Role of Cardiolipin Content and Acyl Chain Composition in Mitochondrial-Mimicking Monolayers

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ABSTRACT

SAHIL DADOO: Role of Cardiolipin Content and Acyl Chain Composition in Mitochondrial-Mimicking Monolayers
(Under the direction of Dr. Saame Raza Shaikh)

Cardiolipin (CL) is a mitochondrial phospholipid that plays a fundamental role in maintaining inner membrane (IMM) structure-function. In several metabolic diseases, CL is shown to undergo alterations in content and acyl chain composition, though there is still debate as to which alteration is the major contributor to pathology. Therefore, this study distinguished the roles of CL content and acyl chain composition on influencing IMM biophysical organization. We utilized Langmuir monolayers to study lipid packing properties of CL, from which we conducted secondary analyses on membrane properties such as elasticity, thermodynamics of mixing, and miscibility. Our data demonstrated that a decrease in CL content and extreme acyl chain remodeling impaired biophysical properties of mitochondrial-mimicking monolayers. However, modest acyl chain remodeling did not have a significant impact on these properties. These findings are crucial for the better design of CL-specific, mitochondrial-targeted therapeutics for metabolic diseases.

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$(14:0)_4$CL	tetramyristoyl cardiolipin
$(18:2)_3$CL	trilinoleoyl cardiolipin
$(18:2)_4$CL	tetralinoleoyl cardiolipin
CL	cardiolipin
IMM	inner mitochondrial membrane
MLCL	monolyso-cardiolipin

CHAPTER I
SPECIFIC AIMS

Cardiolipin (CL) is a polyunsaturated phospholipid that is predominantly localized to the inner mitochondrial membrane (IMM), where it is synthesized and plays a fundamental role in maintaining inner membrane structure-function. However, in several metabolic diseases, in which mitochondrial dysfunction contributes towards pathology, CL's profile is susceptible to considerable alterations. Two well-known alterations to CL's profile include a decrease in CL content and an increase in CL acyl chain remodeling. While many studies have shown that these alterations contribute to mitochondrial dysfunction, there is debate in the field currently as to which is the major contributor to progression of disease. Therefore, the goal of this project is to conduct biophysical studies aimed at better understanding how alterations in CL concentration and CL acyl chain remodeling impair IMM biophysical organization. Results from this study will help to discriminate between the impact of well-known alterations to CL during pathology, which will allow for the better design of CL-targeted therapeutics for metabolic disorders.

Our study will focus on how alterations in CL content and acyl chain composition impact the mitochondrial inner membrane biophysical organization. To accomplish this goal, we will utilize Langmuir monolayers to study how these changes impact lipid dynamics.

Aim 1: Determine the impact of changes in CL content on various biophysical properties of mitochondrial-mimicking monolayers. It is hypothesized that a decrease in CL content, as seen

in various metabolic disorders, will impact the lateral organization and thereby packing of adjacent lipids.

Aim 2: Determine the impact of changes in CL acyl chain composition on various biophysical properties of mitochondrial-mimicking monolayers. It is hypothesized that aberrant CL acyl chain remodeling, as seen in various metabolic disorders, will impact various biophysical properties of mitochondrial-mimicking monolayers.

CHAPTER II
INTRODUCTION

Mitochondria are double-membraned organelles that are critical for many cellular processes including fatty acid oxidation, calcium homeostasis, as well as ATP production, and apoptosis (1, 2). Within mitochondria, the inner membrane is the active site for cellular respiration and thereby ATP production. Thus, mitochondrial structure is intimately associated with optimal function, which is tightly regulated by the supply of membrane constituents such as proteins and phospholipids. For instance, phospholipids are not only important for maintaining the structural integrity of cell membranes, but they are also critical for the compartmentalization of subcellular organelles. The major classes of phospholipids within mitochondrial membranes are similar to other membranes, such as the plasma membrane, and contain large amounts of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (3). However, within the inner mitochondrial membrane, a highly unique phospholipid known as cardiolipin (CL) exists. Studies have shown the importance of cardiolipin for mitochondrial structure-function throughout a range of disease (1, 4-12).

2.1 CL is a hallmark phospholipid of mitochondria with a unique structural and functional role

CL is a highly-curved, anionic phospholipid consisting of two phosphatidic acid groups linked to a central glycerol molecule. CL is found almost exclusively located within the inner mitochondrial membrane (IMM), where it is synthesized (13). While most other phospholipids have two fatty acyl chains attached to one glycerophosphate backbone, CL contains four fatty

acyl side chains connected to two glycerophosphate backbones. The acyl chain composition of CL is very diverse, both among species of organisms and among different cells of the same organism (1, 4, 14). However, in healthy mammalian heart tissue, CL predominately adopts an acyl chain composition in which all four acyl chains are occupied by linoleic acid. This structure occupies nearly 80% of the total CL pool within the IMM (14, 15).

CL is a major component of the IMM, occupying nearly 20% of the IMM phospholipidome. The importance of CL within the IMM arises from its unique structure which allows it to associate with many different proteins. As a result CL regulates many properties of mitochondrial structure-function such as bioenergetics, cristae morphology, respiratory enzymatic activity, cellular signaling, and apoptosis (13, 16-19). Further, CL has a distinct ability to interact with respiratory chain complexes and protein carriers within the IMM, which results from its highly unique structure and ability to form non-bilayer phases (20-27). For example, it has been shown that complex IV requires CL to maintain enzymatic function, which is compromised upon associating with remodeled forms of CL such as monolyso-CL (MLCL) (1, 28). In addition, CL is integral in the optimal functioning of cytochrome c, a membrane-associated protein necessary for the electron transfer between complexes III and IV. However, peroxidation of CL releases cytochrome c from the mitochondria to the cytosol, which initiates cellular apoptosis (29).

Another important property of CL is its ability to form localized membrane domains. This property was first discovered in E. coli, where CL was observed to concentrated near the polar regions of the cytoplasmic membrane (30). Given CL's unique ability to promote negative membrane curvature, it is believed that CL forms enriched microdomains, that are

important for regulating the structure of highly-curved cristae and thereby protein function (13, 20, 21, 31-33).

2.2 The role of CL in the pathogenesis of cardiometabolic diseases

CL is critical for maintaining the integrity and functionality of the IMM. However, due to CL's unsaturated nature, and its close association with electron channeling, CL is susceptible to considerable damage (34, 35). Abnormalities in CL metabolism are causative factors in a variety of diseases, such as Barth Syndrome, and other cardiometabolic pathologies such as diabetes and cardiovascular diseases (9-11, 36). This further underscores the importance of CL in health and disease.

Three well-known alterations to CL include: decreased CL content, acyl chain remodeling, and CL peroxidation (4, 12, 13). Here, we discuss the role of CL content versus CL acyl chain composition. Decreased CL content can occur from increased phospholipase activity, leading to CL degradation, or from decreased cardiolipin synthase activity, leading to decreased CL synthesis (4, 37). CL acyl chain remodeling can occur from lack of $(18:2)_4$CL availability or alterations to proteins involved in CL remodeling, such as tafazzin (12). Various diseases manifest from these alterations in CL content and composition. For example, Barth syndrome and heart failure are both associated with a loss of CL content, specifically $(18:2)_4$CL, whereas ischemia-reperfusion is associated with a loss of CL content and an increase in oxidized CL (4, 38-40).

Mutations in tafazzin lead to a lethal and rare cardiomyopathy known as Barth Syndrome, characterized by decreased $(18:2)_4$CL and increased levels of MLCL (8, 41-44). This specific mechanism was explored by Xu et al., who argue that CL is normally protected

from degradation due to its association with IMM proteins and respiratory chain enzymes. However, with deficiency of tafazzin in Barth Syndrome, this protection is compromised, leading to unstable CL and increased CL turnover. Xu et al. discovered that tafazzin mutation specifically shortens the half-life of CL, which in turn leads to the accumulation of MLCL seen in Barth Syndrome subjects (45).

2.3 Resolving a debate in the field – Is CL content or CL acyl chain composition the more important variable for mitochondrial membrane biophysical organization?

There is debate in the field as to whether CL content versus acyl chain composition contributes more or less towards the progression of mitochondrial dysfunction and disease. Therefore, it is necessary to clearly delineate the role of such CL abnormalities and discriminate between their influence on inner mitochondrial lipid molecular organization. The goal of this study is to examine how CL concentration and CL acyl chain remodeling impact various biophysical properties of mitochondrial membranes. This approach utilizes biomimetic mitochondrial phospholipids monolayers, which allow for tight control of phospholipid composition. It is hypothesized that both lowered CL concentration and products of CL remodeling, such as MLCL, will impact the lateral organization and packing abilities of biomimetic mitochondrial phospholipid monolayers.

The overall significance of this study is that we will resolve a central discrepancy in the field by discriminating between the impact of CL content and CL acyl chain composition on mitochondrial membrane properties and biophysical organization. Better understanding this mechanistic underpinning will allow for the design of CL-specific mitochondrial-targeted therapeutics for various metabolic diseases.

CHAPTER III
MATERIALS AND METHODS

The following materials and methods were adapted from Pennington et al. (2017) (13).

3.1 Materials

The following materials were used to construct monolayers: 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine (18:0 -22:6 PC), 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine (16:0 -20:4 PE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoly-sn-glycero-3-phospho-(1'-myo-inositol) (DOPI), bovine heart cardiolipin [$(18:2)_4$CL)], bovine heart monolyso-cardiolipin [$(18:2)_3$CL], 1,1',2,2'-tetramyristoyl cardiolipin [$(14:0)_4$CL)] and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents and solvents (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hampton, NH). All lipid mixtures were prepared under low light conditions and utilized fresh lipid stocks to minimize lipid oxidation.

3.2 Constructing biomimetic mitochondrial monolayers

Biomimetic mitochondrial monolayers of varying composition were synthesized by premixing lipid stocks in HPLC grade chloroform at the following ratios: 40 mol% 18:0-22:6 PC, 30 mol% 16:0-20:4 PE, 20 mol% (18:2)4CL, 5 mol% DOPI, 3 mol% DOPS, and 2 mol% Chol. For select experiments in which CL's acyl chain composition was altered, $(18:2)_4$CL was replaced with $(18:2)_3$CL or $(14:0)_4$CL in a ratiometric manner. For select experiments in which CL's content was lowered, levels of $(18:2)_4$CL were decreased by 25-50%.

Pressure-area isotherms were created on a Mini Langmuir-Blodgett Trough (KSV NIMA, Biolin Scientific, Paramus, NJ). Isotherms were compressed at a rate of 3.0 mm/min, and data was recorded using a Wilhelmy plate. Prior to spotting the lipid mixtures (1.08×10^{-9} mols total) onto the 10 mM sodium phosphate buffer subphase (pH 7.4), trough barriers were compressed and expanded to ensure the surface pressure remained constant (<0.3 mN/m). Raw isotherms were collected after 10 minutes, in order to allow for the evaporation of chloroform. Prior to collecting each isotherm, the trough was washed three times with 70% ethanol, Milli-Q water, and subphase. Lipid mixtures were produced multiple times to ensure day-to-day reproducibility.

3.3 Analysis of lipid miscibility, elasticity modulus, and Gibbs free energy of mixing in monolayers

The ideal mean molecular area of mixed monolayers was determined at a constant surface pressure (π) by the following equation:

$$A_{ideal} = X_1(A_1)_\pi + X_2(A_2)_\pi + \ \ldots X_n(A_n)_\pi$$

where X_n is the mol fraction of each individual component and A_n is the mean molecular area of each component. The excess area/molecule (A_{ex}) is a measurement of lipid miscibility and was determined at a given surface pressure (π) with the following equation:

$$A_{ex} = (A_{12\ldots n})_\pi - (X_1A_1 + X_2A_2 + \ \ldots X_nA_n)_\pi$$

where X_n is the mol fraction of each component and A_n is the mean molecular area of each component. ($A_{12\ldots n}$) is the mean molecular area of the mixed monolayer at a given surface pressure (π). A negative A_{ex} value indicates phospholipid miscibility and a positive A_{ex} value

indicates phospholipid immiscibility. The surface pressure-area isotherms were also used to calculate the surface elasticity modulus (C_s^{-1}) using the following expression:

$$C_s^{-1} = (-A)(d\pi/dA)_\pi$$

where A is the mean molecular area of the at a given surface pressure (π). The Gibbs free energy of phospholipid mixing (ΔG_{mix}) was calculated by the following expression:

$$\Delta G_{mix} = \Delta G_{ex} + \Delta G_{ideal}$$

where ΔG_{ex} is the excess Gibbs free energy of mixing and ΔG_{ideal} is the ideal Gibbs free energy of mixing. ΔG_{ex} was calculated from pressure-area isotherms using the following expression:

$$\Delta G_{ex} = \int_0^\pi [A_{12\ldots n} - (X_1A_1 + X_2A_2 + X_nA_n)]d\pi$$

where A_n and X_n represent the mean molecular area and mol fraction of each component, respectively, at a given surface pressure (π). ΔG_{ideal} was calculated using the following expression:

$$\Delta G_{ideal} = RT(X_1 lnX_1 + X_2 lnX_2 + X_n lnX_n)$$

where R is the ideal gas constant, T is temperature in Kelvin, and X_n is the mol fraction of each component.

3.4 Statistics

Data were analyzed using GraphPad Prism v5.0b. Data were ensured to display normalized distributions. Statistical significance relied on one-way ANOVA, and was indicated by *p*-values of <0.05.

CHAPTER IV
RESULTS

4.1 Monolayers of differing (18:2)$_4$CL concentration in mitochondrial-mimicking phospholipid mixtures

The biophysical behavior of differing CL concentrations was studied in a mitochondrial-mimicking phospholipid mixture. We chose to study what happens when CL is lowered by 25-50%, which is observed in various cardiometabolic and some neurological diseases (34, 38, 46-50). We start by showing raw monolayer data, which was used to generate secondary analyses that report on quantitative biophysical properties, such as excess area per molecule, elasticity modulus, and the Gibbs free energy of lipid mixing.

We first start by presenting results in which CL concentration was manipulated in mitochondrial-mimicking monolayers. At a physiologically relevant surface pressure of 30 mN/m (51, 52), lowering the level of CL in the mixture decreased the mean molecular area between lipid molecules, indicating a tighter packing of lipids (Figure 1A). Here, a partial loss of CL indicates a 25% loss of CL, whereas an absolute loss of CL is represented by a 50% reduction in CL mass. Our results indicate that lowering CL by 25-50% decreases the mean molecular area of mitochondrial-mimicking monolayers, and a reduction of CL mass by 50% disrupts the mean molecular area to the highest degree.

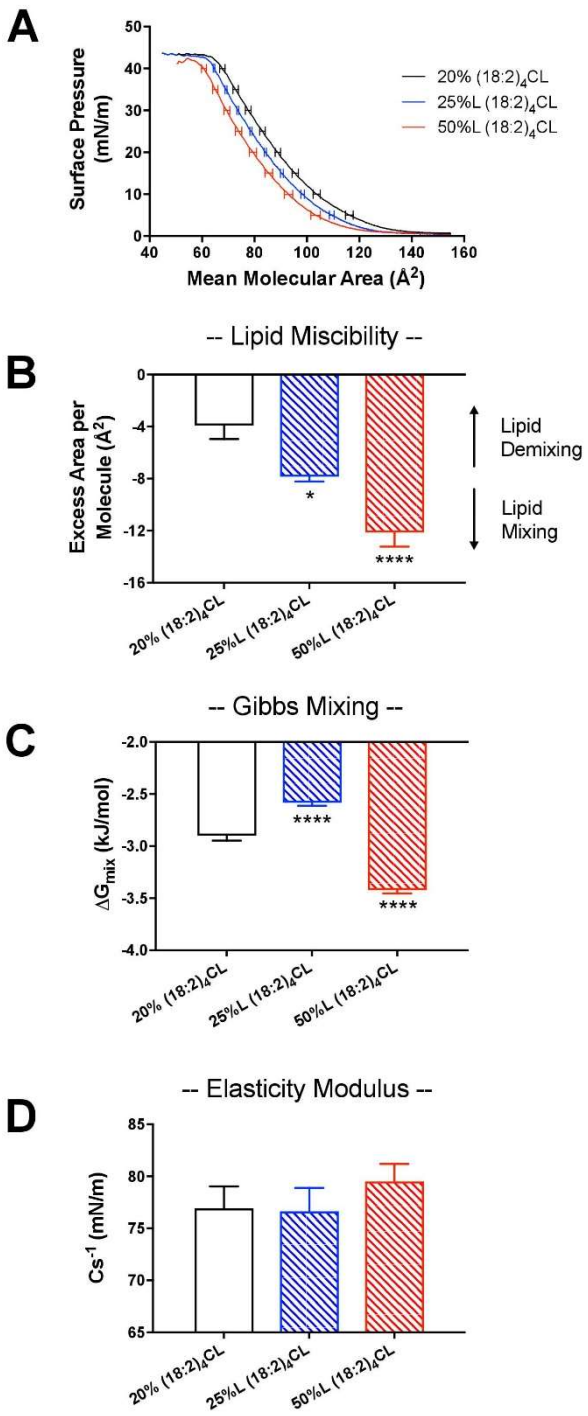

Figure 1. Pressure-area isotherms (A) were acquired at room temperature using a 10 mM sodium phosphate buffer (pH 7.4). Isotherms were analyzed at 30 mN/m and used to calculate excess area/molecule (B), Gibbs free energy of mixing (C), and elasticity modulus (D). Data are average ± SEM from 6 to 8 independent experiments. * = p-value of <0.05, **** = p-value of <0.00005.

Decreasing CL levels disrupted the mixing properties of adjacent phospholipids as indicated by the excess area per molecule calculations. A 25% loss of CL content relative to the control decreased the excess area per molecule by 3.9 $Å^2$, while a 50% loss of CL decreased the excess are per molecule by 8.2 $Å^2$. To assess the thermodynamics of phospholipid interactions, we quantified the Gibbs free energy of mixing. Interestingly, while a 25% loss of CL relative to the control increased the Gibbs free energy by 0.3 kJ/mol, a 50% loss of CL decreased the Gibbs free energy by 0.5 kJ/mol (Figure 1C). Finally, we measured the elasticity modulus, which is an indirect measure of the phospholipid monolayers ability to undergo curvature. While a loss of 25% CL did not impact the elasticity modulus of the monolayer, a loss of 50% CL increased the elasticity modulus by 2.5 mN/m, indicating a decrease in curvature of the monolayer.

4.2 Monolayers of $(18:2)_4$CL remodeling to $(18:2)_3$CL in mitochondrial-mimicking phospholipid mixtures

Lowering CL's concentration would presumably impair the lipid packing properties given the size of CL and its ability to influence the lateral organization of phospholipid mixtures. However, to better understand CL's specific role in phospholipid dynamics, we investigated the role of CL's acyl chain composition on the aforementioned properties of biomimetic mitochondrial monolayers. To do this, we studied the behavior of differing CL acyl chain compositions in mitochondrial-mimicking monolayers. We chose to study what happens when CL is remodeled to $(18:2)_3$CL (or MLCL), as seen in many metabolic diseases, such as Barth syndrome (42, 43, 53). We specifically studied how varying ratios of $(18:2)_4$CL-to-$(18:2)_3$CL impacted properties of monolayers, at two different surface pressures, to better understand the dose-dependent effects of increasing $(18:2)_3$CL levels. Our raw isotherm data

indicates a small step-wise increase in mean molecular area from control to 6% $(18:2)_3$CL (Δ4.1 Å^2), followed by a small step-wise decrease in mean molecular area from 6% $(18:2)_3$CL to 16% $(18:2)_3$CL (Δ2.6 Å^2) (Figure 2).

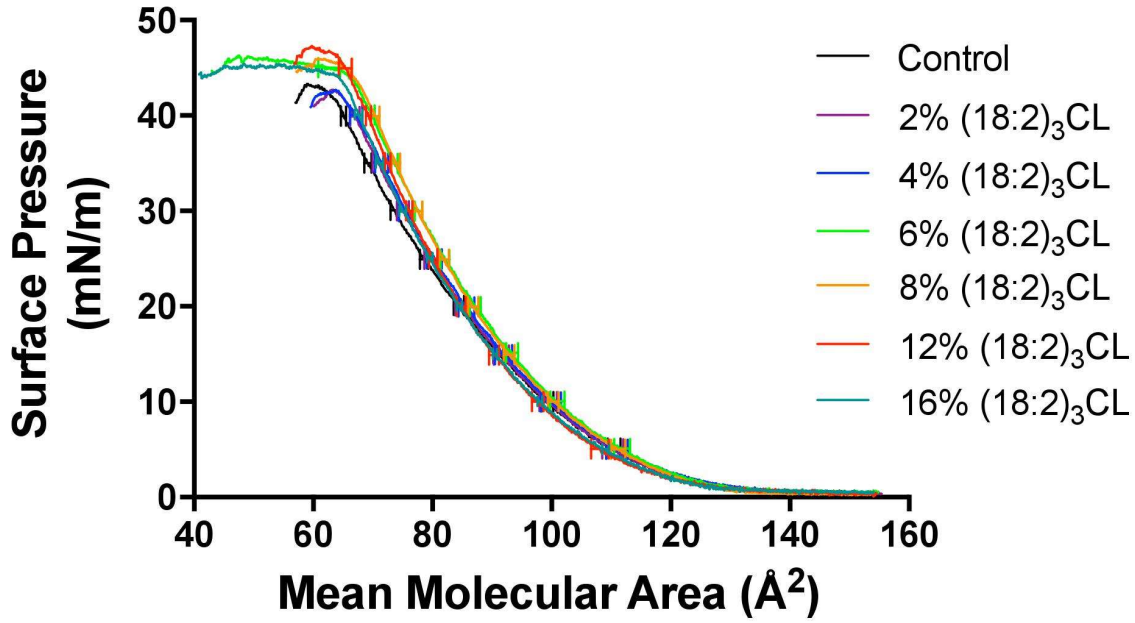

Figure 2. Monolayers containing differing concentrations of $(18:2)_3$CL. Pressure-area isotherms were conducted at ratiometric concentrations of $(18:2)_4$CL to $(18:2)_3$CL, totaling 20% of the total phospholipid mixture. Isotherms were acquired at room temperature using a 10 mM sodium phosphate buffer (pH 7.4). Data are average ± SEM from 6 to 8 independent experiments.

The aforementioned raw pressure-area isotherms (Figure 2) were used to calculate the excess area/molecule, the Gibbs free energy of mixing (ΔG_{mix}), and the elasticity modulus (C_s^{-1}) in response to changes in CL acyl chain composition. All analyses were conducted at

low and high surface pressures of 15 mN/m and 30 mN/m, the latter of which is the physiologically relevant surface pressure of cell membranes (51, 52).

As the amount of $(18:2)_3$CL increased to 6%, the excess area per molecule values increased relative to control, followed by a decrease in excess area per molecule values from 6% $(18:2)_3$CL to 16% $(18:2)_3$CL, relative to control (Figure 3B). These changes implicate that increasing levels of $(18:2)_3$CL differentially impact the lipid miscibility of mitochondrial-mimicking monolayers, which is discussed below in detail. Ironically, extreme levels of $(18:2)_3$CL yielded the smallest increase in excess area per molecule, as compared to the control (Δ0.7 Å^2). At 6% $(18:2)_3$CL, the Gibbs free energy of mixing increased the greatest (Δ0.3 kJ/mol) relative to the control, while the largest decrease in Gibbs free energy occurred at 4% $(18:2)_3$CL (Δ0.5 kJ/mol) (Figure 3D). When 6% $(18:2)_3$CL and 16% $(18:2)_3$CL were incorporated into mitochondrial-mimicking monolayers, it resulted in unfavorable mixing values relative to the control. The elasticity modulus increased in a step-wise manner as CL alteration increased from control to 12% $(18:2)_3$CL (Δ20.0 mN/m). However, at 16% $(18:2)_3$CL, the elasticity modulus decreased, relative to 12% $(18:2)_3$CL (Δ6.2 mN/m) (Figure 3F). Similar results were observed when the monolayer was less compressed at 15 mN/m (Figure 3 A,C,E).

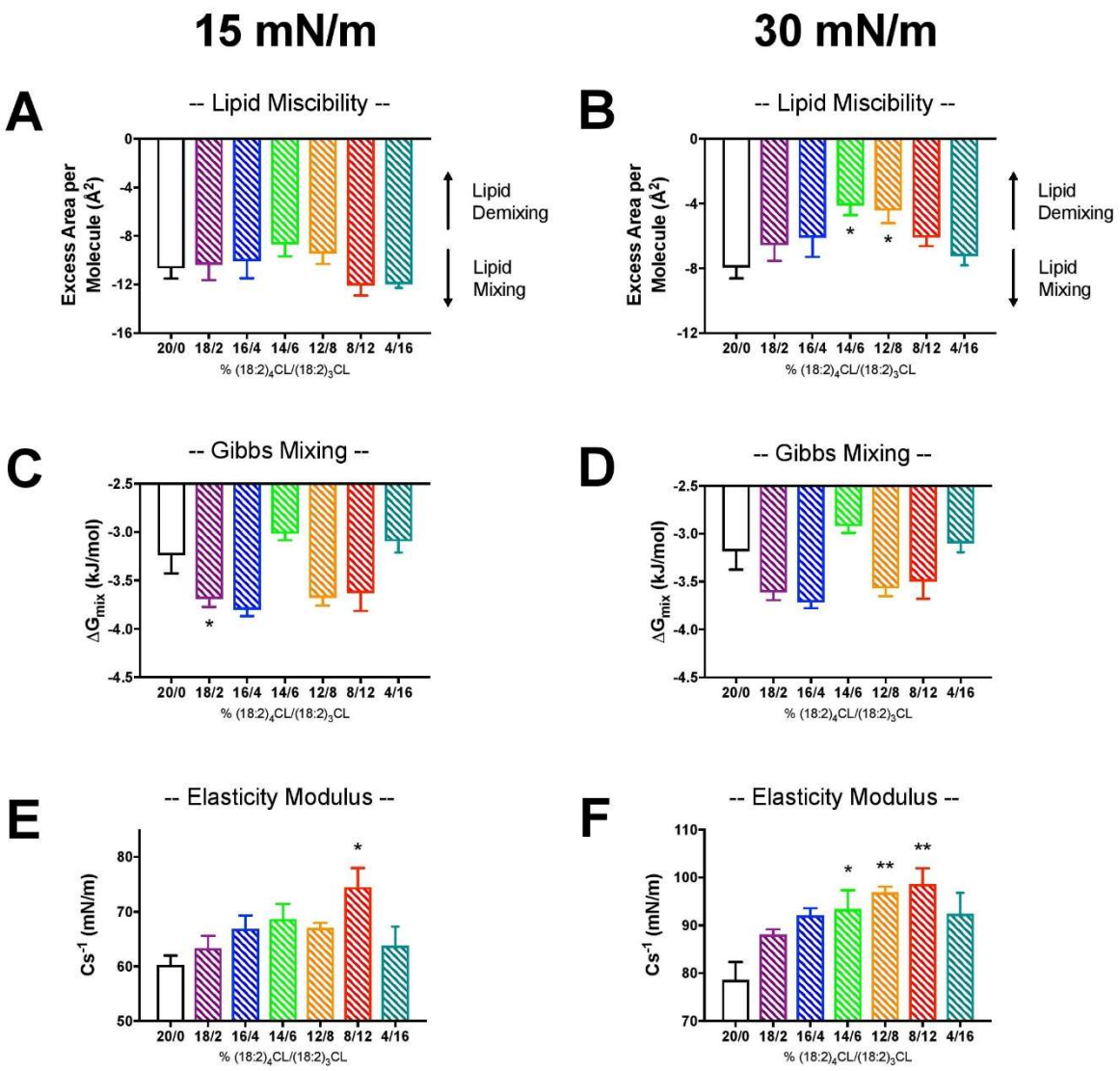

Figure 3. Secondary analyses of monolayers containing differing concentrations of $(18:2)_3$CL, conducted at a low surface pressure of 15 mN/m (A, C, E) and a physiologically relevant surface pressure of 30 mN/m (B, D, F). Raw isotherms (Figure 2) were used to calculate excess area/molecule, Gibbs free energy of mixing, and elasticity modulus. Data are average ± SEM from 6 to 8 independent experiments. * = p-value of <0.05, ** = p-value of <0.005.

4.3 Monolayers of $(18:2)_4$CL remodeling to $(14:0)_4$CL in mitochondrial-mimicking phospholipid mixtures

Finally, we studied the behavior of CL remodeling to $(14:0)_4$CL in a mitochondrial-mimicking phospholipid mixture. While this CL composition is not biologically relevant in humans, it represents a major remodeling event in which all four acyl chains are altered from linoleic acid to myristic acid. This alteration was also completed in a ratiometric manner to determine the impact of varying levels of extreme CL remodeling. Our raw isotherm data indicates a significant step-wise increase in mean molecular area from control to 16% $(14:0)_4$CL (Δ21.3 Å^2) (Figure 4).

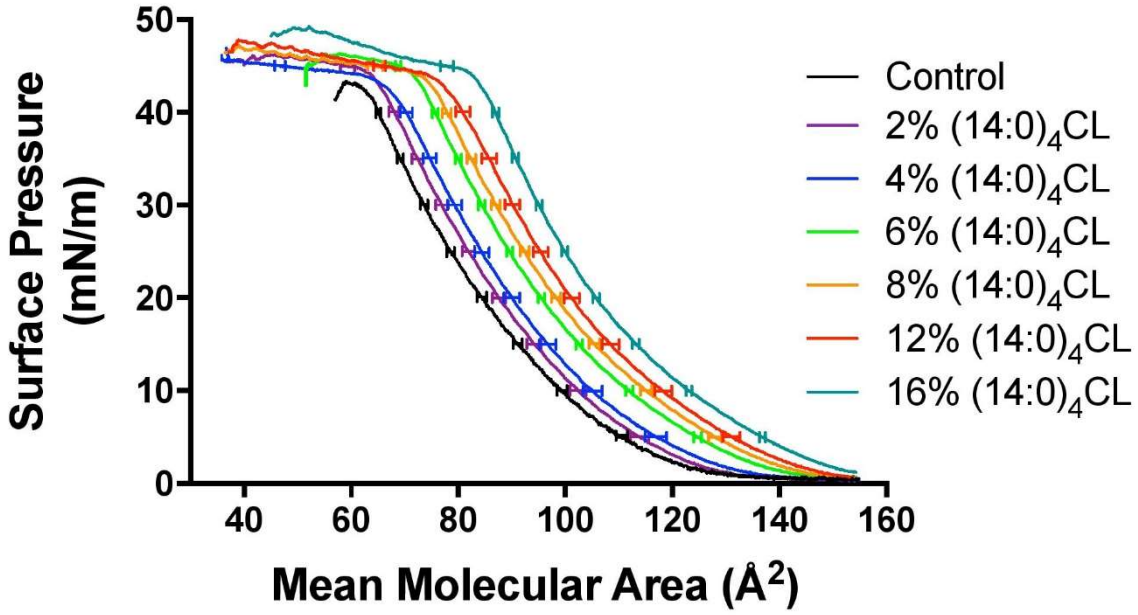

Figure 4. Monolayers containing differing concentrations of $(14:0)_4$CL. Pressure-area isotherms were conducted at ratiometric concentrations of $(18:2)_4$CL to $(14:0)_4$CL, totaling 20% of the total phospholipid mixture. Isotherms were acquired at room temperature using a 10 mM sodium phosphate buffer (pH 7.4). Data are average ± SEM from 6 to 8 independent experiments.

While the incorporation of increasing levels of $(18:2)_3$CL had modest effects on membrane biophysical properties, the incorporation of $(14:0)_4$CL leads to drastic impairments in the organization of mitochondrial-mimicking membranes, relative to control. Excess area per molecule measurements indicated a significant decrease in miscibility as $(14:0)_4$CL was increased to 16% $(14:0)_4$CL (Δ29.1 $Å^2$) (Figure 5B). The Gibbs free energy of mixing also increased as the level of $(14:0)_4$CL increased from control to 16% (Δ1.0 kJ/mol). However, at 6% $(14:0)_4$CL, the change in Gibbs free energy was negligible relative to control (Δ0.1 kJ/mol). All levels of CL alteration yielded a larger Gibbs free energy value, and therefore less favorable lipid mixing (Figure 5D). Finally, the elasticity modulus was significantly increased as $(14:0)_4$CL levels increased from control to 16% $(14:0)_4$CL (Δ25.7 mN/m) (Figure 5F). Nearly identical results were observed when the monolayer was less compressed at 15 mN/m (Figure 5 A,C,E).

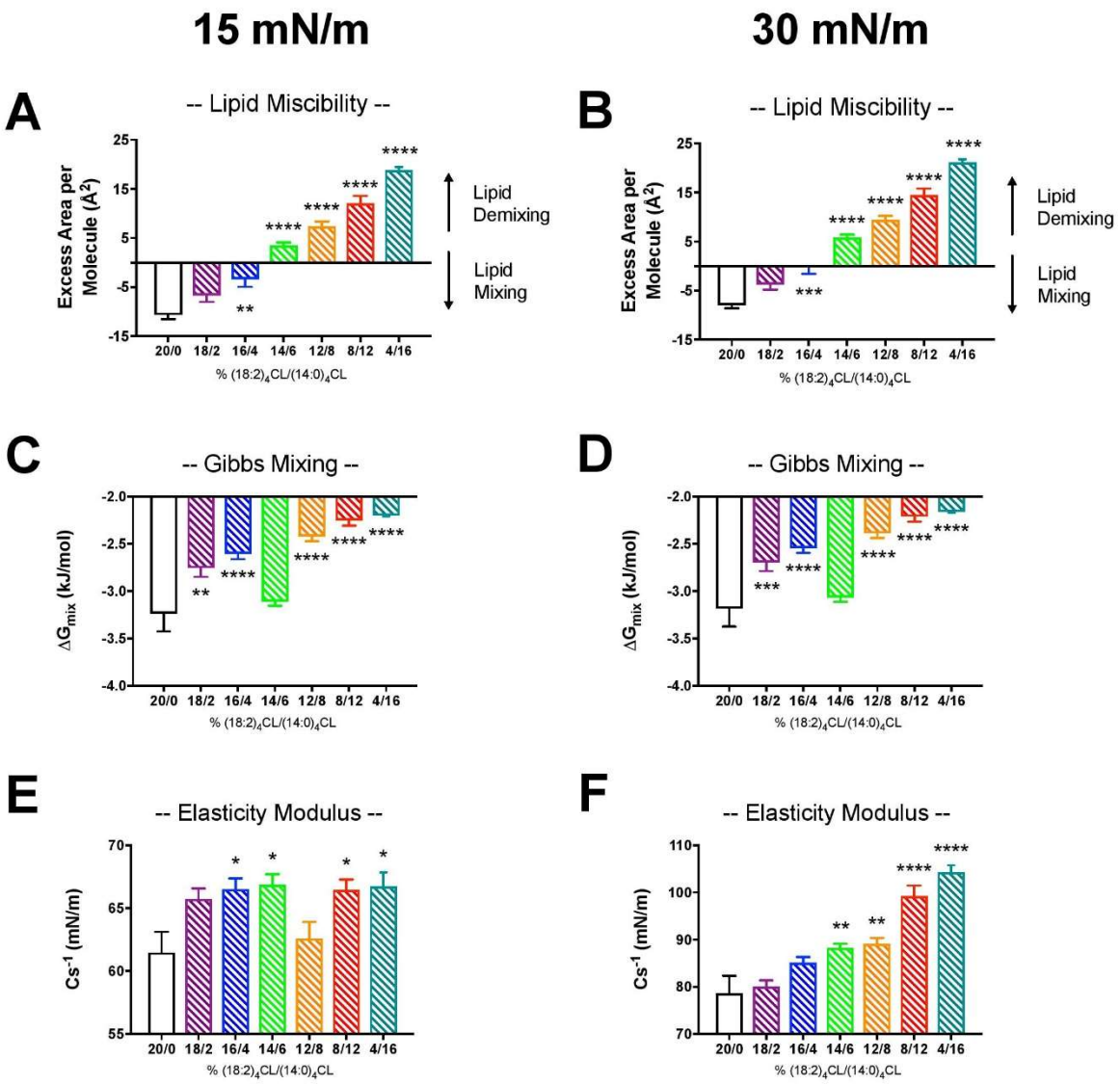

Figure 5. Secondary analyses of monolayers containing differing concentrations of $(14:0)_4$CL, conducted at a low surface pressure of 15 mN/m (A, C, E) and a physiologically relevant surface pressure of 30 mN/m (B, D, F). Raw isotherms (Figure 4) were used to calculate excess area/molecule, Gibbs free energy of mixing, and elasticity modulus. Data are average ± SEM from 6 to 8 independent experiments. * = p-value of <0.05, ** = p-value of <0.005, *** = p-value of <0.0005, **** = p-value of <0.00005.

CHAPTER V
DISCUSSION

5.1. Biological consequences for lowering $(18:2)_4$CL content in mitochondrial-mimicking monolayer studies

In many diseases, CL undergoes alterations in content and acyl chain composition. For example, a loss of CL content occurs in ischemia-reperfusion, whereas various changes occur to acyl chain composition in Barth Syndrome (38-40). It is suggested that these changes to CL would impact biophysical properties of the IMM, thereby impacting the proper functioning of the mitochondria. For this reason, we explored the impact of both CL content and acyl chain composition on mitochondrial-mimicking monolayers.

A loss of CL content was found to decrease the mean molecular area between adjacent lipid molecules in our mitochondrial-mimicking monolayers. This finding suggests that lowered CL content, as seen in many diseases, results in a tighter packing of lipids. This has implications for the stability of specific lipid-protein interactions in the IMM which may impact mitochondrial bioenergetics and thereby ATP production. The excess area per molecule, an inverse measure of lipid miscibility, was found to decrease as CL content was lost from the sample. This finding indicates that a loss of CL results in increased mixing of lipids on the monolayer, which is important because CL may act as a platform for proteins to function optimally in microdomains. A loss of CL content therefore likely has implications for IMM proteins that exist and function in these domains. The elasticity modulus measures the ability of the monolayer to undergo curvature, where a higher value indicates less curvature in

the monolayer. Our findings suggest that a loss of CL content leads to an increase in the elasticity modulus, indicating a decrease in monolayer curvature. This finding may be partially attributed to the fact that CL is a highly-curved phospholipid that regulates lipid microdomains within the IMM. These microdomains are crucial for maintaining the highly-curved structure of the membrane, which helps to explain why a loss of CL results in a decrease in membrane curvature. Failure to maintain the highly-curved structure of the IMM may have important implications for the optimal functioning of respiratory chain enzymes and protein carriers of the IMM.

5.2 Mitochondrial biophysical properties are impacted by the remodeling of $(18:2)_4$CL to $(14:0)_4$CL, but not $(18:2)_3$CL.

We further chose to study the impact of CL remodeling events on biophysical properties, in the hopes of better understanding the current debate over the contributions of CL content versus acyl chain composition on the progression of various metabolic diseases. We first explored the alteration of $(18:2)_4$CL to $(18:2)_3$CL, a common remodeling event in diseases such as Barth Syndrome (54, 55).

Exchange of $(18:2)_4$CL with $(18:2)_3$CL did not strongly influence monolayer excess area/molecule and elasticity modulus. The only exception was with 6% $(18:2)_3$CL, which displayed a significant change in miscibility and elasticity when compared to the control. Interestingly, higher levels of $(18:2)_3$CL began to display properties similar to the control, most notably in excess area/molecule but also with the elasticity modulus. Our hypothesis for this phenomenon is that at a certain threshold and ratio of $(18:2)_4$CL to $(18:2)_3$CL, the monolayer begins to adopt similar biophysical properties relative to control. At higher concentrations of $(18:2)_3$CL, such as 8%, 12%, and 16%, we begin to see these properties shift in value towards

the control, indicating that $(18:2)_3$CL behaves similarly to $(18:2)_4$CL when these lipids are in near equal amounts within mitochondrial-mimicking monolayers. This finding is surprising as we expect alterations to $(18:2)_4$CL will impact biophysical properties of mitochondrial-mimicking monolayers as observed when CL content is lowered. These results clearly implicate that increased levels of $(18:2)_3$CL, as seen in Barth syndrome, may not strongly impact the biophysical properties of the IMM and thereby the activity of proteins that require specific lipid microenvironments for optimal function.

In order to further determine the impact of CL acyl chain remodeling on biophysical properties of mitochondrial-mimicking membranes, we chose to study $(14:0)_4$CL. While this composition is only found in some bacteria and not in mammalian mitochondria (56), and therefore is limited in its biological relevance, our rationale for studying $(14:0)_4$CL was to explore the impact of a major remodeling event for CL. This allowed us to distinguish the results seen from modest remodeling of $(18:2)_4$CL to $(18:2)_3$CL and determine if CL acyl chain composition plays a role in the progression of cardiometabolic disease.

Replacement of $(18:2)_4$CL with $(14:0)_4$CL significantly influenced all biophysical properties studied, especially at higher concentrations of $(14:0)_4$CL. The increase in excess area/molecule as the ratio of $(14:0)_4$CL to $(18:2)_4$CL increased, demonstrating that adjacent phospholipids become more immiscible as more $(14:0)_4$CL is incorporated. This is also demonstrated through the Gibbs free energy of mixing, which also show less favorable mixing interactions as more $(14:0)_4$CL is incorporated. The only exception is the 6% $(14:0)_4$CL, which displayed a negligible change in free energy relative to the control. This may be due to the fact that monolayers containing 6% $(14:0)_4$CL collapsed earlier than other phospholipid mixtures.

The IMM requires a strong membrane potential for optimal electron shuttling and electron transport chain (ETC) function (57). Failure for these lipids to mix properly, due to the lack of $(18:2)_4$CL, would result in abnormal CL microdomain formation. This would further lead to a decrease in the curvature of the membrane, as demonstrated by our findings in the elasticity modulus, and would allow protons to leak through the ETC channels, leading to a lower membrane potential and less ATP production.

Overall, our findings suggest that extreme remodeling events of $(18:2)_4$CL impact the biophysical properties of mitochondrial-mimicking monolayers, however modest remodeling of $(18:2)_4$CL to species such as $(18:2)_3$CL does not seem to majorly impair the biophysical properties of these monolayers.

5.3 Implications of lowered CL content versus CL acyl chain remodeling in the context of health and disease

Loss of $(18:2)_4$CL content and aberrant acyl chain remodeling occur in many diseases, leading to impairment of the IMM structure and function. It is suggested that both of these alterations to the CL profile would impact important lipid-protein interactions in the IMM. As the site of oxidative phosphorylation, the IMM contains numerous respiratory chain enzymes and protein carriers that depend on the surrounding lipid environment for optimal functioning. CL specifically is a crucial lipid for the formation of respiratory chain supercomplexes, acting as a "glue" to hold together these protein structures (20-23, 55). Therefore, it is plausible that any changes to the biophysical properties of the IMM would impact the activity of respiratory chain proteins.

These findings are important in health as a number of pharmaceutical approaches are currently targeting CL in the hopes of restoring metabolic defects as observed in various cardiometabolic diseases (58, 59). For example, a peptide known as SS-31/Bendavia has shown to improve mitochondrial function in animal models, specifically in diseases such as cardiac ischemia-reperfusion (60, 61). Bendavia functions by selectively binding to CL via electrostatic interactions, promoting CL aggregation within the IMM, and thereby improving bioenergetics by lowering ROS (62-64). The development of pharmaceutical approaches targeting CL are very important for helping treat cardiometabolic defects as a result of mitochondrial dysfunction.

5.4 Conclusion

Altogether, the results demonstrate that loss of $(18:2)_4$CL content and extreme acyl chain remodeling impair biophysical properties of mitochondrial-mimicking monolayers, such as lipid miscibility and monolayer elasticity. However, modest acyl chain remodeling, such as that from $(18:2)_4$CL to $(18:2)_3$CL, does not impair these properties. This lays the foundation for future studies which can further examine the impact of CL alterations on the inner membrane, specifically those that incorporate protein and/or occur in bilayer settings. In the context of mitochondrial dysfunction, this study is an important precursor towards the design of CL-specific therapeutics that can target, and ameliorate, the progression of pathologies associated with mitochondrial dysfunction.

REFERENCES

1. Houtkooper R, Vaz F. Cardiolipin, the heart of mitochondrial metabolism. Cellular and Molecular Life Sciences. 2008;65(16):2493-506.

2. Attardi G, Schatz G. Biogenesis of mitochondria. Annu Rev Cell Biol. 1988;4(1):289-331.

3. Fujiki Y, Fowler S, Shio H, Hubbard AL, Lazarow PB. Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: comparison with endoplasmic reticulum and mitochondrial membranes. J Cell Biol. 1982 Apr;93(1):103-10.

4. Chicco AJ, Sparagna GC. Role of cardiolipin alterations in mitochondrial dysfunction and disease. American Journal of Physiology-Cell Physiology. 2007.

5. Sparagna GC, Chicco AJ, Murphy RC, Bristow MR, Johnson CA, Rees ML, et al. Loss of cardiac tetralinoleoyl cardiolipin in human and experimental heart failure. J Lipid Res. 2007 Jul;48(7):1559-70.

6. Schlame M, Kelley RI, Feigenbaum A, Towbin JA, Heerdt PM, Schieble T, et al. Phospholipid abnormalities in children with Barth syndrome. J Am Coll Cardiol. 2003;42(11):1994-9.

7. Paradies G, Paradies V, Ruggiero FM, Petrosillo G. Oxidative stress, cardiolipin and mitochondrial dysfunction in nonalcoholic fatty liver disease. World J Gastroenterol. 2014 Oct 21;20(39):14205-18.

8. Schlame M, Ren M. Barth syndrome, a human disorder of cardiolipin metabolism. FEBS Lett. 2006;580(23):5450-5.

9. Shen Z, Ye C, McCain K, Greenberg ML. The Role of Cardiolipin in Cardiovascular Health. Biomed Res Int. 2015;2015:891707.

10. Dudek J, Hartmann M, Rehling P. The role of mitochondrial cardiolipin in heart function and its implication in cardiac disease. Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease. 2018.

11. Paradies G, Paradies V, Ruggiero FM, Petrosillo G. Cardiolipin alterations and mitochondrial dysfunction in heart ischemia/reperfusion injury. Clinical Lipidology. 2015;10(5):415-29.

12. He Q, Han X. Cardiolipin remodeling in diabetic heart. Chem Phys Lipids. 2014;179:75-81.

13. Pennington ER, Fix A, Sullivan EM, Brown DA, Kennedy A, Shaikh SR. Distinct membrane properties are differentially influenced by cardiolipin content and acyl chain composition in biomimetic membranes. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2017;1859(2):257-67.

14. Pennington ER, Funai K, Brown DA, Shaikh SR. The role of cardiolipin concentration and acyl chain composition on mitochondrial inner membrane molecular organization and function. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2019.

15. Schlame M, Ren M, Xu Y, Greenberg ML, Haller I. Molecular symmetry in mitochondrial cardiolipins. Chem Phys Lipids. 2005;138(1-2):38-49.

16. Claypool SM, Koehler CM. The complexity of cardiolipin in health and disease. Trends Biochem Sci. 2012;37(1):32-41.

17. Petrosillo G, Ruggiero FM, Paradies G. Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria. The FASEB Journal. 2003;17(15):2202-8.

18. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E. Peroxidative damage to cardiac mitochondria: cytochrome oxidase and cardiolipin alterations. FEBS Lett. 1998;424(3):155-8.

19. Ren M, Phoon CK, Schlame M. Metabolism and function of mitochondrial cardiolipin. Prog Lipid Res. 2014;55:1-16.

20. Zhang M, Mileykovskaya E, Dowhan W. Gluing the respiratory chain together. Cardiolipin is required for supercomplex formation in the inner mitochondrial membrane. J Biol Chem. 2002 Nov 15;277(46):43553-6.

21. Pfeiffer K, Gohil V, Stuart RA, Hunte C, Brandt U, Greenberg ML, et al. Cardiolipin stabilizes respiratory chain supercomplexes. J Biol Chem. 2003 Dec 26;278(52):52873-80.

22. Mileykovskaya E, Dowhan W. Cardiolipin-dependent formation of mitochondrial respiratory supercomplexes. Chem Phys Lipids. 2014;179:42-8.

23. Zhang M, Mileykovskaya E, Dowhan W. Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria. J Biol Chem. 2005 Aug 19;280(33):29403-8.

24. Schlame M, Ren M. The role of cardiolipin in the structural organization of mitochondrial membranes. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2009;1788(10):2080-3.

25. Paradies G, Paradies V, De Benedictis V, Ruggiero FM, Petrosillo G. Functional role of cardiolipin in mitochondrial bioenergetics. Biochimica et Biophysica Acta (BBA)-Bioenergetics. 2014;1837(4):408-17.

26. Vos M, Geens A, Deaulmerie L, Swerts J, Craessaerts K, Seibler P, et al. Mitochondrial cardiolipin couples electron transport between ubiquinone and complex I to rescue PINK1 deficiency. Movement Disorders. 2016;31.

27. Sullivan EM, Pennington ER, Sparagna GC, Torres MJ, Neufer PD, Harris M, et al. Docosahexaenoic acid lowers cardiac mitochondrial enzyme activity by replacing linoleic acid in the phospholipidome. J Biol Chem. 2018 Jan 12;293(2):466-83.

28. Robinson NC, Zborowski J, Talbert LH. Cardiolipin-depleted bovine heart cytochrome c oxidase: binding stoichiometry and affinity for cardiolipin derivatives. Biochemistry. 1990;29(38):8962-9.

29. Shidoji Y, Hayashi K, Komura S, Ohishi N, Yagi K. Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. Biochem Biophys Res Commun. 1999;264(2):343-7.

30. Mileykovskaya E, Dowhan W. Visualization of phospholipid domains in Escherichia coli by using the cardiolipin-specific fluorescent dye 10-N-nonyl acridine orange. J Bacteriol. 2000 Feb;182(4):1172-5.

31. Mileykovskaya E, Dowhan W. Cardiolipin membrane domains in prokaryotes and eukaryotes. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2009;1788(10):2084-91.

32. Renner LD, Weibel DB. Cardiolipin microdomains localize to negatively curved regions of Escherichia coli membranes. Proc Natl Acad Sci U S A. 2011 Apr 12;108(15):6264-9.

33. Pennington ER, Sullivan EM, Fix A, Dadoo S, Zeczycki TN, DeSantis A, et al. Proteolipid domains form in biomimetic and cardiac mitochondrial vesicles and are regulated by cardiolipin concentration but not monolyso-cardiolipin. J Biol Chem. 2018 Oct 12;293(41):15933-46.

34. Paradies G, Petrosillo G, Pistolese M, Ruggiero FM. Reactive oxygen species generated by the mitochondrial respiratory chain affect the complex III activity via cardiolipin peroxidation in beef-heart submitochondrial particles. Mitochondrion. 2001;1(2):151-9.

35. Petrosillo G, Ruggiero FM, Pistolese M, Paradies G. Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-

heart submitochondrial particles via cardiolipin peroxidation. Possible role in the apoptosis. FEBS Lett. 2001;509(3):435-8.

36. Schlame M, Ren M. Barth syndrome, a human disorder of cardiolipin metabolism. FEBS Lett. 2006;580(23):5450-5.

37. Malhotra A, Edelman-Novemsky I, Xu Y, Plesken H, Ma J, Schlame M, et al. Role of calcium-independent phospholipase A2 in the pathogenesis of Barth syndrome. Proc Natl Acad Sci U S A. 2009 Feb 17;106(7):2337-41.

38. Heerdt PM, Schlame M, Jehle R, Barbone A, Burkhoff D, Blanck TJ. Disease-specific remodeling of cardiac mitochondria after a left ventricular assist device. Ann Thorac Surg. 2002;73(4):1216-21.

39. Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Serena D, Ruggiero FM. Lipid peroxidation and alterations to oxidative metabolism in mitochondria isolated from rat heart subjected to ischemia and reperfusion. Free Radical Biology and Medicine. 1999;27(1-2):42-50.

40. Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Federici A, Ruggiero FM. Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin. Circ Res. 2004;94(1):53-9.

41. Vreken P, Valianpour F, Nijtmans LG, Grivell LA, Plecko B, Wanders RJ, et al. Defective remodeling of cardiolipin and phosphatidylglycerol in Barth syndrome. Biochem Biophys Res Commun. 2000;279(2):378-82.

42. Schlame M, Towbin JA, Heerdt PM, Jehle R, DiMauro S, Blanck TJ. Deficiency of tetralinoleoyl-cardiolipin in Barth syndrome. Ann Neurol. 2002;51(5):634-7.

43. Gu Z, Valianpour F, Chen S, Vaz FM, Hakkaart GA, Wanders RJ, et al. Aberrant cardiolipin metabolism in the yeast taz1 mutant: a model for Barth syndrome. Mol Microbiol. 2004;51(1):149-58.

44. Claypool SM, Boontheung P, McCaffery JM, Loo JA, Koehler CM. The cardiolipin transacylase, tafazzin, associates with two distinct respiratory components providing insight into Barth syndrome. Mol Biol Cell. 2008;19(12):5143-55.

45. Xu Y, Phoon CK, Berno B, D'Souza K, Hoedt E, Zhang G, et al. Loss of protein association causes cardiolipin degradation in Barth syndrome. Nature chemical biology. 2016;12(8):641.

46. Paradies G, Petrosillo G, Pistolese M, Ruggiero FM. The effect of reactive oxygen species generated from the mitochondrial electron transport chain on the cytochrome c oxidase activity and on the cardiolipin content in bovine heart submitochondrial particles. FEBS Lett. 2000;466(2-3):323-6.

47. Paradies G, Petrosillo G, Pistolese M, Ruggiero FM. Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage. Gene. 2002;286(1):135-41.

48. Eirin A, Ebrahimi B, Kwon SH, Fiala JA, Williams BJ, Woollard JR, et al. Restoration of mitochondrial cardiolipin attenuates cardiac damage in swine renovascular hypertension. Journal of the American Heart Association. 2016;5(6):e003118.

49. Song C, Zhang J, Qi S, Liu Z, Zhang X, Zheng Y, et al. Cardiolipin remodeling by ALCAT1 links mitochondrial dysfunction to Parkinson's diseases. Aging cell. 2019:e12941.

50. Monteiro-Cardoso VF, Oliveira MM, Melo T, Domingues MR, Moreira PI, Ferreiro E, et al. Cardiolipin profile changes are associated to the early synaptic mitochondrial dysfunction in Alzheimer's disease. J Alzheimer's Dis. 2015;43(4):1375-92.

51. Seelig A. Local anesthetics and pressure: a comparison of dibucaine binding to lipid monolayers and bilayers. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1987;899(2):196-204.

52. Demel R, Van Kessel WG, Zwaal R, Roelofsen B, Van Deenen L. Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1975;406(1):97-107.

53. Malhotra A, Edelman-Novemsky I, Xu Y, Plesken H, Ma J, Schlame M, et al. Role of calcium-independent phospholipase A2 in the pathogenesis of Barth syndrome. Proceedings of the National Academy of Sciences. 2009;106(7):2337-41.

54. van Werkhoven MA, Thorburn DR, Gedeon AK, Pitt JJ. Monolysocardiolipin in cultured fibroblasts is a sensitive and specific marker for Barth Syndrome. J Lipid Res. 2006 Oct;47(10):2346-51.

55. McKenzie M, Lazarou M, Thorburn DR, Ryan MT. Mitochondrial respiratory chain supercomplexes are destabilized in Barth Syndrome patients. J Mol Biol. 2006;361(3):462-9.

56. Hoch FL. Cardiolipins and biomembrane function. Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes. 1992;1113(1):71-133.

57. Brown DA, Sabbah HN, Shaikh SR. Mitochondrial inner membrane lipids and proteins as targets for decreasing cardiac ischemia/reperfusion injury. Pharmacol Ther. 2013;140(3):258-66.

58. Murphy MP. Targeting lipophilic cations to mitochondria. Biochimica et Biophysica Acta (BBA)-Bioenergetics. 2008;1777(7-8):1028-31.

59. Walters AM, Porter Jr GA, Brookes PS. Mitochondria as a drug target in ischemic heart disease and cardiomyopathy. Circ Res. 2012;111(9):1222-36.

60. Szeto HH. First-in-class cardiolipin-protective compound as a therapeutic agent to restore mitochondrial bioenergetics. Br J Pharmacol. 2014;171(8):2029-50.

61. Paradies G, Paradies V, Ruggiero FM, Petrosillo G. Mitochondrial bioenergetics and cardiolipin alterations in myocardial ischemia-reperfusion injury: implications for pharmacological cardioprotection. American Journal of Physiology-Heart and Circulatory Physiology. 2018;315(5):H1341-52.

62. Eirin A, Ebrahimi B, Zhang X, Zhu X, Woollard JR, He Q, et al. Mitochondrial protection restores renal function in swine atherosclerotic renovascular disease. Cardiovasc Res. 2014;103(4):461-72.

63. Kloner RA, Hale SL, Dai W, Gorman RC, Shuto T, Koomalsingh KJ, et al. Reduction of ischemia/reperfusion injury with bendavia, a mitochondria-targeting cytoprotective peptide. Journal of the American Heart Association. 2012;1(3):e001644.

64. Brown DA, Hale SL, Baines CP, Rio CLd, Hamlin RL, Yueyama Y, et al. Reduction of early reperfusion injury with the mitochondria-targeting peptide bendavia. J Cardiovasc Pharmacol Ther. 2014;19(1):121-32.