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Circulating ectodysplasin A is a potential biomarker for nonalcoholic fatty liver disease

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ABSTRACT

Background: Ectodysplasin A (EDA), a new hepatokine, may be involved in energy metabolism. This study aims to 1) investigate the role of EDA in hepatic steatosis in C57BL/6 mice and HepG2 cells; 2) evaluate serum EDA in nonalcoholic fatty liver disease (NAFLD) in human.
Methods: This study comprises an experimental study *in vitro* and *in vivo* and a hospital based case-control study. Western blotting, qPCR and ELISA were used to measure EDA levels. siRNA and shRNA were performed to knockdown EDA. An Adipokine Magnetic Bead Panel was performed to measure serum adipokines.
Results: Increased levels of hepatic and secreted EDA were detected in steatosis, *in vivo* and *in vitro*. Steatosis was ameliorated by EDA knockdown *in vitro*, while intrahepatic triglycerides content and liver enzymes were improved *in vivo*. Furthermore, knockdown of EDA upregulated lipolytic genes and suppressed lipogenic genes. Serum EDA in subjects with NAFLD was higher. Moreover, it reveals associations between circulating EDA and higher odds of NAFLD, while circulating EDA presented a practicable performance to identify NAFLD. Lastly, serum EDA level was dependent on BMI, TNF-α, T2DM and obesity.
Conclusions: EDA aggravates steatosis by striking balance between lipid deposition and elimination. It was a potential biomarker of NAFLD.

1. Introduction

Since obesity has become commonplace in the present society, the pattern of chronic liver disease is changing to present a noteworthy growth in patients with features of metabolic disorders, resulting in a rapidly increased prevalence of nonalcoholic fatty liver disease (NAFLD) [1]. NAFLD is believed to be the hepatic manifestation of insulin resistance (IR) and metabolic syndrome (MetS), even though the underlying mechanism of NAFLD is still unclear [2].

Emerging evidence shows NAFLD alters the secretion of proteins from the liver, *e.g.* hepatokines. These hormones function *via* autocrine and paracrine means, as well as through inter-tissue communication to impact energy metabolism in liver and other organs [3]. Ectodysplasin A (EDA), as a type II transmembrane protein, belongs to the tumor necrosis factor superfamily [4]. Firstly, the EDA gene was recognized to be responsible for X-linked hypohidrotic ectodermal dysplasia (XLHED) in human development [5]. Recently, Awazawa et al. [6] found in obese men, hepatic EDA expression increased and correlated with insulin resistance and clinical NASH scores, however it reversed as weight loss. It also indicated hepatic steatosis might promote Eda expression in a peroxisome proliferator-activated receptor (PPAR)γ- and RXR-α-dependent manner. Furthermore, their results supported EDA as a hepatokine impairs systemic insulin sensitivity in obesity, which involves the activation of inflammatory pathway in skeletal muscle. They suggested deregulated hepatic expression of EDA, particularly the EDA-A2 isoform, contributes to obesity-associated deterioration of insulin sensitivity in muscle and glucose homeostasis.

A better understanding the association between steatosis-associated

Abbreviations: ACC, Acetyl-CoA Carboxylase; AMPK, AMP-activated protein kinase; CPT1α, Carnitine palmitoyltransferase 1α; FAS, Fatty acid synthase; FFAs, Free fatty acids; FXR, Farnesoid X receptor; HFD, High-fat diet; H&E, Hematoxylin and eosin; MLYCD, Malonyl-CoA decarboxylase; NAFLD, Non-alcoholic fatty liver disease; PPARα, Peroxisome proliferator-activated receptor α; SCD, Standard chow diet; SREBP1c, Sterol regulatory element binding transcription factor 1; TG, Triglyceride

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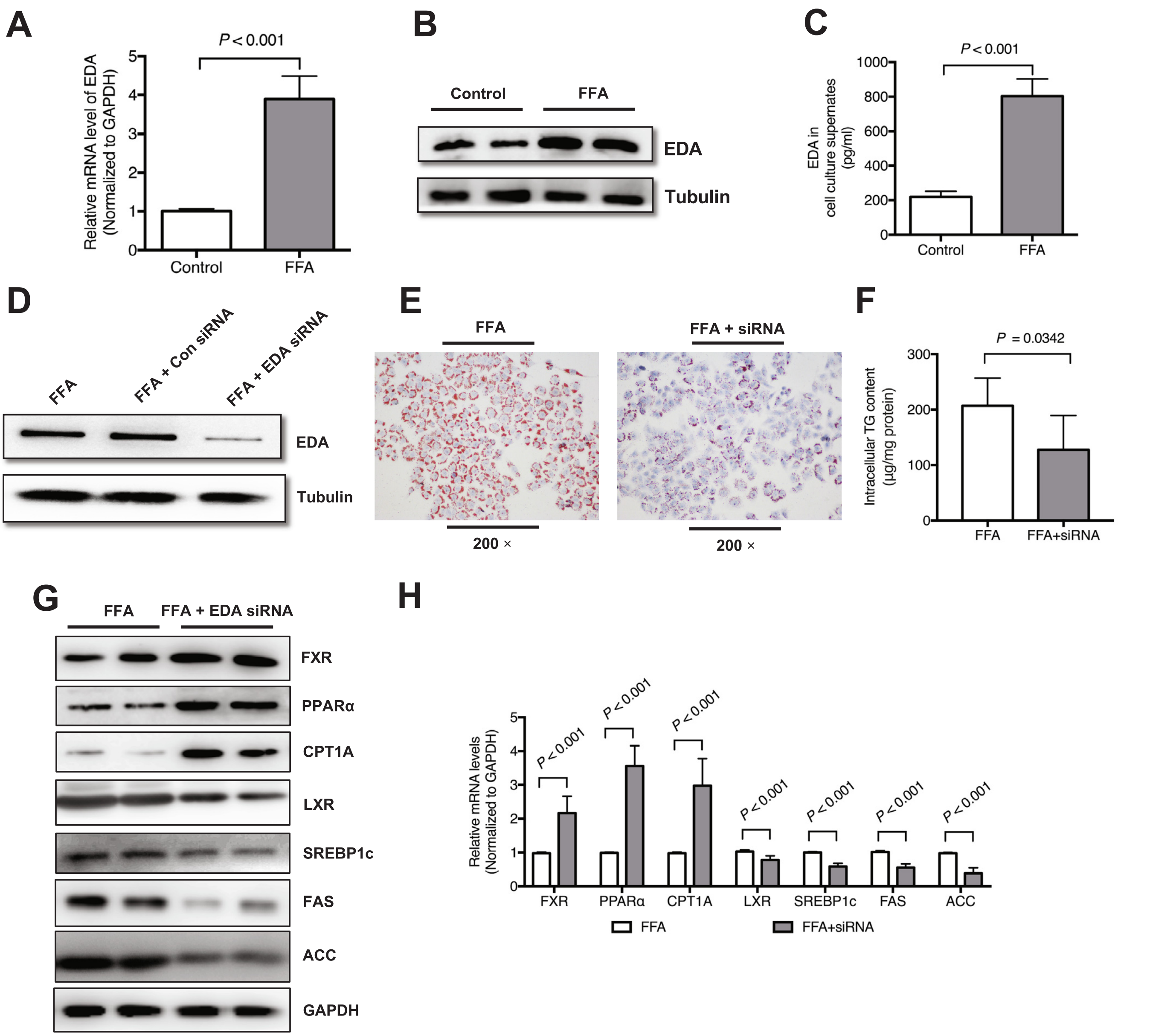

Fig. 1. Expression of EDA in HepG2 cells and EDA knockdown attenuated hepatic lipid accumulation. The levels of EDA mRNA and protein in HepG2 cells and cell culture supernatants increased with FFAs compared with controls.
A: The mRNA levels: Control vs. FFAs ($p < .001$);
B: The protein expression in HepG2 cells;
C: The protein levels in the culture supernatants of HepG2 cells ($p < .001$). EDA knockdown attenuated hepatic lipid accumulation.
D: hepatic EDA was knockdown by siRNA;
E: oil-red stain indicated lipid accumulation was attenuated by EDA knockdown;
F: intercellular TG content was markedly decreased by EDA knockdown ($p = .0342$). EDA regulated hepatic lipid metabolism-related genes
G: the protein levels of lipolytic and lipogenic metabolic nuclear receptors were regulated by EDA knockdown;
H: the mRNA levels of lipolytic and lipogenic genes were regulated by EDA knockdown.

hepatokine and the downstream biological consequences may facilitate the development of effective approaches to the management of metabolic diseases [3]. In this study, we tried to confirm the role of EDA in hepatic steatosis *in vivo* and *in vitro*. Besides, a hospital-based case-control study was conducted to evaluate the role of serum EDA-A2 in subjects with NAFLD.

2. Materials and methods

2.1. Basic experiment

2.1.1. Cell culture and treatment

To establish a cellular model of hepatic steatosis, HepG2 cells were exposed to a mixture of free fatty acids (FFA), including oleate and palmitate at a final ratio of 2:1 and a final concentration of 1 mM for 24 h, as previously reported [7].

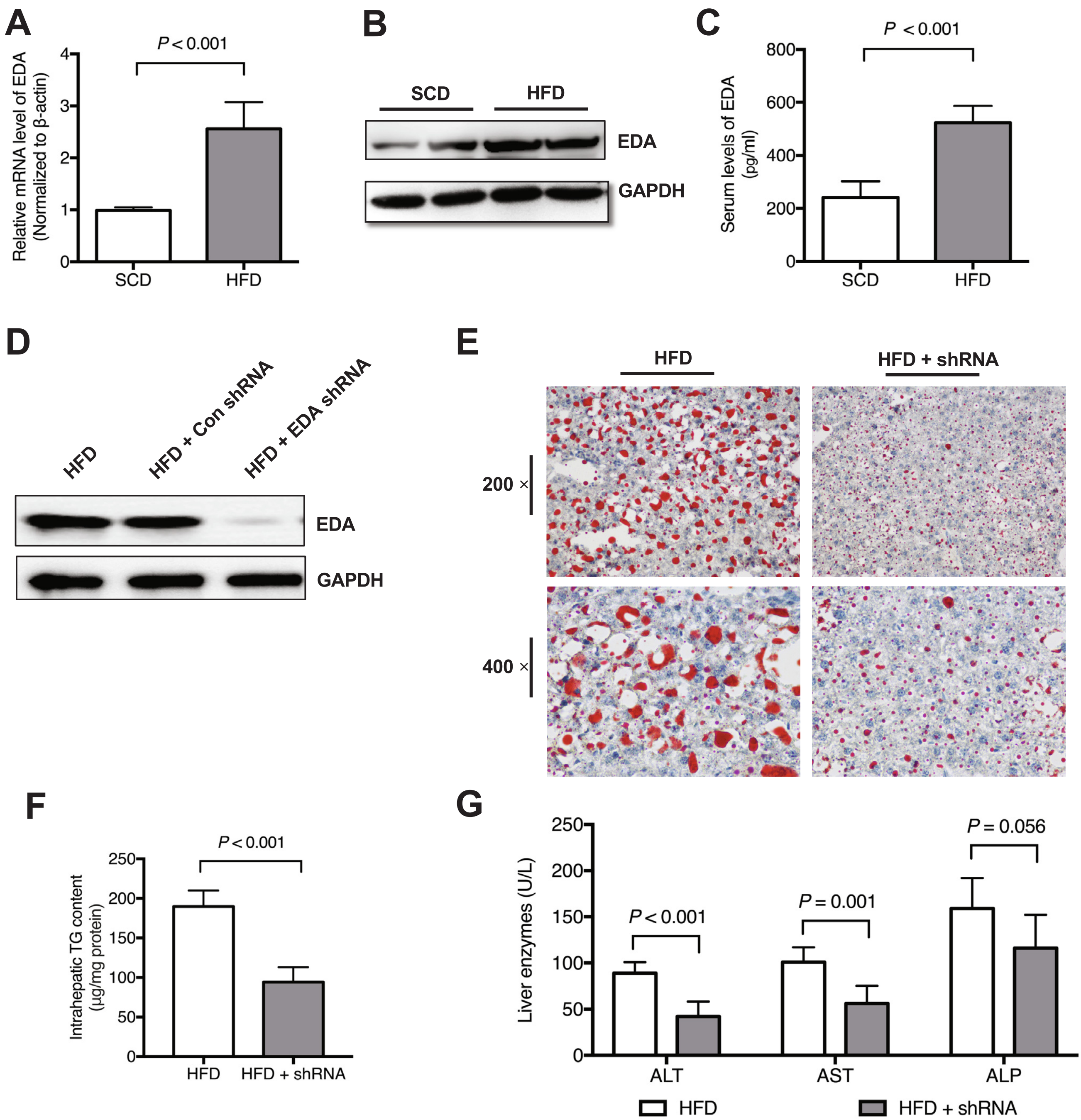

Fig. 2. Expression of EDA in mice and EDA knockdown attenuated hepatic lipogenesis. The levels of EDA mRNA and protein in mice increased in the HFD group compared with the SCD group.
A: The hepatic mRNA level: SCD vs. HFD ($p < .001$);
B: The hepatic protein expression;
C: The serum levels (SCD vs. HFD, $p < .001$). EDA knockdown attenuated hepatic lipogenesis in HFD-fed mice.
D: hepatic EDA was knockdown by EDA shRNA;
E: oil-red stain indicated lipid accumulation was attenuated by EDA knockdown;
F: intercellular TG content was decreased by EDA knockdown ($p < .001$);
G: EDA knockdown alleviated liver enzymes (ALT, $p < .001$; AST, $p = .001$; ALP, $p = .056$).

2.1.2. Animal procedures

Eight- to ten-week-old female, specific pathogen-free C57BL/6 mice were provided by and maintained at the Experimental Animal Center of Zhejiang Province (Hangzhou, China), as previously reported [7]. Mice were randomly divided into four groups, including (1) feeding a standard chow diet (SCD) *vs.* a high fat diet (HFD), (2) HFD *vs.* HFD + short hairpin RNA (shRNA) EDA. All animal studies were approved by the Animal Care and Use Committee of Zhejiang University in accordance with the Chinese guidelines for the care and use of laboratory animals.

2.1.3. Reagents

Rabbit monoclonal GAPDH (ab128915), Tubulin (ab7291) and liver X receptor (LXR) (ab24362) antibodies were purchased from Abcam Inc. (Cambridge, MA). Rabbit polyclonal ACC (ab72046), carnitine

Table 1
Characteristics of subjects according to NAFLD.

Parameters	Control	NAFLD	*P* for trends
No. of subjects	88	88	
Age (years)	52.7 ± 13.2	53.0 ± 13.1	0.882
Male, n (%)	59 (67%)	59 (67%)	1.00
BMI (kg/m^2)	22.8 ± 2.8	26.2 ± 3.4	**< 0.001**
WHR	0.885 ± 0.071	0.932 ± 0.081	**< 0.001**
EDA-A2 (pg/ml)	233.7 ± 197.9	445.6 ± 214.9	**< 0.001**
TG (mmol/l)	1.26 (0.98–1.94)	1.52 (1.11–2.26)	**< 0.001**
TC (mmol/l)	4.44 ± 1.06	4.59 ± 0.99	0.334
HDL-C (mmol/l)	1.24 ± 0.33	1.08 ± 0.32	**0.001**
LDL-C (mmol/l)	2.52 ± 0.80	2.60 ± 0.84	0.562
VLDL-C (mmol/l)	0.72 (0.54–0.99)	0.78 (0.59–1.12)	**0.001**
FPG (mmol/l)	5.56 (4.91–7.58)	5.74 (4.99–7.91)	0.225
HbA1c (%)	6.30 (5.60–8.78)	6.40 (5.70–8.93)	0.376
Fasting insulin (μU/ml)	13.6 ± 7.0	15.1 ± 7.6	0.160
HOMA-IR	3.37 (2.32–4.88)	3.67 (2.49–5.75)	0.058
Fasting C Peptide (ng/ml)	0.96 (0.61–1.39)	1.10 (0.64–1.77)	**0.014**

Data are mean ± SD or median (interquartile range) for continuous variables. BMI, Body mass index; FPG, fasting plasma glucose; HbA1c, Haemoglobin A1C; HDL-C, High-density lipoprotein cholesterol; HOMA-IR, Homeostasis model assessment of insulin resistance; LDL-C, Low-density lipoprotein cholesterol; TC, total cholesterol; TG, Triglycerides; VLDL-C, Very low-density lipoprotein cholesterol; WHR, Waist-to-hip ratio.

palmitoyltransferase 1A (CPT1A) (ab128568), farnesoid X receptor (FXR) (ab51970), PPARα (ab24509) and sterol regulatory element binding transcription factor 1 (SREBP1c) (ab28481) antibodies were purchased from Abcam Inc. (Cambridge, MA). Goat polyclonal EDA-A2 (AF922) antibody were purchased from R&D systems (Beverly, MA). Rabbit polyclonal antibody for EDA (PA1561) was purchased from BosterBio (Pleasanton, CA). Anti-rabbit IgG, HRP-linked Antibody (7074) and Anti-mouse IgG, HRP-linked Antibody (7076) were purchased from Cell Signaling Technology (Minneapolis, MN). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and antibiotics were obtained from Gibco (Shanghai, China). Oleate (O7501) and palmitate (P9767) were provided from Sigma-Aldrich Co. (St. Louis, MO).

2.1.4. ELISA kits

HepG2 cell culture supernatants were taken for the measurement of EDA-A2 levels using an enzyme-linked immunosorbent assay (Catalogue No. ELH-EDAA2–1; RayBiotech Life, Norcross, GA). The intra- and inter-assay variations were 7.8% and 11.7%, respectively. Blood samples from mice were taken for measurement of serum EDA levels using an enzyme-linked immunosorbent assay (Catalogue No. CSB-EL007389MO; CUSABIO Corp., Wuhan, China). The intra- and inter-assay variations were 9.7% and 11.3%, respectively.

2.1.5. Western blot analysis

Proteins were extracted using RIPA buffer (Applygen Technologies Inc., Beijing, China) with added protein and phosphatase inhibitor (Sigma), separated by SDS-PAGE, and electrophoretically transferred to PVDF membranes (Millipore). Chemiluminescence was visualized using an ECL kit (Lianke, Hangzhou, China), as previously reported [7].

2.1.6. Real-time quantitative PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Takara), as previously reported [7]. GAPDH and β-actin served as controls. Primer sequences are indicated in Supplementary File 1.

2.1.7. Small interfering RNA transfection (siRNA)

siRNAs were synthesized by Sigma-Aldrich. HepG2 cells were transfected with EDA siRNA or scramble siRNA as the negative control (NC) using Lipofectamine 2000 (Invitrogen, Shanghai, China). The efficacy of knockdown was determined by real-time PCR and western blot, as previously reported [7].

2.1.8. Triglyceride assay

Intracellular (HepG2) and intrahepatic (mice) triglycerides (TGs) were measured using a TG assay kit (E1013; Applygen Technologies Inc., Beijing, China), as previously reported [7].

2.1.9. Lentivirus target screening for RNAi

An RNAi target sequence was created within the mouse EDA gene along with a shRNA with the sequence shRNA-EDA, 5′-CCGGACGGCA CCTACTTCATCTATACTCGAGTATAGATGAAGTAGGTGCCGTTTT TTG-3′. The EDA RNAi sequence was inserted into a pShuttle vector and co-transduced into 293 T cells to produce the lentivirus. Mice were injected *via* the tail vein with 100 μL of lentivirus expression shRNA-EDA or the negative control on the 4th week before further experiment, as previously reported [7].

2.1.10. Histological analysis

Hematoxylin and eosin (H&E) stain and Oil Red stain were performed as previously reported [7].

2.2. Hospital-based case-control study

2.2.1. Subjects

The 88 NAFLD cases were selected from the patients attended to the outpatient department of the First Affiliated Hospital of Zhejiang University between January 2015 and April 2015. The 88 age−/sex-matched non-NAFLD controls were selected from the subjects attended annually health examinations during the same period.

The study was limited to subjects who had full records of anthropometric and biochemical data, together with result of abdominal ultrasonography. Subjects were excluded if they had cancer, malignancy, severe cardiopulmonary disorders, renal dysfunction, severe inflammatory diseases, thyroid dysfunction, viral/ drug-induced/ autoimmune liver diseases, pregnancy, excessively alcoholic consumption or exposure to antibiotics, steroids, probiotics, and prebiotics.

This study was approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University, in accordance with the Helsinki Declaration. All subjects gave written informed consent before participation.

2.2.2. Anthropometric examinations

Anthropometric examinations, including height, weight, waist circumference, and hip circumference were performed according to standard procedures as previously described [8].

2.2.3. Biochemical examinations

All subjects were informed to complete an overnight fast. About 10 ml whole blood samples were collected from every subject, and then serum samples were separated for immediate analysis or further analysis (stored at −80 °C). Serum analyses were measured using a Hitachi 7600 Auto-Analyzer (Hitachi, Tokyo, Japan) or an Abbott-Architect Immunoanalyzer (Abbott Laboratories, Abbott Park, IL).

2.2.4. Measurement of serum EDA

Serum EDA-A2 level was measured by ELISA (Catalogue No. ELH-EDAA2–1; RayBiotech Life, Norcross, GA). The intra- and interassay variations were 8.6% and 13.0%, respectively. Ten serum samples were selected randomly to validate the ELISA kit. This process included a two-time assessment (with an interval of one week) individually by two investigators.

2.2.5. Diagnosis of NAFLD

Real-time ultrasonography of the upper abdominal organs was performed by 2 experienced physicians using a Toshiba Nemio 20

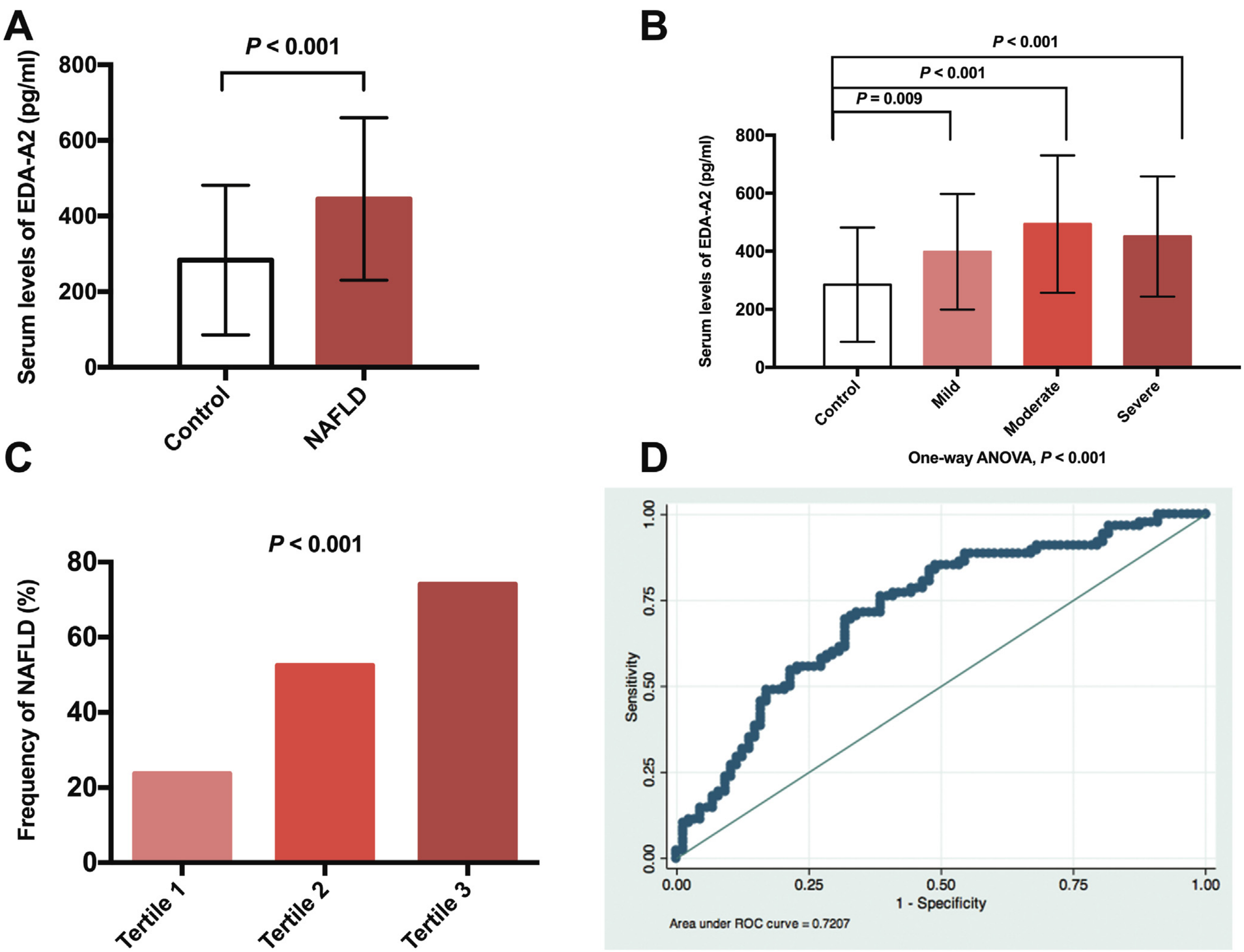

Fig. 3. Circulating levels of EDA in human.
A: comparison of serum EDA (pg/ml) levels by NAFLD ($p < .001$);
B: serum levels of EDA (pg/ml) according to three ultrasonographic degrees of NAFLD (compared with Control: Mild, $p = .009$; Moderate, $p < .001$; Severe, $p < .001$);
C: Frequencies (T1–T3: 23.7, 52.5, and 74.1%; $p < .001$) of NAFLD according to EDA tertiles;
D: ROC curve of EDA to identify NAFLD (0.721 [95% CI 0.645–0.796], $p < .001$).

sonography machine with a 3.5-MHz probe (Toshiba, Tokyo, Japan). NAFLD was diagnosed according to the guidelines for diagnosis and treatment of NAFLD issued by Fatty Liver and Alcoholic Liver Disease Study Group of the Chinese Liver Disease Association [9]. Ultrasonographic degrees of NAFLD were defined according to the Chinese standard (released in 2008) [10].

2.2.6. MILLIPLEX® human Adipokine magnetic bead panel kits

The MILLIPLEX® Human Adipokine Magnetic Bead Panels were performed to measure various specific adipokines in the serum samples. The measurement and analysis was processed according to the manufacturer's protocol. Millipore's MILLIPLEX MAP Human Adipokine Magnetic Bead Panel 1 kit was used for the following adipokines: Adiponectin, Adipsin, Lipocalin-2, Plasminogen activator inhibitor-1 (PAI-1) and Resistin (Cat. # HADK1MAG-61 K). Millipore's MILLIPLEX MAG Human Adipokine Magnetic Bead Panel 2 kit was used for the following: Hepatocyte growth factor (HGF), Interleukin (IL)-6, IL-8, Leptin, Monocyte Chemoattractant Protein-1 (MCP-1), Nerve growth factor (NGF) and Tumor necrosis factor-α (TNF-α) (Cat. # HADK2MAG-61 K).

2.2.7. Statistical methods

Normally distributed variables were presented as mean ± standard deviation (SD); variables with a skewed distribution were presented as median value (interquartile range). Normality of distribution was tested with the Kolmogorov-Smirnov test. The Student's *t*-test or Mann-Whitney *U* test for continuous variables and $\chi 2$ test or Kruskal-Wallis test for categorical variables were used to compare the parameters between the cases and the control. Comparisons among various groups used One-way ANOVA followed by *post hoc test* of LSD. To assess the relationship between EDA and NAFLD, we calculated the adjusted odds ratio (OR) and 95% confidence interval (CI) with a multivariable binary logistic regression model and multinomial logistic regression after adjustments. Bivariate correlation analyses of EDA with anthropometric/biomedical parameters and adipokines were performed using Pearson correlation and Spearman correlation coefficients. Multivariate stepwise linear regression analysis was conducted for EDA (dependent variable), including the variables that correlated with EDA as independent variables. The validation of ELISA kit used Cronbach's Alpha test. All statistical analyses were performed using Stata (version MP 11.2, StataCorp LP, College Station, Texas, USA). Associated data were plotted using GraphPad Prism (6.0, GraphPad Software, Inc., San Diego, CA, USA). Power of sample size was calculated by G*Power (version

Table 2
Odds ratios and 95% confidence intervals of the presence of NAFLD by EDA-A2 tertiles.

Comparisons	Models	Odds ratios (95% CI)	*P* values for trend
T1 *vs.* T2	Model 1	3.56 (1.62–7.83)	**0.002**
	Model 2	3.42 (1.54–7.61)	**0.003**
	Model 3	1.67 (0.65–4.25)	0.286
	Model 4	1.03 (0.98–1.08)	0.204
	Model 5	1.03 (0.98–1.08)	0.207
T1 *vs.* T3	Model 1	3.04 (2.00–4.62)	**< 0.001**
	Model 2	3.23 (2.08–5.02)	**< 0.001**
	Model 3	2.98 (1.74–5.12)	**< 0.001**
	Model 4	1.04 (1.01–1.06)	**0.002**
	Model 5	1.05 (1.02–1.07)	**0.001**

Model 1: unadjusted;
Model 2: adjusted for age and gender;
Model 3: adjusted for age, gender, BMI and WHR;
Model 4: adjusted for age, gender, BMI, WHR, ALT, AST, and GGT;
Model 5: adjusted for age, gender, BMI, WHR, ALT, AST, GGT, TG, HDL-C, LDL-C, and VLDL-C.
ALT, Alanine transaminase; AST, Aspartate transaminase; BMI, Body mass index; FPG, fasting plasma glucose; GGT, γ-Glutamyltransferase; HbA1c, Haemoglobin A1C; HDL-C, High-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; TG, Triglycerides; VLDL-C, Very low-density lipoprotein cholesterol; WHR, Waist-to-hip ratio.

3.1, Heinrich-Heine-Universität Düsseldorf, Germany) [11], as indicated in Supplementary File 2 (based on the base levels of EDA-A2). A two-sided $P < .05$ was considered statistically significant.

3. Results

3.1. Basic experiment

3.1.1. Expression of EDA in HepG2 cells

The mRNA level of EDA increased in human HepG2 cells exposed to FFAs compared with controls (Fig. 1A). The protein levels of EDA in HepG2 cells and cell culture supernatants also increased with FFAs (Fig. 1B and C).

Table 4
Multiple Linear Regression analyses with EDA-A2 as dependent variable.

	Variable	r^2	β	*P* value
Model 1		**0.230**		**< 0.001**
	BMI		0.490	< 0.001
Model 2		**0.455**		**< 0.001**
	BMI		0.484	< 0.001
	T2DM		0.479	< 0.001
Model 3		**0.531**		**< 0.001**
	BMI		0.489	< 0.001
	T2DM		0.395	< 0.001
	TNF-α		0.262	0.002
Model 4		**0.558**		**< 0.001**
	BMI		0.703	< 0.001
	T2DM		0.387	< 0.001
	TNF-α		0.258	0.002
	Obesity		−0.269	0.037

Values are corrected r^2 (r^2), standardized coefficients (β) and associated *P* values;
T2DM and obesity are binary nominal variables.
BMI, Body mass index; T2DM, Type 2 diabetes mellitus; TNF-α, Tumor necrosis factor-α.

3.1.2. EDA knockdown attenuated hepatic lipid accumulation in HepG2 cells

Fig. 1D presented hepatic EDA was knockdown by the treatment of EDA siRNA. As shown in Fig. 1E, lipid accumulation was attenuated by EDA knockdown. The intercellular TG content was markedly decreased in the FFA + siRNA grouped compared with FFAs alone (Fig. 1F, $p = .0342$).

3.1.3. EDA regulated hepatic lipid metabolism-related genes

Here, we found expression of the lipolytic metabolic nuclear receptors FXR and PPARα, the key fatty acid oxidative enzyme CPT1A were significantly elevated in EDA-knockdown HepG2 cell (Fig. 1G/H). Conversely, EDA knockdown obviously suppressed mRNA and protein levels of lipogenic metabolic nuclear receptors LXR and SREBP1c, the key fatty acid synthesis enzymes fatty acid synthase (FAS) and ACC (Fig. 1G/H).

Table 3
Correlations of serum EDA-A2 with various anthropometric/ biochemical parameters and adipokines.

Categories	Parameters	*r* value	*P* value	Categories	Parameters	*r* value	*P* value	Categories	Adipokines	*r* value	*P* value
Anthropometry	**Age**	**0.171**	**0.024**	**Lipid and Cholesterol Metabolism**	TG[a]	0.074	0.327	**Appetite and Energy Homeostasis**	Leptin[a]	0.096	0.443
	Height	0.020	0.797		TC	−0.113	0.135	**Insulin Sensitivity and Vascular Function**	**Resistin**	**0.288**	**0.019**
	Weight[a]	**0.229**	**0.002**		**HDL-C**	**−0.207**	**0.006**		**Adiponectin[a]**	**−0.263**	**0.033**
	BMI	**0.257**	**0.001**		**LDL-C**	−0.101	0.182	**Immunity and Inflammation**	**IL-6[b]**	**0.418**	**< 0.001**
	Hip circumstance	**0.182**	**0.016**		**VLDL-C[a]**	0.145	0.054		IL-8	0.177	0.154
	Waist circumstance	**0.294**	**< 0.001**	**Glucose Metabolism and Insulin Function**	**FPG[b]**	**0.327**	**< 0.001**		MCP-1	−0.072	0.565
	WHR	**0.226**	**0.003**		**HbA1c[b]**	**0.328**	**< 0.001**		**Lipocalin-2**	**0.252**	**0.041**
Liver Enzymes	ALT[a]	−0.045	0.550		FINS[a]	0.077	0.311		**TNF-α**	**0.342**	**0.005**
	AST[a]	−0.108	0.155		**HOMA-IR[a]**	**0.221**	**0.003**		NGF[a]	−0.051	0.684
	GGT[a]	0.133	0.079		**Fasting C Peptide[a]**	**0.213**	**0.005**		Adipsin	0.129	0.303
									HGF	0.177	0.116
								Hemostasis	**PAI-1**	**0.243**	**0.049**

ALT, Alanine transaminase; AST, Aspartate transaminase; BMI, Body mass index; FPG, fasting plasma glucose; GGT, γ-Glutamyltransferase; HbA1c, Haemoglobin A1C; HDL-C, High-density lipoprotein cholesterol; HGF, Hepatocyte growth factor; HOMA-IR, Homeostasis model assessment of insulin resistance; IL, Interleukin; LDL-C, Low-density lipoprotein cholesterol; MCP-1, Monocyte Chemoattractant Protein-1; NGF, Nerve growth factor; PAI-1, Plasminogen activator inhibitor-1; TC, total cholesterol; TG, Triglycerides; TNF-α, Tumor necrosis factor-α; VLDL-C, Very low-density lipoprotein cholesterol; WHR, Waist-to-hip ratio.
[a] lg(x) transformation was performed because of a skewed distribution.
[b] Spearman correlation analysis.

3.1.4. Expression of EDA in mice

The mRNA level of hepatic EDA increased in mice exposed to a HFD compared with a SCD (Fig. 2A). The protein levels in hepatocytes and serum also increased with the HFD (Fig. 2B and C).

3.1.5. EDA knockdown attenuated hepatic lipogenesis in HFD-fed mice

Mice fed eight weeks of a HFD had significantly higher TG contents, whereas Oil Red O staining showed that the increase in lipid droplets caused by HFD was markedly attenuated in mice with EDA knockdown, as shown in Fig. 2D and E. EDA knockdown by shRNA significantly attenuated the intrahepatic TG contents (Fig. 2F). EDA knockdown alleviated liver enzymes, serum alanine transaminase (ALT) and aspartate transaminase (AST), but not alkaline phosphatase (ALP) (Fig. 2G).

3.2. Hospital-based case-control study

3.2.1. General information of human study

A total of 176 subjects were enrolled in this case-control study. The validation of ELISA kit showed an excellent internal consistency (Cronbach's Alpha = 0.998). Given the serum EDA concentrations and the numbers of the case and the control, the power of the sample size was 0.99 (Effect size $d = 1.026$, Supplementary File 2).

3.2.2. Circulating levels of EDA-A2 in NAFLD

The characteristics of the subjects in the study are presented in Table 1 and Supplementary File 3. The serum concentration of EDA-A2 in the control (233.7 ± 197.9 pg/ml) was lower than it in NAFLD (445.6 ± 214.9 pg/ml, $p < .001$, Table 1 & Fig. 3A).

Fig. 3B shows the comparisons of serum EDA-A2 between the control and three ultrasonographic degrees of NAFLD. The control (284.8 ± 197.0 pg/ml) was lower than subjects with mild NAFLD (398.1 ± 199.3 pg/ml, $p = .009$), moderate (493.8 ± 236.8 pg/ml, $p < .001$), and severe (450.8 ± 207.1 pg/ml, $p < .001$), even though it failed to witness difference among the three degrees.

3.2.3. Characteristics of subjects according to EDA-A2 tertiles

All 176 subjects were divided into 3 groups, according to the tertiles of serum EDA-A2 concentrations. NAFLD presented a significant upward trend ($p < .001$) that the frequency of NAFLD increased (23.7%, 52.5%, and 74.1%), as EDA concentration elevated (Fig. 3C).

3.2.4. ORs of NAFLD by EDA-A2 tertiles

In binary logistic regression models (Table 2), compared with the 1st tertile, the 2nd of EDA indicated no association with the presence of NAFLD (adjusted OR = 1.03, 95% CI [0.98–1.08], $p = .207$), after controlling. However, the 3rd tertile revealed its association with higher odds of NAFLD (adjusted OR = 1.05, 95% CI [1.02–1.07], $p = .001$), in comparison with the 1st tertile.

3.2.5. ROC curve of EDA-A2

ROC curve of EDA-A2 was developed to predict the presence of NAFLD (Fig. 3D). Area under ROC was 0.721 ([95% CI 0.645–0.796], $p < .001$), with the sensitivity of 71.6%, the specificity of 65.9% and the accuracy of 68.2%.

3.2.6. Correlations between EDA-A2 and anthropometric/biochemical parameters

In Table 3, among all subjects, correlation analyses indicated the significantly positive associations of EDA with a series of anthropometric parameters, *e.g.* age ($r = 171$, $p = .024$), BMI ($r = 0.257$, $p = .001$) and WHR ($r = 0.226$, $p = .003$).

In terms of lipid and cholesterol metabolism, EDA witnessed an inverse association with HDL-C ($r = -0.207$, $P = .006$). Additionally, EDA correlated with FPG ($r = 0.327$, $p < .001$), Haemoglobin A1C (HbA1c) ($r = 0.328$, $p < .001$), HOMA-IR ($r = 0.221$, $p = .003$), and Fasting C Peptide ($r = 0.213$, $p = .005$), which are parameters of glucose metabolism and insulin function. However, no parameter of liver enzymes witnessed associations with EDA.

3.2.7. Correlations between EDA-A2 and cytokines

Table 3 also reveals the associations between EDA and various adipokines that EDA correlated with resistin ($r = 0.288$, $p = .019$) and adiponectin ($r = -0.263$, $p = .033$), which are related with insulin sensitivity. In terms of adipokines of immunity and inflammation, EDA witnessed correlations with IL-6 ($r = 0.418$, $p < .001$), lipocalin-2 ($r = 0.252$, $p = .041$), and TNF-α ($r = 0.342$, $p = .005$). Lastly, PAI-1, an adipokine of hemostasis, showed an association with EDA ($r = 0.243$, $p = .049$).

3.2.8. Multivariate linear regression

In the multiple linear regression analysis (Table 4), the best model (corrected $r^2 = 0.558$, $p < .001$) that predicted EDA-A2 levels included BMI ($p < .001$), T2DM ($p < .001$), TNF-α ($p = .002$) and Obesity ($p = .037$) as predictive variables.

4. Discussion

The present study shows that EDA, a novel hepatokine, aggravates hepatic steatosis *via* mediating lipolytic and lipogenic genes *in vivo* and *in vitro*. Furthermore, our hospital-based case-control study confirms the association between increased levels of serum EDA and NAFLD. It supports EDA is a potential serum biomarker for the clinical management of NAFLD.

Different splicing of the EDA transcript produces two isoforms [12]. EDA-A1 and EDA-A2 differ by two amino acids and bind to specific receptors, EDAR and XEDAR, respectively [13]. Previously, EDA was mainly known for the function of EDA-A1 in skin development, however, the biological role of EDA in adults, especially EDA-A2, have remained unclear [14]. Inspiration of Awazawa's study [6], we evaluated the role EDA in the development of hepatic steatosis.

In the basic experiment, we found increased levels of hepatic and secreted EDA-A2 under hepatosteatosis. TG accumulation in hepatocytes was induced from an imbalance between *de novo* lipogenesis and TG lipolysis/fatty acid oxidation [15]. The bile acid-activated nuclear receptor FXR and PPARα are essential for TG lipolysis [16]. As the downstream of PPARα, CPT1A transfers fatty acid from cytosol to mitochondria prior to β-oxidation [17]. *De novo* lipogenesis is controlled by several nuclear receptors and transcription factors, including LXR [18,19], which together with SREBP1c belongs to a network of nutrient sensors that are associated with the regulation of fatty acid synthesis and TG accumulation [20,21]. Furthermore, LXR-SREBP1c axis has been regarded as the upstream target of lipogenesis proteins, *e.g.*, fatty acid synthase (FAS), and ACC [22–24].

To confirm if EDA is involved in the development of hepatic steatosis, EDA was knockdown in the livers of HFD-fed mice and FFA-induced HepG2 cells. *In vitro*, TG accumulation was dramatically ameliorated by knockdown of EDA, while intrahepatic TG content and liver enzymes were improved *in vivo*. Furthermore, knockdown of EDA upregulated lipolytic genes and suppressed lipogenic genes. Thus, the results suggest that EDA might aggravate hepatic TG accumulation by striking the balance between *de novo* lipogenesis and TG lipolysis/fatty acid oxidation.

In the hospital-based case-control study, we found the serum concentration of EDA in the subjects with NAFLD was higher. Furthermore, it reveals an association between circulating EDA-A2 and higher odds of NAFLD, while EDA-A2 presented a practicable performance to identify NAFLD. Lastly, positive associations of EDA with a series of anthropometric parameters, *e.g.* age, BMI and WHR. Among variables of lipid and cholesterol metabolism, EDA witnessed an inverse association only with HDL-C. In terms of glucose metabolism and insulin function, EDA correlated with FPG, HbA1c, HOMA-IR, and Fasting C Peptide. The measurement of various serum concentrations of adipokines showed that EDA correlated with Resistin and Adiponectin (insulin sensitivity), IL-6, Lipocalin-2, and TNF-α (immunity and inflammation), and PAI-1 (hemostasis) [25–28]. Additionally, the linear regression analysis revealed a predicting model, including BMI, TNF-α, T2DM and obesity, explained 55.8% of the total variability of serum EDA levels.

Some limitations merit comment. To begin with, the role of EDA-A2's membrane receptor, XEDAR, requires further investigation. Secondly, the case-control design makes it difficult to determine the role of serum EDA in the development of NAFLD. Besides, the study population was all Han Chinese and enrolled from an urban area, which might result in selection bias. Lastly, the golden standard of NAFLD is liver pathological examination rather than ultrasonography that was used to define the degrees of NAFLD, even though ultrasonography has some advantages, *e.g.* safer, economical, repeatable, satisfied sensitivity and specificity [29]. On the meanwhile, other non-invasive tools, *e.g.* computed tomography and magnetic resonance imaging, should be considered in the future.

5. Conclusions

EDA was earlier recognized to involve in skin appendage formation during development, its biological function in adults however has been poorly understood [30–32]. Our study shows that EDA, as a hepatokine, aggravates hepatic steatosis by striking the balance between lipid deposition and elimination. It also suggests the potential of circulating EDA to be a biomarker of NAFLD. The findings might offer translational insights into the clinical management of NAFLD.

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Compliance with ethical standards

All animal studies were approved by the Animal Care and Use Committee of Zhejiang University in accordance with the Chinese guidelines for the care and use of laboratory animals. This study was approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University, in accordance with the Helsinki Declaration. All subjects gave written informed consent before participation.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.09.009.

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