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Reduced circulating levels of sTWEAK are associated with NAFLD and may affect hepatocyte triglyceride accumulation

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ABSTRACT

Context: Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome and is strongly associated with obesity, dyslipidaemia and altered glucose regulation. Previous data demonstrated that low circulating levels of tumor necrosis factor weak inducer of apoptosis (sTWEAK) were associated with obesity, diabetes and insulin resistance, all traits associated with an increased risk of NALFD. Circulating sTWEAK levels are expected to be reduced in the presence of NAFLD.

Objective: We aimed to explore the relationship between NAFLD and circulating sTWEAK levels in obese patients, and to evaluate the effect of sTWEAK on hepatocyte triglyceride accumulation.

Design setting and patients: This is an observational case-control study performed in n=112 severely obese patients evaluated for NAFLD by abdominal ultrasound and n=32 non-obese patients without steatosis. Serum sTWEAK concentrations were measured by ELISA. Multivariable analyses were performed to determine the independent predictors of NAFLD. We analysed TWEAK and Fn14 protein expression in liver biopsies by western blotting and immunohistochemistry. An immortalised primary human hepatocyte cell line (HHL) was used to evaluate the effect of sTWEAK on triglyceride accumulation.

Results: We observed a reduction of serum circulating sTWEAK concentrations with the presence of liver steatosis. On multivariable analysis, lower sTWEAK concentrations were independently associated with the presence of NAFLD (OR=0.023; 95%CI: 0.001-0.579; p<0.022). In human hepatocytes, sTWEAK administration reduced fat accumulation as demonstrated by the reduction in palmitic acid-induced accumulation of triglyceride and the decreased expression of cluster of differentiation 36 (*CD36*) and perilipin 1 and 2 (*PLIN1* and *PLIN2)* genes.

Conclusions: Decreased sTWEAK concentrations are independently associated with the presence of NAFLD. This is concordant with the observation that TWEAK reduces lipid accumulation in human liver cells.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a common condition that is associated with obesity, type 2 diabetes mellitus (T2DM) and insulin resistance. NAFLD encompasses a spectrum of phenotypes ranging from simple steatosis (fatty infiltration) through nonalcoholic steatohepatitis to fibrosis and ultimately cirrhosis [1,2]. NAFLD develops because of an imbalance between lipid supply and demand [3].

Tumor necrosis factor weak inducer of apoptosis (TWEAK) is a cytokine of the TNF superfamily that exists in two forms, a cleaved soluble form (sTWEAK) and a membrane anchored form (mTWEAK). Both forms can bind to fibroblast growth factor-inducible 14 (Fn14), its *bona fide* signal transducing receptor [4]. Cluster of differentiation (CD) 163 has been recently described as a novel scavenger receptor for TWEAK on the surface of monocytes/macrophages, which can be shed to the circulation by pro-inflammatory stimuli [5]. TWEAK regulates a diverse range of cellular processes, including proliferation, differentiation, migration, cell survival, and cell death, and has also been shown to act as a proangiogenic and proinflammatory factor [4]. Additionally, sTWEAK has been described to interfere with tumor necrosis factor alpha (TNFα) activity, down-regulating TNFα-induced inflammatory signals [6] and ameliorating TNFα-induced insulin resistance [7].

The TWEAK/Fn14 axis has been extensively studied in the context of obesity, diabetes and other insulin resistant states [8,9]. Accordingly, reduced circulating sTWEAK concentrations have been found in type 1 diabetes [10] and T2DM [11]. In severe obesity, decreased sTWEAK levels rise after bariatric surgery [6]. Further, decreased sTWEAK levels are significantly and independently associated with an increased risk of T2DM [12] and increased risk of metabolic syndrome [13], collectively highlighting a possible role of sTWEAK in the pathogenesis of metabolic disease.

To our knowledge, the TWEAK/Fn14 axis has not been examined in human NAFLD. Studies using murine models of chronic liver injury and repair suggest that the principal function of TWEAK is to initiate ductal proliferation and liver progenitor cell expansion *via*

activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling. Consequently, TWEAK is proposed as a direct liver progenitor cell expansion mitogen [14,15].

Uptake of free fatty acids by hepatocytes depends on fatty acid transporters such as CD36, which binds lipoproteins, and fatty acid transport protein (FATP) genes such as FATP5 [16,17]. CD36 is also important for lipogenesis because it is responsible for the high affinity uptake of free fatty acids [18]. In the promotion of hepatic steatosis, CD36 is a transcriptional target of orphan nuclear receptors including liver X receptor alpha (LXRα), pregnane X receptor (PXR) and peroxisome proliferator-activated receptor (PPARɣ) [19–21].

Because of the burden of NAFLD in obesity, its association with insulin resistance and the potential role of TWEAK in the liver, we investigated the contribution of TWEAK to the pathogenesis of NAFLD. We explored the TWEAK/Fn14 axis within the context of human steatosis from an observational point of view by: 1) evaluating sTWEAK serum concentrations in a group of severely obese patients with and without steatosis and in a group of non-obese patients without steatosis; 2) analysing TWEAK and Fn14 protein expression in liver biopsies from severely obese patients; and 3) evaluating in *vitro* the effects of sTWEAK on triglyceride accumulation in an immortalised human hepatocyte cell line.

MATERIALS AND METHODS

Study subjects

This is an observational case-control study with an age- and gender-unmatched population. A total of 112 severely obese patients were included. Patients were classified in two groups according to the presence or absence of hepatic steatosis assessed by abdominal ultrasound, and reported that their body weight had been stable for at least three months before the study. Inclusion criteria were age 30–65 years, body mass index (BMI) >30 kg/m^2) and ability to understand study procedures. Exclusion criteria were history of cardiovascular disease (coronary heart disease, stroke, heart failure, peripheral vascular disease or congenital heart disease), chronic inflammatory diseases (Crohn's disease, ulcerative colitis, rheumatoid arthritis), infection in the previous month, ethanol intake >20 g/day or use of medications that

might interfere with insulin action. We excluded any other pre-existing liver condition other than NAFLD, such as hepatitis virus infection (assessed by testing antibodies against hepatitis A and C and HBs Ag (surface antigen of the hepatitis B virus), and other aetiologies including drug-induced, cholestatic, metabolic and genetic liver disease and autoimmune hepatitis (additional testing of anti-mitochondrial, anti-nuclear and anti-smooth muscle antibodies) were also excluded. TNF-α antagonist therapy or treatment with anti-inflammatory drugs (NSAIDs) also constituted exclusion criteria. Dyslipidaemia was diagnosed based on clinical history in patients with active treatment with statins. When indicated for diagnosis, biopsies were taken based on defined clinical and biochemical parameters of suspected advanced stages of NAFLD; some of these biopsies were used for immunohistochemistry and western blotting analysis.

A control group of healthy non-obese subjects without hepatic steatosis were also included (n=32, BMI<30 kg/m^2). The same inclusion/exclusion criteria were used for the control group.

All patients were informed about the purpose and nature of the study and informed consent was obtained. An institutional ethics committee approved the study protocol.

Hepatic steatosis assessment by abdominal ultrasound

We used a Siemens Acuson S2000 (Mochida Siemens Medical System, Tokyo, Japan) ultrasound system with a 3-5 MHz convex transducer to scan the liver. Images were independently evaluated by two radiologists blinded to clinical and laboratory data. Hepatic steatosis was diagnosed according to conventional criteria [22].

Collection, processing and histological study of human liver samples

Biopsies were placed in preservation solution for transport to the laboratory (RNAlater; Sigma-Aldrich, St Louis, MO) and some samples were placed in 10% formol for later paraffin-embedding for pathology diagnosis. After removal of the preservation solution, samples were immediately frozen in liquid nitrogen and then stored at -80° C. Liver biopsies were classified by a pathologist according to the criteria of Brunt [23]. Haematoxylin and eosin staining was

performed using a standard protocol. TWEAK and Fn14 expression in liver biopsies by western blotting and immunohistochemistry were performed as described below.

Analytical methods

Patients underwent anthropometric measurements. Blood was extracted for measurement of plasma lipids, glucose and insulin after an 8h fast. Glucose and lipid levels were determined by standard laboratory methods. Serum insulin was measured in duplicate using a monoclonal immunoradiometric assay (Medgenix Diagnostics, Fleunes, Belgium). The inter-assay CVs were 6.9 and 4.5% at 14 and 89 mU/L, respectively. Glycated haemoglobin (HbA_{1c}) was measured by high-pressure liquid chromatography using a fully automated glycosylated haemoglobin analyzer system (Hitachi L-9100). The homeostatic model assessment insulin resistance index (HOMA-IR) was calculated by the formula: (plasma glucose (mmol/L) x serum insulin (mU/L)) / 22.5). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and ɣ-glutamyltransferase (ɣGT) levels were determined using enzymatic methods. Serum concentrations of sTWEAK and sCD163 were determined in duplicate by ELISA using the commercially available human TWEAK/TNFSF12 kit DY1090 and human CD163 kit DY1607 (R&D Systems Europe, Abingdon, Oxon, UK), respectively. The intra- and inter-assay coefficients of variation were 2.5 and 7.0% for sTWEAK and 2.4 and 6.4% for sCD163, respectively. A standard 75 g oral glucose tolerance test was performed after an overnight fast and venous blood samples were drawn at time points 0, 30, 60, 90 and 120 min for determination of plasma glucose and insulin.

Gene Expression Analysis

RNA was extracted and quantified as described [24,25].The following TaqMan gene expression assays were used: PLIN1 (Hs00160173_m1), PLIN2 (Hs00765634_m1), CD36 (Hs00169627_m1), Nrf2 (Hs00975961_g1), PPARɣ (Hs00234592_m1), LXRα (Hs00172885_m1), FATP5 (Hs00202073_m1), DGAT1 (Hs00201385_m1) SREBP1-c

(Hs01088691_m1), CPT1-B (Hs00992664_m1), FASN (Hs00188012_m1), PXR (Hs01114267_m1) and PPIA (Hs99999904_m1).

Western blot analysis

Cellular proteins and tissue protein extracts were prepared in lysis buffer (25 mM Tris-HCl pH 7.0, 1 mM EDTA and 2% SDS). Proteins were subjected to SDS–PAGE, transferred to nitrocellulose membranes, blocked in 3% dry fat skimmed milk and probed with anti-TWEAK goat polyclonal antibody (R&D Systems), anti-Fn14 affinity purified anti-rabbit (Cell Signaling Technology, Danvers, MA), anti-CD36 affinity purified anti-rabbit (Novus Biologicals, Bionova, Spain) and anti-PLIN2 mouse monoclonal antibody (R&D Systems); all were used at a 1:1000 dilution. Blots were developed with SuperSignal West Femto chemiluminicescent substrate (Pierce Biotechnology, Boston, MA) except for βactin, which was developed with West Pico (Pierce Biotechnology). Proteins were quantified in a VersaDoc Imaging System using Quantity One software (Bio-Rad, Barcelona, Spain).

Immunohistochemistry

Antibody staining was performed on a Roche BenchMark Ultra automated immunostainer (Ventana Medical Systems Inc., Tuscon, AZ). Deparaffined 3-4 μm sections were incubated at 1:100 dilution with anti-Fn14 and anti-TWEAK rabbit polyclonal from Cell Signaling.

Cell culture and treatments

For *in vitro* experiments, we used the HHL cell line immortalised from primary human hepatocytes isolated from a healthy donor and kindly donated by Dr. A. Patel from the Virology Department at the Medical Research Centre (Cambridge, UK) [26,27]. For palmitic acid (PALM) treatments, PALM was conjugated to fatty acid-free bovine serum albumin (BSA) as described [28]. Cells were incubated with or without saturated PALM at a concentration of 0.2 mM for 24 h, or pretreated for 6 h with 100 ng/mL sTWEAK (Peprotech, Barcelona, Spain) followed by

PALM treatment for a further 24 h. Control cells with vehicle were also included in each experiment. Cell viability was not significantly affected by PALM treatment (10-15%) as measured by MTT reduction (data not shown). Triglyceride levels were quantified by Oil red O staining.

For experiments using the PPARɣ agonist rosiglitazone (5 μM) (GlaxoSmithKline, UK) and the LXRα agonist GW3965 (1 μM) (Deltaclon, Spain), cells were pretreated with 100 ng/mL sTWEAK for 6 h prior to agonist treatment for 24 h.

Statistical analyses

We calculated the number of patients needed to find at least a 20% of variation in sTWEAK concentrations between obese patients with and without NAFLD would be 50 in each group (alpha=0.05 and beta=0.20). For clinical, anthropometrical and analytical parameters, all data were tested for normality using the Shapiro-Wilk test. Normally distributed data are shown as mean (SD) and non-normally distributed data are shown as median (25^{th}-75^{th} quartiles). One-way analysis of variance (ANOVA) was used to compare the three study groups. The least square difference test was used for *post hoc* analysis. Non-normally distributed data were used after $\log_{10}$ transformation. Pearson´s and Spearman's correlation coefficients were used to analyse the relationship between normally and non-normally distributed parameters, respectively. To determine if sTWEAK was associated with the presence of NAFLD, multiple logistic regression analyses were employed (stepwise backward selection procedures). All variables associated in the univariate analyses ($p< 0.20$) with these two parameters and those variables known or likely to be associated with them (based on previous literature) were included in those regression models as independent variables. Intra-class correlation coefficients (ICC) were used to determine the degree of agreement between observers on the assessment of steatosis and between the grade of steatosis by ultrasound and histology; agreement was classified as fair (ICC=0.5-0.7), good (ICC=0.7-0.9), or almost perfect (ICC >0.90). *In vitro* experimental results are presented as mean±SE of 3-6 independent experiments. Differences between treatments were analyzed by a paired Student's *t* test. Two-tailed p-values <0.05 were

considered statistically significant. The calculations were made using STATA v.13.1 for Mac (StataCorp LP, College Station, TX).

RESULTS

Decreased sTWEAK concentrations are associated with NAFLD

A total of 112 severely obese patients and 32 healthy non-obese controls were included. The abdominal ultrasound study revealed that 59 severely obese patients had steatosis. The inter-observer agreement was almost perfect for steatosis (ICC=0.97). The observers reached a consensus about discordant ratings before the final analysis. Good agreement was found for steatosis identification between ultrasound and histology (ICC=0.79). Baseline characteristic of the patients included in the first cohort are shown in **Table 1**.

The presence of steatosis was associated with age, male gender, tobacco and with the presence of arterial hypertension, dyslipidaemia, T2DM and OSAS. Weight, waist-to-hip ratio, systolic and diastolic blood pressure, liver function test, triglycerides concentrations, fasting plasma glucose, serum insulin concentrations, HbA_{1c} and HOMA-IR were higher in severely obese patients with steatosis than in those patients without it (**Table 1**). sTWEAK concentrations were significantly lower in severely obese patients with steatosis than in severely obese patients and non-obese patients without steatosis however, no differences were found between non-obese and severely obese patients without steatosis (**Table 1**). A significant reduction of sTWEAK concentrations was also observed when subjects were classified according to their glucose tolerance status (**Figure 1a**). sCD163 levels were significantly higher in severely obese patients with and without steatosis than in non-obese subjects without steatosis (**Table 1**).

Spearman's correlation coefficients for sTWEAK and sCD163 are shown in **Supplementary Table 1**. Considering the most important associations, the univariate analysis showed that sTWEAK was significantly and negatively associated with NAFLD (r=-0.350, p<0.001), waist-to-hip ratio (r=-0.252, p=0.003), triglycerides concentrations (r=-0.262,

p=0.002), HOMA-IR (r=-0.373, p<0.001) and positively associated with HDL-cholesterol (r=0.266, p=0.002), whereas sCD163 concentrations showed a positive correlation with NAFLD (r=-0.245, p=0.004), BMI (r=0.288, p<0.001), systolic blood pressure (r=0.268, p=0.002), triglycerides (r=0.271, p=0.001) and HOMA-IR (r=0.315, p<0.001) and a negative correlation with HDL-cholesterol (r=-0.293, p<0.001) (**Supplementary Table 1**).

The presence of NAFLD was associated with age (OR=1.061, p=0.002), male gender (OR=0.389; p=0.009), tobacco (OR=1.678, p=0.012), hypertension (OR=4.016; p<0.001), dyslipidaemia (OR=1.883; p<0.001), glucose tolerance (IGT/IFG) (OR=4.125; p=0.001) and T2DM (OR=15.00; p<0.001), weight (OR=1.041; p<0.001), BMI (OR=1.119, p<0.001), waist (r=1.064, p<0.001), systolic (OR=1.046; p<0.001) and diastolic (OR=1.065; p<0.001) blood pressure, higher ALT (OR=1.145; p<0.001), AST (OR=1.143; p=0.005) and γGT (OR=1.069; p<0.001), lower HDL-cholesterol concentrations (OR=0.943; p<0.001), higher LDL-cholesterol (OR=1.013, p=0.029), higher triglycerides concentrations (OR=1.019; p<0.001), higher glucose (OR=1.075, p<0.001), insulin (r=1.064, p<0.001), HbA_{1c} (OR=14.538; p<0.001), HOMA-IR (OR=1.275; p=0.001), sCD163 (OR=1.014; p=0.009) and lower sTWEAK concentrations (OR=0.996; p<0.001) **(Supplementary Table 2)**.

Finally, we used multiple logistic regression analyses to determine the independent predictors of the presence of NAFLD in the whole population. The variables included in the final model were age, gender, tobacco, arterial hypertension, dyslipidaemia, hypolipemiant treatment, triglycerides, ALT, HbA1c, HOMA-IR, sTWEAK and sCD163. Results showed that age (p=0.002), BMI (p=0.010), higher concentrations of ALT (p<0.001), triglycerides (p=0.014), and lower concentrations of sTWEAK (p=0.022) were independently associated with the presence of NAFLD (**Table 2**).

Expression of TWEAK and Fn14 in human liver biopsies

Protein expression analysis

Protein expression levels of Fn14 were similar between steatosis and steatohepatitis samples; however, levels of Fn14 were higher in steatosis plus steatohepatitis relative to

samples of severely obese subjects biopsied without steatosis (**Figure 1b**). TWEAK protein levels were not different between the samples analysed.

Immunohistochemistry analysis

Haematoxylin and eosin staining was used to observe the general morphology of the liver samples (**Figure 2a, 2d and 2g**). Immunohistochemistry analysis revealed that TWEAK protein was barely detectable in all liver samples (**Figure 2b, 2e and 2h**). Consistent with the immunoblotting findings, Fn14 staining was strongly detected in steatosis and steatohepatitis samples, and was mainly localized in hepatocytes (**Figure 2f and 2i**). Greater Fn14 staining was detected in hepatocytes from steatohepatitis sections (**Figure 2i**). Liver samples of severely obese subjects biopsied without steatosis were negative for TWEAK and Fn14 staining (**Figure 2b and 2c).**

TWEAK treatment reduces triglyceride accumulation in human liver cells

We employed HHL immortalised primary human hepatocytes as an *in vitro* model to study steatosis. HHL cells were treated with palmitic acid (PALM) from 0.1-0.3 mM and lipid accumulation was measured after 24 h by Oil Red O staining. Results showed that cellular lipid accumulation was significantly higher after treatment with PALM at 0.2 and 0.3 mM than in control (**Supplementary Figure 1a**). Thus, 0.2 mM PALM was used to induce steatosis in HHL cells to achieve maximal fat over-accumulation with minimal cytotoxicity. Further, a progressive increase in Fn14 protein expression was observed relative to fat load (**Supplementary Figure 1b**).

HHL hepatocytes treated with PALM alone exhibited an increase in fatty acid (FA) accumulation relative to vehicle (**Figure 3A**). FA accumulation was reduced up to 22% by pretreatment, but not concurrent treatment, with TWEAK (**Figure 3a**). These results indicate that TWEAK modulates fat accumulation.

To explore the possible mechanism(s) by which TWEAK modulates FA deposition, we analysed the expression levels of hepatic lipid metabolism-related genes. The expression of fatty acid synthesis-related genes diacylglycerol O-acyltransferase 1 (*DGAT1*), fatty acid synthase

(*FASN*) and sterol regulatory element-binding transcription factor 1 (*SREBP1-c*) were unchanged by PALM treatment or by TWEAK pretreatment and PALM stimulation (**Figure 3b**). A similar result was found for carnitine palmitoyltransferase 1B (*CPT1-B*) gene expression, a gene involved in lipid oxidation (**Figure 3b**). By contrast, PALM treatment significantly increased *CD36* and *FATP5* expression (**Figure 3c)**, which are implicated in liver FA transport [17]. Additionally, the expression of the lipid droplet genes *PLIN1* and *PLIN2* was also upregulated by PALM treatment. Cells pretreated with TWEAK exhibited a decrease in *CD36 PLIN1* and *PLIN2* gene expression relative to PALM treatment alone (**Figure 3c**). This effect was also observed in HHL cells treated only with TWEAK. Western blotting confirmed the significant reduction of CD36 protein and a tendency for a decrease in PLIN2 expression with TWEAK treatment (**Figure 3d**).

In the development of hepatic steatosis, *CD36* is a transcriptional target of LXRα, PXR, PPARɣ[19] and nuclear factor erythroid-2 related factor 2 (Nrf2) receptor [29]. To further investigate the mechanisms involved in TWEAK modulation of FA deposition, we evaluated gene expression of *LXRα, PXR, PPARɣ* and *Nrf2* in PALM-treated HLL cells with or without TWEAK pretreatment. PALM treatment upregulated *PPARɣ*, *LXRα*, and *Nrf2* gene expression (**Figure 3e**). Pretreatment of cells with TWEAK significantly reduced *LXRα* gene expression and a trend for *PPARɣ* reduction was also observed (**Figure 3e**). No changes were observed in the expression of *Nrf2*.

To determine if TWEAK is implicated in nuclear factor-induced expression of *CD36*, we utilized synthetic LXR [30] and PPARɣ [31] agonists (GW3965 and rosiglitazone, respectively). As expected, addition of agonists alone increased *CD36* mRNA (**Figure 3f**), which was downregulated in hepatocytes pretreated with TWEAK. These data indicate that TWEAK might be implicated in transcriptional regulation of CD36.

DISCUSSION

This is the first study evaluating the potential role of TWEAK/Fn14 axis in the pathogenesis of NAFLD in humans. We report that lower sTWEAK and higher sCD163

concentrations are associated with steatosis, with lower sTWEAK concentrations emerging as one of the main predictors of NAFLD in severely obese patients. Additionally, liver biopsy analysis indicated that Fn14 expression was higher in obese patients with steatohepatitis than in patients with steatosis. Finally, studies performed in human liver cells demonstrated that TWEAK might act as a modulator of lipid deposition by down-regulating *CD36* transcription.

Cross-sectional studies have demonstrated that reduced sTWEAK concentrations are associated with type 1[10], T2DM [11] and severe obesity [6]. sTWEAK concentrations have been also related with the presence of insulin resistance [32] and an increased risk of metabolic syndrome [13]. Here, we found that sTWEAK concentrations decreased with the presence of steatosis assessed by abdominal ultrasound, and also in parallel with the diabetes status. Considering previous data demonstrating that lower sTWEAK concentrations are associated with obesity, diabetes and insulin resistance, all of them associated with an increased risk of NALFD [33], one might conclude that the association between sTWEAK and NAFLD is spurious. After adjusting for all these potential confounders, however, sTWEAK (negatively) concentrations were still associated with the presence of NAFLD, suggesting a direct relationship between both regardless of diabetes status. High levels of sCD163 have previously been associated with NAFLD and our data corroborate this recent finding[34].

There is no clear explanation for the observed decrease in sTWEAK levels in severely obese patients with steatosis. In general, the mechanisms leading to a reduction in the levels of sTWEAK in diseases associated with cardiovascular risk are not known. A reduction of sTWEAK in serum due to uptake by the Fn14 receptor could be a possibility. We show here an increase in Fn14 expression in human hepatocytes (thus increasing the availability for sTWEAK ligand), which could lead to a peripheral reduction of serum sTWEAK. An alternative hypothesis might be the involvement of the scavenger receptor CD163, known to participate in sTWEAK scavenging[5]. The reduction of sTWEAK could be therefore be related to the presence of sCD163, which we demonstrate to be up-regulated in patients with steatosis.

Fn14 is a highly inducible receptor and PALM serves as an inflammatory stimulus in HepG2 [35] and HHL cells (data not shown). We found that Fn14 but not TWEAK was

immunolocalised in liver section from steatosis and steatohepatitis patients. Fn14 staining was higher in severely obese patients with steatosis than in controls, localizing mainly in hepatocytes. Additionally, Fn14 was more conspicuously expressed in hepatocytes from steatohepatitis sections in agreement with previous results [14]. In patients with alcoholic steatohepatitis, Fn14 was found expressed mainly in a fraction of hepatocytes and in a subpopulation of progenitor cells. We failed to detect Fn14 staining in liver sections without steatosis, although other laboratories have detected Fn14 in bile ducts and in smooth muscle [14].

Studies of TWEAK in the liver have highlighted its involvement in proliferation of oval cells; however, all experimental data have been generated in mouse models [36]. In this setting, it seems that TWEAK selectively promotes proliferation of oval cells without affecting hepatocytes [14,37], and this effect might be mediated through Fn14. Both TWEAK and its receptor increase during liver regeneration but it appears to persist longer in the oval cell.

To question whether TWEAK was beneficial to hepatocytes in a lipid microenvironment, we tested the effect of TWEAK treatment during PALM-induced FA accumulation in HLL cells. Interestingly, TWEAK protected liver cells from fat accumulation. Oil Red O staining analysis revealed that sTWEAK pretreatment significantly ameliorated the excessive triglyceride accumulation in HHL cells induced by PALM. Also, PALM-mediated lipid deposition in HHL cells was linked to the up-regulation of *CD36* expression. *CD36* has an important role in the induction of hepatic steatosis [38] and its overexpression may contribute to the development of insulin resistance in NAFLD [39,40]. *CD36* up-regulation in HHL cells was also mirrored by a significant up-regulation in the expression of the lipid droplet formation genes, *PLIN1* and *PLIN2*, which are closely related to steatosis [39,40]. Interestingly, *CD36*, *PLIN1* and *PLIN2* gene expression were reduced in hepatocytes pretreated with TWEAK prior to a PALM-overload, or TWEAK alone.

Recent studies have demonstrated that LXR, PXR, and PPARɣ cooperate to promote hepatic steatosis by increasing the expression of CD36 [19,43,44]. To gain insight into the mechanisms underlying CD36 regulation by TWEAK, we performed *in vitro* experiments using PPARɣ and LXRα agonists. Agonist treatment increased *CD36* mRNA expression in HLL cells

and this increase was significantly reduced by pre-treatment with TWEAK. These findings suggest that TWEAK plays a protective role in PALM-induced hepatocyte steatosis lipid acumulation by inhibiting *CD36* expression, thereby attenuating fat deposition. Clearly, more studies are needed to corroborate these findings.

Some limitations of our study merit comment. As the subjects were recruited from a study with a different primary outcome objective, there may have been a selection bias. NAFLD was diagnosed by ultrasound, not by histology. Although ultrasound is operator dependent and usually detects steatosis only when more than 30% of hepatocytes are compromised by fat [45], we found good agreement between ultrasound and biopsy histology for steatosis. Our cross-sectional design cannot determine causality; only a longitudinal approach could determine the real associations of sTWEAK and steatosis. Limitations in the assay measurement of sTWEAK of positive patients that were below the detection limits of the assay should also be stated. Finally, the age range of the study subjects could also influence the disease, which would be very different depending on the duration of disease exposure.

In conclusion, we found that decreased sTWEAK concentrations are independently associated with the presence of NAFLD. Additionally, our findings in human hepatocytes broaden our knowledge on the negative regulation of lipid accumulation by sTWEAK, although the specific mechanisms remain to be elucidated. Future studies on animal models with restricted TWEAK transgene expression in the liver, subjected to high fat diet, will lead to a better understanding of the metabolic effects of sTWEAK in the development of hepatic steatosis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Figure Legends

Figure 1

TWEAK/Fn14 in huma hepatic steatosis.

a) sTWEAK circulating levels are reduced according to glucose tolerance status. NGT, Normal glucose tolerance; IGT, Impaired glucose tolerance; IFT, Impaired fasting glucose tolerance T2DM, type 2 diabetes mellitus. Each box plot shows the median, quartiles and extreme values. *p<0.05 compared to NGT, $^{\#}$p<0.05 compared to IGT/IFG. **b) TWEAK and Fn14 expression in steatosis and steatohepatitis human liver biopsies.** Protein extracts were analyzed by western blotting for TWEAK and Fn14 expression. Western blot and densitometric analysis normalized to β-actin are shown. Data are expressed as mean±S.E. *p≤0.05. (without steatosis: n=3 age, 51±4.52, gender, female BMI, 46±5.2; steatosis: n=2 age, 42.5±6.3, gender, female BMI, 45.5±6.3 and steatohepatits n=3 age, 48.6±8.3, gender, 2 female and 1 male, BMI, 46.12±3.36, all had NGT status).

Figure 2

Immunohistochemical localization of TWEAK and Fn14 in steatosis and steatohepatitis human liver sections. Localization of TWEAK and Fn14 protein in: steatosis (e and f, respectively) and steatohepatits of human liver sections (h and i, respectively). Immunostaining demonstrated that Fn14 protein immunolocalized in the hepatocyte of both steatosis (f) and steatohepatitis (i) liver sections. Representative images of n=3 cases in each group (without steatosis: 2 females; age: 44.0±9.0; BMI: 41.5±1.7 kg/m^2, steatosis: 1 male and 2 females, age: 49.0±7.0; BMI: 47.9±3.0 kg/m^2, steatohepatits: 1 male and 2 females; age: 51.5±1.5; BMI: 51.8±8.8 kg/m^2

Figure 3

TWEAK treatment modulates triglyceride accumulation in HHL human hepatocytes

HHL cells were treated with 100 ng/mL TWEAK or left untreated. After 6 h, the medium was replaced with fresh medium. Cells were either left unstimulated (Vehicle) or stimulated for 24 h with 0.2 mM palmitic acid (PALM 0.2) or with 100 ng/mL TWEAK plus 0.2 mM palmitic acid (PreTW+PALM) or palmitic acid and TWEAK concurrent treatment (PALM+TW) or TWEAK treatment alone (TWEAK). Results are expressed as mean±SE of at least 3 independent experiments.

a) Triglyceride accumulation assessed by Oil red O staining. *p≤0.05 compared to vehicle; $^{\#}$p ≤ 0.05 compared to PALM 0.2.

b) TWEAK or PALM treatment does not affect expression of fatty acid synthesis-related genes (*DGAT1*, *FASN* and *SREBP1-c*) or lipid oxidation *CPT1-B* gene expression.

c) TWEAK treatment reduces *PLIN1*, *PLIN2*, *CD36* and *FATP5* gene expression. *p ≤0.05 compared to vehicle; $^{\#}$p ≤0.05 compared to PALM 0.2.

d) CD36 and PLIN2 protein expression in hepatocytes treated with TWEAK. Western blot and densitometric analysis normalised to β-actin levels are shown. *p≤0.05 compared to vehicle and $^{\#}$p ≤0.05, compared to PALM 0.2.

e) *PPARɣ*, *LXRα Nrf2* and *PXR* gene expression. *p ≤0.05 compared with vehicle; $^{\#}$p ≤0.05 compared to PALM 0.2

f) HHL hepatocytes were treated or not with 100 ng/mL TWEAK for 6 h followed by 24 treatment of 5 μM rosiglitazone (PPARɣ agonist) or 1 μM GW501516 (LXRα agonist). *CD36*, *LXRα* and *PPARɣ* gene expression levels were analysed. (Control, medium only; Veh-RGZ and Veh-GW, media plus DMSO; PreTW+RGZ and PreTW+GW, cells pretreated 6 h with 100 ng/mL TWEAK followed by 24 h treatment with different agonists (rosiglitazone or GW3965). *p≤0.05 compared to vehicle; $^{\#}$p ≤0.05, compared to PALM 0.2 or vs agonist only.

Supplementary Figure 1

a) Palmitic acid treatment results in intracellular fat accumulation in HHL human hepatocytes.

HHL cells were treated with 0.2 mM palmitic acid for 24 h. Palmitic acid induced an increase in intracellular lipid accumulation as confirmed by Oil red O staining (×400 magnification). Data are expressed as mean±S.E. of three independent experiments. *p≤0.05 compared to vehicle.

b) Palmitic acid induces a dose-dependent increase in Fn14 protein expression in HHL human hepatocytes.

Results are mean±SE of three independent experiments. * p≤ 0.05 compared to vehicle.

Table 1. Baseline characteristics of the participants in the study according to the study group.

	Non-obese without NAFLD (n=32)	**Severely Obese without NAFLD (n=53)**	**Severely Obese with NAFLD (n=59)**	***p for trend***
Age (years)	40.4 (11.0)	43.5 (9.3)	47.7 (8.8)†	<0.001
Gender (Male/Female)	9/23	12/41	27/32	0.027
Race (caucasian) (n, %)	32 (100)	48 (90.6)	53 (89.8)	0.180
Tobacco				0.019
Non-smoker (n, %)	20 (62.5)	24 (45.3)	23 (39.0)	
Former smoker >1yr. (n, %)	8 (25.0)	18 (34.0)	12 (20.3)	
Current smoker (n, %)	4 (12.5)	11 (20.8)	24 (40.7)	
Hypertension (n, %)	1 (3.1)	16 (30.2)	30 (50.8)	<0.001
Dyslipidaemia (n, %)	2 (6.3)	9 (17.0)	26 (44.1)	<0.001
Glucose tolerance (n, %)				<0.001
Normal	30 (93.8)	36 (67.9)	22 (37.3)	
IGT/IFG	2 (6.3)	14 (26.4)	22 (37.3)	
T2DM	0 (0.0)	3 (5.7)	15 (25.4)	
OSAS (n, %)	0 (0.0)	14 (26.4)	20 (33.9)	0.024
Body composition				
Weight (kg)	64.2 (55.7-71.9)	113.7 (99.0-125.5)*	120.5 (108.5-136.3)†,‡	<0.001
BMI (kg/m^2)	22.7 (21.4-24.5)	43.7 (40.2-47.9)*	45.3 (41.6-49.4)†	<0.001
Waist-to-hip ratio	0.79 (0.74-0.84)	0.89 (0.82-0.98)*	0.97 (0.90-1.03)†,‡	<0.001
Blood pressure				
Systolic (mmHg)	120.5 (11.9)	134.6 (19.1)*	145.1 (19.4)†,‡	<0.001
Diastolic (mmHg)	67.0 (9.3)	73.7 (12.8)*	79.6 (11.7)†,‡	<0.001
Liver function tests				
ALT (U/L)	18 (14-22)	17 (15-24)	28 (22-43)†,‡	<0.001
AST (U/L)	27 (24-31)	18 (15-23)	27 (23-36)†	<0.001
GGT (U/L)	15 (12-26)	20 (14-26)	29 (22-40)†,‡	<0.001
Lipid profile				
Total cholesterol (mg/dL)	190 (165-220)	189 (166-208)	197 (176-229)	0.124
HDL-cholesterol (mg/dL)	67 (57-78)	50 (43-57)*	43 (38-47)†	<0.001
LDL-cholesterol (mg/dL)	104 (91-138)	119 (96-129)	121 (105-143)	0.061
Triglycerides (mg/dL)	69 (49-98)	100 (76-142)*	136 (103-189)†,‡	<0.001
Glucose (mg/dL)	84.0 (78.5-90.0)	90.0 (83.0-96.0)	100.5 (90.0-	<0.001

			111.0)[†,‡]	
Insulin (μIU/mL)	2.0 (0.7-4.3)	9.8 (4.9-16.1)*	17.2 (9.4-25.9)[†,‡]	<0.001
HbA_{1c} (%)	5.5 (5.3-5.6)	5.6 (5.4-5.7)	5.9 (5.6-6.2)[†,‡]	<0.001
HOMA-IR	0.44 (0.24-0.96)	2.28 (1.23-3.87)*	3.94 (2.18-6.62)[†,‡]	<0.001
sTWEAK (pg/mL)	597.0 (483.2-959.7)	603.8 (474.6-744.8)	440.0 (339.1-575.0)[†,‡]	<0.001
sCD163 (ng/mL)	124.1 (100.5-139.2)	139.4 (127.5-156.5)*	154.0 (130.9-173.8)[†]	0.001

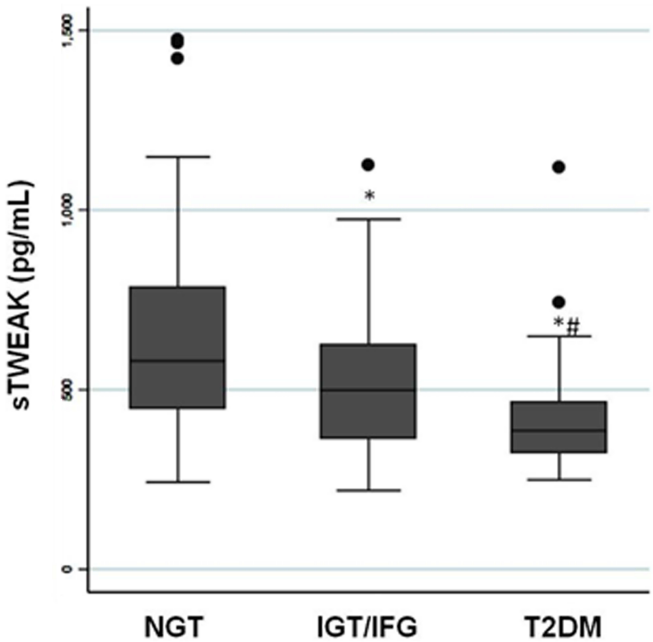
Data are presented as mean (SED) or median (25th-75th) quartiles, as appropriate. IGT, impaired glucose tolerance; T2DM, type 2 diabetes; OSAS, Obstructive sleep apnea syndrome; BMI, body mass index; ALT; alanine aminotransferase; AST aspartate aminotransferase; GGT, γ-glutamyltransferase; HOMA-IR, insulin resistance homeostatic model assessment. *$p<0.05$ for LSD post hoc test compared to the non-obese patients without NAFLD vs. severely obese patients without NAFLD. [†]$p<0.05$ for LSD post hoc test compared non-obese patients without NAFLD vs. severely obese patients with NAFLD. [‡]$p<0.05$ for LSD post hoc test compared severely obese patients without NAFLD vs. severely obese patients with NAFLD.

Table 2. Multivariable ordered logistic regression analyses to evaluate the independent predictors associated with NAFLD for the whole population

	OR	**SD**	**95% CI**	***p***
LR χ^2 99.15; p<0.001				
Age	1.103	0.035	2.036-1.174	0.002
BMI	1.098	0.040	1.022-1.180	0.010
ALT	1.161	0.042	1.081-1,248	<0.001
Triglycerides	1.014	0.038	1.003-1.025	0.014
sTWEAK	0.023	0.038	0.001-0.579	0.022

Figure 1

a)



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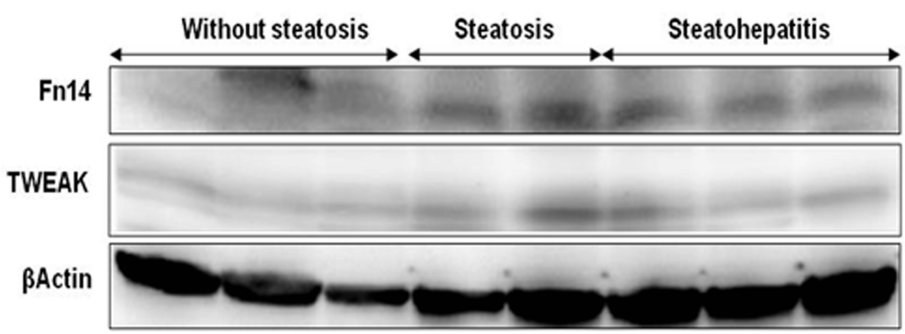

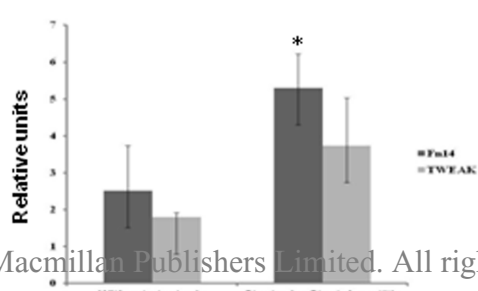

Figure 2