Liver-specific phospholipid transfer protein (PLTP) deficiency reduces high density lipoprotein (HDL) and non-HDL production

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Abstract

Objectives—The liver is one of the critical organs for lipoprotein metabolism and a major source for phospholipid transfer protein (PLTP) expression. The effect of liver-specific PLTP deficiency on plasma lipoprotein production and metabolism was investigated.

Approach and Results—We created a liver-specific PLTP deficient mouse model. We measured plasma high density lipoprotein (HDL) and apolipoprotein B-containing lipoprotein (BLp, or non-HDL) levels and their production rates. We found that hepatic ablation of PLTP leads to a significant decrease in plasma PLTP activity, HDL-lipids, non-HDL-lipids, apoA-I, and apoB levels. In addition, nuclear magnetic resonance (NMR) examination of lipoproteins showed that the deficiency decreases HDL and BLp particle numbers, as well as VLDL particle size which were confirmed by electron microscopy. Moreover, HDL particles from the deficient mice are lipid-poor ones. To unravel the mechanism, we evaluated the apoB and triglyceride production rates. We found that hepatic PLTP deficiency significantly decreases apoB and triglyceride secretion rates. To investigate the role of liver PLTP on HDL production, we set up primary hepatocyte culture studies and found that the PLTP deficient hepatocytes produce less nascent HDL. Furthermore, we found that exogenous PLTP promotes nascent HDL production through an ABCA1-mediated pathway.

Conclusions—Liver-specific PLTP deficiency significantly reduces plasma HDL and BLp levels. Reduction of production rates of both particles is one of the mechanisms.

Keywords
Liver; PLTP; HDL; non-HDL; lipoprotein production; Atherosclerosis

PLTP belongs to a family of lipid transfer/lipopolysaccharide-binding proteins, including cholesteryl ester transfer protein (CETP), lipopolysaccharide-binding protein and bactericidal/permeability increasing protein. It is a monomeric protein of 81 kDa.

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Besides transferring PC, PLTP also efficiently transfers SM, cholesterol, diacylglycerol, α-tocopherol, cerebroside, and lipopolysaccharide. Although CETP can also transfer phospholipids, there is no redundancy in the functions of PLTP and CETP. The liver and small intestine are two important sites of PLTP expression.

In human studies, PLTP activity is inversely associated with HDL levels. PLTP mediates HDL remodeling and enlargement. PLTP KO mice demonstrated a complete loss of phospholipid transfer activities. The deficient mice showed a marked decrease in HDL cholesterol and apoA-I levels. PLTP transgenic mice showed a 2.5- to 4.5-fold increase in PLTP activity in plasma compared with controls. This resulted in a 30-40% reduction of plasma HDL cholesterol levels. Overall, PLTP overexpression or deficiency causes a significant reduction of HDL levels in the circulation, and we still do not have a good explanation for that.

Oram et al. reported that exogenous PLTP can promote removing cholesterol and phospholipids from cells by the ABCA1 pathway. In contrast, PLTP had no effect on lipid efflux from fibroblasts isolated from a patient with Tangier disease, an HDL deficiency syndrome caused by mutations in ABCA1. The same group of researchers also indicated that an amphipathic helical region (aa144-aa163) of PLTP is critical for ABCA1-dependent cholesterol efflux.

We have unexpectedly found that PLTP deficiency causes a significant impairment in hepatic secretion of apoB-containing lipoprotein (BLp). Likewise, it has been reported that animals overexpressing PLTP exhibit hepatic VLDL over-production. Associations of plasma PLTP activity with elevated apoB levels have been found in humans as well. Dr. Largrost's group found that human PLTP transgenic rabbits showed a significant increase of BLp cholesterol in the circulation. Nevertheless, the surprising finding that PLTP affects BLp secretion from the liver has remained unexplained. We believe that PLTP activity is involved in promoting BLp lipidation, since PLTP activity and triglyceride enrichment are two factors for PLTP-mediated HDL enlargement, a process similar to the 2nd step of BLp lipidation. To address the impact of liver-expressed PLTP on lipoprotein metabolism, we created a mouse model that expresses PLTP acutely and specifically in the liver on a PLTP-null background. We found that liver PLTP expression dramatically increases plasma non-HDL-cholesterol, non-HDL-phospholipid, and triglyceride levels, with a slight increase on plasma HDL-lipids, compared with the controls.

To further investigate the impact of liver-PLTP on nascent HDL and BLp production, we prepared a liver-specific PLTP gene knockout mouse model. We found that liver-specific PLTP deficiency significantly reduces both HDL and non-HDL levels. We also explored the possible mechanisms.

**Materials and Methods**

Materials and Methods are available in online-only Supplement.
Results

Liver PLTP deficiency decreases plasma PLTP activity

We prepared homozygous PLTP-Flox mice with a C57BL/6 genetic background. However, the Flox mice have no PLTP activity in the circulation due to Neo cassette insertion in intron 3. Since the Neo cassette is double-flanked by both LoxP and FRT sequences (Fig. 1A), we crossed our PLTP-Flox mice with Flp-transgenic mice. Flp recombinase recognizes the FRT sequences. Through this method, we prepared the PLTP-Flox/Flp transgenic (ΔNeo) mice. We found that the ΔNeo mice have normal plasma PLTP activity (Fig. 1B), as well as plasma cholesterol and phospholipid levels (Supplemental Fig. 1A and 1B).

Next, we took the advantage of the Cre/LoxP system to delete PLTP exon 2 and 3 which are flanked with LoxP sequences. We injected AAV-Cre and AAV-LacZ (controls) into the ΔNeo mice. Since Cre expression was driven by the liver-specific thyroxine binding globulin (TBG) promoter, the PLTP deletion occurred specifically in the liver (Fig. 1C). Moreover, AAV-Cre mediated hepatic PLTP ablation resulted in around 25% reduction of plasma PLTP activity in comparison to the AAV-LacZ controls (Fig. 1D), indicating that liver-expressed PLTP makes a significant contribution to the PLTP activity in the blood. Furthermore, PLTP activity in AAV-Cre injected livers is significantly lower than that of controls (Fig.1E).

PLTP hepatic deficiency significantly decreases both plasma HDL and non-HDL lipid levels

AAV-Cre treated PLTP-Flox female animals significantly decreased plasma levels of cholesterol (21%, P<0.01), phospholipids (22%, P<0.05), and triglyceride (32%, P<0.05, as compared to AAV-LacZ control group (Table 1). We also measured lipid levels in HDL and non-HDL fractions. Our data showed that liver PLTP depletion not only significantly decreases HDL-cholesterol (20%, P<0.05) and HDL-phospholipid (17%, P<0.05), but also non-HDL-cholesterol (29%, P<0.01) and phospholipid (35%, P<0.01) levels (Table 1).

Plasma lipid distributions were also examined by FPLC using pooled plasma. We observed that plasma cholesterol levels are decreased in HDL and non-HDL fractions from the female deficient mice, as compared to controls (Fig. 2A). This is also true for total phospholipid distribution (Fig. 2B), as well as that of triglyceride (Fig. 2C). The same phenomena were also observed in AAV-Cre-treated ΔNeo male mice, as compared with male controls (Supplemental Figs. IIA-C).

Next, we assessed plasma apolipoprotein levels by reducing SDS-PAGE and found that the PLTP deficient mice have a marked decrease of total apoB (60%, P<0.01) (Fig. 2D) and apoA-I (35%, P<0.01) (Fig. 2E), as compared with the control group. This suggests that PLTP deficiency in the liver has impact on both apoA-I-containing lipoprotein (HDL) and BLP (non-HDL) levels.

To investigate the mechanisms responsible for the reduced triglyceride and apoB levels in liver-specific PLTP-deficient mouse plasma, we examined the VLDL production rates in vivo. Both AAV-Cre and AAV-LacZ mice were simultaneously injected with [35S]-
methionine to label apoB. [14C]-oleic acid to label triglyceride, and poloxamer 407 to block the clearance of VLDL from the circulation. We collected plasma 120 minutes after injection and isolated plasma VLDL by ultracentrifugation (density 1.006 g/ml). We found that both [35S]-apoB and [14C]-triglyceride levels were significantly decreased in the VLDL from the deficient mice, as compared to that of the controls (Figs. 2F and G). This suggests that liver PLTP deficiency prevents VLDL secretion.

To further investigate the effect of hepatic PLTP on lipoprotein metabolism, we used NMR to measure total particle number, size, and lipid composition of VLDL, LDL and HDL. We found that the lack of PLTP in the liver decreases the size and particle number of VLDL particles (Table 2). In addition, these particles have less triglyceride in comparison to controls (Table 2). In LDL and HDL, the reduction in particle numbers was also observed; however, we did not detect any noticeable size differences between the deficient animals and controls (Table 2). Moreover, HDL from the deficient animals has less cholesterol compared with controls (Table 2). To confirm that the HDL results, we isolated HDL from the deficient and control mouse plasma by ultracentrifugation. We measured cholesterol and phospholipid levels in the HDLs and found that indeed the HDL particles from the deficient mice is lipid-poor ones (Table 1).

We next sought to examine lipoprotein particles under electron microscopy. As shown in Figure 3, the deficient mice have smaller VLDL particles. After counting 100 particles, the average sizes for VLDL are 52±10 nm and 37±5 nm (Controls vs KO, P<0.05). However, for LDL and HDL particles, there is no significant size difference between the two groups (Fig. 3). These results confirmed what we have observed in NMR analysis (Table 2).

### PLTP promotes nascent HDL production

To further investigate the mechanisms behind the reduction of apoA-I and HDL lipids in plasma, we examined apoA-I production. Both AAV-Cre- and AAV-LacZ mice were simultaneously injected with [35S]-methionine to label proteins. We collected plasma and isolated HDL by ultracentrifugation (density 1.21g/ml). We then separated [35S]-apoA-I on SDS PAGE and found that there is no significant change in [35S]-apoA-I levels between the two groups (Supplemental Figs. 3A and B). We also examined ABCA1 and SR-BI levels in liver homogenates and found no significant change either (Supplemental Figs. IIIC-E).

We next sought to measure nascent HDL production directly. Primary hepatocytes from PLTP KO and control mice were isolated and labeled with 3H-cholesterol. The cells were then incubated with 50 μg/ml human apoA-I. The medium was collected after 5-hours incubation. Non-HDL and HDL were separated by ultracentrifugation. The radioactivity in HDL fraction was determined by liquid scintillation counting. We found that PLTP deficiency significantly reduces hepatocyte nascent HDL production (Fig. 4A).

We further isolate primary hepatocytes from PLTP KO mice and labeled them with 3H-cholesterol. The cells were then incubated with 50 μg/ml human apoA-I together with active recombinant PLTP (rPLTP) (1 μg/ml) or heat inactivated rPLTP. The medium was collected, and non-HDL and HDL were separated. The radioactivity in HDL fraction was
determined by liquid scintillation counting. We found that exogenous active rPLTP significantly promoted hepatocyte nascent HDL production (Fig. 4B).

It has been reported that exogenous PLTP can promote removing cholesterol and phospholipids from macrophages through the ABCA1 pathway. Although PLTP has no effect on lipid efflux from fibroblasts isolated from ABCA1 deficient patients, hepatocytes may react differently. To evaluate whether PLTP-mediated nascent HDL production is ABCA1-dependent or not, we next isolated primary hepatocytes from liver-specific ABCA1 KO mice (Supplemental Fig. 4A), and labeled them with ³H-cholesterol. We then incubated them with apoA-I together with active rPLTP or inactive rPLTP. We did not find significant difference in nascent HDL production (Supplemental Fig. IVB), indicating that indeed rPLTP promotes nascent HDL production from hepatocytes through ABCA1 pathway.

Finally, we performed crosslinking experiment as described in “Material and Methods” to prove that there is an interaction between PLTP and ABCA1. After protein blotting, we found that there are two detectable ABCA1 and PLTP bands, one with 260 KD (complex 1) and one with more than 500 KD (complex 2) (Fig 4C), indicating that rPLTP and ABCA1 are close enough to be crosslinked and suggesting that PLTP can interact with ABCA1, thus promoting nascent HDL formation.

**Discussion**

Adenovirus-associated virus (AAV)-Cre/Loxp, adenovirus (AdV)-Cre/Loxp, Mx1-Cre/Loxp (Cre transgene is controlled by the interferon-inducible Mx1 promoter), and Albumin-Cre/Loxp (Cre transgene expression is controlled by liver specific albumin promoter) are four approaches for liver-specific gene knockout mouse preparation. The former three approaches eliminate the gene in the adult mice, while the latter eliminates the gene in the early stage of the life. As a pilot study of a drug intervention, AAV-Cre, AdV-Cre, and Mx1-Cre approaches are better than albumin-Cre approach, since they mimic drug intervention. PLTP is a potential drug target and we chose to block its activity in the adult mice.

There are also some important differences among AAV-Cre, AdV-Cre, and Mx1-Cre approaches. The concern of AdV-Cre approach is that the virus can mediate immune response. Mx1-Cre approach may not only deplete the gene in the liver, but also in the intestine, at least partially. We chose AAV-Cre approach for the current study, because 1) AAV delivers therapeutic transgenes without inducing toxicity, inflammation or disease in animals and humans; 2) the Cre recombinase expression was driven by the liver-specific thyroxine binding globulin (TBG) promoter; and 3) the AAV approach resulted in sustained Cre expression in the liver for at least 20 weeks.

It is already known that systematic PLTP-deficient mice have less BLp and HDL, compared with the WT controls. Nevertheless, the mechanism of PLTP involvement in BLp production and HDL maintenance is still not quite clear. In our current study, we demonstrated that liver-specific PLTP deficiency leads to: 1) reduction of PLTP activity in
the circulation; 2) reduction of HDL cholesterol and phospholipids, as well as apoA-I protein levels; 3) decrease of non-HDL cholesterol, phospholipids, and triglyceride, as well as total apoB levels; 4) decrease of VLDL production rate accompanied by reduction in VLDL particle size and numbers; and 5) reduction of hepatocyte nascent HDL production.

From this study together with previous studies 17,21, we could clearly dissect out the contribution of the liver to the total PLTP activity in circulation. Previously, we reported that liver-specific PLTP expressed mice with a PLTP-null background have 70-75% less plasma PLTP activity than that of WT mice, indicating that mouse liver makes a small but significant contribution to the plasma PLTP activity 21. In the current study, we showed that liver-specific PLTP deficient mice have about 25% less plasma PLTP activity compared with the controls. In other words, extrahepatic tissues, such as adipose tissue, small intestine, and the lung 6, make major contributions to plasma PLTP activity.

Liver PLTP deficiency prevents BLp production. Liver is one of the major tissues for lipoprotein production and PLTP expression. Our previous work showed that liver PLTP expression with a PLTP-null background dramatically promotes BLp production, through more lipidation and secretion 21. Our finding together with previous findings 17, 19 provide the concept that PLTP-mediated VLDL production per se is one of the driving forces for plasma lipoprotein metabolism. In this context, our liver-specific PLTP deficient mouse is another useful tool to further elucidate the effect of PLTP on VLDL production in the liver. We found that hepatic PLTP deficiency significantly decreases plasma non-HDL lipid levels (Table 1) and BLp production rates (Figs 2F and G). This may be related to: 1) less apoB particle lipidation and secretion 21, and 2) intracellular reactive oxygen species triggered apoB post-ER pre-secretory proteolysis 30.

Our previous study also showed that liver PLTP expression with a PLTP-null background has marginal effect on HDL lipid and apoA-I levels 21. However, in the current study, we found that liver PLTP deficiency (or extrahepatic PLTP expression) decreases HDL lipid and apoA-I levels (Table 1) (Fig. 2E). This cannot be simply explained by the reduction of plasma PLTP activity (about 25%, Fig. 1D), since heterozygous systemic PLTP deficiency (45% reduction of plasma PLTP activity) has no observable HDL lipid phenotypes 3. It is possible that there are multiple PLTP pools which have different types of impact on HDL metabolism.

Liver PLTP deficiency-mediated reduction of HDL levels may be related to lower HDL production from the liver. Nascent HDL was found in mesenteric lymph and hepatic perfusates, where it may arise as a result of PLTP-mediated lipid transfer from cellular plasma membrane into the small intestine- and liver-secreted apoA-I 31-33. ABCA1 mediates the rate-controlling step in HDL particle formation by promoting the efflux of cholesterol and phospholipids to apoA-I 34, 35. Overexpression of hepatic ABCA1 raises HDL cholesterol levels 36, and liver-specific deletion of ABCA1 dramatically decreases plasma HDL cholesterol in mice 27. Oram et al. reported that exogenous PLTP can promote removing cholesterol and phospholipids from macrophages by the ABCA1 pathway 13. In contrast, PLTP had no effect on lipid efflux from fibroblasts isolated from a patient with Tangier disease 14. The same group of researchers also indicated that an amphipathic helical
region (aa144-aa163) of PLTP is critical for ABCA1-dependent cholesterol efflux \(^{15}\), and they found that PLTP can stabilize ABCA1 on cell plasma membrane \(^{13}\).

In line with these reports, we found that liver PLTP is a significant player in nascent HDL production through ABCA1 pathway. Based on our observation, ablation of PLTP in the liver has no effect on apoA-I production (Supplemental Figs. 3A and B) and ABCA1 protein levels in tissue homogenates (Supplemental Figs. 3C and D). However, PLTP deficiency significantly reduces nascent HDL production (Fig. 4A) and exogenous active rPLTP significantly enhances hepatocyte nascent HDL production (Fig. 4B). Furthermore, we found that this PLTP-mediated effect requires the presence of ABCA1, since rPLTP has no effect in HDL production in ABCA1 deficient primary hepatocytes (Supplemental Figs. 4A and B). We believe that, as proposed in macrophages \(^{13}\), PLTP may function to 1) stabilize liver ABCA1; and 2) shuttle lipids between cells and existing HDL particles (formed first through ABCA1 action).

Hepatic PLTP deficiency-mediated reduction of HDL levels might be related to less VLDL production. PLTP can transfer lipids from the surface of BLp into HDL \(^{37,38}\). Systemic PLTP deficiency completely blocks this transfer activity \(^{3,5}\). After mature BLp secreted into the blood and lipoprotein lipase-mediated triglyceride hydrolysis, the core of BLp shrinks \(^{39}\), and the redundant surface constituents (SM and PC, as well as free cholesterol) can be the substrates of PLTP, transferring from BLp to HDL.

PLTP may have an anti-atherogenic function, since it promotes nascent HDL production. However, recent researches challenge the concept that rising of plasma HDL will uniformly translate into reductions in coronary heart diseases. HDL particles are heterogeneous in size and composition \(^{40}\). Understanding the origination of HDL and characterization of HDL, all subclasses, are as important as its plasma concentration in leading to atherosclerotic lesion development. In this study, we showed that PLTP activity promotes nascent HDL production (Fig. 4A and B), through the interaction between PLTP and ABCA1 (Fig. 4C). Whether this PLTP function is anti-atherogenic or pro-atherogenic deserves further investigation. However, PLTP overexpression promotes \(^{41,42}\) and deficiency prevents \(^{16}\) atherosclerosis in mice.

Taken together the results from the current study and our previous one \(^{21}\), we proposed a model for PLTP-mediated HDL and non-HDL production (Fig. 4D). We believe that 1) PLTP may be involved in the 2nd step of BLp lipidation, promoting the fusion of primordial BLp and TG/SM/PC-rich lipid droplets; 2) PLTP may promote ABCA1-mediated nascent HDL production through stabilizing ABCA1 and shuttling lipids between cells and existing HDL particles; and 3) PLTP may transfer BLp surface components into HDL, after LPL-mediated core triglyceride hydrolysis.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgments

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References


Abbreviations

- PLTP: phospholipid transfer protein
- KO: knockout
- AAV: adenovirus associated virus
- HDL: high-density lipoprotein
- BLp: apolipoprotein B-containing lipoprotein
- VLDL: very low-density lipoprotein
- FPLC: fast protein liquid chromatography
- NMR: Nuclear magnetic resonance
- TG: triglycerides
- WT: wild-type
Significance

Our knowledge of atherosclerosis is still limited, and more extensive investigation must provide better understanding of this disease. Exploring the effect of PLTP on lipid metabolism is such an investigation, since PLTP is a well risk factor for the development of the disease. ApoB-containing lipoprotein and HDL production are two processes which are closely related with atherosclerosis. Current study is the first one indicates that liver-specific PLTP deficiency can suppress both apoB-lipoprotein and HDL production. PLTP may accelerate apoB lipidation in the ER lumen of hepatocytes by promoting fusion of lipid droplet and primordial apoB particle. PLTP may increase hepatocyte nascent HDL production by promoting ABCA1-mediated lipid efflux.
Fig. 1. PLTP-LoxP-ΔNeo mouse characterization

Panel A, Strategy for PLTP-LoxP-ΔNeo and PLTP KO mouse preparation. One LoxP site is located in intron 1, and a Neo cassette double flanked with FRT/LoxP sites is placed in intron 3. Flp recombinase recognizes FRT sequences. Flp transgene mediates Neo cassette deletion. Adenovirus associated virus (AAV)-Cre recombinase mediated exon 2 and exon 3 deletions renders PLTP expression deficiency specifically in the liver. Panel B, deletion of Neo cassette restores the plasma PLTP activity. Panel C, PLTP mRNA measurement in different tissues. Panel D, the effect of liver-specific PLTP deficiency on plasma PLTP activity. Panel E, liver PLTP activity measurement. Values are mean ± SD, n=5, *P<0.01.
Fig. 2. Plasma lipid distribution, apolipoprotein, and non-HDL production measurements

Plasma lipid distributions were analyzed by fast protein liquid chromatography (FPLC). A 250-μl aliquot of pooled plasma (from five male animals) was loaded onto the columns and eluted with Tris buffer (50 mM, pH 7.4) at a constant flow rate of 0.35 ml/min. Panel A, cholesterol distribution. Panel B: phospholipid distribution. Panel C: triglyceride distribution. Panel D and E, Plasma (0.2 μl) was separated by 4-15% SDS-PAGE and immunoblotted with polyclonal antibodies against apoB and apoA-I. The results were quantified with ImageJ software. Panel D, apoB Western blot and total apoB quantification. Panel E, apoA-I Western blot and quantification. ApoB-containing triglyceride-rich lipoprotein production in vivo was measured as described in “Materials and Methods.” Panel F, total [35S]-apoB in VLDL and quantification. Total [35S]-albumin (Alb) was a control. Panel G, total [14C]-triglyceride in VLDL and quantification. Values are mean ± SD., n=5, *P<0.01.
Fig. 3. Electron Microscopy
Negative-stain electron microscopy was done as described. After counting 100 articles, the average VLDL sizes are 52±6 nm and 37±5 nm (Controls vs KO, P<0.05). The average LDL sizes are 21.4±1.9 nm and 21.7±2.2 nm (Controls vs KO). The average HDL sizes are 9.6±1.4 nm and 9.4±1.5 nm (Controls vs KO).
Fig 4. Nascent HDL production in cultured primary hepatocytes
The procedure of nascent HDL production is described in “Materials and Methods”. Panel A, nascent HDL production in PLTP KO and wild type primary hepatocytes (no exogenous PLTP treatment). Panel B, nascent HDL production in PLTP KO primary hepatocytes with either active rPLTP or heart inactive (HI) rPLTP treatment. Panel C, Crosslinking between rPLTP and ABCA1. WB: Western blot. The procedure is described in “Materials and Methods”. * p<0.05, ** p<0.01. Panel D, a model of PLTP-mediated lipoprotein production. PLTP is involved in the 2nd step of BLp lipidation in ER lumen, where the primordial BLp is fused with TG/SM/PC-rich lipid droplet. PLTP also can promote ABCA1-mediated nascent HDL production through stabilizing ABCA1. Finally, PLTP can transfer BLp surface lipids into HDL in the circulation. TG, triglyceride; SM, sphingomyelin; PC, phosphatidylcholine.
### Table 1

**Plasma lipid measurement**

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<td>AAV-Cre</td>
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1 All mice are female. Plasma samples were collected after fasting.

Value, Mean ± SD, n=6.

*P<0.05.

2 HDL particles were separated by ultracentrifugation (d<1.21g/ml).

All values are normalized based on protein levels of HDL fractions and presented as Mean±SD, n=3.

*P<0.05.

### Table 2

**NMR analysis of lipoprotein particles**

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All mice are female. Value, Mean of pooled (n=6) samples.

TG: Triglyceride; C: Cholesterol.