Dietary choline increases brown adipose tissue activation markers and improves cholesterol metabolism in female APOE*3-Leiden.CETP mice

Liu, C.; Song, Z.K.; Li, Z.; Boon, M.R.; Schonke, M.; Rensen, P.C.N.; Wang, Y.A.

ARTICLE

Check for updates

Animal Models

Dietary choline increases brown adipose tissue activation markers and improves cholesterol metabolism in female *APOE*3-Leiden.CETP* mice

Cong Liu[1,2], Zikuan Song[1,2], Zhuang Li[1,2], Mariëtte R. Boon[1,2], Milena Schönke[1,2], Patrick C. N. Rensen[1,2,3] and Yanan Wang[1,2,3]✉

OBJECTIVES: Studies in mice have recently linked increased dietary choline consumption to increased incidence of obesity-related metabolic diseases, while several clinical trials have reported an anti-obesity effect of high dietary choline intake. Since the underlying mechanisms by which choline affects obesity are incompletely understood, the aim of the present study was to investigate the role of dietary choline supplementation in adiposity.
METHODS: Female *APOE*3-Leiden.CETP* mice, a well-established model for human-like lipoprotein metabolism and cardiometabolic diseases, were fed a Western-type diet supplemented with or without choline (1.2%, w/w) for up to 16 weeks.
RESULTS: Dietary choline reduced body fat mass gain, prevented adipocyte enlargement, and attenuated adipose tissue inflammation. Besides, choline ameliorated liver steatosis and damage, associated with an upregulation of hepatic genes involved in fatty acid oxidation. Moreover, choline reduced plasma cholesterol, as explained by a reduction of plasma non-HDL cholesterol. Mechanistically, choline reduced hepatic VLDL-cholesterol secretion and enhanced the selective uptake of fatty acids from triglyceride-rich lipoprotein (TRL)-like particles by brown adipose tissue (BAT), consequently accelerating the clearance of the cholesterol-enriched TRL remnants by the liver.
CONCLUSIONS: In *APOE*3-Leiden.CETP* mice, dietary choline reduces body fat by enhancing TRL-derived fatty acids by BAT, resulting in accelerated TRL turnover to improve hypercholesterolemia. These data provide a mechanistic basis for the observation in human intervention trials that high choline intake is linked with reduced body weight.

International Journal of Obesity (2023) 47:236–243; https://doi.org/10.1038/s41366-023-01269-6

INTRODUCTION

Choline is an essential nutrient that has important biological functions, such as being a precursor for phospholipids (PLs) and a regulator of hepatic lipoprotein secretion [1]. However, since 2011 studies in *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice have linked dietary choline to the pathogenesis of cardiometabolic diseases, related to its conversion by the gut–liver axis into trimethylamine N-oxide (TMAO) [2–4]. TMAO was shown to aggravate atherosclerosis via various mechanisms, such as promoting foam cell formation and disturbing cholesterol metabolism [3–6]. However, very recently, we and others reported that a diet rich in choline or its precursor phosphatidylcholine does not aggravate atherosclerosis development in various mouse models of atherosclerosis [7–9]. Furthermore, by using *APOE*3-Leiden.CETP mice*, a well-established mouse model for human-like cardiometabolic diseases, we also showed that plasma TMAO levels do not associate with atherosclerosis development [9].

Moreover, TMAO was previously shown to exacerbate insulin resistance and promote adiposity in diet-induced obese (DIO) and *ob/ob* mice [10–13]. Interestingly, such a correlation between plasma TMAO levels and obesity-associated metabolic disorders is not supported by clinical studies [14–19]. Studies even showed that high choline intake failed to increase plasma TMAO levels in healthy populations [20, 21] and people with obesity [16, 22]. In fact, several clinical trials reported that high choline intake is linked to reduced body weight [23, 24], lowered insulin resistance [25], and decreased cholesterol synthesis [26]. These clinical findings likely indicate a beneficial effect of high dietary choline on obesity in humans.

Obesity, originating from a chronic positive energy balance, is one of the most prevalent public health concerns worldwide. Over the past decades, researchers have searched for interventions that can increase energy expenditure and/or decrease energy intake, thereby effectively combating obesity. Of note, the detection of brown adipose tissue (BAT) in human adults has sparked interest in enhancing energy expenditure through increasing thermogenic activity in brown adipocytes. BAT thermogenesis is primarily mediated by uncoupling protein 1 (UCP-1). UCP-1 transports

[1]Department of Medicine, Division of Endocrinology, Leiden University Medical Center, Leiden, The Netherlands. [2]Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands. [3]Med-X institute, Center for Immunological and Metabolic Diseases, and Department of Endocrinology, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an Jiaotong University, Xi'an, China. ✉email: yanan.wang@xjtu.edu.cn

Received: 9 September 2022 Revised: 23 January 2023 Accepted: 25 January 2023
Published online: 2 February 2023

protons, generated from the electron transport chain resulting from the oxidation of glucose and fatty acids, across the mitochondrial inner membrane in a process uncoupled from adenosine triphosphate (ATP) synthesis, generating heat for non-shivering thermogenesis [27, 28]. As such, BAT activation can induce fat loss, but it remains unclear whether dietary choline promotes weight loss through activation of BAT. Thus, the aim of the present study was to investigate the effect of high dietary consumption on adiposity and BAT function using a well-established humanized mouse model, i.e., *APOE*3-Leiden.CETP* mice.

MATERIALS AND METHODS

Animals

Female *APOE*3-Leiden.CETP* mice (on a C57BL/6J background) were generated as previously described [29]. Mice aged at 8–12 weeks were housed under standard conditions (22 °C; 12/12-h light/dark cycle) with *ad libitum* access to water and a Western-type diet (WTD; 0.15% cholesterol and 16% fat; ssniff, Soest, Germany). All mice were acclimatized to housing and the WTD for 3 weeks prior to the dietary intervention with choline. Then, based on 4-h fasted plasma lipids, body weight and body composition, mice were randomized into two groups using RandoMice [30] to receive either WTD ("Ctrl") or WTD with free choline (1.2% w/w; "Choline") for up to 16 weeks.

In study 1, mice ($n = 16$ per group) were treated for 9 weeks, during which an oral lipid tolerance test was performed at week 6 ($n = 8$ per group). Thereafter, in vivo assays tracing very low-density lipoprotein (VLDL) clearance ($n = 8$ per group) and hepatic VLDL production ($n = 8$ per group) were conducted at week 9. Herein, the sample size was calculated based on the primary outcomes, i.e., hepatic VLDL production and plasma VLDL-like particle clearance. We consider differences in both parameters of 20% to be biological relevant. To achieve the differences with $\alpha = 5\%$ and a power of 80%, 16 animals per group were therefore needed.

To gain more insight into the function of adipose tissue and the liver, various adipose tissue depots and liver samples were collected (for histological analysis, $n = 17$ per group; for mRNA expression analysis, $n = 10$ per group; the sample size was chosen based on a well-established protocol in our group as used in previous studies [9, 31–33]) from study 2 in which mice were treated with choline for 16 weeks. Unless indicated otherwise, mice were group housed (3–5 per cage) during the experimental period to avoid stress caused by single housing.

All animal experiments were carried out according to the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals, and were approved by the National Committee for Animal Experiments (Protocol No. AVD1160020172927) and by the Ethics Committee on Animal Care and Experimentation of the Leiden University Medical Center (Protocol No. PE.18.063.006 and No. PE.18.063.007). All animal procedures conform with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Measurement of body weight and body composition

Body weight was measured weekly with a scale, and body composition of conscious mice was measured every 4 weeks using an EchoMRI-100 analyzer (EchoMRI, Houston, TX, USA).

Plasma lipid profiles and alanine transaminase measurement

Every 4 weeks, after 4-h fasting (9:00–13:00), tail vein blood ($n = 16$ per group) was collected into paraoxon-coated glass capillaries. Plasma was collected and measured for triglyceride (TG) and total cholesterol (TC) levels. In brief, TG and TC levels were measured using Cobas Triglycerides (106571) and Cobas Total Cholesterol (106570) enzymatic kits (both from Roche Diagnostics, Mannheim, Germany), by adding 200 µL reagent (undiluted for TG and 3× diluted in water for TC) to 7.5 µL 5× diluted samples and incubating at room temperature for 30 min prior to measuring absorption at 492 nm versus 650 nm (for TG) or at 505 nm versus 650 nm (for TC). Plasma high-density lipoprotein cholesterol (HDL-C) and non-HDL-C levels were measured using a previously published approach [34]. Plasma alanine transaminase (ALT) levels were determined at the end of the study using Mouse ALT ELISA Kit (Abcam, Cambridge, MA, USA)

Oral lipid tolerance test

At week 6, mice ($n = 8$ per group) were fasted for 4 h. Then, these mice received olive oil (10 mL/kg body weight; Carbonell Traditional, Cordoba, Spain) via oral gavage. Blood was collected into paraoxon-coated glass capillaries at 0, 2, 4, 6, and 8 h following the gavage, spun down, and plasma was used for TG measurement as described above.

In vivo plasma decay and organ uptake of fatty acids derived from very low-density lipoprotein (VLDL)-like particles

VLDL-like particles (average size 80 nm) labeled with glycerol tri[^{3}H]oleate ([^{3}H]TO) and [^{14}C]cholesteryl oleate ([^{14}C]CO) were prepared as described [35]. At week 9, mice ($n = 8$ per group) were fasted for 4 h (9:00–13:00) and intravenously injected ($t = 0$ min) with the VLDL-like particles (1.0 mg TG in 200 µL PBS). Blood samples were collected from the tail vein at 0 min (before injection) and 2, 5, 10, and 15 min after injection to measure the plasma decay of [^{3}H]TO and [^{14}C]CO. After 15 min, all mice were sacrificed by CO_2 inhalation and perfused via the heart with ice-cold PBS. Subsequently, tissues were isolated, transferred into High-Performance glass vials (PerkinElmer, Groningen, The Netherlands) and dissolved overnight at 56 °C in 0.5 mL Solvable (PerkinElmer, Groningen, The Netherlands). Dissolved organs were mixed with 5 mL Ultima Gold scintillation fluid (PerkinElmer, Groningen, The Netherlands), and vials were placed in a Tri-Carb 2910TR Low Activity Liquid Scintillation Analyzer (PerkinElmer, Groningen, The Netherlands) to assess ^{3}H and ^{14}C activity. Disintegrations per minute of ^{3}H and ^{14}C were expressed as percentage of the injected dose per gram tissue and whole organ weight.

Hepatic VLDL production

After 9 weeks of dietary intervention, mice ($n = 8$ per group) were fasted for 4 h (9:00–13:00) and anaesthetized via an intraperitoneal injection (once) of 6.25 mg/kg Acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg Midazolam (Roche, Mijdrecht, The Netherlands), and 0.31 mg/kg Fentanyl (Janssen-Cilag, Tilburg, The Netherlands). Anesthesia was maintained by intraperitoneal injection (3 times; every 45 min) of 0.03 mg/kg Acepromazine, 0.03 mg/kg Midazolam, and 0.001 mg/kg Fentanyl. To ensure deep anesthesia throughout the experimental procedure, the reflexes of mice were checked by pinching the toes of the paws, and body temperature was maintained using a heating pad. Hepatic VLDL production was assessed as previously described [36]. Using commercial kits, TG, TC, and PL (Instruchemie, Delfzijl, The Netherlands) concentrations were determined.

Adipose tissue histology

At week 16, various adipose tissue depots, including interscapular brown adipose tissue (iBAT), subscapular brown adipose tissue (sBAT), subcutaneous white adipose tissue (sWAT) and gonadal white adipose tissue (gWAT), were collected and weighed ($n = 17$ per group). Then, formalin-fixed paraffin-embedded iBAT, sWAT, and gWAT sections (5 µm thickness) were prepared for hematoxylin-eosin (H&E) staining. Moreover, iBAT and sWAT sections were processed for UCP-1 (1:2000; Abcam, Ab10983; Cambridge, MA, USA) staining. Using Image J software (version 1.52a; National Institutes of Health, Bethesda, Maryland), the areas occupied by intracellular lipid vacuoles and UCP-1, as well as the size of adipocyte of gWAT and sWAT were assessed using Image J software.

Liver histology and lipid measurements

Formalin-fixed paraffin-embedded liver samples (collected at week 16; $n = 17$ per group) were stained with H&E. The areas occupied by intracellular lipid vacuoles were quantified using Image J software. Hepatic lipids were extracted from frozen liver samples ($n = 10$ per group) using a modified protocol from Bligh and Dyer [37]. Commercial kits were used for the measurement of hepatic TG, TC, PL, and protein (Pierce, Thermo Fisher Scientific, Waltham, MA, USA). Hepatic lipids were expressed as nmol lipid per mg protein.

Gene expression analysis

Using Tripure RNA isolation reagent (Roche, Mijdrecht, The Netherlands), total RNA was extracted from snap-frozen tissues (collected at week 16; $n = 10$ per group). Using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Leiden, The Netherlands), complementary DNA for quantitative reverse transcriptase-PCR was generated by reverse transcription of total RNA. Then, mRNA expression was normalized to *b-actin* and *Rplp0* mRNA levels and expressed as fold change compared with the Ctrl group. Primer sequences are listed in the Supplementary material online.

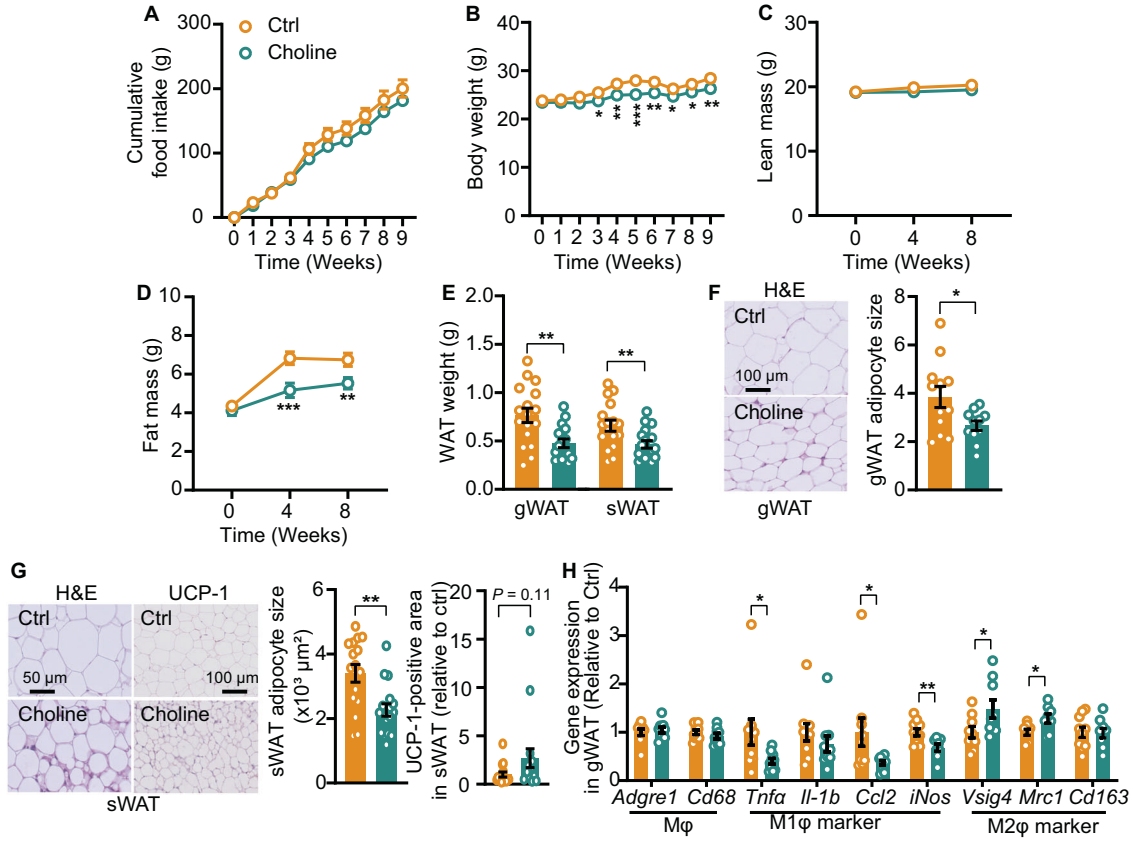

Fig. 1 Choline attenuates fat mass gain and improves WAT function. A cumulative food intake, **B** body weight, **C** body lean mass, and **D** body fat mass were monitored throughout the experimental period. **E** At the end of the study, various white adipose tissue depots were collected and weighed. **F**–**G** white adipocyte enlargement was assessed by hematoxylin-eosin (H&E) staining, and **G** subcutaneous white adipose tissue (sWAT) browning was evaluated by uncoupling protein-1 (UCP-1) immunostaining. **H** Gonadal white adipose tissue (gWAT) inflammation was assessed by quantifying expression of proinflammatory and anti-inflammatory genes. **A**–**D**, data were obtained from study 1; **E**–**H** data were obtained from study 2. Data are represented as mean ± SEM (**A** $n = 4$–5 per group; **B**–**G**, $n = 16$–17 per group; **H** $n = 9$–10 per group). Differences were assessed using **A**–**D** two-way ANOVA followed by Fisher's LSD test or **E**–**H** unpaired two-tailed Student's t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *Adgre1*, adhesion G protein-coupled receptor E1; *Ccl2*, chemokine C–C motif ligand 2; *Cd68*, cluster of differentiation 68; *Cd163*, cluster of differentiation 163; *Il1b*, interleukin-1β; *iNos*, inducible nitric oxide synthase; *Mrc1*, mannose receptor C-type 1; Vsig4, V-set and immunoglobulin domain containing 4; *Tnfa*, tumor necrosis factor α.

Statistical analyses

All samples were blinded and randomly distributed before each assay. Data were normally distributed with equal variances between groups. Comparisons between Ctrl and Choline groups were performed using unpaired two-tailed Student's *t* tests or two-way analysis of variance (ANOVA) when appropriate. Data are presented as mean ± SEM, and a *P* value less than 0.05 is considered statistically significant. Mice/samples were excluded from statistical analysis (exclusion criteria) owing to technical failure, including unsuccessful intravenous injection and poor histological and RNA quality. All statistical analyses were performed with GraphPad Prism 9 (GraphPad Software Inc., CA, USA).

RESULTS

Dietary choline supplementation attenuates fat mass gain

To assess the effects of choline on adiposity, we fed *APOE*3-Leiden.CETP* mice a WTD with or without choline. Dietary choline supplementation did not affect food intake (Fig. 1A). However, choline attenuated WTD-induced body weight gain (−7% at week 9; Fig. 1B), as explained by reduced gain of fat mass (−18% at week 8; Fig. 1D) rather than lean mass (Fig. 1C). Furthermore, choline reduced weights of gWAT (−38%) and sWAT (−29%) (Fig. 1E), attenuated adipocyte enlargement of these WAT depots (gWAT, −31%; sWAT, −33%; Fig. 1F, G), and tended to increase UCP-1 content in sWAT (+169%; Fig. 1G). In gWAT, choline did not affect gene expression of adiponectin i.e., *Adipoq* (Fig. S1A) and common macrophage (*M*φ) surface makers, but downregulated the expression of pro-inflammatory M1φ markers, including tumor necrosis factor α (*Tnfa*; −60%), C-C motif chemokine ligand 2 (*Ccl2*; −63%) and inducible nitric oxide synthase (*iNos*; −32%), and upregulated the expression of anti-inflammatory M2φ markers, including V-set immunoglobulin-domain-containing 4 (*Vsig4*; +48%) and mannose receptor C-type 1 (*Mrc1*; +28%) (Fig. 1H).

Choline reduces hepatic steatosis and inflammation

To evaluate the consequence of attenuated adiposity as induced by choline for hepatic steatosis, histological and biochemistry analyses were performed in livers obtained after 16 weeks of intervention. Choline reduced liver weight (−18%; Fig. 2A), and alleviated WTD-induced hepatic steatosis, as shown by reduced intracellular lipid vacuoles (−40%; Fig. 2B) and reduced hepatic TG (−30%), TC (−24%), and PL (−19%) levels (Fig. 2C). Choline did not affect hepatic expression of genes involved in cholesterol synthesis including 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), mevalonate kinase (*Mvk*) and sterol regulatory element-binding protein 2 (*Srebp2*), apart from slightly increasing sterol regulatory element-binding protein 1c (*Srebp1c*; +37%) (Fig. S1B). In contrast, choline increased hepatic expression of genes involved in fatty acid (FA) oxidation, including carnitine palmitoyl

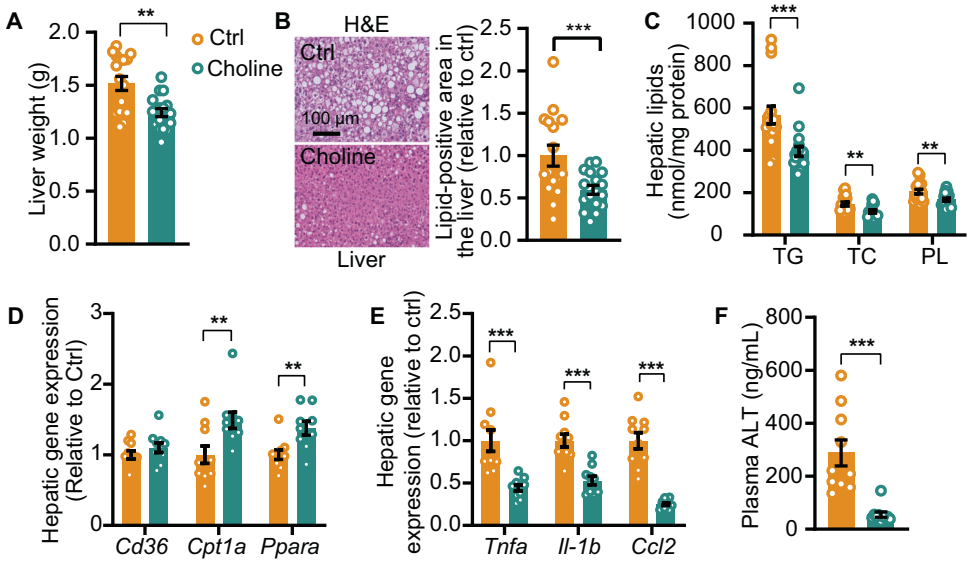

Fig. 2 Choline reduces hepatic steatosis and inflammation. At the end of the study, **A** liver weight was measured, **B** hepatic lipid content was assessed by H&E staining, and hepatic levels of **C** triglyceride (TG), total cholesterol (TC), and phospholipid (PL) were determined. The relative mRNA expression of genes involved in **D** lipid handling and **E** inflammation was determined in the liver. **F** Plasma alanine transaminase (ALT) was also measured. Data were obtained from study 2 and are represented as mean ± SEM (**A**–**C**, $n = 16$–17 per group; **D**–**F**, $n = 9$–10 per group). Differences were assessed using unpaired two-tailed Student's *t* test. $^{**}P < 0.01$, $^{***}P < 0.001$. *Cd36*, cluster of differentiation 36; *Cpt1*, carnitine palmitoyl transferase 1; transcription factor 1; *Ppara*, peroxisome proliferator-activated receptor α; *Tnfa*, tumor necrosis factor α; *Il1b*, interleukin-1β; *Ccl2*, chemokine C–C motif ligand 2.

transferase 1a (*Cpt1a*, +49%) and peroxisome proliferator-activated receptor α (*Ppara*; +38%) (Fig. 2D). Choline also attenuated hepatic inflammation, as evidenced by downregulated hepatic mRNA expression of *Tnfa* (−56%), interleukin 1β (*Il1b*; −47%), and *Ccl2* (−75%) (Fig. 2E). In line with these findings, choline induced a profound reduction of plasma ALT levels (−81%; Fig. 2F).

Choline ameliorates hypercholesterolemia

Next, we evaluated the effects of choline on WTD-induced dyslipidemia. While choline had no effects on plasma TG levels (Fig. S1A), it tended to improve lipid intolerance after 6 weeks of dietary choline supplementation (Fig. S1B). Of note, choline reduced plasma TC levels (−29% at week 8), which was explained by lowered non-HDL-C (−29% at week 8) rather than HDL-C (Fig. 3A–C). To examine the mechanism underlying the choline-induced changes in plasma lipid levels, we examined whether choline affects hepatic VLDL production. Despite choline upregulating the hepatic mRNA expression of microsomal triglyceride transfer protein (*Mttp*; +45%) and apolipoprotein B (*Apob*; +58%), choline did not affect the production rate of VLDL-TG and VLDL-apolipoprotein B (ApoB) (Fig. 3D–F). In contrast, choline decreased the amount of PL (−30%) and TC (−38%) per ApoB, respectively (Fig. 3G). Since each VLDL particle contains a single apoB molecule, these data indicate that choline does not affect VLDL particle production, but largely reduces the hepatic secretion of cholesterol within VLDL.

Choline activates BAT to accelerate TG-derived FA uptake, accompanied by increased hepatic uptake of cholesterol-enriched remnants

Besides profoundly reducing VLDL-cholesterol secretion, we hypothesized that choline may also improve hypercholesterolemia by accelerating VLDL-cholesterol clearance. Therefore, after 9 weeks of treatment, we intravenously injected mice with VLDL-like particles labeled with [^{3}H]TO and [^{14}C]CO. Interestingly, choline accelerated the clearance of [^{3}H]TO from plasma (Fig. 4A), which was primarily caused by increased uptake of [^{3}H]TO-derived [^{3}H]oleate by BAT (iBAT, +66%; sBAT, 75%; Figs. 4B, S2A). Concomitantly, choline increased the plasma clearance of [^{14}C]CO (Fig. 4C), which was attributed to the increased uptake of [^{14}C]CO by the liver (+85%; Figs. 4D, S2B). We did not observe significant effects of choline on the expression of key genes involved in BAT thermogenesis (i.e., *Ucp1*; PRD1-BF1-RIZ1 homologous-domain containing 16; *Prdm16*; and iodothyronine deiodinase 2; *Dio2*; data not shown). However, as the increased FA uptake by BAT is an indicator of activated BAT, we next quantified intracellular lipid vacuoles and UCP-1 protein content in BAT obtained from this study. Consistently, choline reduced BAT weight (−17%; Fig. 4E), and decreased lipid droplet content (−45%; Fig. 4F) and increased UCP-1 level (+11%; Fig. 4F) in BAT, which further evidences BAT activation.

DISCUSSION

Previous studies in mice reported that choline promotes the development of obesity-related metabolic diseases, as caused by the generation of TMAO through the gut–liver axis [2–4]. However, several clinical trials reported that high choline intake is linked to reduced body weight [23, 24] and lowered insulin resistance [25]. Since the underlying mechanisms by which choline affects obesity remain unclear, the present study thus aimed to investigate the role of choline in adiposity by exposing *APOE*3-Leiden.CETP* mice to a WTD supplemented with or without choline. We demonstrated that dietary choline supplementation reduces body fat, ameliorate hepatic steatosis, and lowers plasma cholesterol in this model.

First, by performing mechanistic studies, we demonstrated the reduction in body fat induced by choline is accompanied by activation of BAT. Interestingly, previous mouse studies reported that choline and its metabolite TMAO promote obesity [11–13]. This seeming discrepancy with our study is likely explained by different mouse models. While we used *APOE*3-Leiden.CETP* mice, a well-established model with human-like lipoprotein

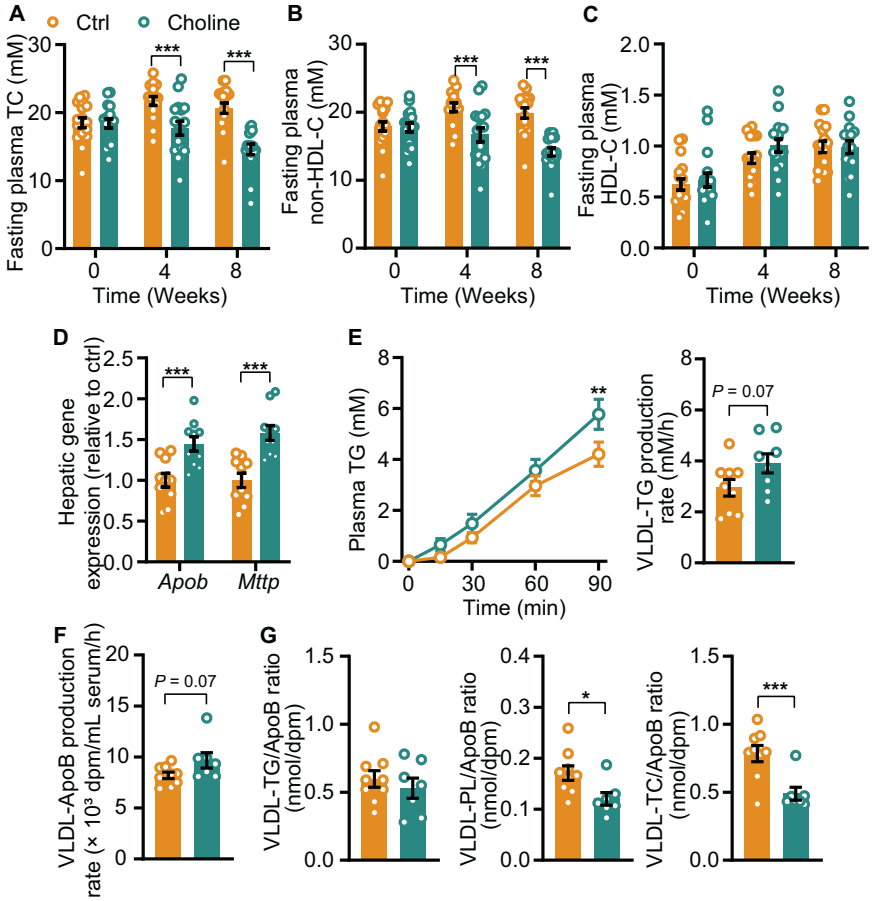

Fig. 3 Choline ameliorates hypercholesterolemia. A–C Plasma cholesterol levels ($n = 14–16$ per group) were measured throughout the study. **D** Hepatic expression of genes involved in very low-density lipoprotein (VLDL) production was quantified ($n = 9–10$ per group). At week 9, the production rate of **E** VLDL-TG and **F** VLDL-apolipoprotein B (ApoB) was determined ($n = 6–8$ per group), and **G** the amount of TG, PL, and TC within VLDL was measured ($n = 6–8$ per group). Data were obtained from study 1 and are represented as mean ± SEM. Differences were assessed using (**A–C** and **E** line graph) two-way ANOVA followed by Fisher's LSD test or unpaired two-tailed Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

metabolism, other studies used either DIO or *ob/ob* mice [11–13]. It should be noted that we recently showed that dietary choline beneficially modulates the gut microbiome and has no impact on plasma TMAO levels in *APOE*3-Leiden.CETP* mice when fed the same diet as in the present study [9]. Similarly, another study showed that by feeding a choline-enriched diet, *Apoe*$^{-/-}$ mice expressing CETP have lower TMAO levels than *ApoE*$^{-/-}$ mice without CETP expression [38]. Likewise, long-term high-choline diet consumption did not increase TMAO levels in clinical trials, possibly as a consequence of human CETP expression [16, 20–22]. In fact, high dietary choline intake has been associated with favorable body composition [23], which might be attributed to increased energy metabolism. In the present study, we observed that choline had no impact on food intake while promoting BAT activation as evidenced by increasing VLDL-derived FA uptake by BAT. BAT has a high metabolic demand, and metabolic substrates are crucial for BAT to initiate and maintain thermogenic function [39]. Activated BAT leads to increased utilization of various metabolic substrates, mainly TG-derived FAs, which results in heat production through uncoupling of mitochondrial respiration from ATP synthesis. Indeed, choline prevented lipid overloading in both BAT and WAT, accompanied by alleviation of WAT inflammation. Given that BAT is present and active in human adults [40], we thus speculate that our data provide a mechanistic explanation for the observation that choline improves body composition in humans. Future studies are needed to investigate the role of dietary choline intake in energy expenditure in relation to BAT activation in various animal models and humans. Besides, in the present study, we used free choline, so it would be also interesting to investigate the effects of choline precursors (e.g., phosphatidylcholine) on adiposity.

We also demonstrated that choline ameliorates liver steatosis and inflammation. In obesity, adipose tissue dysfunction induces a high free FA flux from WAT towards the liver, leading to liver steatosis [41]. Therefore, the fact that choline attenuated adiposity and improved adipose tissue function may already in part explain reduced ectopic lipid deposition in organs such as the liver. In addition, we observed increased hepatic expression of genes involved in hepatic FA oxidation. In line with our findings, choline has been shown to upregulate β-oxidation in an in vitro hepatocellular steatosis model [42]. Although choline had minor effects on hepatic mRNA expression of the transcription factors involved in cholesterol synthesis, we cannot exclude that post-translational modulation of SREBPs may be involved in the observed improvement of hepatic cholesterol metabolism, which could be subject of future studies. In addition, we observed a trend towards increased hepatic TG secretion from the liver as constituent of VLDL, which

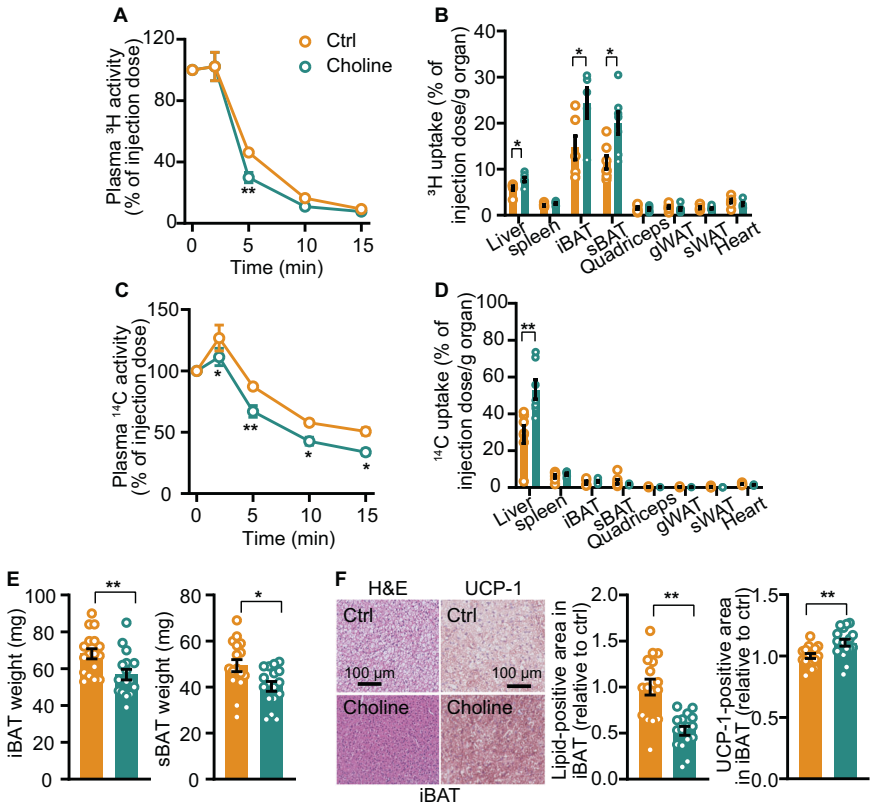

Fig. 4 Choline activates BAT to accelerate TG-derived FA uptake, accompanied by increased hepatic uptake of cholesterol-enriched remnants. At week 9, the clearance of **A** ^{3}H and **C** ^{14}C from plasma and the uptake of **B** ^{3}H and **D** ^{14}C by various tissues were assessed. At week 16, **E** various BAT deports were collected and weighed. In iBAT, **F** lipid droplet content and UCP-1 content were assessed by H&E staining and UCP-1 immunostaining, respectively. **A**–**D** Data were obtained from study 1. **E**, **F** Data were obtained from study 2. Data are represented as mean ± SEM (**A**–**D**, $n = 6$–8; **E**, **F**, $n = 16$–17). Differences were assessed using **A** and **C** two-way ANOVA followed by Fisher's LSD test or unpaired two-tailed Student's t test. *$P < 0.05$, **$P < 0.01$. iBAT, interscapular brown adipose tissue; sBAT, subscapular brown adipose tissue.

would be in line with published data [43], but this effect was not significant. Furthermore, we showed that choline reduces hepatic inflammation, which is consistent with previous studies showing that choline inhibits liver damage [44]. The anti-inflammatory effect of choline is possibly a concomitant effect of the reduced hepatic lipids, confirming a previous study showing that choline alleviated liver injury by normalizing hepatic lipid metabolism [44].

Finally, we demonstrate that choline reduces plasma cholesterol, as shown by a reduction of non-HDL-C rather than HDL-C. These data are in agreement with human studies showing that increased intake of choline-enriched food (i.e., eggs) is associated with an improved plasma lipid profile [24, 26]. Our mechanistic studies revealed that choline both reduced hepatic cholesterol secretion and enhanced lipolytic conversion of VLDL by activated BAT accompanied by increased clearance of cholesterol by the liver. Therefore, the reduction of cholesterol is probably the combined effect of reduced hepatic cholesterol secretion and enhanced hepatic cholesterol clearance via the APOE-LDLR pathway that is fully functional in APOE*3-Leiden.CETP mice. On the other hand, BAT activation for instance via cold exposure, lowers plasma TG [34], an effect that we did not observe in our study. This may imply that the nonsignificant mild increase in hepatic VLDL-TG secretion may in the long term contribute to higher hepatic TG output which counteracts a potential TG reduction induced by BAT activation.

In conclusion, our present study uncovers beneficial effects of choline on adiposity and plasma cholesterol. Mechanistically, choline reduces body fat accompanied by activating BAT, resulting in accelerated hepatic uptake of cholesterol-enriched lipoprotein remnants, which in combination with lower hepatic VLDL-cholesterol secretion and reduces plasma cholesterol.

DATA AVAILABILITY

The data underlying this article will be shared on reasonable request to the corresponding author.

REFERENCES

1. Zeisel SH, da Costa KA. Choline: an essential nutrient for public health. Nutr Rev. 2009;67:615–23.
2. Krueger ES, Lloyd TS, Tessem JS. The accumulation and molecular effects of trimethylamine N-oxide on metabolic tissues: it's not all bad. Nutrients. 2021;13:2873.
3. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature. 2011;472:57–63.
4. Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med. 2013;19:576–85.
5. Seldin MM, Meng Y, Qi H, Zhu W, Wang Z, Hazen SL, et al. Trimethylamine N-oxide promotes vascular inflammation through signaling of mitogen-activated protein kinase and nuclear factor-kappaB. J Am Heart Assoc. 2016;5:e002767.
6. Collins HL, Drazul-Schrader D, Sulpizio AC, Koster PD, Williamson Y, Adelman SJ, et al. L-Carnitine intake and high trimethylamine N-oxide plasma levels correlate with low aortic lesions in ApoE(-/-) transgenic mice expressing CETP. Atherosclerosis. 2016;244:29–37.
7. Aldana-Hernandez P, Azarcoya-Barrera J, van der Veen JN, Leonard KA, Zhao YY, Nelson R, et al. Dietary phosphatidylcholine supplementation reduces atherosclerosis in Ldlr(-/-) male mice(2). J Nutr Biochem. 2021;92:108617.

8. Aldana-Hernandez P, Leonard KA, Zhao YY, Curtis JM, Field CJ, Jacobs RL. Dietary choline or trimethylamine n-oxide supplementation does not influence atherosclerosis development in Ldlr-/- and Apoe-/- male mice. J Nutr. 2020;150:249–55.
9. Liu C, Li Z, Song Z, Fan X, Shao H, Schonke M, et al. Choline and butyrate beneficially modulate the gut microbiome without affecting atherosclerosis in APOE*3-Leiden.CETP mice. Atherosclerosis. 2022;362:47–55.
10. Gao X, Liu X, Xu J, Xue C, Xue Y, Wang Y. Dietary trimethylamine N-oxide exacerbates impaired glucose tolerance in mice fed a high fat diet. J Biosci Bioeng. 2014;118:476–81.
11. Schugar RC, Shih DM, Warrier M, Helsley RN, Burrows A, Ferguson D, et al. The TMAO-producing enzyme flavin-containing monooxygenase 3 regulates obesity and the beiging of white adipose tissue. Cell Rep. 2017;19:2451–61.
12. Chen S, Henderson A, Petriello MC, Romano KA, Gearing M, Miao J, et al. Trimethylamine N-oxide binds and activates PERK to promote metabolic dysfunction. Cell Metab. 2019;30:1141–1151 e5.
13. Schugar RC, Gliniak CM, Osborn LJ, Massey W, Sangwan N, Horak A, et al. Gut microbe-targeted choline trimethylamine lyase inhibition improves obesity via rewiring of host circadian rhythms. Elife. 2022;11:e63998.
14. Golzarand M, Mirmiran P, Azizi F. Association between dietary choline and betaine intake and 10.6-year cardiovascular disease in adults. Nutr J. 2022;21:1.
15. Meyer KA, Benton TZ, Bennett BJ, Jacobs DR, Jr., Lloyd-Jones DM, Gross MD, et al. Microbiota-dependent metabolite trimethylamine N-oxide and coronary artery calcium in the Coronary Artery Risk Development in Young Adults Study (CARDIA). J Am Heart Assoc. 2016;5:e003970.
16. Zhu C, Sawrey-Kubicek L, Bardagjy AS, Houts H, Tang X, Sacchi R, et al. Whole egg consumption increases plasma choline and betaine without affecting TMAO levels or gut microbiome in overweight postmenopausal women. Nutr Res. 2020;78:36–41.
17. Taesuwan S, Thammapichai P, Ganz AB, Jirarattanarangsri W, Khemacheewakul J, Leksawasdi N. Associations of choline intake with hypertension and blood pressure among older adults in cross-sectional 2011-2014 National Health and Nutrition Examination Survey (NHANES) differ by BMI and comorbidity status. Br J Nutr. 2022;128:145–53.
18. Skagen K, Troseid M, Ueland T, Holm S, Abbas A, Gregersen I, et al. The Carnitine-butyrobetaine-trimethylamine-N-oxide pathway and its association with cardiovascular mortality in patients with carotid atherosclerosis. Atherosclerosis. 2016;247:64–9.
19. Koay YC, Chen YC, Wali JA, Luk AWS, Li M, Doma H, et al. Plasma levels of trimethylamine-N-oxide can be increased with 'healthy' and 'unhealthy' diets and do not correlate with the extent of atherosclerosis but with plaque instability. Cardiovasc Res. 2021;117:435–49.
20. Lemos BS, Medina-Vera I, Malysheva OV, Caudill MA, Fernandez ML. Effects of egg consumption and choline supplementation on plasma choline and trimethylamine-N-oxide in a young population. J Am Coll Nutr. 2018;37:716–23.
21. DiMarco DM, Missimer A, Murillo AG, Lemos BS, Malysheva OV, Caudill MA, et al. Intake of up to 3 eggs/day increases HDL cholesterol and plasma choline while plasma trimethylamine-N-oxide is unchanged in a healthy population. Lipids. 2017;52:255–63.
22. Thomas MS, DiBella M, Blesso CN, Malysheva O, Caudill M, Sholola M, et al. Comparison between egg intake versus choline supplementation on gut microbiota and plasma carotenoids in subjects with metabolic syndrome. Nutrients. 2022;14:1179.
23. Mlodzik-Czyzewska MA, Malinowska AM, Szwengiel A, Chmurzynska A. Associations of plasma betaine, plasma choline, choline intake, and MTHFR polymorphism (rs1801133) with anthropometric parameters of healthy adults are sex-dependent. J Hum Nutr Diet. 2022;35:701–12.
24. Thomas MS, Puglisi M, Malysheva O, Caudill MA, Sholola M, Cooperstone JL, et al. Eggs improve plasma biomarkers in patients with metabolic syndrome following a plant-based diet-a randomized crossover study. Nutrients. 2022;14:2138.
25. Gao X, Wang YB, Sun G. High dietary choline and betaine intake is associated with low insulin resistance in the Newfoundland population. Nutrition. 2017;33:28–34.
26. Lemos BS, Medina-Vera I, Blesso CN, Fernandez ML. Intake of 3 eggs per day when compared to a choline bitartrate supplement, downregulates cholesterol synthesis without changing the LDL/HDL Ratio. Nutrients. 2018;10:258.
27. Garlid KD, Jaburek M, Jezek P. The mechanism of proton transport mediated by mitochondrial uncoupling proteins. FEBS Lett. 1998;438:10–4.
28. Nedergaard J, Golozoubova V, Matthias A, Asadi A, Jacobsson A, Cannon B. UCP1: the only protein able to mediate adaptive non-shivering thermogenesis and metabolic inefficiency. Biochim Biophys Acta. 2001;1504:82–106.
29. Westerterp M, van der Hoogt CC, de Haan W, Offerman EH, Dallinga-Thie GM, Jukema JW, et al. Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. Arterioscler Thromb Vasc Biol. 2006;26:2552–9.
30. van Eenige R, Verhave PS, Koemans PJ, Tiebosch I, Rensen PCN, Kooijman S. RandoMice, a novel, user-friendly randomization tool in animal research. PLoS One. 2020;15:e0237096.
31. Liu C, Schonke M, Zhou E, Li Z, Kooijman S, Boon MR, et al. Pharmacological treatment with FGF21 strongly improves plasma cholesterol metabolism to reduce atherosclerosis. Cardiovasc Res. 2022;118:489–502.
32. Liu C, Schonke M, Spoorenberg B, Lambooij JM, van der Zande HJP, Zhou E, et al. FGF21 protects against hepatic lipotoxicity and macrophage activation to attenuate fibrogenesis in nonalcoholic steatohepatitis. Elife. 2023;12:e83075.
33. Zhou E, Li Z, Nakashima H, Choukoud A, Kooijman S, Berbee JFP, et al. Beneficial effects of brown fat activation on top of PCSK9 inhibition with alirocumab on dyslipidemia and atherosclerosis development in APOE*3-Leiden.CETP mice. Pharmacol Res. 2021;167:105524.
34. Berbee JF, Boon MR, Khedoe PP, Bartelt A, Schlein C, Worthmann A, et al. Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development. Nat Commun. 2015;6:6356.
35. Rensen PC, van Dijk MC, Havenaar EC, Bijsterbosch MK, Kruijt JK, van Berkel TJ. Selective liver targeting of antivirals by recombinant chylomicrons–a new therapeutic approach to hepatitis B. Nat Med. 1995;1:221–5.
36. Liu C, Schonke M, Zhou E, Li Z, Kooijman S, Boon MR, et al. Pharmacological treatment with FGF21 strongly improves plasma cholesterol metabolism to reduce atherosclerosis. Cardiovasc Res. 2022;118:489–502.
37. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959;37:911–7.
38. Collins HL, Adelman SJ, Butteiger DN, Bortz JD. Choline supplementation does not promote atherosclerosis in CETP-expressing male apolipoprotein E knockout mice. Nutrients. 2022;14:1651.
39. Wang GX, Zhao XY, Lin JD. The brown fat secretome: metabolic functions beyond thermogenesis. Trends Endocrinol Metab. 2015;26:231–7.
40. Harms M, Seale P. Brown and beige fat: development, function and therapeutic potential. Nat Med. 2013;19:1252–63.
41. Polyzos SA, Kountouras J, Mantzoros CS. Obesity and nonalcoholic fatty liver disease: from pathophysiology to therapeutics. Metabolism. 2019;92:82–97.
42. Zhu J, Wu Y, Tang Q, Leng Y, Cai W. The effects of choline on hepatic lipid metabolism, mitochondrial function and antioxidative status in human hepatic C3A cells exposed to excessive energy substrates. Nutrients. 2014;6:2552–71.
43. Corbin KD, Zeisel SH. Choline metabolism provides novel insights into nonalcoholic fatty liver disease and its progression. Curr Opin Gastroen. 2012;28:159–65.
44. Al Rajabi A, Castro GS, da Silva RP, Nelson RC, Thiesen A, Vannucchi H, et al. Choline supplementation protects against liver damage by normalizing cholesterol metabolism in Pemt/Ldlr knockout mice fed a high-fat diet. J Nutr. 2014;144:252–7.

ACKNOWLEDGEMENTS

We thank T.C.M. Streefland, A.C.M. Pronk, R.A. Lalai, and S. Afkir from the Department of Medicine, Division of Endocrinology, Leiden University Medical Center for technical assistance. This work was supported by the Leiden University Fund/Mulder-Hamelers Fonds (W18307-2-53 to YW), Leiden University Fund/Elise Mathilde Fund (W213045-2 to ZL), the Dutch Diabetes Research Foundation (2015.81.1808 to MRB), an NWO-VENI grant (09150161910073 to MRB), the Netherlands Cardiovascular Research Initiative: an initiative with support of the Dutch Heart Foundation (CVON-GENIUS-2 to PCNR), and the Department of Science and Technology Foundation of Shaanxi province (2021SF-021 to YW). ZL is supported by the China Scholarship Council (CSC 201506170051); MS is supported by Novo Nordisk Foundation (NNF18OC0032394); YW is supported by the China "Thousand Talents Plan" (Young Talents), Shaanxi province "Thousand Talents Plan" (Young Talents), Foundation of Xi'an Jiaotong University (Plan A).

AUTHOR CONTRIBUTIONS

CL designed the study, carried out the research, analyzed and interpreted the results, and wrote and revised the manuscript. ZS carried out the research, interpreted the results, reviewed and revised the manuscript. ZL carried out the research, interpreted the results, reviewed and revised the manuscript, and obtained funding. MRB advised the study and reviewed the manuscript. MS interpreted the results, reviewed and revised the manuscript. PCNR designed and advised the study, interpreted the results, edited, reviewed, and revised the manuscript and obtained funding. YW designed and advised the study, interpreted the results, reviewed and revised the manuscript, and obtained funding.

COMPETING INTERESTS

The authors declared no conflict of interest.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41366-023-01269-6.

Correspondence and requests for materials should be addressed to Yanan Wang.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.