RESEARCH

Role of dysfunctional adipocytes in cholesterol-induced nonobese metabolic syndrome

Jiung-Pang Huang^{1,2}, Sheng-Chieh Hsu^{1,3}, Yaa-Jyuhn James Meir^{1,4,5}, Po-Shiuan Hsieh⁶, Chih-Chun Chang⁷, Kuan-Hsing Chen⁸, Jan-Kan Chen^{2,9} and Li-Man Hung^{1,2,8}

¹Department and Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan

²Center for Healthy and Aging Research, Chang Gung University, Taoyuan, Taiwan

³Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Linkou, Taiwan

⁴Center for Tissue Engineering, Chang Gung Memorial Hospital, Linkou, Taiwan

⁵Department of Ophthalmology, Chang Gung Memorial Hospital, Linkou, Taiwan

⁶Department of Physiology and Biophysics, National Defense Medical Center, Taipei, Taiwan

⁷Department of Clinical Pathology, Far Eastern Memorial Hospital, New Taipei, Taiwan

⁸Kidney Research Center, Chang Gung Memorial Hospital, Linkou, Taiwan

⁹Department of Physiology and Pharmacology, College of Medicine, Chang Gung University, Taoyuan, Taiwan

Correspondence should be addressed to L-M Hung: lisahung@mail.cgu.edu.tw

Abstract

Many studies have reported the causes of obese metabolic syndrome (MS); however, the causes of nonobese MS (NMS) remain unknown. In this study, we demonstrated that inflamed dysfunctional adipose tissue plays a crucial role in cholesterol-induced NMS. Control (C), high cholesterol (HC) and HC with 10% fructose in drinking water (HCF) diets were fed to Sprague–Dawley rats for 12 weeks. After 12 weeks, the body weights of the C- and HC-fed rats were comparable, but the weights of the HCF-fed rats were relatively low. Cholesterol caused metabolic problems such as high blood pressure, hypercholesterolemia and hypoinsulinemia. The HCF-fed rats exhibited whole-body insulin resistance with low circulating high-density lipoprotein levels. Increases in the tumor necrosis factor α level in the plasma, the number of CD68+ macrophages and the free nuclear factor- κ B level in gonadal white adipose tissue (gWAT) resulted in local inflammation, which appeared as inflamed dysfunctional gWAT. Reduced superoxide dismutases (SODs) deteriorate natural antioxidant defense systems and induce reactive oxygen species in gWAT. Dysregulation of plasma levels of catecholamine, adipokines (leptin and adiponectin), hormone-sensitive lipase and perilipin in cholesterol-induced inflamed adipose tissue contributed to increased lipolysis and increased circulating nonesterified fatty acids. Cholesterol activated inflammation, lipolysis and cell death in 3T3-L1 adipocytes. Moreover, Chol-3T3-CM reduced the population of M2-type Raw264.7 macrophages, indicating that the macrophage polarization is mediated by cholesterol. Together, our findings indicate that inflamed dysfunctional adipocytes are critical in NMS, supporting the development of anti-inflammatory agents as potential therapeutic drugs for treating NMS.

Key Words

- ▶ cholesterol
- ▶ nonobese metabolic syndrome
- ▶ inflammation
- ▶ ROS
- ▶ adipose tissue

*Journal of Molecular
Endocrinology*
(2018) **60**, 309–323

Introduction

Obesity is associated with several complications, including diabetes, hypertension and cardiovascular disease (CVD) (Malnick & Knobler 2006, Poirier *et al.* 2006, Nguyen & El-Serag 2010). Chronic low-grade inflammation and increased macrophage infiltration are the major characteristics of hypertrophic adipose tissues, which result in obese insulin-resistant patients (Wellen & Hotamisligil 2003, Murano *et al.* 2008, Guerra *et al.* 2011). However, a proportion of obese patients are considered metabolically healthy, whereas some nonobese patients are considered metabolically unhealthy. Until now, increasing evidence has revealed that inflammation plays an important role in the pathogenesis of insulin resistance (de Luca & Olefsky 2008, Kramer *et al.* 2013, Phillips & Perry 2013).

White adipose tissue (WAT) is composed of lipids with a high ability to preserve triglycerides (TGs) during feeding and to release free fatty acids (FFAs) and glycerol during fasting (Guilherme *et al.* 2008). Therefore, the balance between stored and released lipids is exquisitely controlled by adipose tissue. Dysfunctional adipose tissue loses the function of a metabolic homeostasis regulator and promotes lipolysis. Increased circulating FFAs and ectopic lipid accumulation in the heart, skeletal muscle and liver have been reported to play a role in the development of metabolic diseases, type 2 diabetes mellitus and CVD (Guilherme *et al.* 2008, Hajer *et al.* 2008). Therefore, functional adipocytes with well-controlled lipid homeostasis and adipokine secretion are crucial in the regulation of whole-body metabolism to maintain an ideal body weight.

Obese patients have been reported to show chronic low-grade inflammation and negative effects of inflammation on adipose tissue (Tilg & Moschen 2006, Gustafson *et al.* 2007). However, the metabolic disturbances and inflammation status in patients with nonobese MS (NMS) remain unclear. Recently, cohort studies have reported that inflammation deteriorates the health of both obese and nonobese patients (Pajunen *et al.* 2011, Phillips & Perry 2013). Therefore, we suggest that inflamed dysfunctional adipocytes deteriorate metabolic homeostasis in nonobese patients.

Recent studies and our previous study have shown that dietary cholesterol promotes macrophage accumulation and inflammation in visceral adipose tissue, independent of body weight gain (Deng *et al.* 2008, Subramanian & Chait 2009, Pajunen *et al.* 2011, Chung *et al.* 2014). Moreover, the occurrence of hypercholesterolemia in

underweight and normal-weight unhealthy individuals suggests that the cholesterol level is a crucial factor that distinguishes metabolically healthy and unhealthy conditions (Goday *et al.* 2016). Therefore, animals that are fed a cholesterol-rich diet provide an appropriate animal model for the molecular study of NMS. In our study, we demonstrated that inflamed dysfunctional adipocytes are critical in the pathogenesis of cholesterol-induced NMS.

Materials and methods

Ethical approval

The animal care and experimental procedures used in this study conformed to the recommendations of the Guide for Care and Use of Laboratory Animals (Eighth Edition), published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Chang Gung University in Taiwan (Approval no. CGU12-166 and CGU13-006 to Li-Man Hung).

Experimental animals and diets

Male, 8-week-old Sprague–Dawley (SD) rats (weight, 250–300 g) were purchased from BioLASCO (Taipei, Taiwan). The animals were maintained in a climate-controlled facility under a 12-h light–darkness cycle with water and food *ad libitum*. The animals were randomly divided into three groups and fed either a regular chow diet (C, Labdiet 5001), high-cholesterol diet (Harlan Teklad 2018 plus with 5% coconut oil, 4% cholesterol and 1% cholic acid) or an HC diet with 10% fructose in drinking water (HCF) for 12 weeks. The primary compositions and energy contents of the C and HC diets are listed in [Supplementary Table 1](#) (see section on [supplementary data](#) given at the end of this article).

Plasma biochemistry

After 8 h of fasting, blood samples were collected from the animals by tail amputation and gently mixed with heparin. The samples were subsequently centrifuged at 9300 *g* for 10 min. Total cholesterol, triglycerides (TGs), high-density lipoprotein (HDL) and nonesterified fatty acids (NEFAs) were measured using commercially available kits (Randox reagent kits; Randox Laboratories, Antrim, UK). The insulin level was measured using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia; Uppsala, Sweden). Blood glucose was measured using One

Touch Sure Step strips (Life Scan, Johnson and Johnson, Milpitas, CA, USA). Plasma leptin and adiponectin levels were measured using ELISA, according to the General Sandwich ELISA protocol (ThermoFisher; <https://www.thermofisher.com/tw/zt/home/references/protocols/cell-and-tissue-analysis/elisa-protocol/general-elisa-protocol.html>). The following antibodies and recombinant proteins were used: recombinant rat adiponectin (4903-25, BioVision), rabbit antiadiponectin antibody (AB3269P, Merck Millipore), biotinylated antiadiponectin/Acrp30 antibody (BAF3100, R&D Systems), recombinant rat leptin (400-21, PeproTech, Rocky Hill, NJ 08553 USA), chicken antileptin antibody (AB3521, Merck Millipore) and biotinylated antileptin antibody (500-P185GBt, PeproTech). The circulating tumor necrosis factor (TNF) α level was measured using a commercial kit (rat TNF α ELISA kit, R&D Systems, RTA00).

Mean blood pressure measurement

The animals were anesthetized with inactin (100 mg/kg plus urethane, 0.3 g/kg, i.p.). The core temperature was maintained at 37°C by using heating pads (TC-1000 temperature controller; CWE). A microtip pressure-volume catheter (SPR-838; Millar Instruments, Houston, TX, USA) was inserted into the right common carotid artery for measuring the mean blood pressure (MBP).

Euglycemic-hyperinsulinemic clamp (EHC)

At the end of the study, the rats were anesthetized with pentobarbital (65 mg/kg, i.p.) and subjected to an euglycemic-hyperinsulinemic clamp (EHC) with tracer dilution, as described previously (DeFronzo *et al.* 1979, Meszaros *et al.* 1987, Smith *et al.* 1987, Hsieh 2004, Deng *et al.* 2008).

Histological analysis and CD68⁺ macrophage staining

Gonadal white adipose tissue (gWAT) was fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), dehydrated, embedded in paraffin and sectioned (6–8 μ m). The slides were subjected to hematoxylin and eosin (H&E) and CD68⁺ macrophage staining by using a standard protocol with deparaffinized, rehydrated antigen retrieval and quenching by xylene, ethanol, potassium PBS (KBPS; KH₂PO₄: 0.45 g, K₂HPO₄: 3.81 g, and NaCl: 9 g in 1 L of ddH₂O, pH 7.4), citrate buffer (10 mM C₆H₅O₇, 0.05% Tween 20, pH 6.0) and 0.3% H₂O₂. After H₂O₂

quenching, the slides were washed three times with KPBS and incubated with primary mouse antirat CD68 antibody (MCA341GA, AbD Serotec, NC, USA) for 24 h at 4°C. Secondary biotinylated antimouse IgG antibody (Vector Laboratories, Burlingame, CA, USA) was subsequently added and incubated for 1 h. For visualization, avidin-biotin peroxidase complexes (Vector Laboratories) were added with 3,3-diaminobenzidine tetrachloride (Vector Laboratories).

Western blotting

HNTG buffer was used to extract the gWAT lysate, according to a previously published protocol (HNTG buffer: HEPES, 50 mM; NaCl, 150 mM; glycerol, 10%; Triton X-100, 1%; pH 7.4). Na₃VO₄ (2 mM), NaF (20 mM) and phenylmethanesulfonyl fluoride (1 mM) were added in the buffer as phosphatase and protease inhibitors before use (Cao *et al.* 2002, Inoue *et al.* 2006). The crude homogenate was transferred to an Eppendorf tube and rotated at 4°C for 2 h for further protein extraction. The samples were centrifuged at 15,700 *g* at 4°C for 20 min. The fat cake was removed from the top, and the supernatant was transferred to a new Eppendorf tube. The gWAT lysates were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20, washed and blotted with antibodies. Information on the antibodies is listed in [Supplementary Table 2](#). The membrane was then incubated with horse radish peroxidase-conjugated secondary antibody before chemiluminescence detection (Pierce). ImageJ (RRID:SCR_003070, NIH, Bethesda, MD, USA) was used to quantify the blotting results.

gWAT superoxide anion measurement

The level of superoxide anions in gWAT was measured through modified lucigenin-enhanced chemiluminescence. The chemical specificity of this light-yielding reaction for superoxide anions was determined as previously described (Hung *et al.* 2002).

Reverse transcription polymerase chain reaction

The total RNA was extracted from rat gWAT with TRIzol (Life Technologies) according to the manufacturer's protocol. The target gene primers are as follows:

Leptin: Forward-GATGACACCAAAACCCTCATC,
Reverse-GCCACCACCTCTGTGGAGTAG.
Adiponectin: Forward-TGTTCTCTTAATCCTGCCCA,
Reverse-CCAACCTGCACAAGTTCCCTT.
Actin: Forward-TGGAATCCTGTGGCATCCATGAAAC,
Reverse-TAAAACGCAGCTCAGTAACAGTCCG.
Expected size: leptin, 353bp; adiponectin, 104bp;
and actin, 349bp.

3T3-L1 cell culture

3T3-L1 preadipocytes were purchased from FIRDI (Hsinchu, Taiwan) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum in an atmosphere containing 5% CO₂ at 37°C according to the ATCC-recommended protocol. The cells (P15 to P17) were cultured in MDI medium (high glucose DMEM containing 10% fetal bovine serum (FBS), 0.5mM iso-butylmethylxanthine (I7018, Sigma), 1μM dexamethasone (D4902, Sigma) and 10μg/mL insulin (Novo Nordisk A/S, Actrapid)) for 2 days. The culture medium was subsequently replaced with insulin medium (high glucose DMEM containing 10% FBS and 10μg/mL insulin) for another 6 days. All media contained 1% penicillin–streptomycin (100U/mL). Cholesterol (C3045, Sigma) stock solutions were prepared with 100% ethanol (0.1, 0.3, 1, 3, 10 and 30mg/mL). The cells were then cultured with cholesterol-containing medium (1% ethanol in the final culture medium) for 72h. After 72h, the medium was collected and labeled as 3T3-L1 conditioned medium (Chol-3T3-CM) for a Raw264.7 cell polarization test. The cell viability was assessed using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay.

Oil red staining

The lipid content of the 3T3-L1 cells was determined using oil red staining. Briefly, the cells were fixed in 4% formaldehyde and incubated for 1h in a 0.7% oil red solution (in 1,2-propanediol) with gentle agitation. The

cells were subsequently washed with 85% 1,2-propanediol and PBS. The stained oil red was extracted with isopropanol and measured spectrophotometrically at 490nm by using a microplate spectrophotometer (BioTek Instruments).

Real-time quantitative PCR

Total RNA was extracted from the 3T3-L1 cells after supplying different doses of cholesterol using the TRI reagent (Sigma, T9424). To avoid genomic DNA contamination, the RNA samples were treated with DNase I (Invitrogen, 18068-015). Complementary DNA was synthesized using the ToolScript MMLV RT kit. The mRNA levels were quantitatively assessed using SYBR-green-based RT-qPCR with gene-specific primers (Table 1) in a 7500 Fast Real-Time PCR System (Applied Biosystems). As an internal normalization control, 18s ribosomal RNA was used.

Inflammatory cytokine and glycerol levels in the 3T3-L1 culture medium

The levels of MCP-1, TNF-α, IL-1β, IL-6 and glycerol were measured in the 3T3-L1 culture medium after treatment with different doses of cholesterol for 3 days by using commercial glycerol assay (GY105, Randox Laboratories, Antrim, UK) and ELISA kits (Boster Bio, mouse MCP-1 Picokine ELISA kit EK0568; mouse TNF-α Picokine ELISA kit EK0567; mouse IL-1β Picokine ELISA kit EK0394; mouse IL-6 Picokine ELISA kit EK0411) according to the manufacturers' instructions.

Flow cytometric analysis of Raw264.7 M1-type and M2-type surface markers

Polarization of Raw264.7 macrophages by Chol-3T3-CM was performed through flow cytometry. In brief, Raw264.7 cells were purchased from FIRDI (Hsinchu, Taiwan), seeded at a density of 2 × 10⁴/dish in a 6-cm dish and cultured in 25% Chol-3T3-CM and 75% DMEM (containing 10% FBS) in an atmosphere containing 5% CO₂ at 37°C for 3 days.

Table 1 Primer sets for RT-qPCR.

Gene	Accession number	Forward	Reverse	Amplicon length (bp)
<i>Rn18s</i>	NR_003278.3	CGA GCC GCC TGG ATA CC	CCT CAG TTC CGA AAA CCA ACA A	76
<i>Mcp1</i>	NM_011333.3	CAG CCA GAT GCA GTT AAC GC	GCC TAC TCA TTG GGA TCA TCT TG	73
<i>Tnfα</i>	NM_013693.3	CCC TCA CAC TCA GAT CAT CTT CT	GCT ACG ACG TGG GCT ACA G	61
<i>Il1b</i>	NM_008361.4	ACT CAT TGT GGC TGT GGA GA	TTG TTC ATC TCG GAG CCT GT	199
<i>Il6</i>	NM_031168.2	TAG TCC TTC CTA CCC CAA TTT CC	TTG GTC CTT AGC CAC TCC TTC	76
<i>Casp3</i>	NM_001284409.1	CAG CCA ACC TCA GAG AGA CA	ACA GGC CCA TTT GTC CCA TA	190

The cells were harvested and washed two times with ice-cold PBS. The cells were then stained using antimouse F4/80-FITC (eBioscience, 11-4801-82, as a pan-macrophage marker), antimouse CD80-APC (eBioscience, 17-0801-82, as a M1-macrophage marker) and antimouse CD206-PE-Cy7 (eBioscience, 25-2061-82, as a M2-macrophage marker) antibodies in ice-cold PBS containing 2% FBS and 0.09% sodium azide for 1 h on ice. After staining, the cells were washed two times with ice-cold PBS containing 2% FBS and analyzed using the Attune NxT Acoustic Focusing flow cytometer (Thermo Fisher Scientific). Unstained Raw264.7 cells were used as the negative control.

Statistical analysis

The sample size of experiments were determined empirically based on previous studies and listed in the respective figure captions. Data are expressed as means $\pm$ S.E.M. Significance was analyzed using Student's unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's test. Two-tailed $P < 0.05$ was considered significant.

Results

General metabolic characteristics of rats after HC or HCF feeding

Eight-week-old male SD rats were fed C, HC or HCF diets for 12 weeks. The body weights of HC- and C-fed rats were comparable. However, the body weights of HCF-fed rats had decreased (Table 2). The food intake of the average HCF-fed rat was lower than that of the average C-fed rat; however, the calorie intakes were similar for all three groups because of the increased fructose water intake of HCF-fed rats compensated for their lower food intake.

The MBP was elevated in both HC- and HCF-fed rats. Both HC and HCF diets had no effect on blood glucose but resulted in hypoinsulinemia after 12 weeks, particularly in HCF-fed rats. Elevated plasma cholesterol and reduced plasma TG were observed in cholesterol-rich diet groups. However, plasma HDL had declined in HCF-fed rats, indicating severe metabolic disturbance and higher CVD risk because of the cholesterol–fructose combination. Altogether, these data indicated that rats fed a cholesterol-rich diet constitute an ideal animal model for studying the pathogenesis of NMS.

Cholesterol impaired whole-body insulin sensitivity and abrogated the phosphorylation of insulin-related downstream molecules in gWAT

Traditionally, adipose tissue in the visceral fat depot is more sensitive to the induction of inflammation and insulin resistance than is adipose tissue in the subcutaneous fat depot (Patel & Abate 2013). Therefore, we focused on the question of whether cholesterol induces inflammation and insulin resistance in gWAT. Insulin sensitivity was measured using the EHC method, which is the gold standard to assess whole-body insulin sensitivity *in vivo*. Cholesterol-rich diets, particularly HCF, impaired whole-body insulin sensitivity and blocked the phosphorylation of insulin-related downstream molecules without affecting the fat pad weight and adipocyte size (Fig. 1A, B and C). However, the liver weight and hepatocytes size were increased in the HC- and HCF-fed rats. Moreover, the liver weight of the HCF-fed rats was higher than that of the HC-fed rats, indicating a high level of TG accumulation in the liver after dietary fructose intervention (Fig. 1B and C, and Supplementary Fig. 3). Cholesterol markedly abrogated the expression of p-AKT–Ser473, p-ERK1/2–Thr202/Tyr and p-JNK1/2–Thr183/Tyr185, which are

Table 2 General metabolic characteristics of rats after HC or HCF feeding for 12 weeks.

	C	HC	HCF
Body weight (g)	544 $\pm$ 13	531 $\pm$ 13	506 $\pm$ 13*
Food intake (g/rat/day)	23.4 $\pm$ 1.2	20.5 $\pm$ 1.1	16 $\pm$ 0.7***
Calorie intake (Kcal/rat/day)	79 $\pm$ 4	83 $\pm$ 3.3	76 $\pm$ 4
Water intake (mL/rat/day)	47.6 $\pm$ 3.1	42.2 $\pm$ 2	61.3 $\pm$ 5.6*
MBP (mmHg)	105 $\pm$ 5	122 $\pm$ 5*	127 $\pm$ 5*
Blood glucose (mg/dL)	75 $\pm$ 1.7	80 $\pm$ 1.6	79 $\pm$ 1.6
Insulin (μ g/L)	0.65 $\pm$ 0.13	0.41 $\pm$ 0.03	0.3 $\pm$ 0.04**
Cholesterol (mg/dL)	92 $\pm$ 3.3	204 $\pm$ 10.1**	198 $\pm$ 13.5**
TG (mg/dL)	65 $\pm$ 5.9	45 $\pm$ 3.3*	50 $\pm$ 5.3**
HDL (mg/dL)	39.5 $\pm$ 4.7	35.2 $\pm$ 7.6	17.4 $\pm$ 3.5**

Data are expressed as means $\pm$ S.E.M. ($n = 8$ –10 rats per each group) Significant differences between subject and C is given as *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. HDL, high-density lipoprotein; MBP, mean blood pressure; TG, triglyceride.

the key mediators of the insulin-dependent anabolic metabolism of cell growth and differentiation (Fig. 1D and E and Supplementary Fig. 1A and B). However, the p-p38 level seemed similar after cholesterol feeding. Notably, although the adipocyte size was similar in cholesterol-fed rats, a crown-like structure, the hallmark of adipose tissue inflammation, was observed compared with the control rats (Fig. 1C). Therefore, we suggest that cholesterol-induced macrophage infiltration plays a crucial role in the development of local inflammation, which may further block insulin sensitivity and impair adipocyte function.

Expression of energy-regulatory proteins

Cholesterol-rich diets have no effects on adiposity in adipose tissue expansion and adipocyte enlargement; however, the expression of energy-regulatory proteins was examined to understand energy homeostasis after cholesterol alone or with fructose feeding (Fig. 1F and Supplementary Fig. 1C). ACC1 and FAS are key lipogenic enzymes in the regulation of *de novo* lipogenesis. HC slightly increased ACC1 protein expression but markedly reduced the FAS level. By contrast, HCF upregulated both

Figure 1

Cholesterol-rich diets induced whole-body insulin resistance and impaired insulin signaling. Insulin sensitivity index, GIR/insulin (GIR, glucose infusion rate) (A). Liver and fat pad (gWAT, gonadal white adipose tissue; sub fat, subcutaneous fat; B fat, brown fat) weight (B). Appearance of the liver and hematoxylin and eosin (H&E) staining of the liver (magnification: 400 $\times$) and gWAT (magnification: 200 $\times$). Arrows indicate crown-like structure (CLS) (C). Expression of insulin-related downstream proteins: p-AKT-Ser473, total AKT (D); p-ERK-Thr202/Tyr204, ERK, p-JNK-Thr183/Tyr185, JNK, p-p38-Thr180/Tyr182 and p38 (E) in gWAT. Expression of energy-regulatory proteins: ACC1, FAS, GLUT4, p-AMPK-Thr172 and AMPK in gWAT (actin was used as a normalization control) (F). The representative image is from one mouse from each treatment. ImageJ was used to quantify the Western blotting results, and all the data are expressed as the mean $\pm$ S.E.M., $n=5$ or 6 per group (* $P<0.05$ vs control; # $P<0.05$ vs HC).

ACC1 and FAS, clearly indicating the lipogenic potency of dietary fructose. The blood glucose levels were normal in the three groups; however, the plasma insulin levels observed in the HC- and HCF-fed rats were lower than those observed in the C-fed rats. The GLUT4 expression levels were decreased in the adipose tissues of HC- and HCF-fed rats, showing that a reduced GLUT4 level might be involved in the development of dysfunctional adipose tissues. AMPK and its activated form, p-AMPK-Thr172, regulators of the energy status responsible for maintaining cellular energy homeostasis, were reduced in cholesterol-fed rats. The excessive plasma levels of NEFAs in the HC- and HCF-fed rats (Table 3) were consistent with our hypothesis that cholesterol-induced dysfunction of adipose tissues is involved in the pathogenesis of NMS.

Epinephrine and norepinephrine play a role in cholesterol-induced lipolysis

The expression of PPAR γ , the key activator of adipocyte differentiation and adipogenesis (Sewter *et al.* 2002), was investigated after HC and HCF feeding. The PPAR γ expression levels were comparable in the gWAT in the three groups, indicating that the presence of cholesterol alone or with fructose does not affect adipogenesis (Fig. 2B, and Supplementary Fig. 1D), which is consistent with the similarity in adipocyte size in the three groups. However, a previous study reported that cholesterol may trigger lipolysis (Bhattacharyya *et al.* 1989), and catecholamines have been reported to enhance lipolysis after exercise and fasting (McMurray & Hackney 2005, Duncan *et al.* 2007). Therefore, we next examined whether catecholamines are involved in cholesterol-mediated lipolysis. We found higher levels of plasma catecholamines in the HC- and HCF-fed rats than those in the C-fed rats (epinephrine in HCF-fed rats and norepinephrine in HC- and HCF-fed rats) (Fig. 2A), demonstrating that catecholamines play a role in cholesterol-mediated lipolysis. Furthermore, the expression level of HSL, a regulator of the primary lipase responsible for the initial step of triglyceride hydrolysis (Kershaw *et al.* 2007), was upregulated in the HC- and

HCF-fed rats (Fig. 2B) compared with the C-fed rats; this might explain the excessive release of FFAs. Contrastingly, the expression of perilipin, the lipid droplet-associated protein, was reduced in the gWAT of HC- and HCF-fed rats (Fig. 2B).

Cholesterol triggers inflamed dysfunctional gWAT

Macrophage infiltration induces proinflammatory cytokines, and chronic inflammation is widely considered as a causative mediator of insulin resistance and lipolysis. Therefore, we analyzed the CD68⁺ cell content, which is a specific marker of macrophages, to validate whether macrophages are involved in cholesterol-mediated inflammation. As shown in Fig. 3A, several CD68⁺ cells were recruited in the gWAT of HC- and HCF-fed rats. Furthermore, the plasma levels of the proinflammatory cytokine TNF- α was markedly increased in the HCF-fed rats (HC, $P=0.09$ with an increasing trend, Fig. 3B). To further evaluate the inflammatory status in gWAT, we measured NF κ B-related signal expression, the most important factor for inflammation in gWAT. NF κ B and p-I κ B proteins (p-I κ B-Ser32/36) were highly expressed in gWAT, whereas I κ B expression was reduced in cholesterol-fed rats (Fig. 3C, and Supplementary Fig. 1E). These results revealed that freely activated NF κ B may translocate into nuclei and initiate inflammatory responses in gWAT. In addition, cholesterol reduced Cu/ZnSOD and MnSOD, antioxidant enzymes that prevent attack from reactive oxygen species (ROS) (Fig. 3D, and Supplementary Fig. 1F), which further resulted in the elevation of ROS because of the loss of the antioxidant ability (Fig. 3E). Altogether, macrophage infiltration, NF κ B signaling activation and impaired SOD expression all contribute to the inflamed condition of gWAT. Adipose tissue is the primary site of energy storage and serves as an endocrine organ. It secretes adipokines, such as leptin and adiponectin, to regulate metabolic homeostasis. Dysfunctional adipose tissue impairs adipokine production. As shown in Table 3, cholesterol-rich diets, particularly HCF, upregulated plasma leptin but downregulated plasma adiponectin. Abnormal levels

Table 3 Plasma NEFA, leptin and adiponectin after HC or HCF feeding for 12 weeks.

	C	HC	HCF
NEFA (mmol/L)	0.78 $\pm$ 0.04	0.92 $\pm$ 0.05*	1.02 $\pm$ 0.04**
Leptin (ng/mL)	5 $\pm$ 1.56	18.6 $\pm$ 2.9***	31.2 $\pm$ 3.4***
Adiponectin (μ g/mL)	5.5 $\pm$ 0.9	4.2 $\pm$ 0.7	3.4 $\pm$ 0.4*

Data are expressed as means $\pm$ S.E.M. ($n=8-10$ rats per each group) Significant differences between subject and C is given as *, $P<0.05$; **, $P<0.01$, ***, $P<0.001$. NEFA, non-esterified fatty acid.

Figure 2

Plasma catecholamine level and lipid droplet regulatory protein expression. Cholesterol increases the plasma catecholamine level: epinephrine (Epi) and norepinephrine (NE) (A). PPAR γ , HSL and perilipin expression in gWAT (B). The representative image is from one mouse from each treatment. Actin was used as a normalization control, and ImageJ was used to quantify the Western blotting results. Values are expressed as mean $\pm$ S.E.M., $n=5-6$ per group (* $P<0.05$ vs control).

Figure 3

Cholesterol-triggered inflamed gWAT and loss of SOD-mediated ROS defense ability. Immunohistochemical staining of CD68-positive macrophages in gWAT under 200 $\times$; arrows indicate CD68-positive cells (A). Plasma TNF- α level (B). NF κ B, p-I κ B-Ser32/36, and I κ B (C); Cu/ZnSOD and MnSOD protein expression (D). The representative image is from one mouse from each treatment. Actin was used as a normalization control, and ImageJ was used to quantify the Western blotting results. Gonadal WAT (gWAT) ROS level (E). Values are expressed as mean $\pm$ S.E.M., $n=5-6$ per group (* $P<0.05$ vs control).

of adipokines in gWAT were confirmed through RT-PCR (Fig. 4A and B, and Supplementary Fig. 2A and B). Collectively, the results suggest that cholesterol-rich diets cause inflammatory dysfunction of gWAT in NMS.

Cholesterol increases lipolysis in differentiated 3T3-L1 adipocytes

Our previous results demonstrated that cholesterol causes inflammatory dysfunction of gWAT in SD rats. Thus, we verified whether cholesterol can directly impair the function of 3T3-L1 adipocytes by measuring their lipid content. Well-differentiated 3T3-L1 cells (with lipid droplets) were treated with various doses of cholesterol for 3 days (Fig. 5A). As shown in Fig. 5B, cholesterol

Figure 4

Cholesterol dysregulates the mRNA expression of leptin and adiponectin in inflamed gWAT. Increased leptin (A) and decreased adiponectin mRNA expression (B) in gWAT after cholesterol treatment for 3 days ($n=5$ per group). Data are expressed as mean $\pm$ s.e.m. (* $P<0.05$ vs control).

treatment directly reduced the lipid droplet number and size, suggesting that cholesterol has a lipolytic effect. To further investigate whether cholesterol could trigger lipolysis, oil red staining and a glycerol assay were performed. (Glycerol is a lipolytic product, and the glycerol assay is the most direct method for assessing the extent of lipolysis.) As shown in Fig. 5C, D and E, cholesterol treatment reduced the lipid content of adipocytes and simultaneously increased the glycerol concentration in the 3T3-L1 culture medium, providing strong evidence of cholesterol-mediated lipolysis in 3T3-L1 adipocytes.

Cholesterol triggers inflammation and cell death in 3T3-L1 adipocytes

Inflammation plays a key role in lipolysis in adipocytes; thus, the expression levels of inflammatory cytokine genes were determined using RT-qPCR for evaluating the inflammatory status of the cells after cholesterol treatment. As shown in Fig. 6A, the mRNA levels of inflammatory cytokines (MCP-1, TNF- α , IL-1 β and IL-6) increased after cholesterol treatment. The level of the apoptosis trigger caspase 3 mRNA was also upregulated. These results revealed that inflammation caused by cholesterol plays a critical role in triggering lipolysis, which is independent of macrophage infiltration. Moreover, the augmentation of inflammatory cytokines (MCP-1, TNF- α and IL-6) due to cholesterol in the 3T3-L1 culture medium (Fig. 6B, C, D and E) may exert a deleterious effect on the cells through a paracrine or an endocrine action. The viability of cells was evaluated after cholesterol treatment by using the MTT assay. Cholesterol dose-dependently triggered 3T3-L1 cell death (Fig. 6F and G), demonstrating the lethal effects of cholesterol on 3T3-L1 adipocytes.

Figure 5

Cholesterol reduces the lipid content of 3T3-L1 adipocytes. Well-differentiated 3T3-L1 cells were treated with various concentrations of cholesterol (control, vehicle, 1, 3, 10, 30, 100, and 300 μ g/mL). Before the cholesterol treatment, the lipid droplet size and number were similar in all groups (A). After the treatment for 3 days, the lipid droplet size and number decreased dose-dependently (B). Oil red staining of cholesterol-treated 3T3-L1 cells (C). Lipid content was quantified at 490 nm through spectrophotometry (D), $n=4$ per group. Glycerol, the lipolytic product concentration in the 3T3-L1 cell culture medium, $n=8$ per group (E). Values are expressed as the mean $\pm$ s.e.m. (* $P<0.05$ vs vehicle).

Figure 6

Cholesterol directly triggers adipocyte inflammation and increases cell death. Differentiated 3T3-L1 cells were treated with various concentrations of cholesterol (vehicle, 1, 3, 10, 30, 100, and 300 μ g/mL) for 3 days. MCP-1, TNF- α , IL-1 β , IL-6 and caspase 3 mRNA levels in 3T3-L1 cells were determined through RT-qPCR (A). Levels of MCP-1, TNF- α , IL-1 β and IL-6 in the 3T3-L1 culture medium (B, C, D and E) after cholesterol treatment for 3 days. Cholesterol treatment dose-dependently induced 3T3-L1 cell death, as assessed using the MTT assay (F and G). Values are expressed as the mean $\pm$ s.e.m., $n = 3-4$ per group (* $P < 0.05$ vs vehicle).

Chol-3T3-CM reduces the polarization of Raw264.7 to M2-type macrophages

Macrophage polarization affects the inflammatory status during metabolic disorders. Therefore, the extent of Raw264.7 macrophage polarization was assessed through flow cytometry after Chol-3T3-CM incubation (pan-macrophage marker: F4/80; M1 marker: CD80; and M2 marker: CD206). Chol-3T3-CM-treated Raw264.7 cells (R1 population) exhibited an increase in cell size and granularity (R2 population), especially in the 100 and 300 μ g/mL

groups (Supplementary Fig. 4), demonstrating the effect of Chol-3T3-CM on cell differentiation/polarization. Therefore, two types of double-positive (F4/80 $^{+}$ /CD80 $^{+}$ and F4/80 $^{+}$ /CD206 $^{+}$) macrophage subpopulations (R1 and R2) were further analyzed. Chol-3T3-CM slightly reduced the percentage of M1-type (F4/80 $^{+}$ /CD80 $^{+}$) macrophages in the R1 subpopulation (V-3T3-CM: 94.6% vs 300-3T3-CM: 80.9%), whereas it showed no effect on the R2 subpopulation (V-3T3-CM: 99.7% vs 300-3T3-CM: 98.1%) (Fig. 7A and B). However, Chol-3T3-CM considerably reduced the percentage of M2-type (F4/80 $^{+}$ /CD206 $^{+}$)

Figure 7

Cholesterol reduces the polarization of Raw264.7 to M2-type macrophages. Raw264.7 macrophages were cultured in 25% Chol-3T3-CM (control, vehicle, 1, 3, 10, 30, 100 and 300 µg/mL) and 75% DMEM (containing 10% FBS). The cells were then analyzed through flow cytometry to detect the polarization of Raw264.7 macrophages. Percentages of F4/80⁺/CD80⁺ macrophages in the R1 (A) and R2 subpopulations (B). Percentages of F4/80⁺/CD206⁺ macrophages in the R1 (C) and R2 subpopulations (D). A schematic that illustrates the pathogenesis of how cholesterol-rich diet triggers NMS. TNFR, TNF- α receptor; β AR, β adrenergic receptor (E).

macrophages in the R1 (V-3T3-CM: 35.3% vs 300-3T3-CM: 7.9%) and R2 subpopulations (V-3T3-CM: 87.5% vs 300-3T3-CM: 31.2%). The Chol-3T3-CM-induced reduction in M2-type macrophage polarization might contribute to the abrogation of the regulation of inflammation and exacerbate metabolic disorders.

Discussion

Studies have described that inflammation may cause metabolic disease in both obese and nonobese patients (Kramer *et al.* 2013, Phillips & Perry 2013). Therefore, it is important to understand that either obesity or inflammation can be critical in the regulation of insulin resistance and MS. In the present study, we elucidated the role of inflamed dysfunctional adipose tissue in cholesterol-induced NMS. According to MS criteria, HCF-fed rats exhibited MS with insulin resistance, elevated blood pressure and low HDL. However, HC and HCF diets reduced plasma TG, consistent with studies that reported lower plasma TG after dietary cholesterol intervention in wild-type and ApoE-null mice (Wouters *et al.* 2008, Obama *et al.* 2011). Adipokine levels were also dysregulated in HC- and HCF-fed rats.

Insulin/AKT/mitogen-activated protein kinase (MAPK) signaling regulates insulin sensitivity. Defective activations of AKT and MAPK/ERK are potential contributors to insulin resistance (Zhang *et al.* 2011, Ruiz-Alcaraz *et al.* 2013). The cholesterol treatment considerably reduced the expression of p-AKT and p-ERK in the gWAT of nonobese rats (Fig. 1D and E). This result was similar to that in the gWAT of obese rats. Excessive leptin from inflamed dysfunctional gWAT may play a role in the inhibition of pre-proinsulin gene transcription and insulin secretion (Kieffer & Habener 2000). Moreover, cholesterol-triggered pancreatic β -cell death might directly reduce plasma insulin levels after consumption of a high-cholesterol diet (Zhao *et al.* 2010). Therefore, we speculate that, in our study, cholesterol simultaneously reduced insulin sensitivity and insulin secretion. A high-fat diet enhances adiposity in individuals with hypertrophic adipocytes and hyperinsulinemia (Huang *et al.* 2017). By contrast, the HC and HCF diets demonstrated catabolic reaction characteristics with lipolysis and hypoinsulinemia. Excessive levels of plasma catecholamines play a role in cholesterol-mediated lipolysis. Moreover, AMPK and p-AMPK downregulation might be involved in the lipolysis process. Downregulation of AMPK and p-AMPK expression reduced the suppression of HSL expression,

enhanced lipolysis in adipose tissue and increased the rate of FFA release into the circulation. Excess circulating metabolic substrates (e.g., glucose and FFAs) have been reported to impair AMPK activity (Kraegen *et al.* 2006, Saha *et al.* 2010). Therefore, increased levels of plasma FFAs might abrogate AMPK activity, initiating a vicious cycle.

Cholesterol is an essential component of various cell types and is a precursor of all steroid hormones. It is also responsible for normal body functions (Ikonen 2008). Therefore, the physiological functions of cholesterol mainly occur inside different cells rather than in the plasma. Excessively high levels of cholesterol in the plasma are considered to cause CVD and to be a component of MS. However, the molecular-level understanding of the negative effects of cholesterol in patients with MS is limited. The ATP-binding cassette transporters (ABCA1 and ABCG1) are responsible for cholesterol and lipid efflux to the serum and HDL release from cells (Weibel *et al.* 2014). Low expression levels of ABCA1 increase cholesterol and triglyceride storage in adipose tissues, thereby enlarging the fat pad and increasing body weight (de Haan *et al.* 2014). However, ABCA1 expression is induced by cholesterol loading and decreases after cholesterol removal (Lawn *et al.* 1999, Santamarina-Fojo *et al.* 2001). Therefore, ABCA1 expression induced by high levels of cholesterol may be involved in the pathogenesis of dysfunctional adipose tissue.

The induction of impaired glucose tolerance, insulin resistance, hyperinsulinemia, hyperlipidemia and hypertension by dietary fructose has been extensively studied (Elliott *et al.* 2002, Basciano *et al.* 2005). The liver is the main organ involved in metabolizing fructose and rapidly synthesizing TG through a significantly enhanced rate of *de novo* lipogenesis (Basciano *et al.* 2005). The liver weight of the HCF-fed rats was considerably higher than that of the HC-fed rats, indicating TG accumulation in the liver after fructose intervention; eventually a disturbance in the energy balance between the liver and adipose tissue was observed. The weight of gWAT and the size of adipocytes from HC and HCF are similar. Farina *et al.* have demonstrated that fructose rich diet (FRD) increases the contents of saturated fatty acids (SFAs, C16:0 and C18:0) in a hypertrophic-abdominal adipose tissue (AAT) (Farina *et al.* 2013). In addition, FRD also decreases mono-unsaturated (MUFAs, C16:1) and polyunsaturated (C18:3 n-3 and C20:4 n-6) fatty acids (PUFAs) (Farina *et al.* 2013). Moreover, FRD increases fatty acids release from AAT (Farina *et al.*

2013). Our previous article has also demonstrated the fructose-induced hypertrophic adipocytes (Huang *et al.* 2017). Therefore, the levels of SFA, MUFA and PUFA in HCF may play a role in the induction of hypertrophic adipocytes. By contrast, the lipolytic factors from cholesterol (increased oxidative stress, inflammation and elevated HSL protein level) may counteract the fructose-mediated lipogenesis and trigger the inflamed dysfunctional adipose tissue. HCF-induced NMS was reported previously (Deng *et al.* 2008). Rabbits fed a high-cholesterol diet exhibited pathological features of inclusion body myositis, which could result in muscular dystrophy and weakness (Chen *et al.* 2008). Based on growing evidence, we suggest that the inflammation from cholesterol-induced muscular dystrophy causes body weight loss in HC- and HCF-fed rats.

We observed that cholesterol intervention induces inflammation, independent of the body weight. Our animal and 3T3-L1 cell experiments are the first to address the effects of cholesterol on NMS and clearly demonstrate that inflammation, lipolysis, adipocyte death and macrophage shifting are involved in cholesterol-induced NMS, as illustrated in Fig. 7E. This study provides novel insight, indicating the potential for developing therapeutic strategies for treating NMS with cholesterol-lowering and anti-inflammatory agents.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-17-0194>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by a research grants from Chang Gung Memorial Hospital (CMRPD1F0381, CMRPD1A0203 and CMRPD1C0463), Chang Gung University (EMRPD1D0881) and the Ministry of Science and Technology (MOST 104-2320-B-182-001 and MOST 104-2320-B-182-015) of Taiwan to Dr Li-Man Hung.

Author contribution statement

J P Huang designed and performed experiments and wrote and edited the manuscript. P S Hsieh and C C Chang contributed to data collection. S C Hsu and Y J J Meir provided experimental materials and instruments and contributed to the discussion. K H Chen and J K Chen contributed to the discussion. L M Hung directed the study and wrote and edited the manuscript. All authors have approved the submitted version of the manuscript.

References

- Basciano H, Federico L & Adeli K 2005 Fructose, insulin resistance, and metabolic dyslipidemia. *Nutrition and Metabolism* **2** 5. (<https://doi.org/10.1186/1743-7075-2-5>)
- Bhattacharyya AK, Baker HN, Eggen DA, Malcom GT, Roheim PS & Strong JP 1989 Effect of cholesterol feeding on lipolytic activities in high- and low-responding rhesus monkeys. *Arteriosclerosis* **9** 380–389. (<https://doi.org/10.1161/01.ATV.9.3.380>)
- Cao H, Courchesne WE & Mastick CC 2002 A phosphotyrosine-dependent protein interaction screen reveals a role for phosphorylation of caveolin-1 on tyrosine 14: recruitment of C-terminal Src kinase. *Journal of Biological Chemistry* **277** 8771–8774. (<https://doi.org/10.1074/jbc.C100661200>)
- Chen X, Ghribi O & Geiger JD 2008 Rabbits fed cholesterol-enriched diets exhibit pathological features of inclusion body myositis. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **294** R829–R835. (<https://doi.org/10.1152/ajpregu.00639.2007>)
- Chung S, Cuffe H, Marshall SM, McDaniel AL, Ha JH, Kavanagh K, Hong C, Tontonoz P, Temel RE & Parks JS 2014 Dietary cholesterol promotes adipocyte hypertrophy and adipose tissue inflammation in visceral, but not in subcutaneous, fat in monkeys. *Arteriosclerosis, Thrombosis, and Vascular Biology* **34** 1880–1887. (<https://doi.org/10.1161/ATVBAHA.114.303896>)
- de Haan W, Bhattacharjee A, Ruddle P, Kang MH & Hayden MR 2014 ABCA1 in adipocytes regulates adipose tissue lipid content, glucose tolerance, and insulin sensitivity. *Journal of Lipid Research* **55** 516–523. (<https://doi.org/10.1194/jlr.M045294>)
- de Luca C & Olefsky JM 2008 Inflammation and insulin resistance. *FEBS Letters* **582** 97–105. (<https://doi.org/10.1016/j.febslet.2007.11.057>)
- DeFronzo RA, Tobin JD & Andres R 1979 Glucose clamp technique: a method for quantifying insulin secretion and resistance. *American Journal of Physiology* **237** E214–E223.
- Deng JY, Hsieh PS, Huang JP, Lu LS & Hung LM 2008 Activation of estrogen receptor is crucial for resveratrol-stimulating muscular glucose uptake via both insulin-dependent and -independent pathways. *Diabetes* **57** 1814–1823. (<https://doi.org/10.2337/db07-1750>)
- Duncan RE, Ahmadian M, Jaworski K, Sarkadi-Nagy E & Sul HS 2007 Regulation of lipolysis in adipocytes. *Annual Review of Nutrition* **27** 79–101. (<https://doi.org/10.1146/annurev.nutr.27.061406.093734>)
- Elliott SS, Keim NL, Stern JS, Teff K & Havel PJ 2002 Fructose, weight gain, and the insulin resistance syndrome. *American Journal of Clinical Nutrition* **76** 911–922. (<https://doi.org/10.1093/ajcn/76.5.911>)
- Farina JP, Garcia ME, Alzamendi A, Giovambattista A, Marra CA, Spinedi E & Gagliardino JJ 2013 Antioxidant treatment prevents the development of fructose-induced abdominal adipose tissue dysfunction. *Clinical Science* **125** 87–97. (<https://doi.org/10.1042/CS20120470>)
- Goday A, Calvo E, Vazquez LA, Caveda E, Margallo T, Catalina-Romero C & Reviriego J 2016 Prevalence and clinical characteristics of metabolically healthy obese individuals and other obese/non-obese metabolic phenotypes in a working population: results from the Icaria study. *BMC Public Health* **16** 248. (<https://doi.org/10.1186/s12889-016-2921-4>)
- Guerra S, Boscarì F, Avogaro A, Di Camillo B, Sparacino G & de Kreutzenberg SV 2011 Hemodynamics assessed via approximate entropy analysis of impedance cardiography time series: effect of metabolic syndrome. *American Journal of Physiology-Heart and Circulatory Physiology* **301** H592–H598. (<https://doi.org/10.1152/ajpheart.01195.2010>)
- Guilherme A, Virbasius JV, Puri V & Czech MP 2008 Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nature Reviews Molecular Cell Biology* **9** 367–377. (<https://doi.org/10.1038/nrm2391>)

- Gustafson B, Hammarstedt A, Andersson CX & Smith U 2007 Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **27** 2276–2283. (<https://doi.org/10.1161/ATVBAHA.107.147835>)
- Hajer GR, van Haeften TW & Visseren FL 2008 Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. *European Heart Journal* **29** 2959–2971. (<https://doi.org/10.1093/eurheartj/ehn387>)
- Hsieh PS 2004 Attenuation of insulin-mediated pressor effect and nitric oxide release in rats with fructose-induced insulin resistance. *American Journal of Hypertension* **17** 707–711. (<https://doi.org/10.1016/j.amjhyper.2004.04.006>)
- Huang JP, Cheng ML, Hung CY, Wang CH, Hsieh PS, Shiao MS, Chen JK, Li DE & Hung LM 2017 Docosapentaenoic acid and docosahexaenoic acid are positively associated with insulin sensitivity in rats fed high-fat and high-fructose diets. *Journal of Diabetes* **9** 936–946. (<https://doi.org/10.1111/1753-0407.12505>)
- Hung LM, Su MJ, Chu WK, Chiao CW, Chan WF & Chen JK 2002 The protective effect of resveratrol on ischaemia-reperfusion injuries of rat hearts is correlated with antioxidant efficacy. *British Journal of Pharmacology* **135** 1627–1633. (<https://doi.org/10.1038/sj.bjp.0704637>)
- Ikonen E 2008 Cellular cholesterol trafficking and compartmentalization. *Nature Reviews Molecular Cell Biology* **9** 125–138. (<https://doi.org/10.1038/nrm2336>)
- Inoue M, Chiang SH, Chang L, Chen XW & Satiel AR 2006 Compartmentalization of the exocyst complex in lipid rafts controls Glut4 vesicle tethering. *Molecular Biology of the Cell* **17** 2303–2311. (<https://doi.org/10.1091/mbc.E06-01-0030>)
- Kershaw EE, Schupp M, Guan HP, Gardner NP, Lazar MA & Flier JS 2007 PPARGamma regulates adipose triglyceride lipase in adipocytes in vitro and in vivo. *American Journal of Physiology-Endocrinology and Metabolism* **293** E1736–E1745. (<https://doi.org/10.1152/ajpendo.00122.2007>)
- Kieffer TJ & Habener JF 2000 The adipoinsular axis: effects of leptin on pancreatic beta-cells. *American Journal of Physiology-Endocrinology and Metabolism* **278** E1–E14. (<https://doi.org/10.1152/ajpendo.2000.278.1.E1>)
- Kraegen EW, Saha AK, Preston E, Wilks D, Hoy AJ, Cooney GJ & Ruderman NB 2006 Increased malonyl-CoA and diacylglycerol content and reduced AMPK activity accompany insulin resistance induced by glucose infusion in muscle and liver of rats. *American Journal of Physiology-Endocrinology and Metabolism* **290** E471–E479. (<https://doi.org/10.1152/ajpendo.00316.2005>)
- Kramer CK, Zinman B & Retnakaran R 2013 Are metabolically healthy overweight and obesity benign conditions?: a systematic review and meta-analysis. *Annals of Internal Medicine* **159** 758–769. (<https://doi.org/10.7326/0003-4819-159-11-201312030-00008>)
- Lawn RM, Wade DP, Garvin MR, Wang X, Schwartz K, Porter JG, Seilhamer JJ, Vaughan AM & Oram JF 1999 The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. *Journal of Clinical Investigation* **104** R25–R31. (<https://doi.org/10.1172/JCI8119>)
- Malnick SD & Knobler H 2006 The medical complications of obesity. *QJM* **99** 565–579. (<https://doi.org/10.1093/qjmed/hcl085>)
- McMurray RG & Hackney AC 2005 Interactions of metabolic hormones, adipose tissue and exercise. *Sports Medicine* **35** 393–412. (<https://doi.org/10.2165/00007256-200535050-00003>)
- Meszaros K, Lang CH, Bagby GJ & Spitzer JJ 1987 Contribution of different organs to increased glucose consumption after endotoxin administration. *Journal of Biological Chemistry* **262** 10965–10970.
- Murano I, Barbatelli G, Parisani V, Latini C, Muzzonigro G, Castellucci M & Cinti S 2008 Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. *Journal of Lipid Research* **49** 1562–1568. (<https://doi.org/10.1194/jlr.M800019-JLR200>)
- Nguyen DM & El-Serag HB 2010 The epidemiology of obesity. *Gastroenterology Clinics of North America* **39** 1–7. (<https://doi.org/10.1016/j.gtc.2009.12.014>)
- Obama T, Nagaoka S, Akagi K, Kato R, Horiuchi N, Horai Y, Aiuchi T, Arata S, Yamaguchi T, Watanabe M, *et al.* 2011 Dietary cholesterol reduces plasma triacylglycerol in apolipoprotein E-null mice: suppression of lipin-1 and -2 in the glycerol-3-phosphate pathway. *PLoS ONE* **6** e22917. (<https://doi.org/10.1371/journal.pone.0022917>)
- Pajunen P, Kotronen A, Korpi-Hyovalti E, Keinänen-Kiukkaanniemi S, Oksa H, Niskanen L, Saaristo T, Saltevo JT, Sundvall J, Vanhala M, *et al.* 2011 Metabolically healthy and unhealthy obesity phenotypes in the general population: the FIN-D2D Survey. *BMC Public Health* **11** 754. (<https://doi.org/10.1186/1471-2458-11-754>)
- Patel P & Abate N 2013 Body fat distribution and insulin resistance. *Nutrients* **5** 2019–2027. (<https://doi.org/10.3390/nu5062019>)
- Phillips CM & Perry IJ 2013 Does inflammation determine metabolic health status in obese and nonobese adults? *Journal of Clinical Endocrinology and Metabolism* **98** E1610–E1619. (<https://doi.org/10.1210/jc.2013-2038>)
- Poirier P, Giles TD, Bray GA, Hong Y, Stern JS, Pi-Sunyer FX & Eckel RH 2006 Obesity and cardiovascular disease: pathophysiology, evaluation, and effect of weight loss: an update of the 1997 American Heart Association Scientific Statement on Obesity and Heart Disease from the Obesity Committee of the Council on Nutrition, Physical Activity, and Metabolism. *Circulation* **113** 898–918. (<https://doi.org/10.1161/CIRCULATIONAHA.106.171016>)
- Ruiz-Alcaraz AJ, Lipina C, Petrie JR, Murphy MJ, Morris AD, Sutherland C & Cuthbertson DJ 2013 Obesity-induced insulin resistance in human skeletal muscle is characterised by defective activation of p42/p44 MAP kinase. *PLoS ONE* **8** e56928. (<https://doi.org/10.1371/journal.pone.0056928>)
- Saha AK, Xu XJ, Lawson E, Deoliveira R, Brandon AE, Kraegen EW & Ruderman NB 2010 Downregulation of AMPK accompanies leucine- and glucose-induced increases in protein synthesis and insulin resistance in rat skeletal muscle. *Diabetes* **59** 2426–2434. (<https://doi.org/10.2337/db09-1870>)
- Santamarina-Fojo S, Remaley AT, Neufeld EB & Brewer HB Jr 2001 Regulation and intracellular trafficking of the ABCA1 transporter. *Journal of Lipid Research* **42** 1339–1345.
- Sewter C, Blows F, Considine R, Vidal-Puig A & O'Rahilly S 2002 Differential effects of adiposity on peroxisomal proliferator-activated receptor gamma1 and gamma2 messenger ribonucleic acid expression in human adipocytes. *Journal of Clinical Endocrinology and Metabolism* **87** 4203–4207. (<https://doi.org/10.1210/jc.2002-011511>)
- Smith D, Rossetti L, Ferrannini E, Johnson CM, Cobelli C, Toffolo G, Katz LD & DeFronzo RA 1987 In vivo glucose metabolism in the awake rat: tracer and insulin clamp studies. *Metabolism* **36** 1167–1174. ([https://doi.org/10.1016/0026-0495\(87\)90244-7](https://doi.org/10.1016/0026-0495(87)90244-7))
- Subramanian S & Chait A 2009 The effect of dietary cholesterol on macrophage accumulation in adipose tissue: implications for systemic inflammation and atherosclerosis. *Current Opinion in Lipidology* **20** 39–44. (<https://doi.org/10.1097/MOL.0b013e32831bef8b>)
- Tilg H & Moschen AR 2006 Adipocytokines: mediators linking adipose tissue, inflammation and immunity. *Nature Reviews Immunology* **6** 772–783. (<https://doi.org/10.1038/nri1937>)
- Weibel GL, Drazul-Schrader D, Shivers DK, Wade AN, Rothblat GH, Reilly MP & de la Llera-Moya M 2014 Importance of evaluating cell cholesterol influx with efflux in determining the impact of human serum on cholesterol metabolism and atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **34** 17–25. (<https://doi.org/10.1161/ATVBAHA.113.302437>)

- Wellen KE & Hotamisligil GS 2003 Obesity-induced inflammatory changes in adipose tissue. *Journal of Clinical Investigation* **112** 1785–1788. (<https://doi.org/10.1172/JCI20514>)
- Wouters K, van Gorp PJ, Bieghs V, Gijbels MJ, Duimel H, Lutjohann D, Kerkseik A, van Kruchten R, Maeda N, Staels B, *et al.* 2008 Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. *Hepatology* **48** 474–486. (<https://doi.org/10.1002/hep.22363>)
- Zhang W, Thompson BJ, Hietakangas V & Cohen SM 2011 MAPK/ERK signaling regulates insulin sensitivity to control glucose metabolism in *Drosophila*. *PLoS Genetics* **7** e1002429. (<https://doi.org/10.1371/journal.pgen.1002429>)
- Zhao YF, Wang L, Lee S, Sun Q, Tuo Y, Wang Y, Pei J & Chen C 2010 Cholesterol induces mitochondrial dysfunction and apoptosis in mouse pancreatic beta-cell line MIN6 cells. *Endocrine* **37** 76–82. (<https://doi.org/10.1007/s12020-009-9275-y>)

Received in final form 15 March 2018

Accepted 26 March 2018

Accepted Preprint published online 26 March 2018