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Targeting inter-organ cross-talk in cardiometabolic diseases

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Citation

Liu, C. (2023, May 16). *Targeting inter-organ cross-talk in cardiometabolic diseases*. Retrieved from <https://hdl.handle.net/1887/3618361>

Version: Publisher's Version

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

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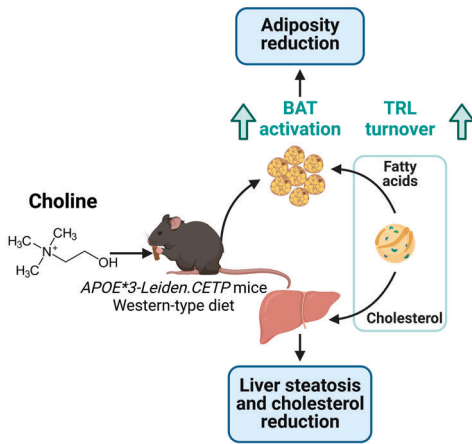
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Int J of Obes 2023; in press.

ABSTRACT



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. 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. 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. 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.

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 [22, 23]. In fact, several clinical trials reported that high choline intake is linked to reduced body weight [24, 25], lowered insulin resistance [26], and decreased cholesterol synthesis [27]. 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 protons, generated from the electron transport chain resulting from 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 [28, 29]. 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 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 were generated as previously described [30]. Mice aged at 8-12 weeks were housed under standard conditions (22°C; 12/12-hour 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-hour fasted plasma lipids, body weight and body composition, mice were randomized into two groups using RandoMice [31] 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 (OLTT) 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.

To gain more insight in 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) 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 were 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-hour 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 μ L reagent (undiluted for TG and 3x diluted in water for TC) to 7.5 μ L 5x 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 [32]. 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 hours. 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 hours 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) labelled with glycerol tri 3 H]oleate (3 H]TO) and 14 C]cholesteryl oleate (14 C]CO) were prepared as described[33]. At week 9, mice (n=8 per group) were fasted for 4 hours (9:00–13:00) and intravenously injected (t=0 min) with the VLDL-like particles (1.0 mg TG in 200 μ L 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 CO₂ 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 hours (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 a 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 [34]. 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 μ m thickness) were prepared for hematoxylin-eosin (H&E) staining. Moreover, iBAT and sWAT sections were processed for UCP-1 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 [35]. 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.

Statistical analyses

Comparisons between Ctrl and Choline groups were performed using unpaired two-tailed Student's *t*-tests or two-way analysis of variance (ANOVA). Data are presented as mean \pm SEM, and a *P* value less than 0.05 is considered statistically significant. 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 M1 ϕ 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 M2 ϕ 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 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 1 β (*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%

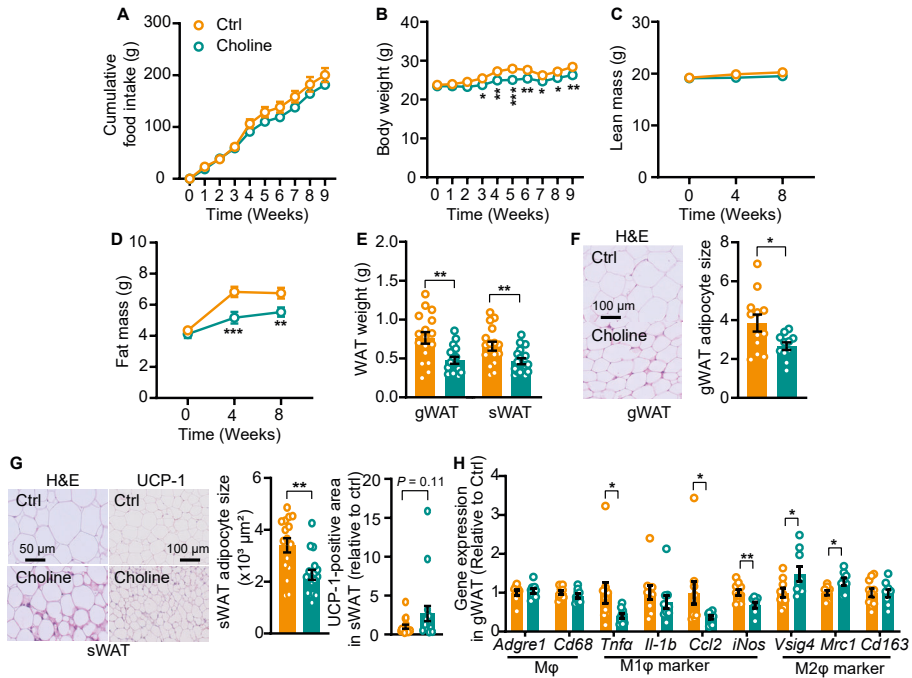


Fig 1. Choline attenuates fat mass gain and improves WAT function. (A) cumulative food intake, (B) body weight, (C) body lean mass and (D) body fat mass were monitored throughout the experimental period. (E) At the end of the study, various white adipose tissue depots were collected and weighed. (F and 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 \pm 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-1 β ; *iNos*, inducible nitric oxide synthase; *Mrc1*, mannose receptor C-type 1; *Vsig4*, V-set and immunoglobulin domain containing 4; *Tnfa*, tumor necrosis factor α -

at week 8) rather than HDL-C (Fig. 3A–C). To examine the mechanism underlying the choline-induced changes of 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.

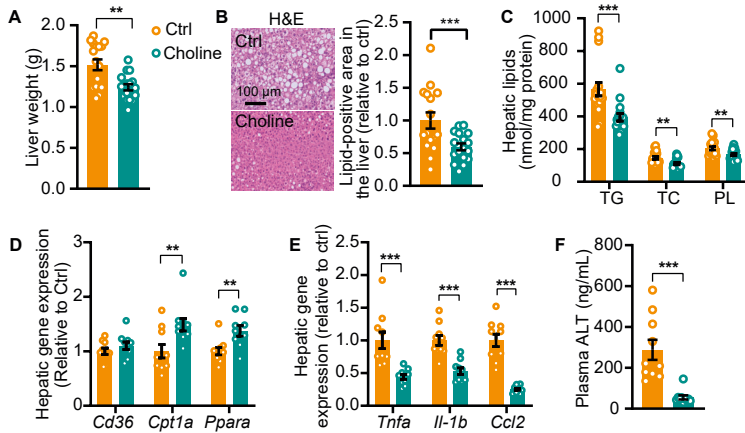


Fig 2. Choline reduces hepatic steatosis and inflammation. At the end of the study, (A) liver weight was measured, (B) hepatic lipid content was assessed by H&E staining, and hepatic levels of (C) triglyceride (TG), total cholesterol (TC) and phospholipid (PL) were determined. The relative mRNA expression of genes involved in lipid handling (D) and inflammation (E) was determined in the liver. (F) Plasma alanine transaminase (ALT) was also measured. Data were obtained from study 2 and are represented as mean \pm 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; *Cpt1a*, carnitine palmitoyl transferase 1; transcription factor 1; *Ppara*, peroxisome proliferator activated receptor α ; *Tnfa*, tumor necrosis factor α ; *Il1b*, interleukin-1 β ; *Ccl2*, chemokine C-C motif ligand 2.

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 labelled 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%; Fig. 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%; Fig. 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.

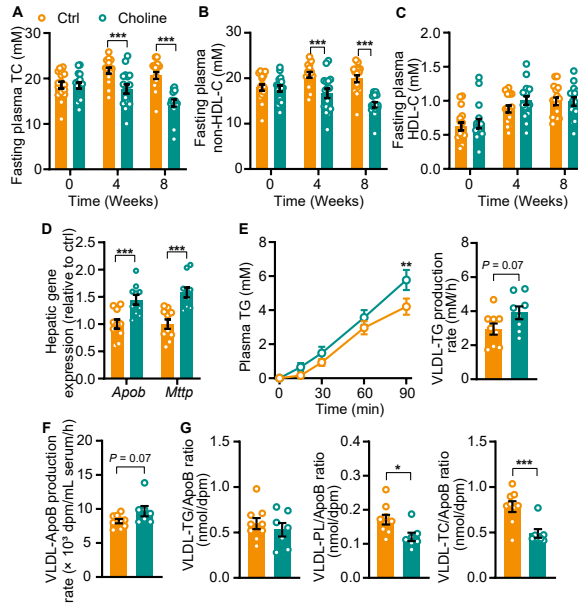


Fig 3. Choline ameliorates hypercholesterolemia. (A-C) Plasma cholesterol levels (n=14-16 per group) were measured throughout the study. (D) Hepatic expression of genes involved in very low-density lipoprotein (VLDL) production was quantified (n=9-10 per group). At week 9, the production rate of (E) VLDL-TG and (F) VLDL-apolipoprotein B (ApoB) was determined (n=6-8 per group), and (G) the amount of TG, protein, PL and TC within VLDL was measured (n=6-8 per group). Data were obtained from study 1 and are represented as mean \pm 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.

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 [24, 25] and lowered insulin resistance [26]. 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 seemingly discrepancy with our study is likely explained by different mouse models.

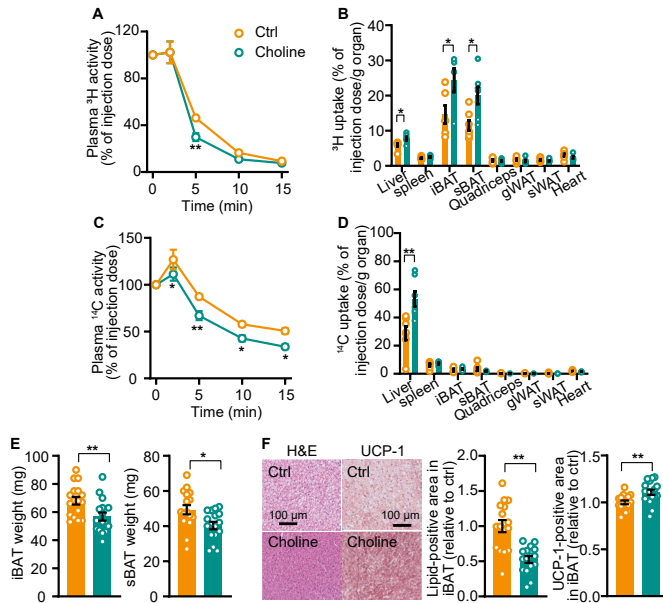


Fig 4. Choline activates BAT to accelerate TG-derived FA uptake, accompanied by increased hepatic uptake of cholesterol-enriched remnants. At week 9, the clearance of ^3H (A) and ^{14}C (C) from plasma and the uptake of ^3H (B) and ^{14}C (D) by various tissues were assessed. At week 16, various BAT depots were collected and weighed (E). In iBAT, lipid droplet content and UCP-1 content were assessed by H&E staining and UCP-1 immunostaining, respectively (F). (A-D) Data were obtained from study 1. (E-F) Data were obtained from study 2. Data are represented as mean \pm 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.

While we used *APOE*3-Leiden.CETP* mice, a well-established model with human-like lipoprotein 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 [36]. Similarly, another study showed that by feeding a choline-enriched diet, *ApoE^{-/-}* mice expressing CETP have lower TMAO levels than *ApoE^{-/-}* without CETP expression [37]. Likewise, long-term high-choline diet consumption did not increase TMAO levels in clinical trials, possibly as a consequence of human CETP expression [20-23]. In fact, high dietary choline intake has been associated with favorable body composition [24], 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 [38].

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 [39], 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 [40]. 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 [41]. 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 would be in line with published data [42], 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 [43]. 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 [43].

Finally, we demonstrate that choline reduces plasma cholesterol, as shown by a reduction of non-HDL-C rather than HDL-C. This data is in agreement with human studies showing that increased intake of choline-enriched food (i.e., eggs) is associated with an improved plasma lipid profile [25, 27]. 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 [32], 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.

Acknowledgments

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.

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.

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SUPPLEMENT

Supporting table 1. List of polymerase chain reaction primer sequences used in mRNA expression analysis.

Gene	Forward primer (5'-3')	Reverse Primer (5'-3')
<i>Actb</i>	AACCGTGAAAAGATGACCCAGAT	CACAGCCTGGATGGCTACGTA
<i>Adgre1</i>	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
<i>Adipoq</i>	CTCCACCCAAGGGAACCTTGT	TAGGACCAAGAAGACCTGCATC
<i>Apob</i>	GCCCATTTGTGGACAAGTTGATC	CCAGACTTGGAGGTCTTGGA
<i>Rplp0</i>	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
<i>Ccl2</i>	GCATCTGCCCTAAGGTCTTCA	TTCACTGTACACTGGTCACTCCTA
<i>Cd36</i>	GCAAAGAACAGCAGCAAAATC	CAGTGAAGGCTCAAAGATGG
<i>Cd68</i>	ATCCCCACCTGTCTCTCTCA	TTGCATTTCCACAGCAGAAG
<i>Cd163</i>	CTCAGGAAACCAATCCAGA	CAAGAGCCCTCGTGGTAGAC
<i>Cpt1a</i>	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
<i>Hmgcr</i>	CCGGCAACAACAAGATCTGTG	ATGTACAGGATGGCGATGCA
<i>Il1b</i>	GCAACTGTTCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
<i>iNos</i>	CGGGCATCTGGTAGCCAGCG	TGGCAACATCAGGTCGGCCAT
<i>Mrc1</i>	GAGAGCCAAGCCATGAGAAC	GTCTGCACCCTCCGGTACTA
<i>Mvk</i>	GCCTTGAACCTGAGAACCTTCC	CACCTGCTTAATACCGATGTTG
<i>Mttp</i>	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGATAGCCGCTCATT
<i>Ppara</i>	ATGCCAGTACTGCCGTTTTTC	GGCCTTGACCTTGTTTCATGT
<i>Srebp1c</i>	AGCCGTGGTGAGAAGCGCAC	ACACCAGTCCCTCAGTGATTGCT
<i>Srebp2</i>	TGAAGCTGGCCAATCAGAAAA	ACATCACTGTCCACCAGACTGC
<i>Tnfa</i>	AGCCCACGTCGTAGCAAACCAC	TCGGGGCAGCCTTGTCCTT
<i>Vsig4</i>	GGATCCCACCCACCTAAAACA	CTCGAGTCAGCAGGAGGAATAGA

Actb, β -actin; *Adgre1*, adhesion G protein-coupled receptor E1; *Adipoq*, Adiponectin; *Apob*, apolipoprotein B; *Ccl2*, C-C motif chemokine ligand 2; *Cd36*, cluster of differentiation 36; *Cd68*, cluster of differentiation 68; *Cd163*, cluster of differentiation 163; *Cpt1a*, carnitine palmitoyl transferase 1 α ; *Hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *Il1b*, interleukin-1 β ; *iNos*, inducible nitric oxide synthase; *Mrc1*, mannose receptor C-type 1; *Mvk*, mevalonate kinase; *Mttp*, microsomal triglyceride transfer protein; *Ppara*, peroxisome proliferator-activated receptor α ; *Srebp*, sterol regulatory element-binding protein; *Rplp0*, ribosomal protein lateral stalk subunit p0; *Tnfa*, tumor necrosis factor α ; *Vsig4*, V-set immunoglobulin-domain-containing 4.

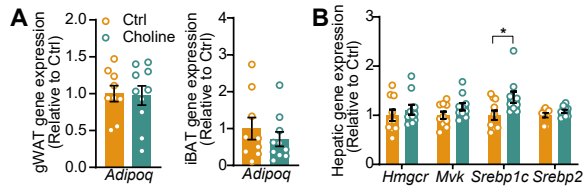


Fig. S1. Choline does not affect adipose tissue expression of adiponectin and has minor effects on hepatic expression of cholesterol synthesis genes. *APOE*3-Leiden.CETP* mice were fed a Western-type diet without and with choline for 16 weeks. (A) At the end of the study, gonadal white adipose tissue (gWAT) and interscapular brown adipose tissue (iBAT) was collected, and gene expression of adiponectin was determined in these adipose tissue depots. (B) In addition, the liver was also collected, and the expression of genes involved in cholesterol synthesis was measured in the liver. Data were obtained from study 2 and are represented as mean \pm SEM (n=8-10 per group). Differences were assessed using unpaired two-tailed Student's *t*-test. * P <0.05.

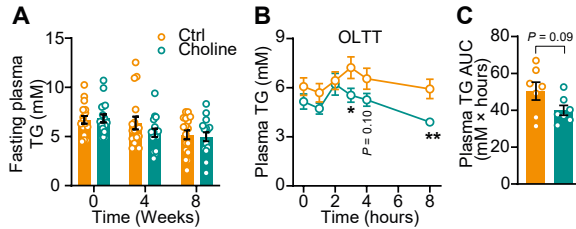


Fig. S2. Choline tends to improve lipid intolerance. *APOE*3-Leiden.CETP* mice were fed a Western-type diet without and with choline for 9 weeks. (A) At 0, 4 and 8 weeks, plasma triglyceride (TG) levels were measured. At week 6, (B) an oral lipid tolerance test (OLTT) was performed, and the area under the curve (AUC) of plasma TG during OLTT was calculated. Data were obtained from study 1 and are represented as mean \pm SEM (A, n=15-16 per group; B, n=6-8 per group). Differences were assessed using (A and B line graph) two-way ANOVA followed by Fisher's LSD test or (B bar graph) unpaired two-tailed Student's *t*-test. ** P <0.01.

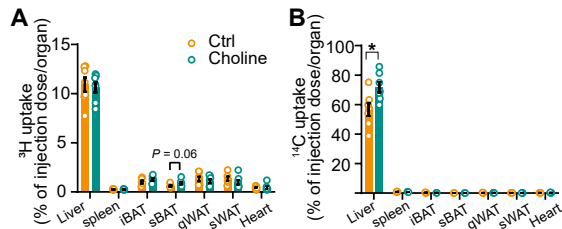


Fig. S3. Choline increases cholesterol-enriched remnant uptake by the liver. *APOE*3-Leiden.CETP* mice were fed a Western-type diet without and with choline for 9 weeks. At week 9, uptake of (A) ^3H and (B) ^{14}C by indicated organs was evaluated. Data were obtained from study 1 and are represented as mean \pm SEM (n=6-8 per group). Differences were assessed using unpaired two-tailed Student's *t*-test. * P <0.05. iBAT, interscapular brown adipose tissue; sBAT, subscapular brown adipose tissue; subcutaneous white adipose tissue (sWAT), gonadal white adipose tissue (gWAT).