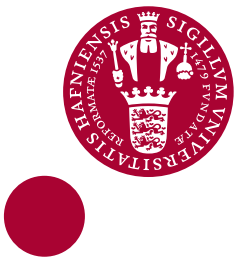

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Increased plasma apoM levels impair triglyceride turnover in mice

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ABSTRACT

Objective: Apolipoprotein M (apoM) is an essential transporter of plasma Sphingosine-1-Phosphate (S1P), typically attached to all lipoprotein classes, but with a majority bound to high density lipoproteins (HDL). ApoM-deficient mice display an increased activity in brown adipose tissue and a concomitant fast turnover of triglycerides. In what manner apoM/S1P affect the triglyceride metabolism is however still unknown and explored in the present study.

Methods: Triglyceride turnover and potentially associated metabolic pathways were studied in the female human apoM transgenic mouse model (apoM-Tg) with increased plasma apoM and S1P levels. The model was compared with wild type (WT) mice.

Results: ApoM-Tg mice had a reduced plasma triglyceride turnover rate and a lower free fatty acid uptake in subcutaneous adipocytes compared to WT mice. Screening for potential molecular mechanisms furthermore revealed a reduction in plasma lipase activity in apoM-Tg animals. Overexpression of apoM also reduced the plasma levels of fibroblast growth factor 21 (FGF21).

Conclusions: The study features the significant role of the apoM/S1P axis in maintaining a balanced triglyceride metabolism. Further, it also highlights the risk of inducing dyslipidaemia in patients receiving S1P-analouges and additionlly emphasizes the apoM/S1P axis as a potential therapeutic target in treatment of hypertriglyceridemia.

1. Introduction

Surplus calories are typically converted to triglycerides, further deposited in white and brown adipose tissues (WAT and BAT, respectively) [1]. WAT stores non-utilized energy as triacylglycerols which can be released for energy consumption in metabolic active organs under catabolic conditions. BAT is characterized by the expression of uncoupling protein 1 (UCP1), which is regulated by a complex network of transcription factors including Peroxisome proliferator-activated receptor gamma (PPARG), PPARG coactivator 1 alpha (PGC1A) and PR domain containing 16 (PRDM16) [2]. The considerably more abundant WAT however plays the pivotal role in plasma triglyceride clearance [3]. Adipocytes in close proximity to the endothelium interact with triglyceride-rich lipoproteins, such as chylomicrons and very low-density lipoproteins (VLDL), to mediate lipid uptake into BAT and WAT through the lipoprotein lipase (LPL) and CD36 pathway [4]. Glycocalyx associated glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 (GPIHBP-1) thereby links LPL with the vascular endothelium [5]. Loss of either GPIHBP-1 or glycocalyx integrity thus affects LPL availability and consequently lipid turnover. A recent study in mice, for instance, demonstrated that loss of syndecan (SDC) 4 affects lipid metabolism and body composition substantially [6]. In addition to LPL, also hepatic lipase (HL) hydrolyses triglyceride-rich particles to free fatty acids (FFA), which are consequently taken up by the liver and stored as fat [7,8]. Whereas the activity of HL is predominantly regulated by lipoproteins such as intermediate-density

Abbreviations: ANGPTL4, angiopoietin like protein family 4; apo, Apolipoprotein; apoM-KO, apoM knock-out; apoM-Tg, apoM transgenic; BAT, Brown adipose tissue; epiWAT, epididymal WAT; FAP-α, Fibroblast activation protein α; FFA, Free fatty acid; FGF21, Fibroblast growth factor 21; FGFR, fibroblast growth factor receptor-1c; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GPIHBP1, Glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1; HDL, High-density lipoprotein; HL, Hepatic lipase; isBAT, interscapular BAT; KLB, β-klotho; LDL, Low-density lipoprotein; LPL, Lipoprotein lipase; MMP, Matrix metalloproteinase; PGC1A, Peroxisome proliferator-activated receptor-gamma coactivator-1α; PPARG, Peroxisome proliferator-activated receptor γ; PRDM16, PR domain containing 16; S1P, Sphingosine-1-Phosphate; $S1P_{1-5}$, S1P-receptor 1–5; SDC, Syndecan; subBAT, subscapular BAT; subWAT, subcutaneous WAT; WAT, White adipose tissue; UCP1, Uncoupling protein 1.

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lipoproteins (IDL) and high-density lipoproteins (HDL) [8], members of the angiopoietin like protein family (ANGPTL) regulate the activity of LPL [9]. Common apolipoproteins (apo) play an additional role in regulating plasma lipase activities including apoC-I (inhibitor), apoC-II (activator), apoC-III (inhibitor) and apoA-V (activator) [10–12].

A recent study highlighted the beneficial role of apolipoprotein M (apoM) in plasma triglyceride turnover [13]. Plasma apoM circulates at a concentration of ~0.9 μmol/L in humans and mice [14,15]. Although the liver and kidneys play the pivotal role in apoM gene expression [16], minor expression levels in adipocytes [17] and porcine brain capillary endothelial cells [18] are additionally suggested. ApoM is ubiquitously distributed among lipoproteins, the majority (95%) resides however in HDL [16,19]. ApoM facilitates binding of sphingosine-1-phosphate (S1P) [20]. ApoM-associated S1P accounts for ~70% of total plasma S1P whereas the residual amount resides in albumin and to a significantly lesser extent in apoA-IV [21–23]. S1P levels furthermore correlate with apoM gene dosage and thus plasma apoM abundance [20,24]. S1P interacts with five S1P-receptors ($S1P_{1–5}$), thereby modulating a wide spectrum of intra- and extracellular signalling pathways [25–27]. Among those, S1P regulates matrix metalloproteinases (MMPs) which cleave proteoglycans (i.e. SDCs) directly from the cellular membrane [28]. Hence, S1P plays a role in maintaining glycocalyx integrity and SDC shedding, potentially important for LPL mediated triglyceride hydrolysis [29,30].

Previous studies identified a role of the apoM/S1P system in triglyceride metabolism [31–34]. Most imperative, apoM-deficient (apoM-KO) mice with characteristically low plasma S1P levels revealed an apoM/S1P-mediated protection against diet-induced obesity and an accelerated postprandial plasma triglyceride clearance rate [13]. Administration of the functional S1P antagonist FTY720 to wild type mice (WT) furthermore mimicked the beneficial effects of apoM deficiency [13]. To which extent an increased plasma apoM and S1P concentration affects triglyceride metabolism, as well as the mechanism(s) underneath, are however unknown.

Similar to the apoM/S1P system, fibroblast growth factor 21 (FGF21) also plays a significant role in regulating triglyceride turnover [35]. The triglyceride lowering effect of FGF21 is however primarily attributed to an increased FFA and whole lipoprotein uptake into WAT, among others. FGF21 typically signals through fibroblast growth factor receptor-1c (FGFR) and its joint co-receptor β-klotho (KLB). Loss of either results in elevated plasma triglyceride levels [36,37].

In the present study, we investigated the impact of human *APOM* overexpression on triglyceride metabolism in the apoM transgenic (apoM-Tg) mouse model [38]. A remarkably delayed plasma triglyceride clearance rate, caused by reduced plasma lipase activities, was discovered. Elevated plasma concentrations of apoM and S1P furthermore affected the FGF21 system. Together with recent findings in apoM-KO mice, this study highlights the crucial role of the apoM/S1P complex in maintaining a balanced triglyceride metabolism.

2. Material and methods

2.1. Animals

Mice were housed at the Panum Institute (University of Copenhagen) in a temperature-controlled facility with a 12-h dark/light cycle and fed standard chow (Altromin number 1314; Brogaarden) or high-fat containing diet (Research Diet D12492). ApoM-Tg mice, with ~38 copies of human *APOM*, were back-crossed with C57B6/J wild type (WT) animals for >10 generations [38]. Only female mice were used throughout the study. WT mice (C57B6/J) were obtained from a separate colony and served as the control group for all experiments. Exclusion criteria were loss of body weight, bite wounds, malnutrition, and signs of infections. The required sample size was determined by a priori knowledge on biological variation for each experimental setup. When applicable, mice from different cages were randomly assigned to either treatment or control group and each mouse was assigned an experimental number across groups and genotypes throughout animal procedures and laboratory analysis to blindly perform the experiments. Depending on the type of the experiment, *in vivo* and corresponding downstream *in vitro* procedures were performed by different researchers. All procedures have been approved by the Danish Authorities and were performed in accordance to the ARRIVE guidelines.

2.2. Blood and chylos sampling

Blood samples from the tail vein or venous plexus in the orbital cavity were collected in Na_2EDTA or EDTA coated tubes and kept on ice. Plasma was isolated via centrifugation (3000 rpm; 4 °C; 10 min) and immediately used or stored at −80 °C for further analysis. For chylos sampling, animals where anesthetized 30 min after an oral gavage of 200 μL olive oil. The ductus thoracicus was localized and cannulated at cisterna chyli to collect 3–8 μL chylos.

2.3. Isolation of tissue samples

Animals aged between 14 and 18 weeks were anesthetized (0.1 mL/g body weight) by a mixture of Zoletil 50 Vet (3.26 mg/mL), Xylazine (2.61 mg/mL) and Butorphanoltartrat (0.065 mg/mL). After retro-orbital blood sampling, mice were perfused with 20 mL ice-cold saline through the left ventricle of the heart. Depending on the experimental setup, blood, liver, heart, lung, spleen, kidney, epididymal WAT (epiWAT), subcutaneous WAT (subWAT), interscapular BAT (isBAT), subscapular BAT (subBAT), renal artery, aortic arch (down to the 6th rib) and femur bone were isolated after careful removal of adherent tissues, weighted, snap frozen in liquid nitrogen, and stored at −80 °C for downstream processing. Organ weights were normalized to femur bone length.

2.4. Plasma analysis

Each analysis was performed in duplicates. Plasma apoM levels were determined by an in-house ELISA assay as described previously [39]. The plasma triglyceride and cholesterol concentration was assayed by an enzymatic kit according to the manufacturer's specifications (GPO Trinder, Sigma-Aldrich; Cholesterol CHOD-PAP Method (11491458), Roche). Plasma samples from the tail vein were subjected to SDC4 (ELISA; E03S0064, BlueGene Biotech, China) and FGF21 (Mouse FGF-21 ELISA Kit; ab212160; Abcam) plasma measurements, following the manufacturer's instructions.

2.5. Triglyceride turnover

After overnight fasting (6 h), mice received a single oral gavage of 300 μL olive oil, supplemented with or without ^{3H}-triolein. Blood samples were collected at baseline as well as 0.5, 1, 2, 4 and 8 h post gavage followed by sacrifice and perfusion with 0.9% NaCl. Tissue biopsies were prepared in Solvable (PerkinElmer) and analysed by scintigraphy in a Wallac 1409 Liquid Scintillation Counter (PerkinElmer). Triton WR-1339 (0.5 mg/g body weight) was injected intravenously after overnight fasting (6 h) and blood samples were taken at 0, 30, 60, 90, and 120 min. A separate cohort of mice also received Triton WR-1339 (0.5 mg/g body weight), immediately followed by an oral gavage of olive oil (12 μL/g body weight). Blood sampling was performed after 0, 30, 60 and 120 min. Fingolimod (FTY720; Sigma-Aldrich) or saline was given by oral gavage (0.5 μg/g body weight) followed by blood sampling at 0 and 2 h. After overnight fasting (6 h), W146-hydrate, solved in (2-Hydroxypropyl)-β-cyclodextrin, was administered by intraperitoneal injection (10 μg/g body weight). After 1 h, the animals were given an oral gavage of olive oil (12 μL/g body weight). Blood was collected at the time points 0, 60 and 120 min. Intralipid® (10%; Fresenius-Kabi, Uppsala, Sweden) was administered intravenously (5 μL/g body weight), and blood

samples were taken at 2, 10, 20, 60, and 120 min after injection.

2.6. Lipase activity

To determine lipase activities, non-fasted WT and apoM-Tg animals received 0.6 IE/g body weight unconserved heparin. Blood samples were taken before the injection (pre-heparin) and 10 min afterwards (post-heparin). Enzyme activities were measured by an enzymatic lipase activity assay (#700640, Cayman Chemical) in duplicates. Immunoblotting with pre- and post-heparin plasma was additionally performed. Plasma samples (1.0–1.5 μL) were reduced and prepared using NuPAGE (Invitrogen) reagents, resolved on an 12% Bis-Tris gel (NuPAGE, Invitrogen), and transferred to a PVDF membrane. After blocking in 5% skim milk, the membrane was incubated with the primary antibody against LPL (R&D Systems, AF7197), HL (LSBio, LS-C295163), ANGPTL3 (Abcam, ab126718), ANGPTL4 (Novus Biologicals, NB300-590), ANGPTL8 (Abcam, ab180915) or apoC-III (LSBio, LS-C372831) overnight, washed, incubated with a corresponding HRP labelled secondary antibody for 1 h, and developed using SuperSignal West Pico PLUS substrate (Invitrogen).

2.7. Glucose tolerance test

ApoM-Tg and WT mice received either a chow or a high-fat diet (HFD) for 10 consecutive weeks. Total body weight was assessed weekly. After 10 weeks, the animals were fasted overnight, before i.p. injection of glucose (2 μg/g body weight). Blood glucose was measured after 0, 30, 60, and 120 min.

2.8. Gene expression analysis

mRNA from selected tissues was isolated with TRIZOL reagent (Invitrogen) and cDNA was synthesized with a cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qPCR) was then performed in triplicates by an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using either SYBR Green PCR Master Mix (Applied Biostystems) or TaqMan Gene Expression Assay (FAM; Applied Biosystems). The average gene expression levels of Hypoxanthine-guanine phosphoribosyltransferase (HPRT), TATA-binding protein (TBP) or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. Primer sequences are provided in Supplementary table 1.

2.9. Hepatic triglycerides

Liver biopsies (~30 mg) were weighted and lipids extracted as described previously [40]. After evaporation, lipids were solved in isopropanol with 1% triton-x-100. Glycerol and C.F.A.S. (Calibrator for Automated Systems; Roche) were mixed 1:1, extracted as samples and used as standards. Triglycerides were determined by an enzymatic assay (GPO Trinder, Sigma-Aldrich). The experiment was performed in duplicates.

2.10. Statistical analysis

GraphPad Prism version 6 (GraphPad Software) and R [41] were used for statistical analyses. Differences between groups of two were assessed using a student's *t*-test with Welch-Satterthwaite correction whenever appropriate. The area under the curve (AUC) at the indicated range was calculated using trapezoid interpolation. Data points exceeding the recommended limitations for each assay resulted in exclusion of the entire animal from that test. A *p*-value <0.05 was considered significant.

3. Results

3.1. Excessive plasma apoM and S1P levels reduce plasma triglyceride clearance

When compared to human individuals, the genotype of apoM-Tg mice results in a 12-times increased human plasma apoM concentration (0.9 μmol/L vs 10.9 μmol/L) [38]. In parallel, plasma S1P increased 2-times, compared to WT mice (3.6 ± 0.1 μmol/L versus 1.8 ± 0.1 μmol/L, $p < 0.001$; Fig. 1A), also comparable to previous reports [20,38].

Loss of apoM accelerates triglyceride turnover in mice [13]. In the present study, apoM overexpressing mice were challenged with a single bolus of olive oil, preceding a 6 h fasting period. Plasma triglyceride clearance in those mice was substantially delayed when compared to WT animals (AUC: 273.5 ± 81.2 vs 772.7 ± 178.3, $p < 0.05$; Fig. 1B). Cholesterol turnover was however not affected (Fig. 1C). To evaluate tissue specific lipid uptake, olive oil was supplemented with radiolabelled triolein and administered by oral gavage. ApoM-Tg mice showed 80% reduced lipid uptake in subcutaneous WAT (subWAT, $p < 0.01$) and 336% increased hepatic uptake ($p < 0.05$), combined with a 61% increased plasma triolein concentration ($p < 0.01$; Fig. 1D) after 2 h. Lipid uptake in BAT was not affected. Moreover, changes in plasma triglyceride clearance could not be subscribed to changes in total bodyweight (Supplemental Fig. 1A), nor specific organ mass normalized to tibia bone length (Supplemental Fig. 1B).

3.2. A high-fat diet elevates plasma triglycerides in apoM-Tg mice

To investigate lipid metabolism in a chronic setting, we challenged WT and apoM-Tg mice with a high fat diet (HFD) for 10 consecutive weeks. Weight gain in apoM-Tg mice was significantly reduced when compared to WT animals (1.0 ± 0.1 g/week vs 0.8 ± 0.1 g/week; AUC HFD: 59.4 ± 5.2 vs 41.50 ± 5.6, $p < 0.05$; Fig. 2A). No differences between genotypes were observed when animals were fed a regular chow diet. To verify whether the apoM-Tg genotype also affected glucose turnover, an oral glucose tolerance test was performed. No differences between WT and apoM-Tg mice were however observed (Fig. 2B). After 10 weeks on a HFD, plasma triglycerides in the apoM-Tg model were increased by 40%, compared to corresponding WT animals ($p < 0.01$; Fig. 2C). Independent of the diet, total plasma cholesterol was increased by 25% in the apoM-Tg mice (Fig. 2D), in line with a previous study [38]. Plasma FFAs was not affected in apoM-Tg mice (Fig. 2E).

3.3. Reduced lipase activities delay plasma triglyceride clearance in apoM-Tg mice

Changes in intestinal lipid transport (i.e. formation of chylomicrons) may delay plasma triglyceride turnover in apoM-Tg mice. To verify whether increased apoM/S1P concentrations also affect triglyceride metabolism after by-passing intestinal processing, WT and apoM-Tg animals received a bolus of intralipids intravenously. Already after 2 min, apoM-Tg mice displayed a tendency towards a delayed plasma triglyceride turnover rate, however without reaching statistical significance (Fig. 3A). Intestinal triglyceride processing can be thus not excluded as a contributor to a reduced turnover in the apoM-Tg animals. Of note, apoM was also present in chylos, isolated after administering an oral gavage of olive oil (Supplemental Fig. 2). Hence, apoM is already associated with native formed chylomicrons from intestinal compartments, and may therefore potentially affect their formation.

To establish whether the apoM/S1P complex changes the hepatic secretion rate of triglyceride-containing VLDL, the lipolytic activity of LPL was blocked by an intravenous injection of Triton WR-1337 (Fig. 3B). Plasma triglyceride levels in apoM-Tg and WT mice however diverged only after 120 min without reaching statistical significance. To investigate a potentially increased secretion rate of chylomicrons, we administered Triton WR-1337 combined with a bolus of olive oil

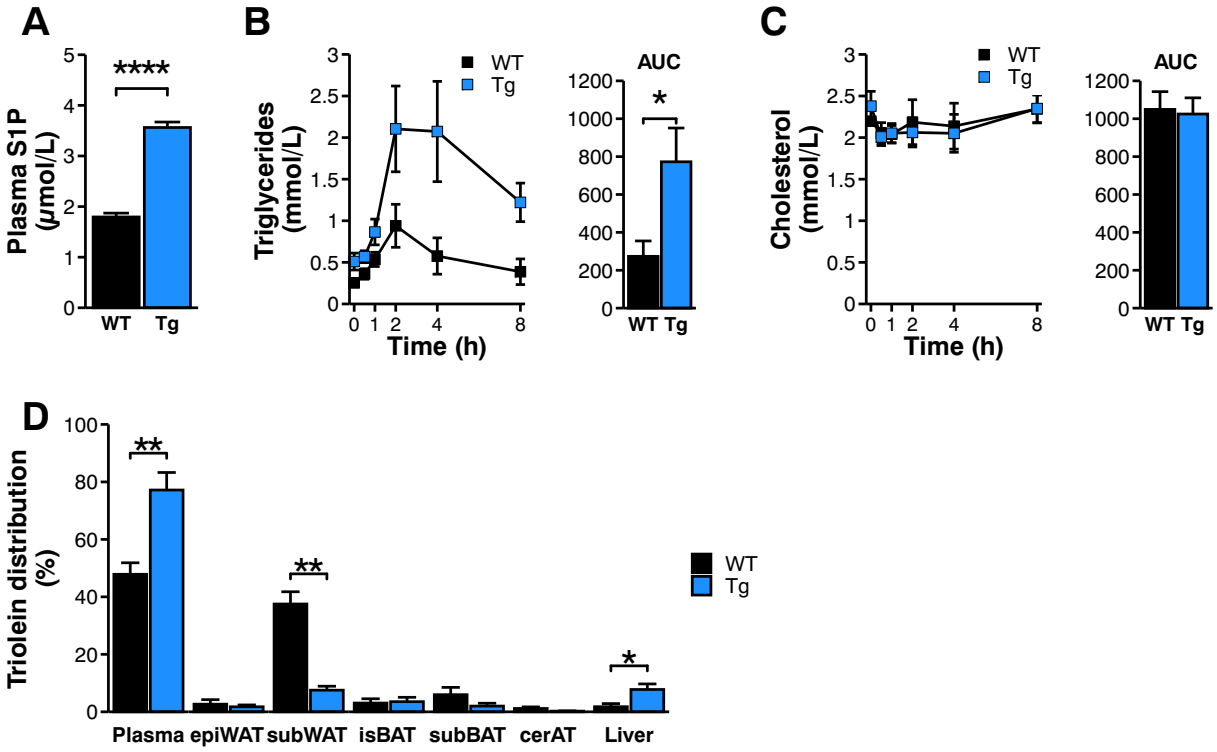

Fig. 1. Baseline characteristics and triglyceride metabolism in the apoM-Tg mouse model. (A) Plasma S1P concentration in WT ($n = 9$) and apoM-Tg ($n = 11$) mice. Plasma (B) triglycerides and (C) total-cholesterol, measured after oral administration of 300 μL olive oil in WT ($n = 6$) and apoM-Tg (n = 6) mice. (D) To explore tissue specific triglyceride uptake, 300 μL of olive oil supplemented with ^{3}H-triolein were orally administered and the mice were sacrificed after 2 h. Triolein uptake is shown as percentage of the total dose (WT: $n = 4$; apoM-Tg: n = 4). Groups of two were compared by a student's *t*-test. Data is presented as Mean ± SE and no datapoints were excluded. WAT = white adipose tissue, BAT = brown adipose tissue, epiWAT = epididymal WAT, isBAT = interscapular BAT, subBat = subscapular BAT, cerAT = cervical adipose tissue, Tg = apoM-Tg, WT = Wild type. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$.

(Fig. 3C). Differences between apoM-Tg and WT mice were absent.

While the production rate of chylomicrons per se may be independent of apoM/S1P, their processing via lipase mediated pathways could be affected. Lipases play a crucial role in triglyceride hydrolysis at the vascular endothelium. Reduced activities may impede triglyceride clearance in apoM-Tg mice and were therefore assessed by a heparin assay. Compared to WT mice, apoM-Tg mice had 81% reduced plasma lipases activities ($p < 0.05$) (Fig. 3D). Immunoblotting against the plasma triglyceride lipases, LPL and HL, in addition to the enzymatic regulators ANGPTL3, ANGPTL4, ANGPTL8 and apoC-III showed no quantitative differences (Fig. 3E) between the genotypes. Changes in the LPL gene expression in epiWAT, subWAT and muscle were furthermore absent (Fig. 3F). Proteins of the ANGPTL family play a central role in energy metabolism and are regulated by the prandial state. Compared to non-fasted animals, ANGPTL-3, −4 and − 8 concentrations did not differ between apoM-Tg and WT mice after 6 h fasting (Supplemental Fig. 3). We can however not exclude that prolonged fasting or methodological aspects could induce a difference between the animals.

LPL availability at the vascular lumen relies on glycocalyx integrity. In apoM-Tg mice, the hepatic gene expression of the two major plasma membrane proteoglycans, *SDC1* and *SDC4*, was significantly increased (*SDC1*: 40%, $p = 0.02$; *SDC4*: 30%, $p = 0.04$; Fig. 3G). The plasma SDC4 concentration was also 52% higher in apoM-Tg mice (Fig. 3H) suggesting increased shedding of SDC4. In contrast, gene expression analyses of the most common MMPs in the aorta and *A. renalis* did not reveal any differences between genotypes (Fig. 3I). The expression of MMP13 in the aorta of apoM-Tg mice however suggested a reduction by 65% ($p = 0.07$), and may therefore contribute to an altered glycocalyx composition, thereby negatively affecting plasma lipase activities.

3.4. Potential pathways affecting triglyceride turnover in apoM-Tg mice

ApoM deficient mice show an accelerated triglyceride turnover rate, likely due to low plasma S1P levels and compromised S1P-signalling via $S1P_1$ [13]. We thus quantified the gene expression of relevant S1P receptors in different tissues. Most striking, the $S1P_1$ expression in epiWAT, but not subWAT, from apoM-Tg mice was 47% reduced, compared to WT animals ($p < 0.01$; Fig. 4A). Differences in liver and muscle tissue were not evident (Supplemental Fig. 4A and B). Next, we explored the effect of treatment with the functional S1P-antagonist FTY720 (Fig. 4B). As expected, FTY720 administered to WT animals reduced the plasma triglyceride concentration by 57% after 2 h ($p < 0.001$). In the apoM-Tg mice however, no statistically significant effect was observed when compared to baseline values, despite a trend towards 36% further increased plasma triglycerides. Such marginal divergence between WT and apoM-Tg mice may be due to a lifelong exposure of S1P. S1P receptor sensitization or changes in its degradation rate may bias the outcome of FTY720 stimulation. Hence, we performed a study with W146, a specific $S1P_1$ antagonist, and thus independent of receptor sensitization and degradation. Compared to animals without treatment (Fig. 1B), we observed an accelerated triglyceride turnover in WT (27%) as well as apoM-Tg (48%) mice after 120 min (Fig. 4C). This dataset suggests that the delayed triglyceride clearance in non-treated apoM-Tg mice could be due to increased S1P-$S1P_1$ signalling.

Beyond the apoM/S1P axis, also the FGF21 system plays a vital role in triglyceride turnover and energy homeostasis [35,42]. Gene expression analyses identified increased mRNA transcripts of FGF21 (124%; $p < 0.05$) and β-Klotho (18%; $p < 0.05$; Fig. 4D) in apoM-Tg mice, compared the WT animals. Changes in the FGFR1c gene expression were however absent. In contrast to the hepatic FGF21 gene expression, the plasma FGF21 concentration in apoM-Tg animals was 57% decreased (p

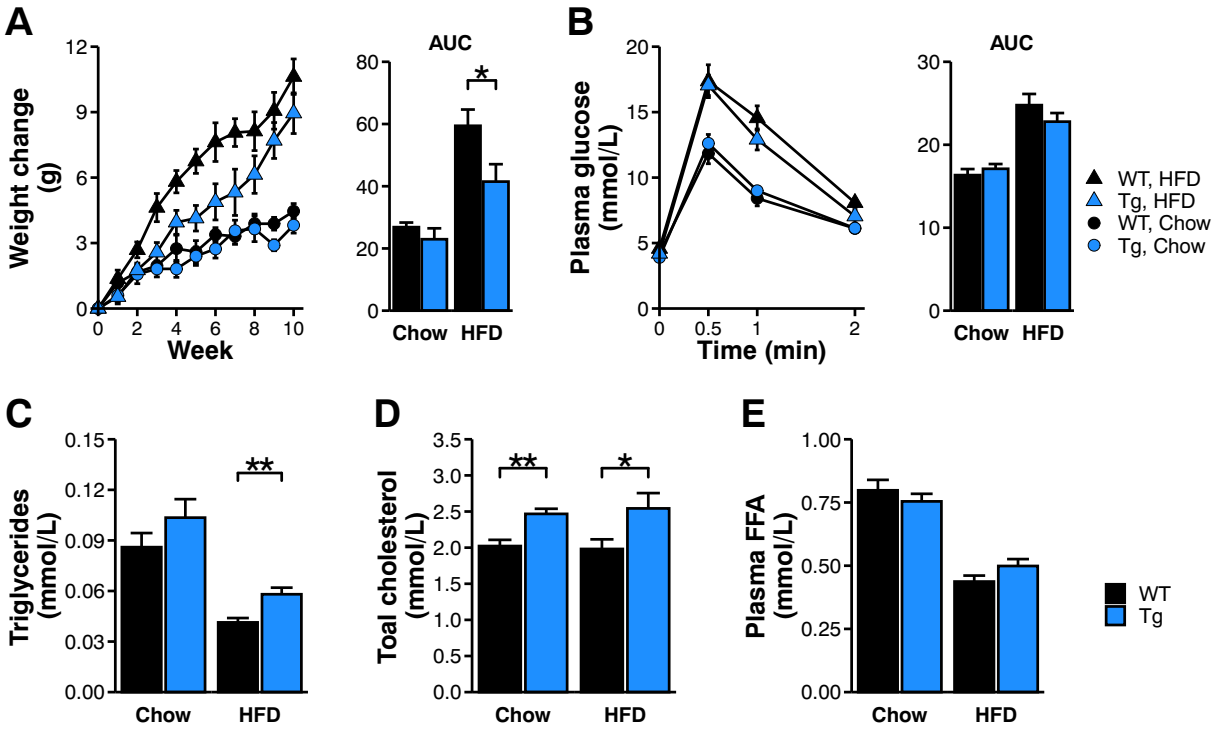

Fig. 2. HFD effects body weight and plasma lipids. WT and apoM-Tg mice either received a standard chow diet (n = 6) or a high fat diet (HFD; $n = 8$). (A) Change in body weight over 10 consecutive weeks, normalized to baseline measurements (Week 0 = 22.7 g (±0.3)). (B) After 10 weeks, an oral glucose tolerance test (2 μg glucose per g body weight) was performed after 12 h of fasting. Plasma levels of (C) triglycerides, (D) total cholesterol and (E) free fatty acids were assessed after 10 weeks on a chow or HFD. Groups of two were compared by a student's t-test. Data is presented as Mean ± SE and no datapoints were excluded. HFD=High fat diet, Tg = apoM-Tg, WT = Wild type. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$.

< 0.05), compared to WT mice (Fig. 4E). We hypothesized that accelerated enzymatic degradation by fibroblast activation protein α (FAP) [43]) may explain such reduction, differences between WT and apoM-Tg mice were however absent (Supplemental fig. 5). FTY720 mediated S1P receptor activation did furthermore not affect the plasma FGF21 concentration, 2 h post injection (Fig. 4E). The observed FGF21 phenotype is thus either independent of S1P receptor signalling or emerged as a result of chronic S1P receptor activation. FGF21 accelerates disposal of triglyceride-rich particles into adipose tissues, thus lowering the particles secretion from the liver, and resulting in a lower hepatic triglyceride content [35]. In the present study, quantification of hepatic triglycerides showed a 15% increase in apoM-Tg mice compared to WT animals (98.8 ± 2.1 vs 85.1 ± 3.3 nmol/L/mg tissue; $p < 0.01$; Fig. 4F). Moreover, genotypical differences between animals receiving a HFD were not evident.

BAT utilizes vast amounts of triglycerides to generate heat. Since apoM deficient mice displayed enhanced BAT activity, we also quantified the gene expression of BAT associated marker in the apoM-Tg mouse model (Fig. 4G). While differences between WT and apoM-Tg mice in WAT and subBAT were absent, the gene expression of UCP1 as well as PRDM16 ($p = 0.02$) was significantly increased (43%, $p = 0.02$ and 30%, $p = 0.02$, respectively). Moreover, histological sections of BAT from apoM-Tg mice did not deviate from WT mice (Supplemental fig. 6).

4. Discussion

Herein we report a reduction of the plasma triglyceride clearance rate in apoM-Tg mice, caused by increased human plasma apoM and subsequently increased S1P concentrations, when compared to WT animals. Lipid uptake into subWAT was furthermore decreased, whereas hepatic lipid uptake and content were significantly increased. The phenotype may be explained by a reduction in plasma lipase activity and plasma FGF21 concentration, as well as potential changes in plasma SDC4. Combined, the data of the present study highlights the potent role of the apoM/S1P complex in triglyceride metabolism.

Specifically, triglyceride metabolism is regulated by a vast repertoire of already described and to date elusive factors [44]. In support to our findings, apoM-deficiency and reduced plasma S1P concentrations were recently linked to accelerated plasma triglyceride clearance [13]. Reports also emphasize a connection between the S1P system and triglyceride metabolism [31,45]. In the present study, apoM-Tg mice showed 80% reduced triolein uptake into subWAT when compared to WT mice. In apoM-KO mice, triolein uptake into WAT and BAT is increased compared to WT mice [13]. It is well described that activated BAT contributes to an enhanced triglyceride turnover [46]. Although changes in apoM-Tg BAT volume were not evident in the present study, the gene expression of UCP1 and its transcription factor PRDM16 in isBAT was significantly elevated. Those changes in apoM-Tg BAT physiology may indicate a compensatory mechanism to handle excessive plasma triglyceride concentrations. Further mechanisms may play an additional role in apoM/S1P mediated triglyceride turnover. In support, apoM-KO mice housed at thermoneutral conditions (30 °C), and therefore without activation of BAT, still displayed a significantly increased plasma triglyceride clearance rate [13]. Additional factors besides BAT thus contribute to an apoM/S1P regulated triglyceride metabolism.

LPL and HL play a fundamental role in clearing triglycerides from the blood circulation. In the present study, we identified 81% reduced total lipase activities in our apoM-Tg mouse model. For comparisons, apoM-KO mice display an 40% increased lipase activities [13]. Protein immunoblotting against LPL and HL, as well as the lipase mediators apoC-III and members of the ANGPTL family, furthermore excluded significant quantitative difference between WT and apoM-Tg animals. Our data suggests that increased apoM/S1P levels directly affect plasma lipase activities, thereby delaying the triglyceride clearance rate. It is generally accepted that rodent pre-heparin samples represent HL activity and the difference to post-heparin samples represent LPL [47,48].

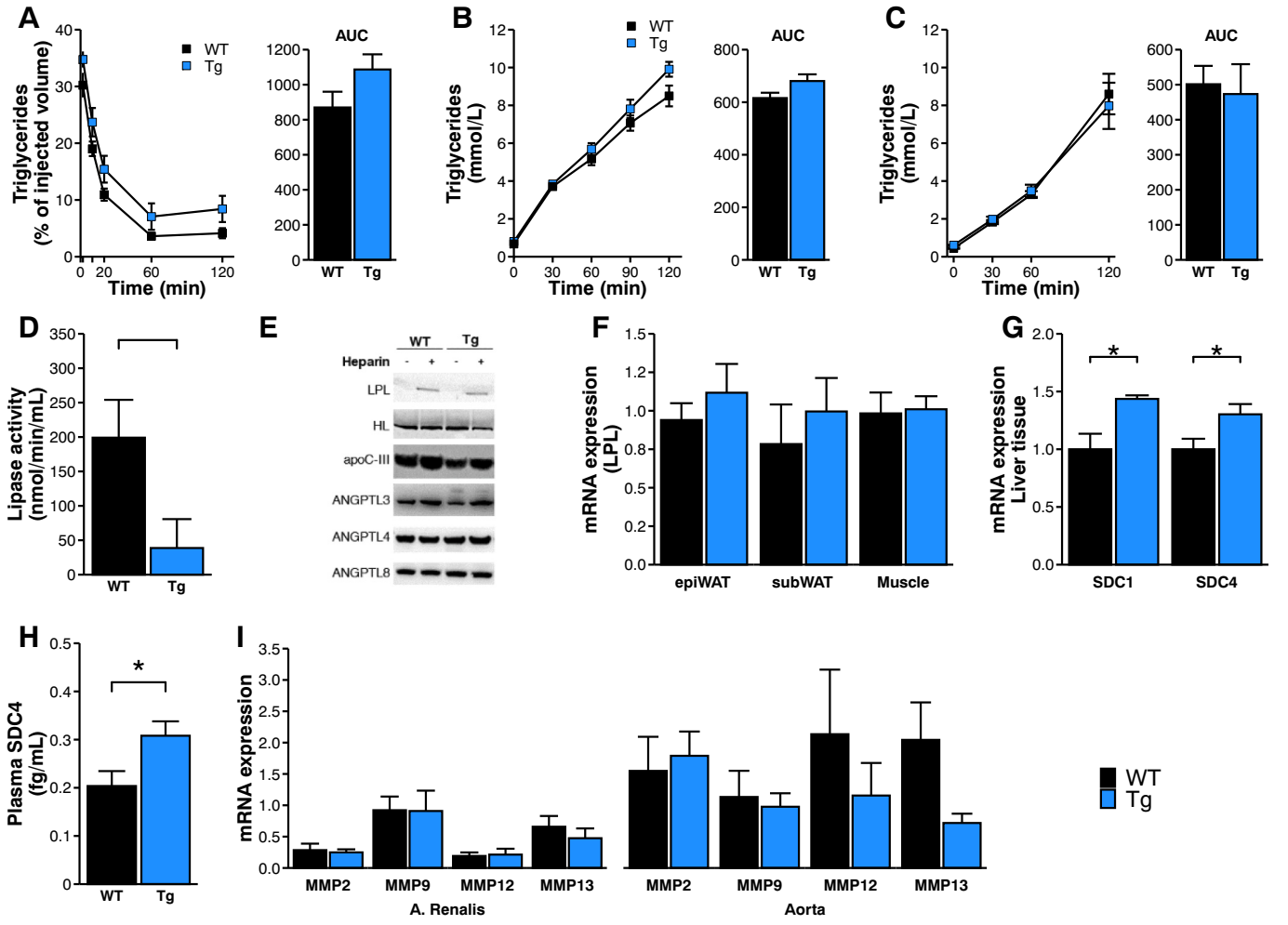

Fig. 3. Triglyceride clearance in the apoM-Tg mouse model. (A) Intralipids (10%) were administered intravenously (5 μL/g bodyweight) to by-pass intestinal lipid uptake, and plasma triglycerides were determined at indicated time points (WT: $n = 5$; apoM-Tg: n = 5). Plasma triglyceride concentration measured after inhibition of LPL activity by intravenous injection of Triton WR-1339 (0.5 mg/g body weight), (B) without (WT: n = 5; apoM-Tg: n = 5) and (C) with (WT: $n = 7$; apoM-Tg: n = 6) subsequent olive oil gavage (12 μL/g body weight). (D) Plasma lipase activity in WT (n = 8) and apoM-Tg mice (n = 11) mice. Enzyme activities were calculated by subtracting pre-heparin lipase activities from the corresponding post-heparin samples. (D) Representative immunoblot of plasma lipases and modulating plasma proteins pre (−) and 10 min post (+) heparin administration. (F) LPL gene expression in epiWAT, subWAT and muscle (WT: n = 7; apoM-Tg: n = 6). (G) Hepatic gene expression of SDC1 and SDC4 (WT: n = 7; apoM-Tg: n = 5). (H) Plasma concentration of SDC4 as indicator for SDC4 shedding (WT: n = 7; apoM-Tg: n = 8). (H) MMP gene expression in a. renalis (WT: n = 6–7; apoM-Tg: n = 4) and aorta (WT: n = 6–7; apoM-Tg: n = 6). Groups of two were compared by a student's t-test. Data is presented as Mean ± SE and no datapoints were excluded. MMP = Matrix metalloproteinase, WAT = white adipose tissue, epiWAT = epididymal WAT, subWAT = subcutaneous WAT, Tg = apoM-Tg, WT = Wild type. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$.

Reduced plasma lipase activities in the apoM-Tg mouse strain thus likely reflect LPL. In addition, endothelial lipase hydrolyses HDL more efficiently than triglyceride rich particles, thus playing a minor role in triglyceride turnover [49]. Efficient triglyceride hydrolysis, as well as the consequent FFA uptake into adipocytes, rely on a stable interaction between the GPIHBP-1/LPL complex and the glycocalyx surface of endothelial cells [5]. Here, triglyceride containing particles bind to the endothelial surface inbetween patches of the glycocalyx [50]. The number of gaps between such patches and interactions with the glycocalyx structure itself are equally important for an efficient triglyceride metabolism. Syndecans are a major constituent of the glycocalyx. Interestingly, female SDC4$^{-/-}$ mice had increased plasma lipid and glucose levels, in addition to an elevated hepatic triglyceride content [6]. In the present study, the increased SDC4 gene expression (30%) and plasma concentration (52%) in apoM-Tg mice contradict such observations. ApoM-Tg mice had increased plasma cholesterol and postprandial triglyceride levels. Moreover, S1P protects the glycocalyx by inhibiting MMPs, thereby preventing SDC1 and SDC4 shedding [51]. The increased plasma S1P concentrations in apoM-Tg mice did however not affect the MMP gene expression, although we discovered a marginal reduction in MMP-13. We speculate that increased apoM and S1P signalling may increase or modulate the glycocalyx structure, as indicated by an elevated plasma SDC4 concentration and gene expression. Reduced gaps between glycocalyx patches may lower the possibility of interactions between triglyceride containing lipoproteins and the GPIHBP1/LPL complex, consequently lowering LPL activity and thereby increasing post prandial triglyceride levels. This needs however further investigations to elucidate the exact mechanisms.

The liver plays a pivotal role in lipid metabolism, including uptake, synthesis, secretion and minor storage of triglycerides [52]. Experiments with radioactive labelled triolein revealed an increased uptake in the liver 2 h post oral gavage. Whether the effect arises as a compensatory mechanism due to a prolonged plasma triglyceride circulation time (i.e. reduced plasma lipase activities) or whether the apoM-S1P axis affects the system directly is unknown. Triglycerides taken up by the liver are typically secreted in VLDL [52]. To assess hepatic VLDL secretion, we injected Triton WR-1339 to block LPL activity. Differences between WT and apoM-Tg mice were not evident. Changes in the apoM-S1P system

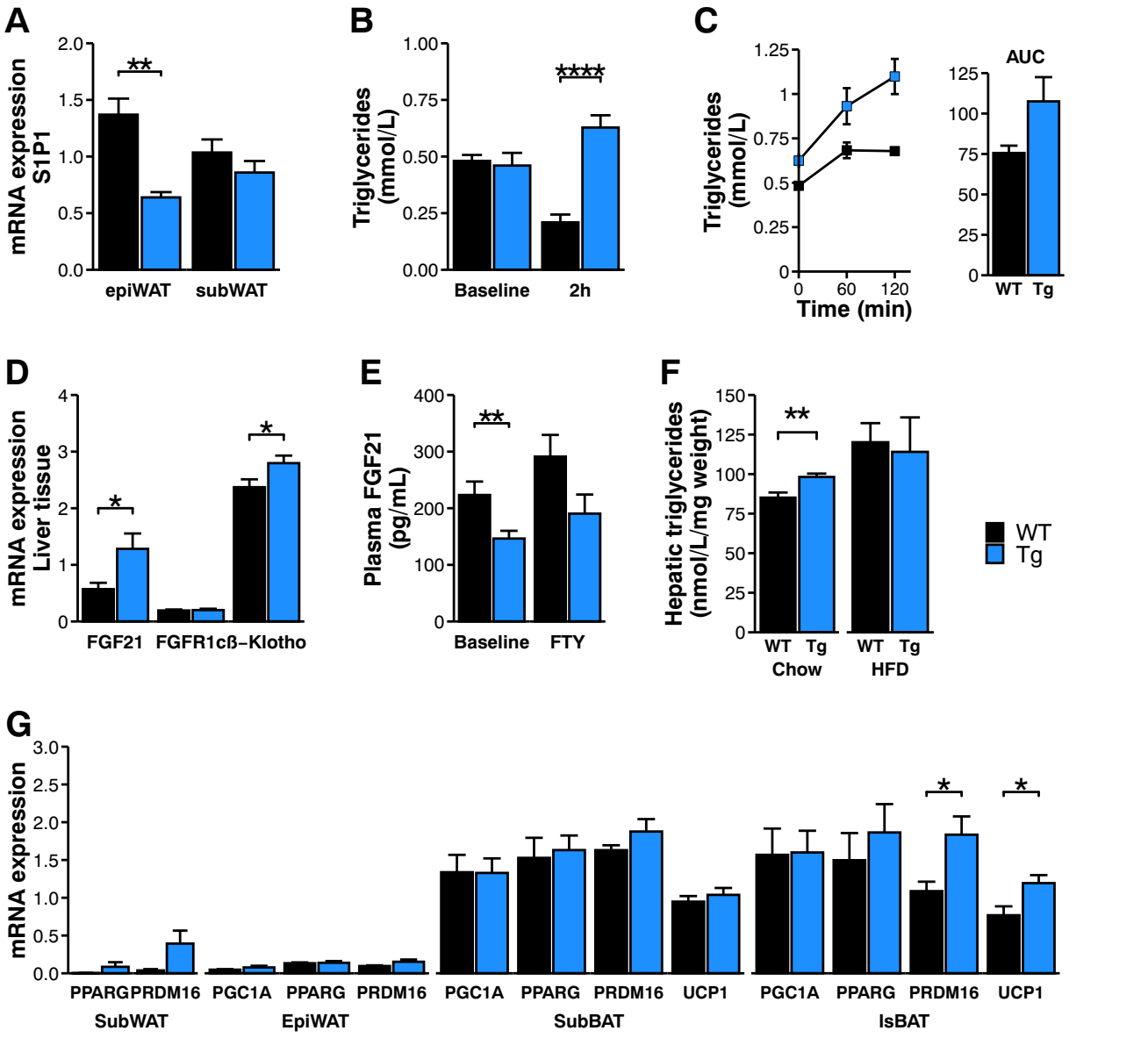

Fig. 4. Pathways in triglyceride turnover. (A) $S1P_1$ gene expression in epiWAT and subWAT (WT: n = 5; apoM-Tg: *n* = 13). (B) Plasma triglycerides before and after oral gavage of FTY720 (0.5 μg/g body weight) in WT (n = 5) and apoM-Tg mice (n = 5). (C) Plasma triglycerides before and after W146 induced $S1P_1$ inhibition (WT: n = 7, apoM-Tg: n = 6). (D) Hepatic gene expression of FGF21 and the corresponding receptors FGFR1c and β-Klotho (WT: n = 5, apoM-Tg: n = 13). (E) Plasma FGF21 concentration in WT and apoM-Tg mice before (WT: *n* = 22, apoM-Tg: *n* = 26) and 2 h after injection of FTY720 (0.5 μg/g body weight; WT: n = 5, apoM-Tg: n = 5). (F) Hepatic triglyceride content in mice on a chow diet (WT: n = 6, apoM-Tg: n = 5) and high fat diet (HFD; WT: n = 8, apoM-Tg: n = 8). (G) Expression of thermogenic genes in WAT and BAT (WT: n = 8–9, apoM-Tg: n = 8–11). Groups of two were compared by a student's t-test. Data is presented as Mean ± SE and no datapoints were excluded. HFD=High fat diet, PGC1A = PPARG coactivator 1 alpha, PPARG = Peroxisome proliferator-activated receptor gamma, PRDM16 = PR domain containing 16, S1P1-3 = S1P receptor 1-3, Tg = apoM-Tg, WAT = white adipose tissue, epiWAT = epididymal WAT, subWAT = subcutaneous WAT, UCP1 = uncoupling protein 1, WT = Wild type. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$.

may therefore not affect VLDL secretion, although apoM-Tg mice, compared to WT animals, showed an increased hepatic triglyceride content and uptake in mice fed chow diet.

Intestinal transport of dietary lipids may be an additional factor contributing to a delayed triglyceride turnover in apoM-Tg mice. Although the liver synthesizes the majority of plasma apoM, the protein is typically attached to all types of lipoproteins (~95% HDL, ~3% LDL, ~2% other lipoproteins), including chylomicrons [16,53], and is quickly exchanged between subtypes of lipoproteins [19,54]. In our study, we detected apoM in chylos which suggests intestinal production of apoM-containing chylomicrons. To evaluate whether intestinal lipid uptake and chylomicron formation contribute to the delayed triglyceride turnover in apoM-Tg mice, plasma triglyceride clearance was determined after an intravenous injection of intralipids. Significant differences between WT and apoM-Tg mice were however absent, even though a trend towards delayed triglyceride turnover in apoM-Tg animals was observed. Second, we combined Triton WR-1339 with an oral bolus of olive oil. Since no differences between the genotypes were evident, we conclude that the production rate of chylomicrons may be independent of the apoM/S1P complex. Intralipids are metabolized via interacting with LPL [55,56]. In our study, plasma triglyceride turnover in apoM-Tg mice did not deviate from WT animals after intralipid administration, although their significant reduction in LPL activity. It is unknown whether intralipid particles interact with apoM-containing lipoproteins to thereby gain apoM. Intralipids without apoM may accelerate triglyceride turnover, compared to endogenous apoM-

containing chylomicrons. Taken together, our data suggests that the apoM/S1P complex plays a role in metabolizing chylomicrons via the LPL metabolic pathway.

Starvation typically stimulates the FGF21 gene expression and protein secretion, to thereby induce lipolysis and glucogenesis, adjusting the organism for nutrition shortages [42,57]. Moreover, the hormone reduces hepatic VLDL secretion and plasma triglycerides by accelerating FFA uptake into WAT and BAT [35,42]. The exact biological function and regulation of FGF21 is however complex and controversially discussed. Although administration of FGF21 reduces plasma triglycerides and glucose in ob/ob and db/db mice [57], an increased FGF21 plasma concentration simultaneously correlates with diabetes and obesity in mice and humans [42]. In the present study, increased apoM and S1P concentrations associated with a reduction in plasma FGF21. Nutrition deficiencies are however unlikely, since plasma glucose concentrations did not deviate between WT and apoM-Tg mice and apoM-Tg mice had ~50% higher plasma triglyceride after 12 h fasting. Since FGF21 is released by passive diffusion [58], accelerated enzymatic degradation, by other factors than FAP-α [43], and/or renal clearance [59] in apoM-Tg mice may contribute to reduced plasma FGF21 concentrations. Reduced levels of FGF21 may also impair FFA uptake into adipose tissues of apoM-Tg mice, thereby increasing plasma triglycerides. Further experiments are however required to elucidate the exact mechanism.

Other signalling pathways besides FGF21 may also contribute to a delayed triglyceride turnover in apoM-Tg mice. The functional S1P antagonist FTY720 (Fingolimod) accelerates triglyceride turnover in mice [13]. In the present study, we confirmed a 2.3-times reduced plasma triglyceride concentration, 2 h after FTY720 administration in WT animals. Interestingly, treating apoM-Tg mice with FTY720 showed a trend towards a further impaired triglyceride turnover. The precise mechanism by which FTY720 triggers S1P signalling and possible unspecific targets are unknown to date. FTY720 interacts with all known S1P receptors, except S1P2 [60]. The molecule is furthermore a two-faced mediator. Whereas binding to S1P receptors induces S1P signalling, it subsequently promotes receptor internalisation and degradation [61]. Whether this response is altered in apoM-Tg mice is unknown. We showed that the $S1P_1$ antagonist W146 accelerated plasma triglyceride turnover in both apoM-tg and WT mice, compared to untreated animals. This suggest that inhibition of $S1P_1$ could play a role in triglyceride metabolism. When Fingolimod is used to treat patients with multiple sclerosis, dyslipidaemia is a well-accepted side effect [62], but not all clinical studies reported such an outcome [63,64]. S1P signalling furthermore stimulates lipolysis in WAT [33], additionally confirmed by FTY720 in vitro [34]. Conclusively, more attention towards S1P-recetor signalling induced dyslipidaemia will be needed together with further studies exploring the exact treatment potential of apoM/S1P mimicking drugs on lipid disturbances.

5. Conclusion

Enhanced apoM/S1P signalling reduces the activity of plasma lipases, thus perturbing plasma triglyceride clearance. The study furthermore suggests a complex interplay between the FGF21 and apoM-S1P axis, as well as glycocalyx integrity. Additional studies are however demanded to completely understand the potential of those pathways in regulating plasma triglyceride turnover in humans.

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CRediT authorship contribution statement

SH and CC conceptualized the study design. Data acquisition and interpretation was performed by SH, SE, AB, and CC. The first draft was written by SH, and critically revised by SE, AB, and CC. Funding was acquired by CC. All authors read and approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2021.158969.

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