SUPPLEMENTAL DATA

Material and methods

Patient selection

In order to analyze the effect of obesity and T2D on the gene expression levels of TNC, adipose tissue samples from 75 subjects (18 males and 57 females) recruited from healthy volunteers and patients attending the Departments of Endocrinology & Nutrition and Surgery at the Clínica Universidad de Navarra were used. Patients underwent a clinical assessment including medical history, physical examination, body composition analysis and co-morbidity evaluation by a multidisciplinary consultation team. BMI was calculated as weight in kilograms divided by the square of height in meters and body fat (BF) was estimated by air-displacement-plethysmography (Bod-Pod®, Life Measurements, Concord, CA) (1). The waist-to-hip ratio (WHR) was measured as the quotient between the circumference of the waist (at the midway level between the margin of the lowest rib and the iliac crest) and the hip (at the widest trochanters). Obese patients were further subclassified according to the established diagnostic thresholds for diabetes [normoglycemia (NG): fasting plasma glucose (FPG) concentration < 100 mg/dL and PG < 140 mg/dL 2-h after an oral glucose tolerance test (OGTT); T2D: FPG > 126 mg/dL or PG ≥ 200 mg/dL 2-h after OGTT)] (2). T2D subjects were not on insulin therapy or on medication likely to influence endogenous insulin levels. In addition, an intraoperative liver biopsy was performed in the obese patients during bariatric surgery to establish a histological diagnosis of the hepatic state as well as to analyze TNC gene expression levels. This procedure is not clinically justified in lean subjects. It has to be highlighted that liver sample biopsies are not a procedure performed on a routine basis.

The samples were collected from patients undergoing either Nissen fundoplication [for hiatus hernia repair in lean (LN) volunteers] or RYGB [for morbid obesity treatment in obese (OB) subjects] at the Clínica Universidad de Navarra. Both interventions were carried out via a laparoscopic approach. Tissue samples were immediately frozen in liquid nitrogen and stored at -80 ºC for subsequent analyses. The study was approved, from an ethical and scientific standpoint, by the Hospital's Ethical Committee responsible for research and the written informed consent of participants was obtained.

Blood assays

Plasma samples were obtained by venipuncture after an overnight fast. Glucose was analyzed based on enzymatic spectrophotometric reactions by an automated analyzer

(Hitachi Modular P800, Roche, Basel, Switzerland). Insulin was measured by means of an enzyme-amplified chemiluminescence assay (IMMULITE®, Diagnostic Products Corp., Los Angeles, CA) with intra- and inter-assay coefficients of variation of 4.2 and 5.7%, respectively. Insulin resistance and sensitivity were calculated using the HOMA and QUICKI indices, respectively (3). Total cholesterol, high-density lipoprotein (HDL-cholesterol) and low-density lipoprotein (LDL-cholesterol) levels were calculated as previously described (3). Uric acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ-glutamyltransferase (γ-GT) and creatinine were measured by enzymatic tests (Roche) in an automated analyzer (Roche/Hitachi Modular P800). High sensitivity C-reactive protein (CRP), fibrinogen and von Willebrand factor antigen (vWF) concentrations were determined as previously reported (3). Leptin was measured by a double-antibody RIA method (Linco Research, Inc., St. Charles, MO); intra- and inter-assay coefficients of variation were 5.0 and 4.5%, respectively.

Multiplex immunoassays

The circulating concentrations of different inflammatory markers were quantified with a Luminex™ 200 platform (Luminex Inc., Austin, TX) using microsphere-based multiplexing technology. The human immunoassay kit (Millipore Iberica, Madrid, Spain) encompassing analyte-specific components for the measurement of the MMP-1, -2, -7, -9 and -10 was used in the study. The standard curve was calculated using five-parameter-curve fitting, and results were analysed using the *Luminex IS™ 2.3* software (Luminex Inc.). Intra-assay precision ranged from 6.0 to 11.0%, while inter-assay precision was <17.0%. Calibrators, controls and samples were run in duplicate throughout the study.

RNA extraction and Real-time PCR

Adipose tissue RNA isolation was performed by homogenization with an ULTRA-TURRAX® T 25 basic (IKA Werke GmbH, Staufen, Germany) using QIAzol® Reagent (Qiagen, Valencia, CA). Samples were purified with the RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's directions and treated with DNase I (RNase-free DNase Set, Qiagen) in order to remove any trace of genomic DNA. For first strand cDNA synthesis constant amounts of 1 μg of total RNA were reverse transcribed in a 40 μL final volume using random hexamers (Roche) as primers and 200 units of M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) (4).

The transcript levels for *TNC*, *TLR4*, *MMP-2* and *MMP-9* were quantified by Real-Time PCR (7300 Real Time PCR System, Applied Biosystem, Foster City, CA). Primers and probes (Supplemental Table 1) were designed using the software *Primer Express 2.0* (Applied Biosystems) and purchased from Genosys (Sigma-Aldrich, Madrid, Spain). Primers or TaqMan® probes encompassing fragments of the areas from the extremes of two exons were designed to ensure the detection of the corresponding transcript avoiding genomic DNA amplification. The cDNA was amplified at the following conditions: 95 ºC for 10 min, followed by 45 cycles of 15 s at 95 ºC and 1 min at 59 ºC, using the TaqMan® Universal PCR Master Mix (Applied Biosystems). The primer and probe concentrations for gene amplification were 300 nmol/L and 200 nmol/L, respectively. All results were normalized to the levels of the ribosomal *18S* rRNA (Applied Biosystems) and relative quantification was calculated using the ΔΔCt formula. Relative mRNA expression was expressed as fold expression over the calibrator sample (average of gene expression corresponding to the LN group) as previously described (5). All samples were run in triplicate and the average values were calculated.

Western blot studies

Tissues were homogenized and protein content was measured as previously described (6). Equal amounts of protein (25 μg) were run out in 12% SDS-PAGE, subsequently transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA) and blocked in Tris-buffered saline (10 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.00) with 0.05% Tween 20 (TBS-T) containing 5% non-fat dry milk for 1 h at room temperature. Blots were then incubated overnight at 4 ºC with a murine monoclonal anti-TNC antibody (Abcam, Cambridge, UK) or murine monoclonal anti-β-actin (Sigma). The antigen-antibody complexes were visualized using horseradish peroxidase-conjugated anti-mouse IgG antibodies (1:5,000) and the enhanced chemiluminescence ECL detection system (Amersham Biosciences, Buckinghamshire, UK). The intensity of the bands was determined by densitometric analysis with the Gel Doc™ gel documentation system and the *Quantity One 4.5.0* software (Bio-Rad) and normalized with β-actin density values. All assays were performed in duplicate.

Immunohistochemistry of TNC

The immunohistochemistry of TNC was carried out using the indirect immunoperoxidase method as previously described (5). Sections (6 μm) of formalin-

fixed paraffin-embedded VAT were dewaxed in xylene, rehydrated in decreasing concentrations of ethanol and treated with 3% H_2O_2 (Sigma) in absolute methanol for 10 min at RT to quench endogenous peroxidase activity. Then, slides were blocked during 1 h with 1% goat or murine serum (Sigma) diluted in Tris-buffer saline (TBS) (50 mmol/L Tris, 0.5 mol/L NaCl, pH 7.36) to prevent non-specific adsorption. Sections were incubated overnight at 4 ºC with rabbit anti-human TNC (Sigma) antibody diluted 1:50 in TBS. After three washes (5 min each) with TBS, sections were incubated with horseradish peroxidase-conjugated anti-rabbit (Amersham Biosciences) polyclonal IgG diluted 1:100 in TBS for 1 h at RT. After washing in TBS, the peroxidase reaction was visualised with a 3,3'-diaminobenzidine (DAB, Amersham Biosciences)/H_2O_2 solution (0.5 mg/mL DAB, 0.03% H_2O_2 diluted in 50 mmol/L Tris-HCl, pH 7.36), as chromogen and Harris hematoxylin solution (Sigma) as counterstaining. Sections were dehydrated, coverslipped and observed under a Zeiss Axiovert 40 CFL optic microscope (Zeiss, Göttingen, Germany). Negative control slides without primary antibody were included to assess non-specific staining.

Cell culture

Human stromovascular fraction cells (SVFCs) were isolated from omental adipose tissue from obese normoglycemic subjects as previously described (6). SVFCs were seeded at 2 x 10^5 cell/cm^2 and grown in adipocyte medium [DMEM/F-12 [1:1] (Invitrogen), 17.5 mol/L glucose, 16 µmol/L biotin, 18 µmol/L panthotenate, 100 µmol/L ascorbate and antibiotic-antimycotic] supplemented with 10% newborn calf serum (NCS). After 4 days, the medium was changed to adipocyte medium supplemented with 3% NCS, 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX), 0.1 µmol/L dexamethasone, 1 µmol/L BRL49653 and 10 µg/mL insulin. After a 3-day induction period, cells were fed every 2 days with the same medium but without IBMX and BRL49653 supplementation for the remaining 7 days of adipocyte differentiation.

Differentiated human omental adipocytes and SVFC were serum-starved for 24 h and then treated with increasing concentrations of TNF-α (1, 10 and 100 ng/mL) (Sigma), LPS (10, 100 and 1,000 ng/mL) (Sigma) and TNC (1, 10, 100 nmol/L) (R &D systems) for 24 h.

Study in animals

In the first obesity model, 12-week-old male C57BL/6 mice were maintained during 20 weeks on a commercial high-fat diet [n=10; rodent diet with 60% fat calories, 23.0 kJ/g:

58.7% fat, 26.7% carbohydrate and 14.7% protein (diet F3282, Bio-Serv, Frenchtown, NJ, USA)] or on a normal diet [(n=8; rodent diet with 14 % fat calories, 12.1 kJ/g: 13% fat, 67% carbohydrate and 20% protein (diet 2014, Teklad, Harlan Laboratories, Madison, WI, USA)]. Both diets were isoproteic and contained a similar amount of sodium and phytates (7). Mice were purchased from The Jackson Laboratory (Bar Harbor, ME). In the second obesity model, 10-week-old male wild type (C57BL/6J) (n=9) and genetically obese *ob/ob* mice (C57BL/6J) (n=8) supplied by Charles River (L'Abresle, France) were used. All mice were maintained with controlled temperature of 22 ± 2 °C on a 12:12 h light-dark cycle (lights on at 08:00 am) under pathogen-free conditions. Body weight was recorded on a regular basis to monitor progression of the diet-induced and genetically obese mice. After 12 weeks on a high-fat diet, mice exhibited a higher final body weight than those on a normal diet (47.3 ± 1.3 vs 29.7 ± 0.7 g; $P<0.001$). Simialr results were found for *ob/ob* mice as compared to their wild type littermates (48.3 ± 1.8 vs 23.5 ± 1.1 g; $P<0.001$). Mice were sacrificed after 6 h of fasting by CO_2 inhalation. The epididymal adipose tissue depot was carefully dissected out, frozen in liquid nitrogen and stored at -80 ºC. Both murine obesity models also exhibited significantly increased epididymal fat depot weights ($P<0.01$). All experimental procedures conformed to the European Guidelines for the Care and Use of Laboratory Animals (directive 86/609), and the study was approved by the Ethical Committee for Animal Experimentation of the University of Navarra.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Differences in the proportion of subjects within groups regarding gender were assessed by using a contingency test (Chi-square test). Due to their non-normal distribution CRP and MMPs concentrations were logarithmically transformed. The normal distribution of the other variables was adequate for the use of parametric tests. Differences between groups were assessed by one-way ANOVA followed by Tukey's *post hoc* tests and two-tailed unpaired Student's *t*-test as appropriate. Differences between groups adjusted for age were analyzed by ANCOVA. Pearson's correlation coefficients (r) were used to analyze the association between variables. The calculations were performed using the SPSS/Windows version 15.0 statistical package (SPSS, Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

1. **Ginde SR, Geliebter A, Rubiano F, et al.** 2005 Air displacement plethysmography: validation in overweight and obese subjects. Obes Res. 13:1232-7.
2. **Genuth S, Alberti KG, Bennett P, et al.** 2003 Follow-up report on the diagnosis of diabetes mellitus. Diabetes Care. 26:3160-7.
3. **Gómez-Ambrosi J, Salvador J, Rotellar F, et al.** 2006 Increased serum amyloid A concentrations in morbid obesity decrease after gastric bypass. Obes Surg. 16:262-9.
4. **Gómez-Ambrosi J, Catalán V, Diez-Caballero A, et al.** 2004 Gene expression profile of omental adipose tissue in human obesity. FASEB J. 18:215-7.
5. **Catalán V, Gómez-Ambrosi J, Rodríguez A, et al.** 2011 Increased circulating and visceral adipose tissue expression levels of YKL-40 in obesity-associated type 2 diabetes are related to inflammation: impact of conventional weight loss and gastric bypass. J Clin Endocrinol Metab. 96:200-9.
6. **Rodríguez A, Gómez-Ambrosi J, Catalán V, et al.** 2009 Acylated and desacyl ghrelin stimulate lipid accumulation in human visceral adipocytes. Int J Obes. 33:541-52.
7. **Frühbeck G, Alonso R, Marzo F, Santidrian S** 1995 A modified method for the indirect quantitative analysis of phytate in foodstuffs. Anal Biochem. 225:206-12.

Supplemental Table 1. Sequences of the primers and TaqMan® probes

Gene (GenBank accession)	Oligonucleotide sequence (5'-3')
CCL2 (NM_002982)	
Forward	GCTCATAGCAGCCACCTTCATT
Reverse	TCTGCACTGAGATCTTCCTATTGGT
TaqMan® Probe	FAM-TCGCTCAGCCAGATGCAATCAATGC-TAMRA
CD68 (NM_001251)	
Forward	CACGCAGCACAGTGGACATT
Reverse	CGAGTTGCTGCAACTGAAGCT
TaqMan® Probe	FAM-TCGGCTCAGAATGCATCCCTTCGA-TAMRA
MMP2 (NM_004530)	
Forward	CCATTTTGATGACGATGAGCTATG
Reverse	GTTGTACTCCTTGCCATTGAACAA
TaqMan® Probe	FAM-CTTGGGAGAAGGCCAAGTGGTCCGT-TAMRA
MMP9 (NM_004994)	
Forward	GCCCGGACCAAGGATACAGT
Reverse	CCCCTCAGTGAAGCGGTACA
TaqMan® Probe	FAM-ACGCGCTGGGCTTAGATCATTCCTCA-TAMRA
TLR4 (NM_138554)	
Forward	CTGCGTGGAGGTGGTTCCTA
Reverse	CAGGTCCAGGTTCTTGGTTGAG
TaqMan® Probe	FAM-TTTCTACAAAATCCCCGACAACCTCCCCT-TAMRA
TNC (NM_002160)	
Forward	AGGCGATCCCAGACAGTCAGT
Reverse	TCCAGCTGACAGTAGCCGAATT
TaqMan® Probe	FAM-AACAACAGCCATGGGCTCCCCAAA-TAMRA
TNFA (NM_000594)	
Forward	CCCCAGGGACCTCTCTCTAATC
Reverse	ACATGGGCTACAGGCTTGTCA
TaqMan® Probe	FAM-CCTCTGGCCCAGGCAGTCAGATCAT-TAMRA

CCL2, chemokine (C-C motif) ligand 2; *CD68*, CD68 antigen; *MMP2*, matrix metalloproteinase-2; *MMP9*, matrix metalloproteinase-9; *TLR4*; toll-like receptor 4; *TNC*, tenascin C, *TNFA*, tumor necrosis factor α.

Supplemental Table 2. Anthropometric and biochemical characteristics of subjects included in the study classified according to the absence or presence of non-alcoholic fatty liver disease.

	Non-NAFLD	NAFLD
n	18	25
Age (years)	40 ± 15	41 ± 14
BMI (kg/m^2)	37.2 ± 7.1	40.0 ± 2.7
Body fat (%)	52.0 ± 4.9	49.5 ± 6.4
Waist (cm)	115.7 ± 10.7	119.1 ± 9.63
Waist-to-hip ratio	0.91 ± 0.07	0.94 ± 0.09
SBP	121 ± 16	126 ± 17
DBP	78 ± 7	77 ± 9
Fasting glucose (mg/dL)	92.5 ± 13.0	106.4 ± 31.43
2h OGTT glucose (mg/dL)	122.7 ± 23.7	158.3 ± 60.9*
Fasting insulin (μU/mL)	13.1 ± 6.3	14.3 ± 7.5
2h OGTT insulin (μU/mL)	94.5 ± 67.3	100.6 ± 65.0
HOMA	2.9 ± 1.6	3.5 ± 1.8
QUICKI	0.338 ± 0.038	0.325 ± 0.028
Triglycerides (mg/dL)	83 ± 28	114 ± 44*
Cholesterol (mg/dL)	184 ± 38	191 ± 41
LDL-cholesterol (mg/dL)	114 ± 32	115 ± 32
HDL-cholesterol (mg/dL)	55 ± 13	50 ± 13
Leptin (ng/mL)	58.5 ± 20.6	44.2 ± 22.2
CRP (mg/L)	7.9 ± 3.0	8.2 ± 4.1
Fibrinogen (mg/dL)	373 ± 94	351 ± 89
von Willebrand factor (%)	127 ± 54	119 ± 51
Homocysteine (μmol/L)	8.0 ± 2.2	9.5 ± 2.8
AST (UI/L)	13 ± 4	15 ± 6
ALT (UI/L)	18 ± 9	22 ± 11
ALP (UI/L)	93 ± 31	98 ± 25
γ-GT (UI/L)	13 ± 7	28 ± 6*
MMP-1 (pg/mL)	207 ± 85	150 ± 31
MMP-2 (ng/mL)	19.33 ± 6.17	19.58 ± 4.59
MMP-7 (ng/mL)	2.91 ± 1.18	3.24 ± 1.44
MMP-9 (ng/mL)	3.40 ± 2.13	3.54 ± 1.21
MMP-10 (pg/mL)	201 ± 153	232 ± 79

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; DBP, diastolic blood pressure; γ-GT, γ-glutamyltransferase; HOMA, homeostatic model assessment; MMP, matrix metalloproteinase; NAFLD, non-alcoholic fatty liver disease; OGTT, oral glucose tolerance test; QUICKI, quantitative insulin sensitivity check index; SBP, systolic blood pressure. Data are mean ± SD. Differences between groups were assessed by two-tailed unpaired Student's *t*-test. *P<0.05 vs subjects without NAFLD.

Legends for supplemental figures

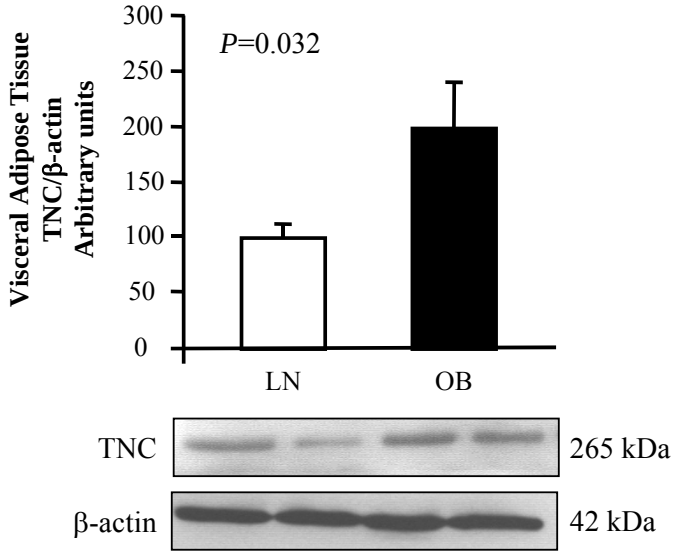
Supplemental Figure 1. Western-blot analysis of TNC levels in visceral adipose tissue (VAT) (A). Bars represent the mean ± SEM of the ratio between TNC to β-actin. The expression in LN subjects was assumed to be 1 (LN: n=9; OB: n=10). Differences between groups were analyzed by two-tailed unpaired Student's *t*-test. $^{*}P<0.05$ vs LN. Immunohistochemistry of TNC in VAT (B). A strong positivity (brown staining) was observed for TNC in cells of the SVFC. No immunoreactivity was found without the primary antibody (negative control). Images are representative of immunostaining in VAT from obese subjects (n=6; *Magnification, x400*).

Supplemental Figure 2. Analysis of mRNA levels of tenascin c (*Tnc*) and Toll-like receptor 4 (*Tlr4*) in epididymal adipose tissue of two different murine models of obesity. Gene expression levels of *Tnc* in a model of diet-induced obesity (normal diet: n=10; high-fat diet: n=8) (A) and in the leptin-deficient (*ob/ob*) mice (wild type: n=9; *ob/ob*: n=8) (B). Bars represent the mean ± SD of the ratio between the gene expression to *18S* rRNA. The expression level in wild type mice was assumed to be 1. Differences between groups were analyzed by two-tailed unpaired Student's *t*-test. $^{*}P<0.05$, $^{**}P<0.01$ vs wild type.

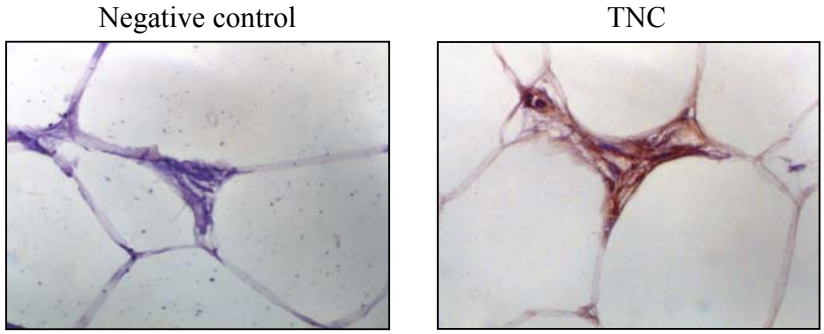
Supplemental Figure 3. Gene expression levels of *MMP9* (A) and *MMP2* (B) in VAT of LN, obese NG and obese T2D volunteers. Bars represent the mean ± SD of the ratio between the gene expression to *18S* rRNA. The expression level in LN subjects was assumed to be 1 (LN: n=13; OB-NG: n=32; T2D: n=30). Differences between groups were analyzed by one-way ANOVA followed by Tukey's tests. $^{*}P<0.05$, $^{**}P<0.01$ vs LN.

Supplemental Figure 4. Gene expression levels of *TNC* in hepatic biopsies (n=35) of obese NG and obese T2D volunteers (A) and obese patients with and without non alcoholic fatty liver disease (NAFLD) (B). Bars represent the mean ± SD of the ratio between the gene expression to *18S* rRNA. Differences between groups were analyzed by two-tailed unpaired Student's *t*-test.

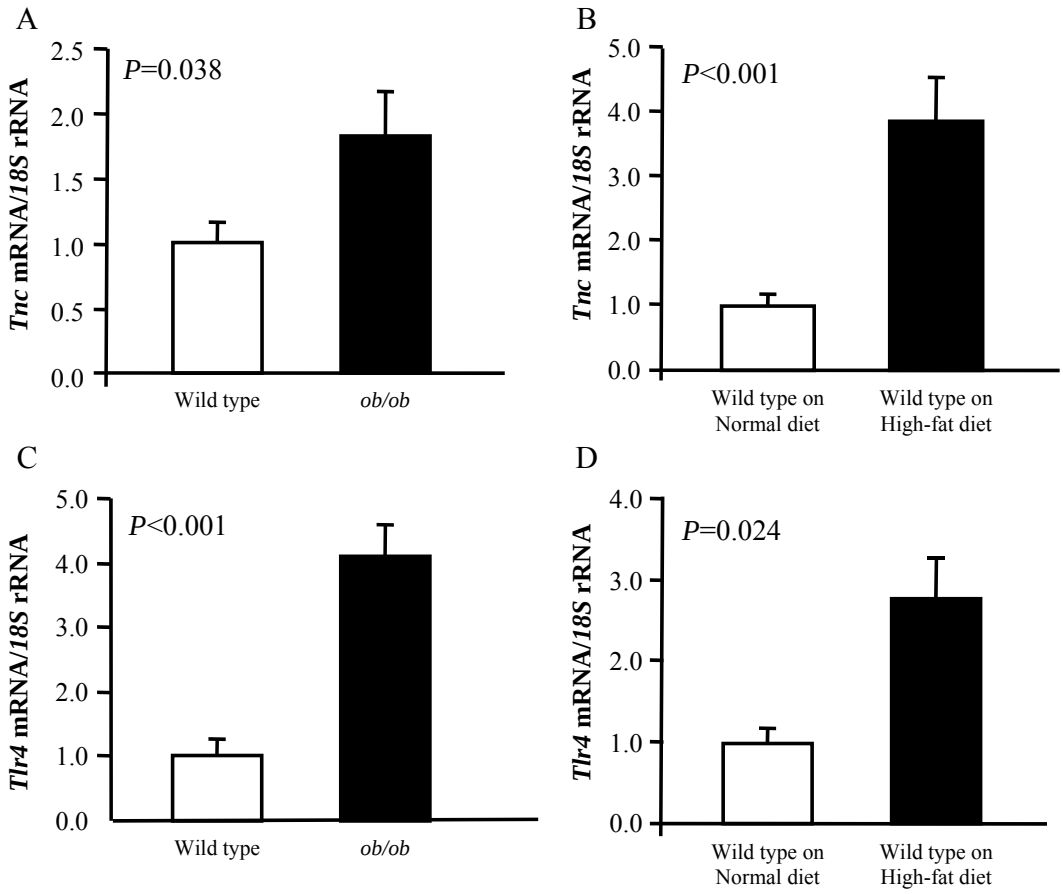
A



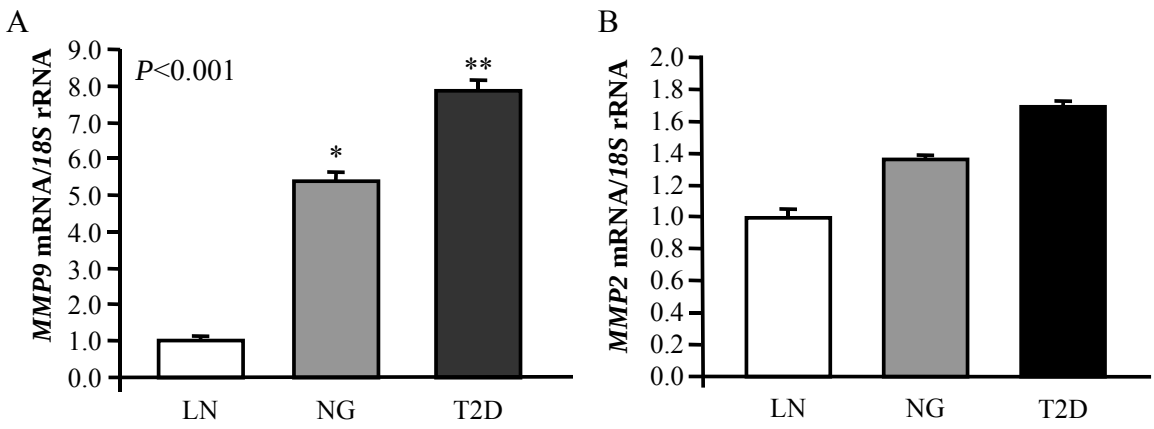
B



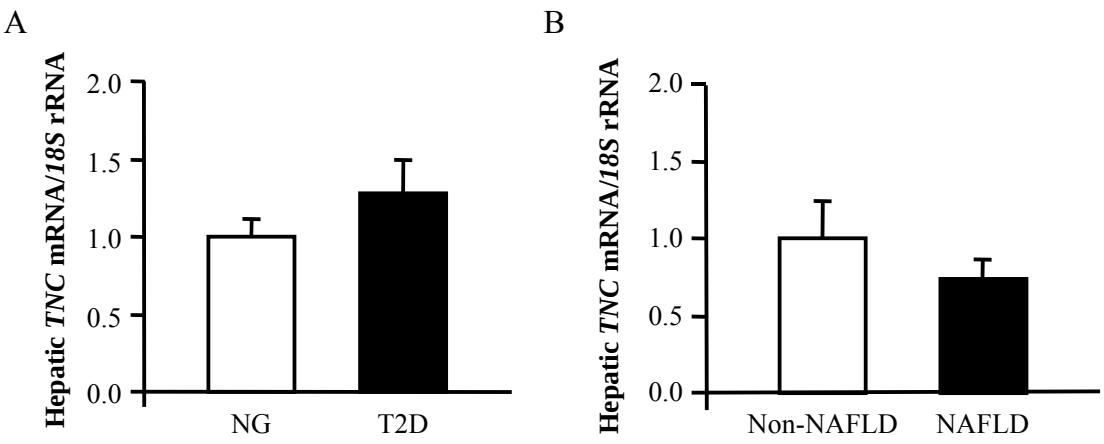
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4