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Dietary linoleic acid supplementation fails to rescue established cardiomyopathy in Barth syndrome

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Abstract

Barth syndrome (BTHS) is a mitochondrial lipid disorder caused by mutations in *TAFAZZIN* (*TAZ*), required for cardiolipin (CL) remodeling. Cardiomyopathy is a major clinical feature, with no curative therapy. Linoleic acid (LA) supplementation is proposed to ameliorate BTHS cardiomyopathy by enhancing linoleoyl group incorporation into CL. While the beneficial effect of dietary LA supplementation in delaying the development of BTHS cardiomyopathy has been recently tested, its potential to reverse established BTHS cardiomyopathy remains unclear. Our study revealed that LA supplementation cannot rescue established BTHS cardiomyopathy in mice, highlighting the importance of early initiation of LA supplementation for maximum benefits.

Keywords

Barth syndrome; Linoleic acid; Cardiomyopathies; Dietary supplements

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CRediT authorship contribution statement

Siting Zhu: Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Jing Pang:** Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Anh Nguyen:** Investigation, Data curation. **Helen Huynh:** Validation, Investigation, Data curation. **Sharon Lee:** Methodology, Investigation, Data curation. **Yusu Gu:** Visualization, Methodology, Investigation, Data curation. **Frederic M. Vaz:** Visualization, Methodology, Investigation, Data curation. **Xi Fang:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

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.

1. Introduction

Barth syndrome (BTHS) is an X-linked mitochondrial disorder caused by mutations in tafazzin (*TAZ*) [1,2] . Cardiomyopathy is a major clinical feature in BTHS and plays significant roles in the outcomes and progression of BTHS [1,2]. In recent years, life expectancy has risen among BTHS patients due to improved disease diagnosis and management [3]. Patients who survive infancy and live into their late forties with impaired but stabilized cardiac function [3], highlighting the importance to investigate therapies for adult BTHS cardiomyopathy. Current treatment for BTHS cardiomyopathy includes the use of standard heart failure medications. Thus far, there is no curative therapy for BTHS cardiomyopathy [1].

TAZ is essential for the remodeling and maturation of cardiolipin (CL), a signature phospholipid of mitochondria. CL is composed of two phosphatidylglycerol backbones and four fatty acyl chains. In mammalian heart, the major mature CL species is tetralinoleoyl-CL, which contains four linoleic acid (LA) side chains [1,4]. CL de novo synthesis occurs exclusively in the inner mitochondrial membrane (IMM) [1,4]. CL synthesis is initiated upon the formation of phosphatidic acid (PA) and is catalyzed by a series of enzymes to produce nascent CL [1,4]. Newly synthesized nascent CL contains a mixture of fatty acyl side chains [1,4]. TAZ functions as a phospholipid-lysophospholipid acyltransferase and remodels nascent CL to mature CL containing more unsaturated fatty acyl side chains [1,4]. TAZ deficiency in BTHS causes inefficient transacylation from linoleoyl-phospholipid to monolysocardiolipin (MLCL), resulting in decreased levels of mature CL, accumulation of both MLCL and nascent CL, and concomitantly elevated ratios of MLCL to total CL (MLCL/CL) [1,2].

Owing to a lack of acyl specificity in de novo CL synthesis, the fatty acyl composition in nascent CL is largely determined by concentrations of specific free fatty acids [5]. In BTHS patient derived-fibroblasts, LA supplementation increases mature CL levels by increasing incorporation of linoleoyl groups into newly synthesized CL in a time- and dose-dependent manner [5]. Moreover, LA supplementation significantly mitigated contractile defects of BTHS-iPSC derived cardiomyocytes [6]. Thus, LA supplementation was proposed to mitigate BTHS by increasing the incorporation of linoleoyl groups into nascent CL towards mature CL composition without requiring the remodeling process. However, due to longstanding difficulties in generating a *Taz* knockout mouse model for BTHS, the effects of LA supplementation on BTHS cardiomyopathy in vivo remains largely unknown.

LA is one of two essential fatty acids that must be obtained through diet. Given that over 70 % of safflower oil is composed of LA, dietary LA supplementation through safflower oil offers a convenient and accessible method to assess its effect on BTHS cardiomyopathy. Our recent study reported that dietary LA supplementation to *Taz* cardiomyocyte-specific knockout (cKO) mice at one month of age, before observed cardiac dysfunction, completely prevented dilated cardiomyopathy (DCM) at 4 months, but the beneficial effects declined at 6 and 8 months of age [7]. This study suggests that LA supplementation has beneficial effects in delaying the development of BTHS cardiomyopathy. However, it

remains unknown whether LA supplementation could rescue established cardiomyopathy in BTHS. To address this question, in this study, dietary LA supplementation for *Taz* cKO mice was initiated at 4 months of age, when established cardiomyopathy was observed, and maintained throughout the entire study until 10 months of age. Our results revealed that, while dietary LA supplementation partially ameliorated CL and mitochondrial abnormalities, it did not rescue the established cardiomyopathy in *Taz* cKO mice.

2. Materials and methods

2.1. Mouse model and dietary LA supplementation

Taz cKO and control (Ctrl) mice were generated as previously described [7–10] and were fed with high-LA oil diet (LAD) or low-LA control diet (CD) starting at 4 months of age continued until 10 months of age. Dietary LA supplementation was achieved by using safflower oil as an ingredient in rodent diet [11,12]. Palm oil, which contains low LA, was used in control diets. In compliance with dietary recommendations to prevent cardiovascular disease, total fat content of all diets was 28 % of the total kcal intake [13]. LAD and CD contained 10 % and 1 % LA (LA weight/chow weight) respectively.

2.2. Animal procedures and echocardiography

The UC San Diego (UCSD) Animal Care Program maintained all animals and the UCSD Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures. Echocardiography was performed as previously described [7–10].

2.3. CL profile analysis

CL profile analysis was performed as described previously [7,8,14]. The raw LC/MS data were converted to mzXML format using MSConvert as previously described [7,8,14]. The dataset was processed using an in-house developed metabolomics pipeline [14].

2.4. Statistical analyses

Data are presented as the mean ± SEM unless indicated otherwise. Statistical analysis was performed using GraphPad Prism 10.0 (Graph-Pad Software), with mixed-effects ANOVA for comparisons among groups, Tukey's multiple comparison test was used for multiple pairwise comparisons following ANOVA analysis. P values of <0.05 were considered statistically significant.

3. Results

3.1. Dietary LA supplementation fails to rescue established cardiomyopathy in Taz cKO mice

To explore the potential rescuing effect of LA supplementation on established cardiomyopathy, we fed *Taz* cKO mice with a high LA safflower oil diet (LAD) after the development of cardiomyopathy. Our previous studies revealed that *Taz* cKO mice developed cardiomyopathy and heart failure at 4 months of age [8]. Thus, we performed baseline echocardiographic measurement for *Taz* cKO mice and control at 4 months of age (Fig. 1A). To ensure the development of cardiomyopathy in *Taz* cKO mice, we specifically

chose mice with fractional shortening below 30 % for our study. LAD and CD was administered to *Taz* cKO and control mice after baseline echocardiographic measurements and was maintained throughout the entire study. We performed follow-up echocardiographic measurements at 6, 8, and 10 months of age to assess cardiac function in both *Taz* cKO and control mice post-treatment. Surprisingly, we did not observe any improvement in the cardiac function of *Taz* cKO mice even after 6 months of treatment (Fig. 1B–E). Cardiac dysfunctions in *Taz* cKO mice were comparable between the LAD and CD groups (Fig. 1B–E). Histological analysis at 10 months of age further confirmed that LAD treatment initiated at 4 months of age was unable to reverse left ventricle dilation in *Taz* cKO mice (Fig. 1F–G). Consistent with previous observations in *Taz* cKO mice on normal chow [8], we did not observe increases in the ventricular weight to body weight or ventricular weight to tibia length ratio (Fig. 1H–I). Interesting, our transmission electron microscopy (TEM) analysis revealed that LA supplementation initiated at 4 months of age ameliorated the disorganization of cristae structures in the cardiomyocyte of *Taz* cKO mice at 10 months of age (Fig. 1J). In LAD-treated *Taz* cKO mice, we observed fewer mitochondria with abnormal cristae compared to the CD control group (Fig. 1K). However, this improvement in mitochondrial cristae did not mitigate cardiac dysfunctions in *Taz* cKO mice. Our results suggest that dietary LA supplementation fails to rescue established cardiomyopathy in *Taz* cKO mice.

3.2. Dietary LA supplementation partially mitigates CL abnormalities in Taz cKO mice

Given that LAD exhibits significant effects in preventing BTHS cardiomyopathy when the treatment is initiated before the development of cardiomyopathy [7], the lack of a beneficial effect from LAD on established cardiomyopathy in *Taz* cKO mice was surprising. Given the long half-life of CL in the adult heart [15], we doubted the incorporation of linoleoyl group into CL is limited when treated at 4 months of age. To assess whether LAD mitigates CL abnormalities, we performed lipidomic analyses in *Taz* cKO and control hearts under LAD or CD treatments at 10 months of age. Similar to our previous findings [7,8], we observed “BTHS-like” CL profiles in *Taz* cKO hearts in both the LAD and CD groups (Fig. 2A–E). However, LAD slightly ameliorated the severity of CL abnormalities in *Taz* cKO hearts. As shown in Fig. 2A, the levels of total CL were slightly increased in *Taz* cKO hearts in the LAD group, compared with the CD group. While there was no difference in the levels of MLCL (Fig. 2B), the elevated ratio of MLCL to CL in *Taz* cKO hearts was ameliorated in the LAD group compared with the CD group (Fig. 2C). We further quantified the levels of mature CL with longer or more unsaturated acyl groups and nascent CL with shorter or more saturated acyl groups. As shown in Fig. D, LAD increased the levels of mature CL (72:8, 72:7, 74:7) in *Taz* cKO hearts, compared with the CD group. Notably, the levels of tetralinoleoyl-CL (72:8) were dramatically increased in control mice, suggesting that the incorporation of linoleoyl group occurs largely in the *Taz*-mediated remodeling process (Fig. 2D). We also observed that the increased nascent CL (66:1, 66:2, 68:1, 68:2, 70:2, 70:3, 72:2, 72:3, 72:4) in *Taz* cKO hearts were partially diminished in LAD group (Fig. 2E). These results suggest that initiating LAD treatment at 4 months of age partially mitigates CL abnormalities in *Taz* cKO mice. However, the improvements in CL profile do not rescue the DCM phenotype in *Taz* cKO mice.

4. Discussion and limitations of the current study

Our results demonstrate that dietary LA supplementation is unable to rescue established cardiomyopathy in *Taz* cKO mice, a murine BTHS cardiomyopathy model, when the treatment is initiated after the onset of cardiomyopathy. Our analysis of CL profiles revealed that LAD initiated at 4 months of age partially ameliorates CL abnormalities in *Taz* cKO mice. This result suggests that, despite the relatively slow turnover in adult hearts [15], linoleoyl groups are still able to incorporate into newly synthesized CL molecules. However, future studies comparing the incorporation of supplemented linoleoyl groups in adult stages at 4 months of age with adolescent stages at one month of age might be helpful in assessing whether the decreased CL turnover contributes to the inefficiency of LAD in adult stages. Moreover, it remains unclear whether the increased mature CLs are incorporated into the inner mitochondrial membrane (IMM). Given that CL synthesis exclusively occurs in the IMM and LA supplementation increases mature CLs during CL synthesis, it is likely that the increased mature CLs are present in the IMM. However, direct assessment of this issue requires analysis of CL profiles in isolated mitochondria or IMM.

Interestingly, we did observe that LA supplementation improves the disorganization of cristae structures at 10 months of age, even when LAD was initiated after the onset of cardiomyopathy at 4 months of age. However, it remains unknown whether the cristae abnormalities are improved at 6 and 8 months of age. It is possible that the improvement of cristae abnormalities is a slow process in adult hearts and therefore could not rescue the established cardiomyopathy in *Taz* cKO mice. It is also possible that preexisting myocardial damage due to the existing mitochondrial defects in *Taz* cKO mice, contributing to the DCM phenotype, cannot be rescued when treatment is initiated after the establishment of the phenotype. It's also worth noting that, in our current study, we tested only one concentration of LAD, using safflower oil as the fatty acid source, amounting to a 10 % ratio of LA weight to total chow weight. It remains unknown whether a diet with a higher LA concentration would offer enhanced therapeutic effects on BTHS cardiomyopathy. Moreover, while dietary LA supplementation using safflower oil offers a convenient method to increase the supply of linoleoyl groups for CL synthesis, the intake of LA is constrained by food consumption. Therefore, it is of great interest to determine whether pharmacological supplementation of high LA could confer beneficial effects in BTHS mouse models, and importantly, in BTHS patients. In addition, it remains unknown whether LA supplementation can provide additional benefits on top of standard heart failure treatments for BTHS cardiomyopathy.

Taken together, in line with our previous discovery that dietary LA supplementation markedly delays the development of a murine model of BTHS cardiomyopathy when administered at an early stage of life, our current findings strongly advocate for the early initiation of LA supplementation to maximize its potential benefits.

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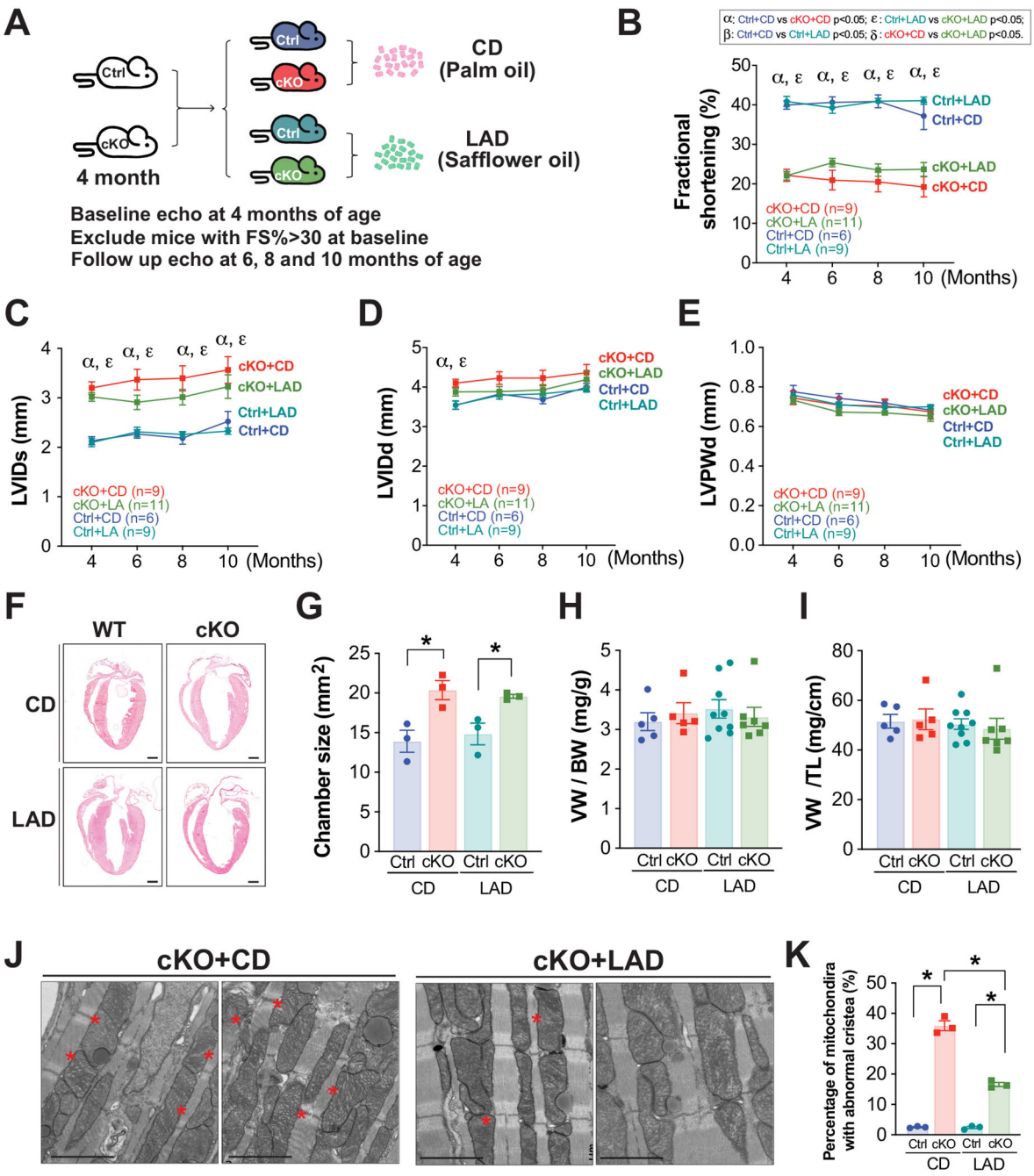

Fig. 1. Dietary LA supplementation does not rescue established cardiomyopathy in *Taz* cKO mice.

A. Schematic of experimental protocol for dietary LA supplementation in *Taz* cardiomyocyte-specific knockout (cKO) and Cre-negative control (Ctrl) mice. *Taz* cKO and Ctrl mice were fed with LAD or CD starting at 4 months of age, and continued until 10 months of age. Echocardiography analysis was performed at baseline (4 months of age) and followed up at 6, 8, and 10 months of age. *Taz* cKO mice with fractional shortening (% FS) of >30 % at baseline were excluded from this study. **B-E.** Echocardiographic measurements

of left ventricular percentage of fractional shortening (% FS) (B), end-systolic left ventricle (LV) internal diameter (LVIDs) (C), end-diastolic LV internal diameter (LVIDd) (D), and LV posterior wall thickness at the end-diastolic (LVPWd) (E) for Ctrl and *Taz* cKO mice in LAD and CD groups at 4, 6, 8, and 10 months of age. $n = 6$ for CD-treated Ctrl mice (blue color); $n = 9$ for LAD-treated Ctrl mice (cyan color); n = 9 for CD-treated cKO mice (red color); $n = 11$ for LAD-treated cKO mice (green color). **F-G.** Representative four chamber-sectional views of Hematoxylin and Eosin (H&E)-stained sections (F) and quantification of left ventricle (LV) chamber sizes (G) from Ctrl and *Taz* cKO mice in LAD and CD groups at 10 months of age. $n = 3$ for each group. Scale bar: 1 cm. **H-I.** Ratios of ventricle weight to body weight (LV/BW) (H) and ventricle weight to tibia length (I) of Ctrl and *Taz* cKO mice in LAD and CD groups at 10 months of age. $n = 5$ for CD-treated Ctrl mice; n = 9 for LAD-treated Ctrl mice; n = 5 for CD-treated cKO mice; $n = 7$ for LAD-treated cKO mice. **J.** Representative electron micrographs of *Taz* cKO heart in LAD and CD groups at 10 months of age. Red asterisks: disorganized inner mitochondrial membranes. Scale bar: 1 μm. **K.** Quantification of the percentage of abnormal mitochondria in Ctrl and *Taz* cKO mice heart in LAD and CD groups at 10 months of age. n = 3 for each group. Data are represented as the mean ± SEM. α: $P < 0.05$ CD-treated Ctrl vs CD-treated cKO. β: $P < 0.05$ CD-treated Ctrl vs LAD-treated Ctrl. ε: $P < 0.05$ LAD-treated Ctrl vs LAD-treated cKO. δ: $P < 0.05$ CD-treated cKO vs LAD-treated cKO, by mix-effects ANOVA (4 group comparison with repeated observations for Figs. C-F). *$P < 0.05$ between indicated groups, by two-way ANOVA (four group comparison for Figs. H and J). Tukey's multiple comparison test was used for multiple pairwise comparisons following ANOVA analysis.

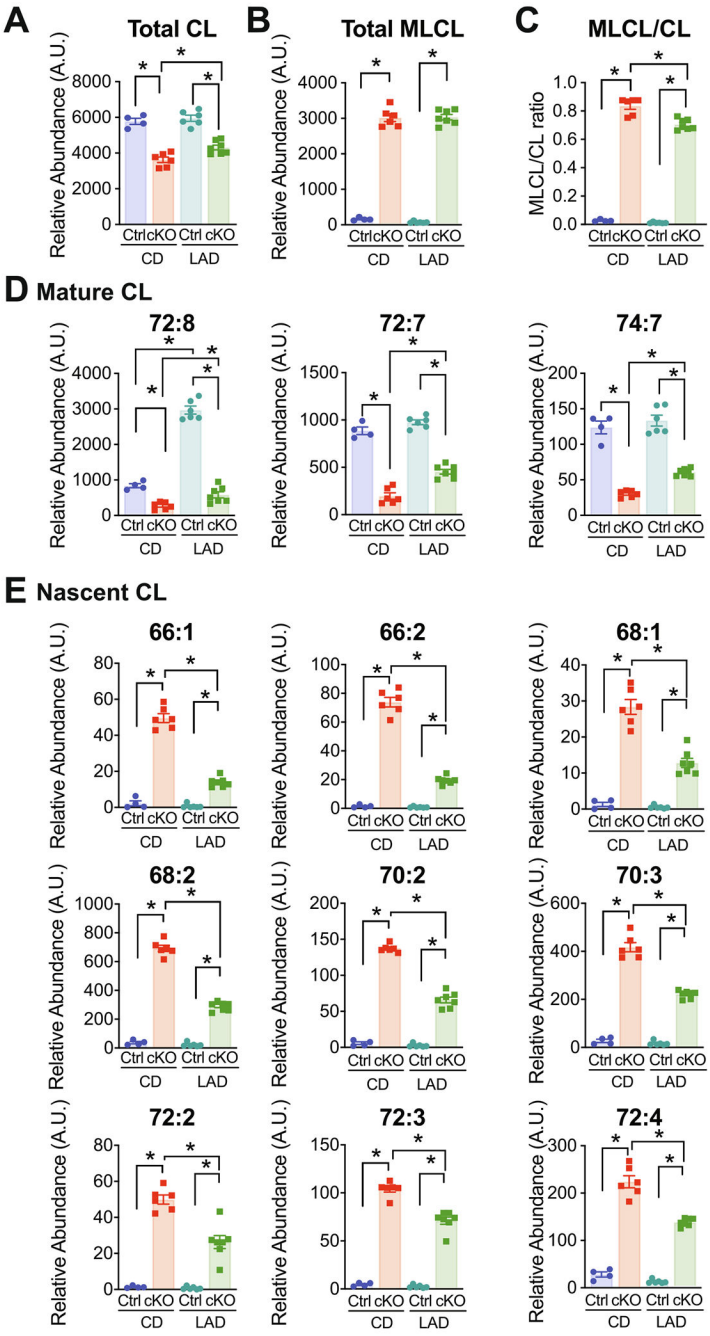

Fig. 2. Dietary LA supplementation partially mitigates CL abnormalities in *Taz* cKO mice.

A-B. Levels of total CL (A) and monolyso-CL (MLCL) (B) in *Taz* cKO hearts and Ctrl hearts under either CD (red or deep blue) or LAD (green or light blue) treatments at 10 months of age. **C.** The ratio of MLCL to CL was calculated from the total MLCL and CL levels. **D.** Levels of mature CL molecular species 72:8, 72:7, and 74:7 in *Taz* cKO hearts and Ctrl hearts under either CD (red or deep blue) or LAD (green or light blue) treatments at 10 months of age. **E.** Levels of nascent CL molecular species 66:1, 66:2, 68:1, 68:2, 70:2, 70:3, 72:2, 72:3, and 72:4 in *Taz* cKO hearts and Ctrl hearts under either CD (red or deep blue) or

LAD (green or light blue) treatments at 10 months of age. $n = 4$ for CD-treated Ctrl mice; $n = 6$ for LAD-treated Ctrl mice; n = 6 for CD-treated cKO mice; $n = 7$ for LAD-treated cKO mice. Data are represented as the mean ± SEM. *: $P < 0.05$ between indicated groups, by two-way ANOVA. Tukey's multiple comparison test was used for multiple pairwise comparisons following ANOVA analysis.