (B) Wet weight of different organs. scWAT: subcutaneous adipose tissue; eWAT: epididymal adipose tissue; BAT: brown adipose tissue. (C) Representative photos of H&E staining and Oil Red O staining of liver sections (200x, Scale bar: 100um). (D) Hepatic triglyceride (TG) content determined by
biochemical analysis. (E) Serum levels of ALT and AST. (F) Real-time PCR analysis for genes involved in hepatic lipid metabolism. Acox-1: acyl-CoA oxidase 1; Cpt1α: carnitine palmitoyltransferase IA; Lecd/Mecd/Scad: long/medium/short-chain acyl coenzyme-A dehydrogenase; Scd-1: stearoyl-CoA desaturase-1; Fasn: fatty acid synthase; Ldlr: low density lipoprotein receptor. Genes are normalized with beta-actin and shown as fold change relative to SC-STC group. (G) Fatty acid oxidation (FAO) rate in livers. Oxidation of fatty acid is coupled to NADH-dependent reduction of INT (iodonitrotetrazolium) to INT-formazan. FAO activity was shown as production of INT-formazan (mol) per gram protein. n=6-8; For (A), (B), and (D)-(G), statistical calculations were performed by one-way ANOVAs followed by Tukey’s multiple comparisons using Prism 6. *p<0.05, **p<0.01, ***p<0.001, data are presented as the mean ± SEM.

Figure 5: Characterization of the neutralizing activity of anti-autotaxin IgG and evaluation of its effects on body weight, fat mass and food intake in mice. (A) Concentration-dependent response curves for the inhibitory effect of anti-mouse autotaxin IgG (Anti-ATX) on serum lysoPLD activity (shown as % of inhibition). Rabbit non-immune IgG (Control) was used as a control. The sera used were from 6-week-old C57BL/6N male mice (n=3 in each time point); (B) Concentration-dependent effects of anti-ATX IgG on lysoPLD activity of recombinant mouse autotaxin (rmATX) (n=3 in each time point). (C) Dose-dependent effect of anti-ATX IgG on autotaxin-induced phosphorylation of the MAP kinase ERK1/2 in HepG2 cells. Cells grown in serum-free medium with the addition of 50 μM 14:0 LPC were treated without or with 200 ng/ml rmATX in the absence or presence of different concentrations of anti-ATX or non-immune IgG for 10 minutes and were then harvested for Western blot analysis (n=3 in each group). HSP90 was used as a loading control. (D) Dynamic changes in circulating anti-ATX IgG after a single i.v. injection (1 mg/kg body weight) into 14-week-old C57BL6/N mice (n=4) measured with western blot (insert). For the determination of serum levels of anti-ATX neutralizing IgG, a standard curve was generated by densitometry using a series of rabbit non-immune IgG standards with known quantity, and concentration of anti-ATX neutralizing IgG was calculated based on the equation generated from the standard curve. ***p<0.001. Data are presented as the mean ± SEM. (E-G) 6-week-old C57BL/6N male mice (n=6-8) were fed with either standard chow diet (STC) or high-fat diet (HFD) for 8 weeks, and subsequently injected with 1mg/kg body weight Anti-ATX or non-immune IgG (control)) every 10 days for another 7 weeks (five injections in total). Body weight (E) and fat mass (F) measured at indicated time points; average daily food intake shown as kcal per day (kcal/day) (G). Data are presented as the mean ± SEM. For (E)-(G), statistical calculations were performed by one-way ANOVAs followed by Tukey’s multiple comparisons using Prism 6. For (A) and (B), statistical calculations were performed by Student’s t test using Prism 6.

Figure 6: Treatment of anti-autotaxin neutralizing antibody ameliorates obesity-induced dyslipidemia and NAFLD. 6-week-old C57BL/6N male mice (n=6-8) were fed with either standard chow diet (STC) or high-fat diet (HFD) for 8 weeks, and subsequently injected with 1mg/kg body weight antibody (anti-autotaxin (Anti-ATX) or non-immune IgG (control)) every 10 days for another 7 weeks (five i.v. injections in total). Mice are sacrificed at the 15th week. (A) Serum lysoPLD (LPD) activity at different time points; (B) Serum levels of triglyceride (TG), cholesterol and free fatty acid levels; (C) Wet weight of different organs. scWAT: subcutaneous adipose tissue; eWAT: epididymal adipose tissue; BAT: brown adipose tissue. (D) Representative photos of H&E staining and Oil Red O staining of liver sections (200x, Scale bar: 200μm); (E) Heparic triglyceride content; (F) Serum levels of ALT and AST. (G) Real-time PCR analysis for genes involved in hepatic lipid metabolism. Acox-1: acyl-CoA oxidase 1; Cpt1a: carnitine palmitoyltransferase IA; Lecd/Mecd/Scad: long/medium/short-chain acyl coenzyme-A dehydrogenase; Scd-1: stearoyl-CoA desaturase-1; Fasn: fatty acid synthase; Ldlr: low density lipoprotein receptor. Genes are normalized with beta-actin and shown as fold change against STC-Control group. (H) Fatty acid oxidation (FAO) rate in livers. Oxidation of fatty acid is coupled to NADH-dependent reduction of INT (iodonitrotetrazolium) to INT-formazan. FAO
activity was shown as production of INT-formazan (mol) per gram protein. n=6-8. For (A)-(C) and (E)-(H), statistical calculations were performed by one-way ANOVAs followed by Tukey’s multiple comparisons using Prism 6. *p<0.05, **p<0.01, ***p<0.001, data are presented as the mean ± SEM.

Figure 7: Autotaxin-LPA axis suppresses hepatic production of FGF21 via ERK-mediated inhibition of PPARα. 6-week-old C57BL/6N male mice (n=6-8) were fed with either standard chow diet (STC) or high-fat diet (HFD) for 8 weeks, and subsequently injected with 1mg/kg body weight antibody (anti-autotaxin (Anti-ATX) or non-immune IgG (control)) every 10 days for another 7 weeks as in Figure 6. Mice are sacrificed at the 15th week. (A) Real-time PCR analysis of mRNA expression levels of several hepatokines in liver (shown as fold change against STC-Control group). Angptl-3/4/6: angiopoietin-like proteins-3/4/6; fgf-1: fibroblast growth factor 1; fgf15: fibroblast growth factor 15; dpp4: dipeptidyl-peptidase 4; lcn-2: lipocalin-2. (B) Real-time PCR analysis for mRNA expression levels of several adipokines in epididymal adipose tissue (shown as fold change against STC-Control group). Fabp4: fatty acid-binding protein 4. n=6-8. Data are presented as the mean ± SEM. (C) Serum levels of FGF21; (D) Hepatic mRNA expression levels of Fgf21 determined by real-time PCR analysis; (E) Serum levels of adiponectin. (F-H) 6-week-old C57BL/6N male mice were fed with standard chow diet (STC) or high-fat diet (HFD) for 15 weeks after intravenous injected with 1x10^11 v.g. (vector genome) of adeno-associated virus (AAV) encoding shRNA specific to autotaxin (KD) or scramble control (SC) as in Figure 4. (F) Serum levels of FGF21; (G) Hepatic mRNA expression levels of FGF21 determined by real-time PCR analysis; (H) Serum levels of adiponectin. (I-J): Huh7 cells grown in serum-free DMEM were treated with or without indicated dosage of rhATX, PF8380 (inhibitor of autotaxin), LPA or Ki16425 (LPA receptor antagonist) for 24 hours. The mRNA expression levels of FGF21 (I, expressed as fold changes relative to vehicle (DMSO)-treated controls) and its protein concentrations in the conditioned medium (J) were determined by real-time PCR and ELISA, respectively. n=5, *p<0.05, **p<0.01. Data are presented as the mean ± SEM. (K-L) Huh7 hepatocytes were pre-treated with rhATX, LPA, PF8380 (inhibitor of autotaxin), Ki16425 (LPA receptor antagonist), for 30 minutes, followed by treatment with or without fenofibrate for 24 hours. FGF21 protein secreted into conditioned medium (K) and hepatic FGF21 mRNA (L) were determined by ELISA and real-time PCR respectively. (M-O) Huh7 hepatocytes were pretreated with U0126 (ERK inhibitor), wortmannin (PI3K), or LPA for 30 minutes, followed by treatment with or without fenofibrate for 30 minutes to collect cell lysates for Western blot analysis (M, N); or 24 hours to measure the relative mRNA abundance of FGF21, PPARα and VLCAD (Very-long-chain acylcoenzyme-A dehydrogenase, a downstream target of PPARα) (O). (P): Relative luciferase activity in Huh7 hepatocytes transfected with a luciferase reporter vector carrying PPAR response element (PPRE) and treated with LPA, U0126, wortmannin in the presence or absence of fenofibrate for 48 hours. Data is normalized against human BETA-ACTIN and expressed as fold change relative to DMSO-treated controls. For (A)-(L) and (N)-(P), statistical calculations were performed by one-way ANOVAs followed by Tukey’s multiple comparisons using Prism 6. *p<0.05, **p<0.01, ***p<0.001. n=5. Data are presented as the mean ± SEM.

Figure 8: FGF21-deficient mice are refractory to the therapeutic benefits of anti-autotaxin antibody against obesity-induced dyslipidemia and fatty liver. 6-week-old male FGF21 knock-out (KO) mice in C57BL/6N background and their wild type (WT) littermates (n=6-8) were fed with HFD for 8 weeks, and subsequently treated with 1mg/kg body weight anti-autotaxin (Anti-ATX) IgG or non-immune IgG (control)) every 10 days for another 7 weeks by 5 i.v. injections as in Figure 3. (A) Serum lysoPLD activity; (B) Serum levels of triglyceride, cholesterol, and free fatty acid; (C) Wet weight of liver tissues. (D) Representative images of H&E staining and Oil Red O staining of liver sections (200x, Scale bar: 200 μm). (E) Hepatic triglyceride content. (F) Serum levels of ALT and AST. (G) Serum adiponectin levels. (H) Real-time PCR analysis for genes involved in hepatic lipid metabolism. Acox-1: acyl-CoA oxidase 1; Cpt1α: carnitine palmitoyltransferase 1A;
Figure 9: Dynamic changes of autotaxin expression in mice with CDAHF60 diet-induced NASH. Ten-week-old C57BL/6N male mice were fed with standard chow diet (STC) or CDAHF60 diet for 10 weeks (n=5/time point). (A-B) Serum autotaxin levels (A) and serum lysoPLD activity (B) measured at different time points after initiation of CDAHF60 diet. (C) Real-time PCR analysis for mRNA expression levels of autotaxin in liver and epididymal white adipose tissue were expressed as fold changes relative to STC group. (D) Representative images of liver sections showing the histopathological development of CDAHF60-induced NASH, as evaluated by H&E, Oil red O, Sirius red and Masson’s trichrome staining. Scale bar: 100 μm. (E-G) 10-week-old male FGF21 KO mice and their wild type (WT) littersmates were fed with anti-ATX or non-immune IgG (1 mg/kg body weight, 5 injections) every 10 days for 7 weeks upon CDAHF60 diet feeding (n=6-8). Body weight (E) and fat mass (F) measured at indicated time points; average daily food intake shown as kcal per day (kcal/day) (G). For (A) and (B), statistical calculations were performed by one-way ANOVAs followed by Tukey’s multiple comparisons using Prism 6. *p<0.05, **p<0.01, ***p<0.001, data are presented as the mean ± SEM.

Figure 10: Anti-autotaxin neutralizing antibody alleviates CDAHF60 diet-induced NASH by inducing FGF21 production. 10-week-old male FGF21 knock-out (KO) mice and their wild type (WT) littersmates were fed with choline-deficient, L-amino acid-defined, 60% high-fat (CDAHF60) for 3 weeks, followed by 5 i.v. injections of 1mg/kg body weight anti-autotaxin (Anti-ATX) or non-immune IgG (Control)) every 10 days for 7 weeks upon CDAHF60 feeding. (A) Serum lysoPLD activity in mice; (B-C) Serum levels of FGF21 (B) and adiponectin (C). (D) Serum concentrations of triglyceride, cholesterol and free fatty acids; (E) Liver weights. (F) Representative photos of inflammatory loci (shown as 4x magnified photos of H&E, indicated by black arrows), H&E, Oil Red O (ORO) and Sirius red staining of liver sections (200x, Scale bar: 200um). Blue arrows: collagen formation. (G) NASH scores (NAS) as determined by histological examination. (H) Hepatic triglyceride content. (I) Serum levels of ALT and AST. (J) Fatty acid oxidation (FAO) rate in livers. Oxidation of fatty acid is coupled to NADH-dependent reduction of INT (iodonitrotetrazolium) to INT-formazan. FAO activity was shown as production of INT-formazan (mol) per gram protein. (K) Real-time PCR analysis of genes involved in hepatic lipid metabolism (Cpt1a, Acox1, L/M/Scad, Scd-1, Fasn, Cd36, Ldlr); fibrosis and inflammatory responses (Acta2, Colla1, Timp-1, Fn1, Tnfa, Il-1b); Genes are normalized with beta-actin and shown as fold change relative to WT-control group. Acta2: α actin alpha 2; Colla1: alpha-1 type I collagen; Timp-1: TIMP Metallopeptidase Inhibitor 1; Fn1: Fibronectin 1; Tnfa: tumor necrosis factor alpha; Il-1b: Interleukin 1 Beta. n=6-8, for (A)-(E) and (G)-(K), statistical calculations were performed by one-way ANOVAs followed by Tukey’s multiple comparisons using Prism 6. *p<0.05, **p<0.01, ***p<0.001, data are presented as the mean ± SEM.

Figure 11: A schematic summary of the role of hepatocyte-secreted autotaxin in exacerbating NAFLD through its autocrine actions. Obesity or other risk factors promote increased production and secretion of autotaxin, thereby catalyzing the conversion of LPC to LPA, which in turn binds to its receptors (LPARs) to
trigger ERK activation and subsequent PPARα inhibition, leading to decreased production of FGF21 and adiponectin. Anti-autotaxin neutralizing antibodies reverse these pathological pathways and increase FGF21 production, thereby exerting its therapeutic benefits on alleviation of hepatic steatosis, steatohepatitis, and fibrosis.
Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal (n=14)</th>
<th>Steatosis (n=31)</th>
<th>B. NASH (n=62)</th>
<th>NASH (n=44)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.61 ± 2.82</td>
<td>31.64 ± 1.36</td>
<td>30.23 ± 0.91</td>
<td>28.90 ± 1.14</td>
<td>0.303</td>
</tr>
<tr>
<td>Sex(M: F)</td>
<td>4:10</td>
<td>12:19</td>
<td>29:33</td>
<td>21:23</td>
<td>0.000</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>33.38 ± 1.62</td>
<td>39.77 ± 1.61</td>
<td>40.52 ± 0.92</td>
<td>42.34 ± 1.19</td>
<td>0.002</td>
</tr>
<tr>
<td>Neck Circumference (cm)</td>
<td>38.43 ± 1.02</td>
<td>42.41 ± 0.77</td>
<td>43.10 ± 0.57</td>
<td>44.19 ± 0.72</td>
<td>0.000</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>106.36 ± 3.14</td>
<td>124.35 ± 3.15</td>
<td>123.70 ± 1.77</td>
<td>128.44 ± 2.55</td>
<td>0.000</td>
</tr>
<tr>
<td>Hip Circumference (cm)</td>
<td>115.05 ± 2.95</td>
<td>125.78 ± 2.93</td>
<td>125.24 ± 1.54</td>
<td>129.94 ± 2.13</td>
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</tr>
<tr>
<td>Lactate Dehydrogenase (U/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine deaminase (U/l)</td>
<td>5.27 ± 0.23</td>
<td>6.45 ± 0.58</td>
<td>6.55 ± 0.35</td>
<td>6.82 ± 0.36</td>
<td>0.274</td>
</tr>
<tr>
<td>Ferritin (mg/l)</td>
<td>12.17 ± 0.70</td>
<td>14.02 ± 0.87</td>
<td>15.12 ± 0.52</td>
<td>17.10 ± 0.70</td>
<td>0.000</td>
</tr>
<tr>
<td>TCHOL (mg/dl)</td>
<td>4.82 ± 0.17</td>
<td>4.98 ± 0.18</td>
<td>5.14 ± 0.12</td>
<td>5.17 ± 0.16</td>
<td>0.524</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>1.09 ± 0.05</td>
<td>1.08 ± 0.05</td>
<td>1.02 ± 0.02</td>
<td>0.97 ± 0.23</td>
<td>0.114</td>
</tr>
<tr>
<td>Lipoprotein a (mg/dl)</td>
<td>194.12 ± 75.35</td>
<td>188.66 ± 45.74</td>
<td>187.43 ± 29.67</td>
<td>91.05 ± 14.62</td>
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<tr>
<td>Apolipoprotein B (g/l)</td>
<td>0.92 ± 0.04</td>
<td>0.98 ± 0.03</td>
<td>1.09 ± 0.23</td>
<td>1.05 ± 0.04</td>
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<tr>
<td>Lactate Dehydrogenase (U/l)</td>
<td>176.40 ± 10.08</td>
<td>202.93 ± 9.91</td>
<td>212.54 ± 6.10</td>
<td>222.89 ± 9.36</td>
<td>0.024</td>
</tr>
</tbody>
</table>

Table 1: Clinical characteristics of the obese subjects included in this study. Data are expressed mean ± SEM. BMI: Body mass index; HbA1c: glycated hemoglobin (A1c); HOMA-IR: Homeostasis model assessment of insulin resistance; HOMA-B: HOMA of β-cell function index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TCHOL: total cholesterol; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol.
Table 2. Association of serum autotaxin levels with histological severity of biopsy-confirmed NAFLD (n=125).

<table>
<thead>
<tr>
<th>Severity of pathohistology</th>
<th>Pseudo R</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>0.318</td>
<td>0.001**</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0.353</td>
<td>0.000***</td>
</tr>
<tr>
<td>Ballooning</td>
<td>0.297</td>
<td>0.003**</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.657</td>
<td>0.252</td>
</tr>
<tr>
<td>Histology Diagnosis</td>
<td>0.383</td>
<td>0.000***</td>
</tr>
</tbody>
</table>

(Normal liver, simple steatosis, borderline NASH and NASH)

The scores of steatosis (0-3), hepatocellular ballooning (0-2), lobular inflammation (0-3), and fibrosis (0-4) were evaluated according to the NASH Clinical Research Network (NASH CRN) scoring system. **p<0.01, ***p<0.001. R: Correlation coefficient.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Kind</th>
<th>Primer Sequences (5’-3’)</th>
<th>Gene Name</th>
<th>Kind</th>
<th>Primer Sequences (5’-3’)</th>
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<td>Murine</td>
<td>Forward</td>
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<tr>
<td>Enpp2</td>
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<td>Acta2</td>
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**Table 3. Primers used for Real-time PCR**