# The Effect of Cardiolipin on Vascular Smooth Muscle Cell Dedifferentiation, Function, and Mitochondrial Respiration

Deema Galambo

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By: Deema Galambo

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Signed by the final Examining Committee:

Dr. Grant Brown Chair

Dr. Alisa Piekny Examiner

Dr. Christopher Brett Examiner

Dr. Vladimir Titorenko External Examiner

Dr. Andreas Bergdahl Supervisor

Approved by _____

(Chair of Department or Graduate Program Director)

_____ 2017 _____

(Dean of Faculty)

# ABSTRACT

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Deema Galambo

The initial phases of atherosclerosis involve the transition of normal vascular smooth muscle cells (VSMCs) to a synthetic, dedifferentiated phenotype, which can migrate and proliferate. This could potentially obstruct blood flow, which, when destined to the cardiomyocytes, can lead to myocardial infarction. Studies have demonstrated that cardiomyocytes, undergoing apoptosis, release a phospholipid molecule called cardiolipin (CL) into the systemic circulation. The objective of this project was to investigate the impact of cardiolipin on VSMCs, specifically addressing cellular dedifferentiation, migration, proliferation, and whether these modifications are mitochondria-mediated. The effect of CL on aortic contractility was also examined. Using adult mice aortas, we quantified a number of differentiation and dedifferentiation markers (α-smooth actin, calponin1, calpain I, and MMP14), following 48 hours of organ culture with physiological concentrations of CL (1μM and 10 μM) through immunoblotting. These CL concentrations were also used to investigate VSMC migration and proliferation over six days using cell culture, and to study blood vessel contraction following 48 hours of organ culture. Additionally, the acute effects of cardiolipin on VSMC mitochondria were examined through the use of high-resolution respirometry and selected substrate-inhibitor-uncoupler titration protocols. We found that calponin1 protein expression has significantly decreased by CL, while the other proteins remained unaffected. VSMC migration was ceased when treated with CL, while proliferation and contraction seemed unaffected. Mitochondrial respiration was significantly decreased for complex I and III. In conclusion, our results demonstrate that CL significantly reduces VSMC migration, calponin levels, and mitochondrial respiration, which could potentially act as an anti-atherosclerotic factor.

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# Introduction

# General Introduction

## *Cardiolipin*

Cardiolipin (CL) is a highly conserved (Mileykovskaya and Dowhan, 2009; Balasubramanian *et al*., 2015), negatively charged phospholipid molecule (Allegrini, 1984; Kates *et al*., 1993; Haines and Dencher, 2002; Lewis and Mcelhaney, 2009; Paradies *et al*., 2014). Unlike most phospholipids (Zhong *et al*., 2014), CL is composed of four fatty acyl chains, three glycerol units, and two phosphate molecules (Haines and Dencher, 2002; Houtkooper and Vaz, 2008; Zhong *et al*., 2014). CL resides in the inner mitochondrial membrane (Schlame and Haldar, 1993; Hatch, 1998), where it provides structural support to the inner membrane and protein complexes (Gonzalvez and Gottlieb, 2007; Claypool *et al*., 2008; Singh *et al*., 2010; Paradies *et al*., 2014; Szeto, 2014; Planas-Iglesias *et al*., 2015). CL also acts as a proton trap (Haines and Dencher, 2002), and promotes mitophagy (Chu *et al*., 2013; Balasubramanian *et al*., 2015) by serving as an external signal on damaged mitochondria (Smith *et al*., 2011). CL triggers cellular apoptosis (McMillin, and Dowhan, 2002) as CL electrostatically anchors cytochrome c, which, when released, initiates downstream apoptotic cascades (McMillin and Dowhan, 2002; Tuominen *et al*., 2002). In many prokaryotes, CL acts as a pathogen defense mechanism against hosts' immunity (Balasubramanian *et al*., 2015). Prokaryotic CL pairs with the extracellular lipopolysaccharides and acts to inhibit the production of macrophage cytokine (Balasubramanian *et al*., 2015). The involvement of CL in such functions indicates that its presence is highly regulated, and a disruption of CL content affects cells' fate (McMillin, and Dowhan, 2002; Tuominen *et al*., 2002). Alteration in CL content is observed in various pathologies, including myocardial infarction (Vreken *et al*., 2000; Schlame and Ren, 2006; Dorio, *et al*., 2007; Nakajima *et al*., 2008; Goldmann and Medina, 2013).

Myocardial infarction and subsequent ischemia trigger cardiomyocytes' perforation, necrosis, and apoptosis, all of which release various intracellular components, including CL-positive membrane fragments (De Windt *et al*., 1998; Paradies *et al*., 1999; Lesnefsky *et al*., 2001; Carnevale and Bergdahl, 2015). Released CL affects neighboring cells, and elevates anti-CL antibodies in the blood circulation (Beauregard *et al*., 1980; Hamsten *et al*., 1986; Rossen *et al*., 1994; Erta *et al*., 2013; Cernevale and Bergdahl, 2015). A recent study suggests that physiological concentrations of CL inhibit endothelial cell proliferation and microvessel

formation, contributing to anti-angiogenesis (Carnevale and Bergdahl, 2015). However, the effects of physiological concentrations of CL on vascular smooth muscle cells (VSMCs) were not yet been explored.

### *Vascular Smooth Muscle Cells*

VSMCs are highly specialized (Zhang *et al*., 2015), differentiated cells that reside in the middle layer of the vascular walls, called tunica media, as well as in other hollow organ walls (Chiong, *et al*., 2013). Under normal physiological conditions, VSMCs express contractile proteins (Miano, 2003; Owens *et al.* 2004; Zhang *et al*., 2015) to regulate vasculature function (Chiong *et al*., 2013). VSMCs contract through various contractile proteins, such as myosin and α-actin (Fitridge, 2011; Shi and Chen, 2015). These proteins give VSMC its elongated, spindle-like shape, and allows it to contract, contributing to pressure regulation (Hellstrand and Albinsson, 2005; Fitridge, 2011; Chiong *et al*., 2013; Chiong *et al*., 2014; Shi and Chen, 2015). Differentiated VSMCs also have low migration, proliferation, and extracellular matrix secretory rates (Rzucidlo *et al*., 2007; Cecchettini *et al*., 2011; Chistiakov *et al*., 2015; Zhang *et al*., 2015).

Unlike skeletal myocytes and cardiomyocytes, VSMC retain plasticity (Babaev *et al*., 1990; Bobryshev and Lord, 1996; Perry and Rudnick, 2000; Rzucidlo *et al*., 2007; Gomez and Owens, 2012; Zhang *et al*., 2015), meaning they dedifferentiate from the contractile state to a synthetic phenotype in response to environmental cues (Gomez and Owens, 2012; Shi and Chen, 2015; Zhang *et al*., 2015). During vascular disease and injury (Campbell and Campbell, 1985; Owens GK., 1995; Rzucidlo *et al*., 2007; Cecchettini *et al*., 2011), inflammatory signals stimulate VSMC dedifferentiation in response to wound healing (Wang *et al*., 2006). Dedifferentiated VSMCs have high rates of DNA and protein synthesis and possess high rates of proliferation, migration, and extracellular matrix secretion (Rzucidlo *et al*.. 2007; Cecchettini *et al*., 2011; Fitridge, 2011), but low contractility (Campbell and Campbell, 1985; Gomez and Owens, 2012). These morphological and functional changes of VSMC are a result of altered expression of essential proteins, such as α-smooth muscle actin, calponin, calpain, and MMP14 (Owens *et al*., 2004; Rzucidlo *et al*., 2007; Cecchettini *et al*., 2011).

### *VSMC Protein Markers*

Actin filaments and myosin motors, as well as their associated proteins, make up the filamentous network in VSMCs that allows the cells to perform their mechanical functions in the vascular walls (Fritzsche, *et al*., 2013). α-actin is a conserved protein in mammals and in birds, and is expressed in VSMC (Perrin and Ervasti, 2010). α-actin makes up the actin microfilament bundles in VSMC and is involved in VSMC contraction (Hinz *et al*., 2001; Cherng *et al*., 2008; Lehman and Morgan, 2012; Yuan, 2015). Thus, disruption or degradation of α-actin proteins can affect vasculature elasticity and integrity (Schildmeyer *et al*., 2000; Suh *et al*., 2011; Fritzsche, *et al*., 2013; Yuan, 2015).

Calponin binds to α-actin to regulate VSMC contraction (Sugenoya *et al*., 2002; Cecchettini *et al*., 2011; Gomez and Owens, 2012). Calponin exists in three isoforms, of which only one, known as basic calponin or calponin-1, is expressed in differentiated VSMC (Jiang *et al*., 1997; Sugenoya *et al*., 2002). Calponin induces actin polymerization and stability, while it inhibits actin depolymerization (Jiang *et al*., 1997; Sugenoya *et al*., 2002). Calponin also inhibits myosin head ATPase activity (Sugenoya *et al*., 2002), and suppresses VSMC proliferation and DNA synthesis when overexpressed (Jiang *et al*., 1997; Sugenoya *et al*., 2002).

Calpain is a conserved $Ca^{2+}$-dependant protease that participates in intracellular signaling pathways (Yan *et al*., 2016). It promotes VSMC proliferation, migration, and differentiation, and is expressed during wound healing (Nassar *et al*., 2012). Inhibiting calpain's activity reduces tissue damage in cardiovascular diseases (Nassar *et al*., 2012).

MMP14 is a membrane-anchored matrix metalloproteinase that degrades extracellular matrix formed by VSMCs (Lehti *et al*., 2008). Upregulation of MMP14 protein expression is required for VSMC dedifferentiation and migration, which promotes neointimal formation (Lehti *et al*., 2008; Yan *et al*., 2016).

Upregulation of MMP14 and downregulation of α-actin, calponin, and calpain is observed in synthetic VSMCs, which allows the cells to migrate to the site of inflammation, proliferate and secrete extracellular matrix (Campbell and Campbell, 1985). This behavior contributes to the development of atherosclerosis and cardiovascular diseases (Campbell and Campbell, 1985; Chiong, 2014).

### *Atherosclerosis*

Cardiovascular diseases are diseases related to the heart and blood vessels, which account for 33 deaths per minute, globally (American Heart Association, 2015). The major cause of cardiovascular diseases is atherosclerosis, which is the hardening and thickening of blood vessels (Noll, 1998; Guyton and Hall, 2006; Wick, 2012; Shi and Chen, 2015). During atherosclerosis development, factors such as cytokines trigger VSMC dedifferentiation and subsequent migration to the inflammatory area. Migrated VSMCs cannot return to their original location, which commits atherosclerosis to the irreversible stages of its development.

Various risk factors are associated with atherosclerosis development, such as hyperlipidemia, hypertension, hyperglycemia, infections, gender, oxidative stress, genetics, tobacco smoke, and allergies (Noll, 1998; Bergmann and Sypniewska, 2011). Vascular regions that experience non-uniform blood flow directionality, such as branch points and curvatures, are more prone to atherosclerosis development compared to unidirectional blood flow regions (Davies *et al*., 2010). This is because unidirectional blood flow induces a steady shear stress that evokes nitric oxide production, which in turn promotes vasodilation and anti-inflammation (Qiu *et al*., 2013). Unidirectional flow also downregulates pro-inflammatory and pro-VSMC dedifferentiation factors, such as interleukin-8 and vascular adhesion molecules (Goldman *et al.* 2007; Davies *et al.* 2013), while these factors are upregulated during non-uniform blood flow (Malek *et al.* 1999; Davies *et al*., 2013). All of these contribute to atherosclerosis formation, called atherogenesis, by evoking vascular endothelial damage, edema, or vasoconstriction.

### *Stages of Atherosclerosis Progression*

Atherosclerosis is a slow progressive disease that develops in six main stages (Wick, 2012). During the first stage in atherogenesis, endothelial cells become damaged as these cells are in direct contact with the blood, making them the most susceptible cells to blood changes associated with the atherosclerotic risk factors (Simionescu and Sima, 2011). When the endothelial lining, called tunica intima, become damaged, low-density lipoproteins increase transcytosis (Simionescu and Sima, 2011), allowing low-density lipoproteins to translocate through the epithelia and accumulate in the sub-endothelial region (Guyton and Hall, 2006; Wick, 2012), where they interact with matrix proteins and other factors, and become oxidized. These

modifications further increase endothelial cells' permeability and adhesiveness (Simionescu and Sima, 2011).

In the second stage, oxidized lipoproteins stimulate endothelin-1 generation, which triggers vasoconstriction and hypertension (Noll, 1998). Healthy endothelial cells generate anti-atherogenic factors, such as nitric oxide, to inhibit platelet adhesion (Noll, 1998). Oxidized lipoproteins disrupt nitric oxide production, and cause further endothelial dysfunctions, such as increased expression of leukocyte-adhesion molecules and chemotactic factors that recruit leukocytes (Noll, 1998; Guyton and Hall, 2006; Wick, 2012). Nitric oxide also promotes VSMC relaxation, while inhibiting VSMC migration and proliferation. This results in vasodilation and healthy vasculature (Noll, 1998). Thus, during atherogenesis, endothelial damage and nitric oxide disruption promote vasoconstriction, platelet adhesion, as well as VSMC proliferation and migration at later stages (Noll, 1998).

During the third stage, endothelial cytokines recruit platelets to the sub-endothelial injury site, where they attach to the endothelial binding molecules, become activated, and secret inflammatory cytokines that attract more platelets and leukocytes to the damaged area. Leukocytes, such as monocytes, are recruited, adhered, and translocated through the endothelia to the sub-endothelial injury site (Noll, 1998). Platelet-monocyte interactions form aggregates at the injury site, which triggers inflammatory responses (Noll, 1998; Guyton and Hall, 2006; Wick, 2012) in the intima, where monocytes mature into macrophages via the influence of oxidized lipoproteins and cytokines. Macrophages engulf the modified lipoproteins and become foam cells, which are considered a hallmark of fatty streak lesion formation (Noll, 1998; Wick, 2012).

During the fourth stage, foam cells release growth factors and cytokines that mediate VSMC switch from the contractile to the synthetic phenotype (Noll, 1998; Lacolley *et al*., 2012). These changes that occur in the sub-endothelial thicken the intimal layer and contribute to symptomatic coronary diseases.

Stage five marks the beginning of the irreversible stage of atherosclerosis development. At this stage, dedifferentiated VSMCs migrate from the tunica media to the inflammatory site in the tunica intima, where they proliferate (Lacolley *et al*., 2012). Synthetic VSMCs secret extracellular matrix that stabilizes foam cells and hardens the plaque (Noll, 1998; Lacolley *et al*., 2012). This causes the endothelial lining to become bulged into the vascular lumen, forcing

blood to pass through a narrower lumen, contributing to blood pressure elevation (Noll, 1998). Calcium ions are also withdrawn to the plaque's site, calcifying the bulge, and increasing its stiffness and susceptibility of the plaque to rupture (Noll, 1998; Guyton and Hall, 2006).

At stage six, macrophages and other leukocytes express-inflammatory cytokines and proteases that digest the matrix, which cannot be replenished because VSMC collagen-synthesis becomes downregulated (Simionescu and Sima, 2011). This thins the fibrous cap and weakens the plaque, compromising the vascular wall's ability to withstand high blood pressure (Simionescu and Sima, 2011). Blood flow against the plaque injures the endothelial cells and exposes the pro-inflammatory and pro-coagulant factors within the extracellular matrix (Simionescu and Sima, 2011). This, in turn, triggers platelets to further adhere to the injury site, where they become activated and form a thrombus. Depending on the thrombus site, myocardial infarction or stroke might occur, which could be lethal (Guyton and Hall, 2006; Wick, 2012).

It is well-established that ischemia and heart failure are associated with CL loss and mitochondrial dysfunction (Paradies *et al*., 1999; Lesnefsky *et al*., 2001; Chicco and Sparagna, 2006; Paradies *et al*., 2014). This led to shift research focus on mitochondria and its role in these pathologies.

### *Mitochondria*

A mitochondrion is the 'power plant' organelle in eukaryotic cells (Chiong *et al*., 2014), consisting of an outer membrane and an inner membrane, separated by an intermembrane space. The inner membrane has a large surface area as it is highly crumpled into folds known as cristae, which are involved in ATP production through oxidative phosphorylation (Okumura *et al*., 1991; Scheffler, 2001; Heerdt *et al*., 2002; Paradies *et al*., 2014).

Oxidative phosphorylation is the metabolic process that generates ATP in the presence of oxygen. Five complexes are involved in oxidative phosphorylation, complex I-V, which transfer electrons in a step-wise manner. NADH and $FADH_2$, which are generated through glycolysis, fatty acid oxidation, and citric acid cycle, are energy-rich molecules that donate electrons to complex I and II respectively, and simultaneously mediating proton transfer from the mitochondrial matrix to the intermembrane space through complex I, III, and IV. Proton

accumulation in the intermembrane space generates a proton motive force that drives ADP phosphorylation to ATP via complex V, also known as ATP synthase.

To generate ATP via oxidative phosphorylation, mitochondria first utilizes substrates derived from glucose and fatty acids via glycolysis and beta-oxidation in the cytosol. These processes ultimately produce Acetyl-coenzyme A, which is composed of two acetyl carbons that are bound to coenzyme A. Acetyl coenzyme A is an important molecule involved in cellular metabolism as it allows acetyl carbons to enter the citric acid cycle in the mitochondrial matrix (Paradies *et al*., 2014). The citric acid cycle produces coenzymes (NADH and $FADH_2$) that play key roles in generating large amounts of ATP in the electron transport chain complexes (Scheffler, 2001; Chiong *et al*., 2014).

### *Mitochondria and Cardiolipin*

Mitochondria are fundamental organelles in eukaryotic cells. Mitochondrial respiration generates reactive oxygen species (ROS), whereas mitochondrial dysfunction can overproduce ROS (Zhong *et al*., 2014). ROS signals for automated cell death, thus is associated with neurodegenerative diseases, aging, and other pathologies (Chu *et al*, 2013; Zhong *et al*., 2014). To avoid this, eukaryotic cells have established a process to degrade defected mitochondria in order to preserve cells' viability (Zhong *et al*., 2014). This process is known as mitophagy, which is not completely understood (Chu *et al*, 2013; Zhong *et al*., 2014).

It was shown in cortical neurons and in SH-SY5Y cell line that an essential provoker of mitophagy is the externalization of CL from the mitochondrial inner membrane to the mitochondrial outer membrane (Chu *et al*, 2013). Different cells have different thresholds of externalized CL to induce mitophagy, but they seem to agree in concept (Chu *et al*, 2013). CL externalization is stimulated as a response to several pro-mitophagy factors, such as rotenone (Chu *et al*, 2013).

Rotenone was found to increase the presence and the mitochondrial co-localization of the microtubule-associated-protein-1 light chain 3 (LC3), an autophagy protein. LC3 covalently binds CL and autophagosomes to mediate autophagy (Chu *et al.*, 2013). LC3 also binds directly to the externalized CL of the cargo, signaling mitophagy and mediating mitochondrial engulfment (Chu *et al.*, 2013). This indicates that rotenone is associated specifically with

increasing the mitochondria-targeted autophagy, mitophagy (Chu *et al.*, 2013). LC3 presence is directly associated with autophagosomes (Chu *et al.*, 2013). The cortical treatment with rotenone had increased the percentage of only CL, but not other phospholipids, in the mitochondrial outer membrane by ten-folds, relative to the basal CL amount of 0.8%. This shows that CL was externalized (Chu *et al*, 2013). In contrast to the well-studied lipids that become oxidized upon apoptosis, CL molecules are not peroxidized upon mitophagy, suggesting mitophagy occurs to prevent the accumulation of pro-apoptotic signals (Chu *et al*, 2013).

In contrast to mitophagy, ROS oxidizes polyunsaturated CL acyl chains during apoptosis (Paradies *et al*., 2014; Zhong *et al*., 2014). CL oxidation is related to mitochondrial dysfunction, apoptosis regulation, and several pathologies (Zhong *et al*., 2014). Here we discuss CL-triggered apoptosis, and CL's link to cytochrome c, a positively charged molecule (Kooijman *et al*., 2017). Cytochrome c is a mitochondrial protein involved in electron transfer from complex III to complex IV in the electron transport chain (Paradies *et al*., 2014; Ren *et al*., 2014). Cytochrome c plays an instrumental role in triggering apoptosis (Paradies *et al*., 2014) when it binds to CL (Chicco and Sparagna, 2006; Paradies *et al*., 2014).

In the outer leaflet of the mitochondrial inner membrane, CL binds to cytochrome c (Ren *et al*., 2014), which is a dormant peroxidase (Kooijman *et al*., 2017). Cytochrome c is anchored to the inner membrane through two CL binding sites, the A-site and the C-site (Paradies *et al*., 2014). The A-site binds to CL through electrostatic interactions (Chicco and Sparagna, 2006; Kapralov *et al*., 2007; Muenzner *et al*., 2013; Paradies *et al*., 2014), while the C-site binds to CL through hydrogen and hydrophobic interactions (Paradies *et al*., 2014; Zhong *et al*., 2014). The latter induces partial unfolding of cytochrome Met-80 residue from its associated heme-iron (Paradies *et al*., 2014).

Peroxidized CL introduces conformational changes in cytochrome c and destabilizes the tertiary structure of its heme group (Paradies *et al*., 2014; Ren *et al*., 2014; Kooijman *et al*., 2017). This CL-cytochrome c complex pro-apoptotic peroxidase complex becomes activated, which further oxidizes CL (Kagan *et al*., 2005; Paradies *et al*., 2014; Ren *et al*., 2014; Kooijman *et al*., 2017). Oxidized CL, in turn, enhances the oxidation ability of the peroxidase (Paradies *et al*., 2014; Ren *et al*., 2014), which further oxidizes polyunsaturated in a continuous positive-feedback loop (Tyurina *et al*., 2006; Ren *et al*., 2014). The unfolded cytochrome c escapes to the cytosol, where

it activates cellular pathways that orchestrate apoptosis (Kagan *et al*., 2005; Chicco and Sparagna, 2006; Paradies *et al*., 2014; Zhong *et al*., 2014). The release of cytochrome c to the cytosol has been linked to permeabilizing the mitochondrial membrane, which facilitates the release of the apoptotic factors from the mitochondria (Belikova *et al*., 2007; Paradies *et al*., 2009; Paradies *et al*., 2014), but this remains unclear (Kagan *et al*., 2005; Raemy and Martinou, 2014).

Several strategies have been studied to inhibit CL oxidation, which relates to atherosclerosis and other diseases (Zhong *et al*., 2014). Inhibiting the cytochrome c-CL peroxide complex inhibited apoptosis, and overexpressing lipid-reducing factor have lowered liver apoptosis, cytochrome c release, and CL oxidation (Zhong *et al*., 2014).

It has been shown that under the sub-lethal threshold, ROS levels are enough to trigger CL translocation, but not CL peroxidation – a characteristic observed in mitophagy (Chu *et al*., 2013; Ren *et al*., 2014). Thus, unoxidized CL is translocated from the inner to the outer leaflet of the mitochondrial inner membrane, rather than oxidized CL (Chu *et al*., 2013; Ren *et al*., 2014). Through this, mitophagy is initiated, but not apoptosis in sub-lethal mitochondrial injuries, preserving the cell's viability (Chu *et al*., 2013; Ren *et al*., 2014).

CL is also involved in structural support of the mitochondrial components. CL is negatively charged and have a long hydrophobic tail, allowing it to form strong ionic and hydrophobic interactions with neighboring molecules (Lewis and Mcelhaney, 2009; Paradies *et al*., 2014; Ren *et al*., 2014). CL tightly binds to the CL-affinity sites on the mitochondrial respiratory complexes I-V, via hydrophobic and ionic bonds (Eble *et al*., 1989; Lesnefsky *et al*., 2001; Claypool *et al*., 2008; Singh *et al*., 2010; Paradies *et al*., 2014).

Complex I, also known as NADH-ubiquinone oxidoreductase, is involved in electron transport from NADH to ubiquinone (Paradies *et al*., 2014), and is associated with CL (Chicco and Sparagna, 2006; Paradies *et al*., 2014). Complex I activity was lost upon CL loss in bovine heart and was only restored upon exogenous addition of CL (Paradies *et al*., 2014). 22% of CL loss have resulted in diminishing 15% of complex I activity in rat brains (Chicco and Sparagna, 2006). CL oxidation by ROS has also been shown to inactivate complex I (Paradies *et al*., 2014). This effect was encountered upon the addition of exogenous CL (Paradies *et al*., 2014).

Complex II, also known as succinate dehydrogenase, oxidizes succinate from the Krebs cycle and provides electrons to the electron transport chain (Paradies *et al*., 2014). The presence of CL in the phospholipid environment of complex II is required for the stability and enzymatic activity for the complex (Paradies *et al*., 2014). CL presence around complex II seems to restrict ROS production, even though this remains unclear (Paradies *et al*., 2014).

CL stabilizes the quaternary structure of complex III, also known as ubiquinol cytochrome c oxidoreductase (Haines and Dencher, 2002; Chicco and Sparagna, 2006; Paradies *et al*., 2014). Multiple CL molecules bind to complex III to stabilize and maintain its enzymatic activity (Haines and Dencher, 2002; Paradies *et al*., 2014). In the absence of CL, the complex was destabilized and its enzymatic activity was significantly diminished (Haines and Dencher, 2002; Chicco and Sparagna, 2006; Paradies *et al*., 2014). CL oxidation by ROS have also deactivated complex III (Paradies *et al*., 2014). Complex III full activity and stability were rescued only upon the administration of CL, signifying the importance of CL and its association to complex III (Paradies *et al*., 2014).

Complex IV, also known as cytochrome c oxidase, tightly binds to three CL molecules through CL-specific binding sites (Paradies *et al*., 2014). The complex's binding to unsaturated CL molecules promotes for its optimal enzymatic activity (Lesnefsky *et al*., 2001; Haines and Dencher, 2002; Chicco and Sparagna, 2006; Paradies *et al*., 2014). Absence of CL has been shown to destabilize complex IV and reduced its activity (Paradies *et al*., 2014). CL peroxidation by ROS also inactivated complex IV (Paradies *et al*., 2014). Its activity was rescued upon the addition of exogenous CL (Paradies *et al*., 2014).

Complex V, also referred to as $F_0F_1$ ATP-synthase, uses proton gradient-derived energy to phosphorylate ADP to ATP (Paradies *et al*., 2014). In complex V, at least four sites were found to bind exclusively to CL with high affinity (Eble *et al*., 1989; Chicco and Sparagna, 2006; Paradies *et al*., 2014). Even though the exact contribution of CL to complex V is not yet well studied, but it is evident that CL supports the enzymatic activity of complex V (Haines and Dencher, 2002). The removal of phospholipids surrounding complex V have significantly diminished its activity, and the restoration of complex V activity was more effective upon the addition of CL compared to other phospholipids (Paradies *et al*., 2014).

It has also been shown that CL is associated with numerous other proteins and that it is required for their optimal enzymatic activities (Eble *et al*., 1989; Paradies *et al*., 2014). These proteins include: mitochondrial glycerol-3-phosphate dehydrogenase, the phosphate transporter, ADP/ATP carrier protein (Beyer and Klingenberg, 1985; Eble *et al*., 1989; Haines and Dencher, 2002; Chicco and Sparagna, 2006; Claypool *et al*., 2008; Paradies *et al*., 2014; Ren *et al*., 2014), mitochondrial creatine kinase, carbamoyl phosphate synthetase I, carnitine/acylcarnitine carrier, nucleoside diphosphate kinase, phosphate transporter, pyruvate transporter, tricarboxylate carrier (Chicco and Sparagna, 2006; Paradies *et al*., 2014). The binding sites and the affinity of CL to these proteins have not yet been determined (Eble *et al*., 1989; Chicco and Sparagna, 2006; Paradies *et al*., 2014).

In our lab, CL was found to inhibit endothelial cell proliferation and migration (Carnevale and Bergdahl, 2015). Endothelial cells are involved in angiogenesis, a physiological process that forms new capillaries from pre-existing blood vessels. Angiogenesis is important during ischemic conditions to promote healing (Carnevale and Bergdahl, 2015). This process was significantly suppressed by the addition of physiological CL concentrations in a dose-dependent manner (Carnevale and Bergdahl, 2015). This indicates that CL has anti-proliferative properties on vascular endothelial cells, and that CL disrupts endothelial cells migration and degradation of the basement membrane (Carnevale and Bergdahl, 2015).

# Project Overview

Excluding work done in our lab, all previous studies that involved CL were based on eliminating CL. Alternatively, we decided to explore the effects of adding physiological concentrations of CL. Specifically, to study the effects of CL on VSMCs, as these cells determine the progression of atherosclerosis into the irreversible stages, and because the link between CL and VSMC has not been explored before.

Thus, we studied the effects of physiological concentrations of CL on VSMC phenotypic protein markers, as well as its effect on VSMC bioenergetics and aortic contraction. We hypothesized that CL would inhibit VSMC proliferation and migration as it did in endothelial cells. Consequently, we expected CL to downregulate VSMC dedifferentiation markers and upregulate

VSMC differentiation markers. Due to the involvement of mitochondria in various cellular functions, we expected that acute addition of CL would affect mitochondrial bioenergetics.

# Techniques

*In situ* and *ex-vivo* studies were performed on adult male mice aortic tissues to study the effects of physiological concentrations of CL. The effect of CL on VSMC phenotypic switch was studied by quantifying various differentiation and dedifferentiation markers using immunoblotting. We also studied the effect of CL on VSMC migration and proliferation rates using migration and proliferation assays. Given that CL resides in the mitochondria, we suspected that CL addition affects mitochondrial activity. Thus, we studied VSMC mitochondrial respiration using the high-resolution respirometry technique. To test whether CL has an effect on the overall function of the aortic tissue, isometric force measurement was used to examine aortic contractility. CL used in these experiments was derived from bovine heart as CL is highly conserved across species (Chicco and Sparagna, 2006).

*Immunoblotting*

Immunoblotting, also referred to as Western blotting, is a technique used to quantify proteins using antibodies. We used immunoblotting to determine the amount of differentiation and dedifferentiation markers present in VSMCs following organ culture incubation for 48 hours at 37$^{o}$C in 0µM, 1µM, and 10µM CL.

To extract the proteins of interest from VSMCs in the aortas, we used lysis buffer and physical force to lyse the vessels and release their protein content. This lysis mixture was then centrifuged and the pellets were discarded, while the supernatants were preserved for the study.

To prevent the protein tertiary structure from interfering with the protein migration distance during the electrophoresis separation stage, we pipetted DTT into the samples to break the protein disulfide bridges.

The samples were loaded into the electrophoresis gel wells along with a standard protein ladder, which allows us to identify the approximate distance travelled by the proteins. The gel was supplied with electric current to separate the proteins according to their size. Proceeding protein migration, the bands were transferred from the gel into a cellulose membrane, and the membrane

was stained with Ponceau dye to visualize the protein bands. The membrane was then immersed in blocking buffer to decrease the background interference. To achieve the first stage of labeling and quantifying the proteins of interest, the membrane was incubated in a buffer solution containing primary antibodies that are specific to these proteins' epitopes. Excess primary antibodies were washed off, and the membrane was incubated with secondary antibodies. The secondary antibodies are specific to the epitopes of the primary antibodies. Multiple secondary antibodies can bind one primary antibody, which amplifies the antibody tag per protein. Because antibodies are invisible, the membrane was immersed in ECL solution, which is a fluorescence solution that tags secondary antibodies. Signals emitted from the ECL-containing bands were detected by a light-sensitive, silver-based film, which was then developed and scanned to quantify the bands.

### *Tissue Preparation for Migration and Proliferation*

Under a light microscope, fat streaks surrounding the aortas were detached under a dissection microscope, then the aortas were bilaterally dissected, and the endothelial cells were gently scraped off, while the VSMC-containing layer was plated.

### *Migration Assay*

Migration assay is a technique used to assist cell migration rate. VSMCs present along the diameter of the confluent plates were scraped off and supplied with serum-free media (Jones *et al*., 1993) containing 0 μM, 1 μM, and 10 μM CL. The serum-free media restricts VSMC proliferation, eliminating this parameter from interfering with the migration results. The scrapes were observed under phase-contrast microscopy and the width of the scrapes were blindly measured.

### *Proliferation Assay*

Proliferation assay is a technique used to measure the amount of viable cells over time. Plated cells were supplied with media containing 0 μM, 1 μM, and 10 μM CL. The cells were detached from the plates, and the cells were counted using a hemocytometer under a phase-contrast microscopy.

*High-Resolution Respirometry*

High-resolution respirometry is often used to study mitochondrial respiration in isolated, highly-oxygenated chambers. This technique requires permeabilized samples (Pesta and Gnaiger, 2012) to measure the overall mitochondrial respiration performance by calculating samples' respiration rate, which is the rate at which mitochondria consume oxygen and convert ADP to ATP, through measuring oxygen consumption level, i.e. the drop in oxygen level in the isolated chamber (Kuznetsov, 2008). The tissues were treated with 0 μM, 1 μM, and 10 μM CL acutely, and the performance of individual phosphorylation complexes can be assessed by using a stepwise multiple substrate-uncoupler-inhibitor titration protocols (Pesta and Gnaiger, 2012). This method provides a deeper insight into the samples' metabolism and physiology, narrowing down to the complexes that might be involved in a mitochondrial defect in case of a dyscoupling when compared to control specimens (Pesta and Gnaiger, 2012).

*Isometric force measurement*

To assist the functional implications of CL on aortic contraction, we used the isometric force measurement technique. The aortas incubated in 0 μM, 1 μM, and 10 μM CL with were mounted on a myograph, where two wires pass through the vessel. One wire connects to a micrometer screw to restrict the wire movement. The second wire is free to move and is connected to a force transducer to measure the force that the vessel generates upon its contraction. High potassium solution was added to the vessel chambers to induce their maximum contraction.

# II

# The Effect of Cardiolipin on Vascular Smooth Muscle Cell Dedifferentiation, Migration, and Proliferation

Deema Galambo and Andreas Bergdahl

# Abstract

The initial phases of atherosclerosis involve the transition of normal vascular smooth muscle cells (VSMCs) to a synthetic, dedifferentiated phenotype, which can migrate and proliferate. This potentially obstructs blood flow, which, when destined to the cardiomyocytes, can lead to myocardial infarction. Studies have demonstrated that cardiomyocytes, undergoing apoptosis, release a phospholipid molecule called cardiolipin (CL) into the systemic circulation. The objective of this project was to investigate the impact of cardiolipin on VSMCs, specifically addressing cellular dedifferentiation, migration, proliferation, as well as aortic contractility. Using adult mice, we quantified a number of differentiation and dedifferentiation markers (α-smooth actin, calponin, calpain, and MMP14), following 48 hours of organ culture with physiological concentrations of CL (1μM and 10 μM). These CL concentrations were also used to investigate the migration and proliferation of VSMCs over six days using cell culture, and blood vessel contraction following 48 hours of organ culture. We found that calponin protein expression has significantly decreased by CL addition, while the other proteins remained unaffected. VSMC migration was ceased when treated with CL, while proliferation and contraction were non-significantly affected. In conclusion, our results demonstrate that CL significantly reduces VSMC calponin-content and migration, which potentially act as an anti-atherosclerotic factor.

# Introduction

Cardiolipin (CL) is a conserved (Mileykovskaya and Dowhan, 2009; Balasubramanian *et al*., 2015) phospholipid molecule with an approximate half-life of 2 days in rodent cardiomyoblast (Zachman *et al*., 2010; Xu and Schlame, 2014). CL resides in the inner mitochondrial membrane (Schlame and Haldar, 1993; Hatch, 1998), where it provides structural support to the membrane (Szeto, 2014) and its protein complexes (Gonzalvez and Gottlieb, 2007; Claypool *et al*., 2008; Singh *et al*., 2010; Paradies, 2014). CL electrostatically anchors cytochrome c, which, when released, initiates downstream apoptotic cascades (McMillin and Dowhan, 2002; Tuominen *et al*., 2002). The involvement of CL in such functions indicates that its presence is highly regulated, and a disruption of CL content affects cell fate (McMillin and Dowhan, 2002; Tuominen *et al*., 2002).

Myocardial infarction and subsequent ischemia trigger perforation, necrosis, and apoptosis of cardiomyocytes, consequently releasing various intracellular components, including CL-positive membrane fragments (Chicco and Sparagna, 2006; Carnevale and Bergdahl, 2015). Released CL affects neighboring cells and elevates anti-CL antibodies in the blood circulation (Beauregard *et al*., 1980; Hamsten *et al*., 1986). A recent study suggests that physiological concentrations of CL inhibits endothelial cell proliferation and microvessel formation, contributing to anti-angiogenesis (Carnevale and Bergdahl, 2015). However, the effects of physiological concentrations of CL on vascular smooth muscle cells (VSMCs) were not yet been explored.

VSMCs are highly specialized, differentiated cells that reside in the middle layer of the tunica media (Hellstrand and Albinsson, 2005; Zhang *et al*., 2015). Under normal, physiological conditions, VSMCs express contractile proteins, which regulate vasculature function (Miano, 2003; Owens *et al*., 2004; Chiong *et al*., 2013; Chistiakov *et al*., 2015; Zhang *et al*., 2015). VSMC contractibility is required for vascular elasticity and contributes to blood pressure maintenance. VSMC contraction involves many pathways and that depends on actomyosin force, as well as on $Ca^{2+}$ influx, which upregulates some contractile proteins through binding to their transcription factors (Brozovich *et al*., 2016).
In addition, VSMCs retain plasticity (Babaev *et al*., 1990; Bobryshev and Lord, 1996), meaning they switch from contractile to synthetic phenotype in response to environmental cues (Gomez and Owens, 2012; Shi and Chen, 2015; Zhang *et al*., 2015) during vascular disease and injury

(Campbell and Campbell, 1985; Rzucidlo *et al*., 2007; Cecchettini *et al*., 2011). Dedifferentiated VSMCs have high rates of DNA and protein synthesis (Fitrifge, 2011), and possess high rates of proliferation, migration, and extracellular matrix secretion (Rzucidlo *et al*., 2007; Lehti *et al*., 2008; Cecchettini *et al*., 2011; Lacolley *et al*., 2012; Brozovich *et al*., 2016), but low contractility (Campbell and Campbell, 1985; Gomez and Owens, 2014). Proteins, such as MMP14, calpain, calponin, and α-smooth muscle actin, participate in these morphological and cellular functions (Owens *et al*., 2004; Rzucidlo *et al*., 2007; Lehti *et al*., 2008; Cecchettini *et al*., 2011).

Dedifferentiated VSMC also proliferates during vascular wall remodeling, as well as during atherosclerosis development. Proliferation proceeds through the cell cycle, and is finalized by cytokinesis through actomyosin force.

Furthermore, cell migration is needed for VSMCs during inflammatory responses and vascular inflammation (Bunnell *et al*., 2011; Artman *et al*., 2014). VSMC migration occurs as a result of cytoskeletal movement through actin microfilament remodeling and its association with myosin II, which plays an early role in VSMC migration by aligning the cytoskeletal actin bundles, maintaining their length, and establishing cell polarity (Artman *et al*., 2014; Brozovich *et al*., 2016).

Because CL was found to inhibit endothelial cell migration and proliferation, we hypothesize that VSMC migration and proliferation can also be inhibited by CL addition. Given the involvement of actins in VSMC contraction, migration, and proliferation, we hypothesize that CL could disrupt calponin1 or actin expression, which would lead to a restriction in the contraction, migration, and proliferation ability of VSMCs. Thus, we quantified VSMC protein markers and tested for VSMC functions using various techniques.

## Methods

### *Animal Care*

Male C57B1/6 mice, 7-8 months old were obtained from the breeding colony at Concordia University, and randomly assigned to either control, 1 μM CL, or 10 μM CL groups. The mice were kept on a 12:12 h photoperiod at a thermos-neutral environment (22$^{o}$C), and had free access to standard dry rodent chew and water. All procedures were approved by the Animal Ethics

Committee of Concordia University (Ethics: #30000259) and were conducted according to the Canadian Council on Animal Care guidelines.

*Tissue Extraction*

The mice were euthanized in $CO_2$ according to the animal ethics protocol. The abdominal cavity and the thorax were exposed, and the aortas were extracted by cutting the connective tissues along the spine. The aortas were kept on ice in tubes containing physiological salt solution (nominally $Ca^{2+}$-free Krebs sterile solution containing (135.5 mM NaCl; 5.9 mM KCl; 1.2 mM $MgCl_2$; 11.6 mM glucose; 11.6 mM HEPES; pH 7.35, penicillin 100 U/ml and streptomycin 100 Ig/ml). Fat streaks and connective tissues surrounding the vessels were detached under a dissection microscope.

*Immunoblotting Tissue Preparation*

The dissected, cleaned samples were placed in DMEM/F-12 media plates containing 0 µM, 1 µM, and 10 µM CL, and incubated at 37ºC under 5% $CO_2$ for 48 hours (corresponding to CL's half-life) (Xu and Schlame, 2014). Post-incubation, the aortas were bilaterally dissected and the endothelial cell layer was gently scraped off with a sponge, leaving behind only the VSMC-containing layer.

*Immunoblotting*

To extract cell lysate, the aortic tissues were grinded in 120 µl lysis buffer (250 mM NaCl; 50 mM HEPES; 10% glycerol; 1% trion X-100; 1.5 mM $MgCl_2$; 1 mM EGTA; 10 mM $Na_4P_2O_7$; 1 mM NaF; 800 µM $Na_3VO_4$). The lysis mixture was centrifuged at 13000 rpm for 10 minutes, and the supernatants were preserved for the study. 20 µl of the lysate was mixed with 2 µl DTT (1M 1,4-dithio-DL-threitol, D0632-5G, Sigma-Aldrich; 10mM sodium acetate; pH 5.2) and 4 µl of sample buffer (347 mM SDS; 0.25 M Tris, pH 6.8; 1.49 mM bromophenol blue, 501 mM DTT; 6.84 M glycerol). The mixture was incubated at 37ºC for 20 minutes, then separated on 10% SDS-PAGE along with a standard ladder in running buffer (2.27 M Tris base; 17.5 M glycine; 315 mM SDS; pH 8.3), and subsequently transferred to a nitrocellulose membrane (0.45 µm, Bio-Rad, Germany) in sodium tetraborate decahydrate solution (10 µM, Sigma-Aldrich, St. Louis, MO) for 40 minutes on 120 mV. The protein bands in the membrane were visualized using Ponceau dye (1.49 mM Ponceau; 0.87 mM acetic acid) and then scanned to quantify the

proteins in each lane using Image J software. The membranes were blocked with 5% skimmed milk in 0.1% TBS-T (0.1% of 0.1 M Tris-base and 1.5 M NaCl; 1 ml Tween-20, 0777-1L, VWR International, Solon, OH) for 1 hour, then the membranes were incubated with primary antibodies diluted in 0.1% TBS-T at 4$^{o}$C overnight. Primary antibodies used: anti-α-actin (1:2000, Ab7817 Abcam); anti-calponin (1:20,000, ab46794 Abcam); anti-calpain (1:2000, ab28258 Abcam); anti-MMP14 (1:2000, ab3644 Abcam). The membranes were then washed in 0.1% TBS-T for 3 x 10 minutes, then incubated in secondary antibodies for 1 hour at room temperature. Secondary antibodies used: ab6721 Abcam rabbit polyclonal for MMP14, calponin, calpain; and ab6728 Abcam mouse monoclonal for α-actin. The membranes were re-washed in 0.1% TBS-T for 3 x 10 minutes. The membranes were covered with ECL fluorescence solution (Amersham ECL Western Blotting Detection Reagents; RPN2106; GE Healthcare, UK) for 6 minutes, and the films were developed and the bands were analyzed using Image J software. Each protein was tested for n = 9 - 12.

*Tissue and Culture Preparation for Migration and Proliferation*

The dissected, cleaned samples were bilaterally cut-open, the endothelial cells were gently scraped off with a sterilized metal surface. The sample tissues were further cut into small pieces (approximately 0.5 $cm^2$), mixed, and randomly placed (under sterile conditions in a biological safety cabinet) on multiple media-containing Petri dishes: (DMEM/F-12, 1:1, with serum and antibiotics). The plates were then kept in an incubator at 37$^{o}$C under 5% $CO_2$ in the air and the cells were allowed to grow to confluency (approximately 1 week). Next, the plates were trypsinized (0.05% Trypsin, 0.53 mM EDTA; 25-052-Cl; Corning, Manassas, VA, USA) in a biological safety cabinet. The media in the plates was pipetted out, and its traces were washed away with PBS solution (1.36 M NaCl, 26.8 mM KCl, 101.4 mM $Na_2HPO_4$, 17.6 mM $KH_2PO_4$, pH 7.4). Approximately 4 ml of trypsin-EDTA was added to the plates and incubated at 37$^{o}$C for about 8 minutes. Trypsin was neutralized with DMEM/F-12 media (approximately 4 ml) and the cell mixture was centrifuged at 3500 rcf for 6 minutes. The supernatant was discarded and the pellet was re-suspended in DMEM/F-12 media (around 10 ml).

*Migration Assay*

Around 0.5 ml of the cell suspension was added to P15.6 plates, which were supplemented with 1 ml DMEM/F-12 media and the cells were allowed to reach confluency at 37ºC under 5% $CO_2$ in air for a week. Cells that align the diameter of the plates were scraped off using a sterile P100 micropipette tip, making a horizontal strip on the plates' bottom. Then the media was exchanged for 0 μM, 1 μM, and 10 μM CL in serum-free media (six plates per condition). The serum-free media keeps the cells differentiated, as well as it restricts the cells from proliferating, limiting and observed changes to migration but not proliferation. The width of the scrapes were observed under phase-contrast microscopy and (approximately 10) images were captured per plate every 48 hours for 6 days, and the width of the scrapes, or the "wounds," were blindly measured (Approximately 3 measurements per image).

*Proliferation Assay*

120 μl of the cell suspicion solution was added onto P60 plates, which were further supplemented with 1.5 ml DMEM/F-12 media. The cells were incubated at 37ºC under 5% $CO_2$ in air for 1-2 days to attach to the plates and grow. The media was then exchanged for DMEM/F-12 media containing 0 μM, 1 μM, and 10 μM CL. The plates were trypsinized (in the same manner as in the migration assay) every 48 hours for six days, and the cells were counted using a hemocytometer under a phase-contrast microscopy. This was done for n = 3.

*Isometric force measurement*

Aortas cleaned from fat streaks were mounted on a wire myograph in high sodium solution, which consists of (in mM): NaCl 135.5, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11.6, HEPES 11.6, pH 7.35. The wires were set to 700 mg basal tension, which is considered within the optimal resting tension range to obtain the maximal contractile tension when the muscle is induced to contract by high potassium solution (Jiang *et al*, 1999). The high sodium solution was exchanged to high potassium solution, which consists of (in mM): KCl 141.4, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11.5, HEPES 11.6, pH 7.35. The muscle contraction was recorded for 7 minutes, and then the high potassium solution was exchanged back to high sodium solution until the readings stabilized. This was repeated and a second high potassium reading was measured. Upon the second reading stabilization, the high sodium solution was exchanged with 10 nm

endotheline-1 in high sodium solution and the muscle contraction was recorded for 7 minutes. The results were analyzed using the LabChart 7 software. This experiment was repeated for n = 11 – 13.

*Statistics*

Data in figures are presented as means ± SE, while the aortic contraction measurement is the only one presented as ±SD. All statistical evaluations were considered significant if $P < 0.05$. One way *ANOVA* test was done to recognize significant data.

## Results

To investigate whether physiological concentrations of CL affect VSMC dedifferentiation or its synthetic features, we quantified VSMC differentiation and dedifferentiation markers. Additionally, we studied VSMC migration and proliferation using migration and proliferation assays. The effect of physiological concentrations of CL on the aortic contractile functional properties was studied using wire myography.

As seen in Figure 1-4, there were no significant differences ($p > 0.05$) in the quantity of calpain, MMP14, or α-actin proteins upon the addition of either 1 µM or 10 µM of CL compared to the control samples. The *ANOVA* test revealed that the expression of calponin (Figure 4) was significantly decreased ($p < 0.005$) upon the administration of 1 µM or 10 µM of CL when compared to control.

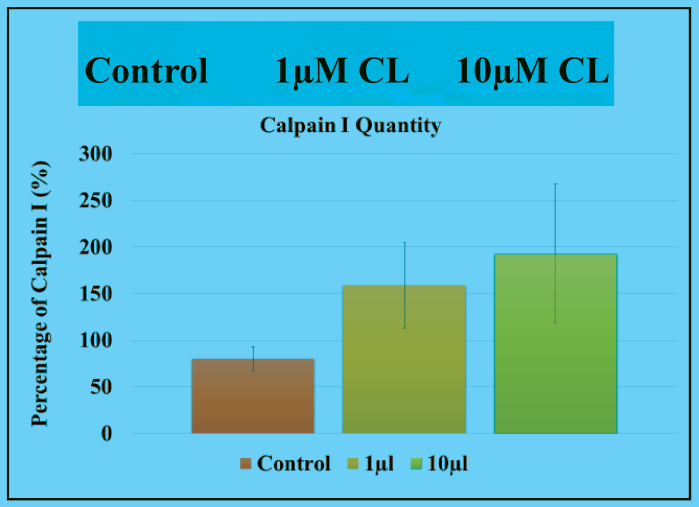


**Figure 1. Calpain level.** (Top) Visual illustration of calpain immunoblotting results. There is a non-significant difference in Calpain I quantity in CL-incubated samples vs. control (Bottom) Calpain I levels non-significantly increased ($p=0.314$) as CL concentration increases from 0 to 10 µM CL. This chart depicts the mean values of n=11 for control and 1µM CL samples, and n=12 for 10 µM CL samples. Standard Error Mean measurement was used to calculate the error bars.

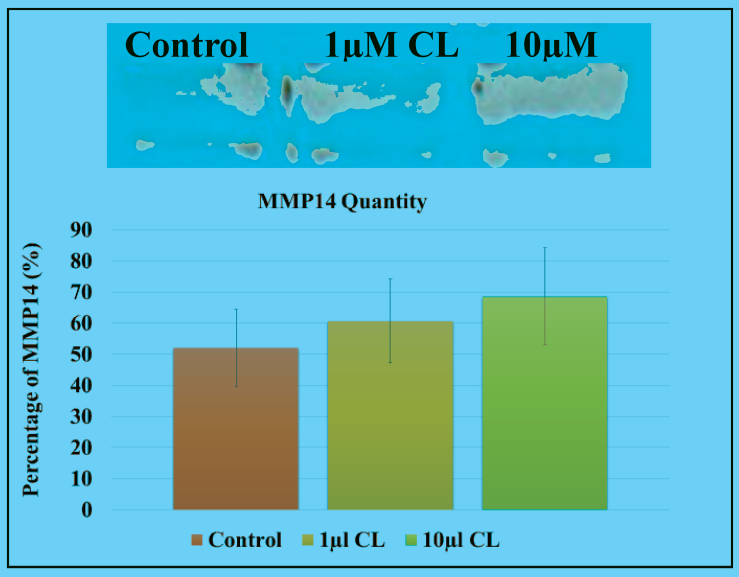


**Figure 2. MMP14 level.** (Top) Visual illustration of MMP14 immunoblotting results. The upper band is the dephosphorylated, active MMP14 protein band, while the lower band is the phosphorylated, inactive MMP14 protein band. There is no significant difference in the MMP14 protein band intensity in the presence and absence of CL. (Bottom) MMP14 levels non-significantly show an increasing trend (p=0.702) as CL concentration increases from 0 to 10 µM. This chart depicts the average values of n=11 for control, 1µM CL, and 10 µM CL samples. Standard Error Mean measurement was used to calculate the error bars.

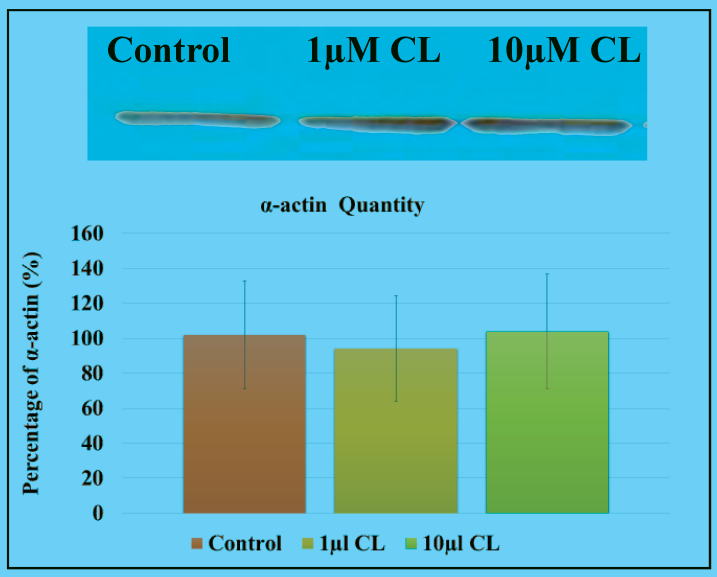


**Figure 3. α-actin level.** (Top) Visual illustration of α-actin immunoblotting results. There is no significant difference in the α-actin protein band intensity in the presence and absence of CL. (Bottom) α-actin levels change non-significantly (p=946) as CL concentration increases from 0 to 10 µM. This chart depicts the average values of n=11 for control, 1µM CL, and 10 µM CL samples. Standard Error Mean measurement was used to calculate the error bars.

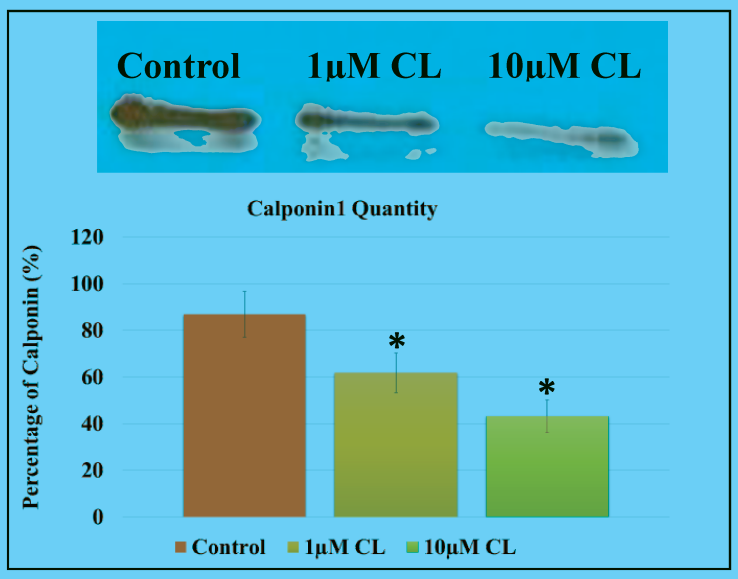


**Figure 4. Calponin level.** (Top) Visual illustration of Calponin1 immunoblotting results. There is an obvious decrease in the Calponin1 protein band intensity in the presence and absence of CL. (Bottom) Calponin1 levels significantly decreases (p=0.004) as CL concentration increases from 0 to 10 µM. This chart depicts the average values of n=9 for control, 1µM CL, and 10 µM CL samples. Standard Error Mean measurement was used to calculate the error bars.

Figure 5 shows a significant decrease in the wound gap size in the control samples compared to the 1 or 10 µM CL samples with an overall drop in the wound size. The ANOVA test revealed a significant difference on day 2, 4, and 6, with p values 0.006, 0.038, and 0.010 respectively. The turkey post-hoc test revealed a significant difference between the CL-treated groups and the control, but not between the 1 and 10 µM CL-treated samples.

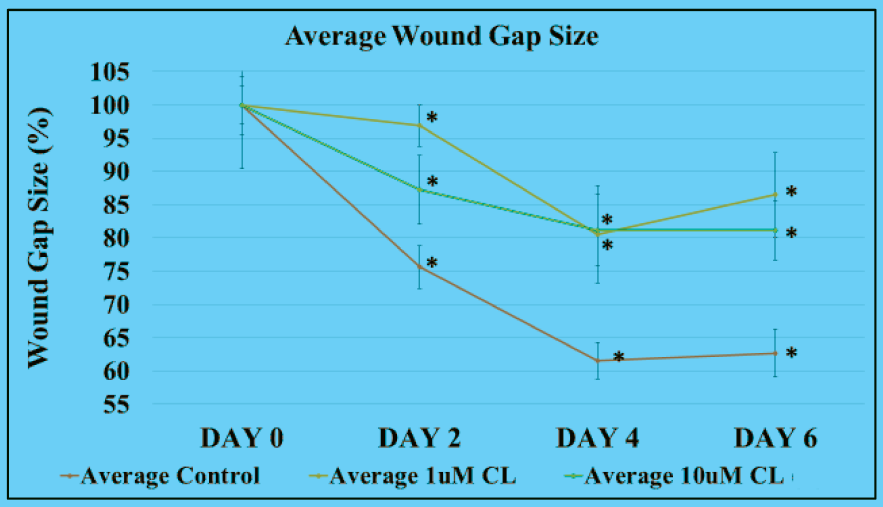


**Figure 5. Migration Assay (Wound Gap Size).** VSMC migration rate as CL concentration increases from 0 to 10 µM. There is a significant decrease in the gap size in control vs. CL-treated plates (day 2 p-value = 0.006; day 4 p-value = 0.038; day 6 p-value = 0.01). Six plates were prepared per condition, and approximately 10 images were captured per plate, and each image was analyzed at 3 distinct regions. Therefore, n-value is approximately = 180. Standard Error Mean measurement was used to calculate the error bars.

Table 1 depicts images of scraped VSMC plates incubated in control, 1 µM and 10 µM CL treated media. The snapshots were taken every 48 hours for six days. The images show that the induced gap has decreased more in the control plates compared to the CL-treated plates.

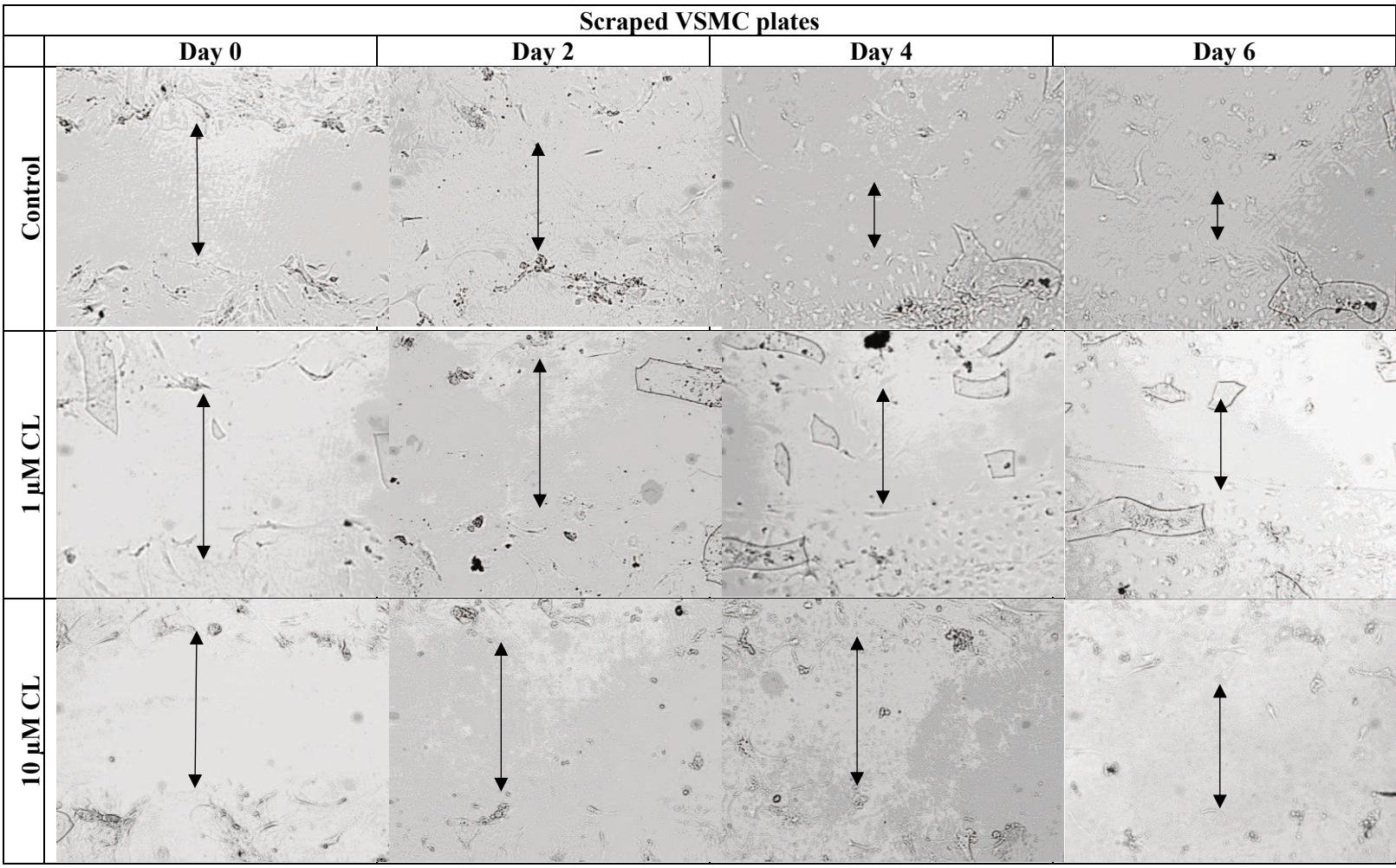


**Table 1. Scraped VSMC culture plates.** Images of scraped VSMC culture plates taken every 48 hours for six days in control, 1 µM and 10 µM CL treated media. The images show that the induced gap has decreased significantly in the control plates compared to the CL-treated plates. Six plates were prepared per condition, and approximately 10 images were captured per plate, and each image was analyzed at 3 distinct regions. Therefore, n-value is approximately = 180. Images captures under total magnification of x100.

Figure 6 depicts an overall increase in cell proliferation for the control, 1 µM CL, and 10 µM CL groups. However, there was no significant difference between the three groups as revealed by the ANOVA test ($p > 0.05$).

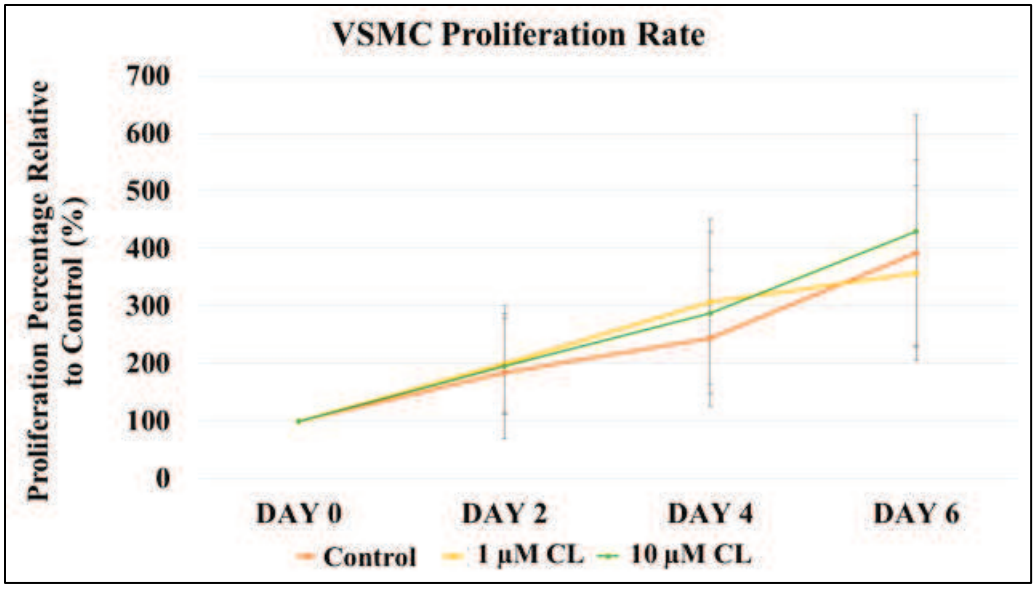


**Figure 6. Proliferation Assay.** VSMC Proliferation rate as CL concentration increases from 0 to 10 µM. There was no significant difference in proliferation in the presence or absence of CL. This graph represents n=3. Standard Error Mean measurement was used to calculate the error bars.

Figure 7 depicts no significant difference in the maximal high-potassium contraction force between any of the three groups, with ANOVA p-value > 0.05. Our results (data not shown) also depicts no significant change in the endotheline-1-induced contraction in any of the three groups (ANOVA p > 0.05).

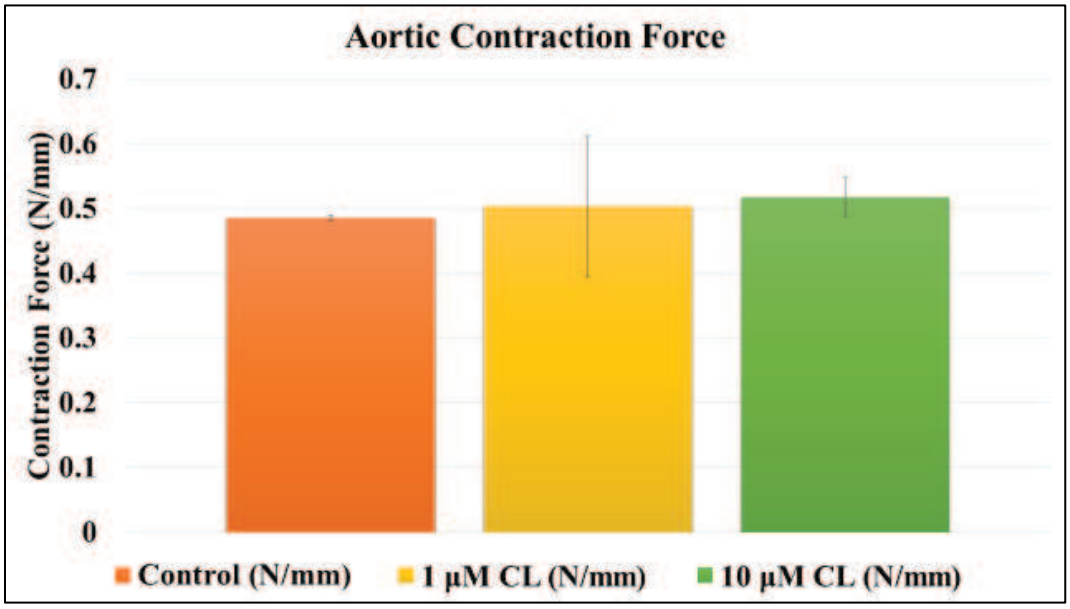


**Figure 7. Aortic Contraction.** Vessel contraction force as CL concentration increases from 0 to 10 µM. There was no significant difference in aortic contraction force in the presence or absence of CL. This graph represents n = 11 for 1 µM CL treated samples, and n = 13 for control and 10 µM treated samples. Standard Deviation measurements were used to calculate the error bars.

# Discussion

CL is the only phospholipid molecule that is significantly released out of the mitochondria upon cell perforation (De Windt *et al*., 1998; Paradies *et al*., 1999; Lesnefsky *et al*., 2001). The recent discovered role of CL on endothelial cells (Carnevale and Bergdahl, 2015) has led us to study the effect of CL on VSMCs dedifferentiation, migration, and proliferation. We also studied the functional implications of CL on mice aorta. SMCs are the only myocytes that retain plasticity and can change morphology due to altered expression of essential proteins, such as MMP14, calpain, calponin, and α-smooth muscle actin (Owens *et al*., 2004; Rzucidlo *et al*., 2007; Lehti *et al*., 2008; Cecchettini *et al*., 2011). We studied the expression of these proteins as they would provide a wide spectrum of the effect of CL on VSMCs since MMP14 is a dedifferentiation marker, while calponin, calpain, and α-actin are differentiation markers. These proteins also vary in their turnover rates, providing an insight of CL's effects on these proteins' expression.

MMP14 is a membrane-anchored matrix metalloproteinase that degrades extracellular matrix formed by VSMCs (Lehti *et al*., 2008). MMP14 expression upregulation is required for VSMC dedifferentiation, and migration, which promotes neointimal formation (Lehti *et al*., 2008; Yan *et al*., 2016). MMP14 has an mRNA half-life of 26-27 hours in rat microvascular endothelial cells, while its mature, activated protein has a half-life of 1 hour (Litwack, 2009). Expression of MMP14 is upregulated in dedifferentiated VSMCs (Lehti *et al*., 2008). Despite the slight increasing trend observed as CL concentration increased, our results reveal no significant difference in MMP14 quantity in any of the CL groups versus the control group. This suggests that CL does not suppress MMP14 protein expression because if it did, MMP14 (at least its active form) would have had enough time to be degraded. Since MMP14 protein quantity remained stable throughout the experiment in all three conditions and that its expression would have increased in dedifferentiated VSMCs, we concluded that CL does not promote VSMCs dedifferentiation.

Calpain I, which has a half-life of 5 days in WI-38 human diploid fibroblasts (Zhang *et al*., 1996), is a conserved $Ca^{2+}$-dependant protease that participates in intracellular signaling pathways. Calpain I is expressed during wound healing as it promotes VSMC proliferation, migration, and differentiation (Nassar *et al*., 2012). Consequently, inhibiting calpain activity reduces tissue damage in cardiovascular diseases (Nassar *et al*., 2012). Our results reveal that

calpain's quantity remained unchanged upon VSMCs incubation in CL-containing media compared to control, even though an increasing pattern was observed at increasing CL concentrations. This further suggests that CL does not induce VSMC dedifferentiation upon incubation for 2 days.

Calponin1, which has an mRNA half-life of 16 hours in human mesangial cells (Sugenoya *et al*., 2002; Owens *et al*., 2004; Rzucidlo *et al*., 2007; Cecchettini *et al*., 2011), exists in three isoforms, of which only one, known as basic calponin or calponin1, is expressed in differentiated VSMCs (Jiang *et al*., 1997; Sugenoya *et al*., 2002). The expression of calponin1 has significantly dropped in a dose-dependent manner upon increasing concentrations of CL. Since actin-associated proteins have a half-life of 4-5 days (Hossain *et al*., 2006), and that our calpain and MMP14 data suggest that CL does not cause VSMC dedifferentiation and that MMP14's half-life is shorter than calponin's, the drop in calponin1 quantity indicates a specific effect of CL on calponin1. Thus, we further examined the functions that calponin1 inhibition could potentially affect.

Calponin1 associates with actin, whose monomers, referred to as G-actins, are proteins that polymerize to form a filamentous structure, known as F-actin (Artman *et al*., 2014). Actin filaments are required for many cellular functions, such as vesicle trafficking, cell shape, cell proliferation, chromatin organization, cell migration, and contraction (Perrin and Ervasti, 2010; Almuzzaini *et al.,* 2016). In mammals, there are six isoforms of actin, some are muscular and some are not, where each participates in different tasks (Perrin and Ervasti, 2010; Yuan, 2015).

α-smooth actin, which is a muscular actin, has a half-life of 72 hours in human myofibroblasts (Sugenoya *et al*., 2002; Baouz, 2005). α-actin is a conserved protein in mammals and birds, and is strongly expressed in differentiated VSMCs (Perrin and Ervasti, 2010). α-smooth actin makes up the actin microfilament bundles (Cherng *et al*., 2008; Lehman and Morgan, 2012; Yuan, 2015), which are associated with myosin and other proteins (Fritzsche, *et al*., 2013). This α-actinomyosin filamentous network is involved in VSMCs contractility (Owens *et al*., 2004; Rzucidlo *et al*., 2007; Hinz *et al*., 2001; Cecchettini *et al*., 2011; Yuan, 2015). This led us to quantify α-actin and measure aortic contractility. Our data shows that α-actin quantity is unaffected by the addition of CL, further suggesting that CL does not initiate VSMC dedifferentiation and that the effect of CL is calponin-specific. Our isometric force measurement

results indicate that the aorta maximum contractibility created by high $K^+$ or endotheline-1 (data not shown) was unaffected by its incubation in CL-containing media for 48 hours. This supports our conclusion that CL affects neither α-actin's quantity, nor its contractile function in aortas, even though we observed an increasing trend in VSMC maximum contractility. This trend could be explained by the potential increase in myosin ATPase function as myosin ATPase inhibition decreases as calponin level decreases, leading to more or stronger contractions (Jiang *et al*., 1997). The contractions induced by endothline-1, which is a potent vasoconstrictor, are not significant with no apparent trend (data not shown). This also excludes the possibility that CL affects α-actin or aortic contraction. Since calponin is involved in α-actin polymerization and that CL decreases calponin's level but not α-actin's, it is possible that there might be another player that compensates for calponin's reduction and α-actin polymerization.

Calponin also associates with β-actin, which has a half-life of approximately 2 days (Cheever *et al*., 2011). β-actin is a non-muscular, cytoplasmic actin molecule (Jiang *et al*., 1997; Artman *et al*., 2014). β-actin monomers are involved in regulating gene expression as they interact with RNA polymerases and chromatin-remodeling complexes (Bunnell *et al*., 2011). β-actin inhibits the serum response factor gene-regulatory pathway that regulates the expression of G-actins and actin-binding proteins (Bunnell *et al*., 2011). β-actin polymerizes to form cytoskeletal microfilaments involved in cell migration and cell proliferation (Almuzzaini *et al*., 2006; Perrin and Ervasti, 2010; Artman *et al*., 2014).

To maintain the cellular functions α- and β-actin participate in, the levels of these actins as well as their polymerization and depolymerization rates, must be regulated. calponin1 induces actin polymerization and actin thin-filament stability, while it inhibits their depolarization (Jiang *et al*., 1997; Sugenoya *et al*., 2002). It also inhibits myosin ATPase activity and lowers the actin filament translocation rates towards myosin (Jiang *et al*., 1997). Calponin1 also inhibits cell division signaling, which contributes to diminishing DNA synthesis and cell proliferation in muscular and non-muscular cells (Jiang *et al*., 1997; Sugenoya *et al*., 2002), although the exact mechanism remains incompletely understood (Jiang *et al*., 1997). Thus, a disruption in calponin1 level impacts β-actin monomer: polymer balance, as well as their actomyosin functions, such as migration and proliferation (Jiang *et al*., 1997; Schildmeyer *et al*., 2000; Suh *et al*., 2011;

Fritzsche, *et al*., 2013). Thus, we decided to study the effect of CL of these two functions in VSMCs (Yuan, 2015).

VSMC migration is initiated during vascular inflammation and is required for the repair process (Artman *et al*., 2014). VSMC migration is accomplished through cytoskeletal movement, which is based on the constant β-actin microfilament remodeling and its association with myosin II, which plays an early role in VSMC migration (Artman *et al*., 2014; Brozovich *et al*., 2016). VSMC migration is initiated by receiving pro-migratory external signals, which trigger myosin II to align the cytoskeletal β-actin bundles, maintain their length, and establish cell polarity (Artman *et al*., 2014; Brozovich *et al*., 2016). These signals also initiate cytoskeletal remodeling through actin polymerization and depolymerization (Artman *et al*., 2014; Brozovich *et al*., 2016).

β-actin is the predominant actin isoform required for cell migration (Artman *et al*., 2014). Actin polymerization occurs at the leading edge of the cell, known as lamelliopodia (Brozovich *et al*., 2016), through the assembly of ATP-bound β-actin monomers. This allows the cell to proceed towards the stimuli by extending its lamelliopodia frontwards and establishing new focal contact sites (Brozovich *et al*., 2016). The proximal end of the cell detaches from its original place through degrading the mature focal contact sites by metalloproteinases and calpain (Brozovich *et al*., 2016). Actin filaments depolymerize at the trailing edge as ATP hydrolyzes into ADP, which makes the actin monomers lose affinity and become more prone to breakage (Artman *et al*., 2014). This drags the trailing end forward to the leading edge (Artman *et al*., 2014; Brozovich *et al*., 2016). β-actin deficiency disrupts the actomyosin contractile function, actin polymerization, and creates persistent adhesive forces (Bunnell *et al*., 2011). This was demonstrated by our results. Plates treated with CL had no significant wound closure distance compared to non-treated plates. This implies that VSMCs in the control plates were free to migrate compared to the treated plates, where VSMCs mobility was hindered. This could be an implication of β-actin polymerization-deficiency due to calponin1 reduction. Unlike the dose-effect observed in calponin's quantity when the tissues were treated with CL, there seem to be no CL dose-effect on VSMC migration. This could be because VSMC migration requires a threshold amount of calponin, and a reduction of this amount significantly ceases this cellular function. According to the literature, polymerization can be reversed in 150 seconds (Verlag, 2013), indicating that the effect caused by a disruption to the polymerization pathway is instantaneous. Our migration

results also potentially provide an explanation to the not significant increasing trend observed in MMP14 and calpain protein expressions. These proteins promote VSMC migration, and so their expression could have been upregulated to compensate for the hindered mobility.

In addition to migration, β-actin along with γ-actin, another cytoplasmic actin, play a role in proliferation. Dedifferentiated VSMCs proliferate during vascular wall remodeling, as well as during atherosclerosis development. Proliferation proceeds through the cell cycle, and is finalized by cytokinesis. β-actin activates RNA polymerase I gene transcription to support cell cycle progression and the proliferative state of cells. β-actin also tethers nuclear myosin I and assembles RNA Polymerase I complex to rDNA genes (Almuzzaini *et al*., 2006). Nuclear myosin I motor, in turn, generates tension that stabilizes RNA polymerase I to rDNA during transcription (Almuzzaini *et al*., 2006). Thus, β-actin is required for transcription during cell cycle. β-actin deficiency abolishes rRNA levels and affects cell cycle progression and cell proliferation, which could explain the increase in multinucleated cells observed by Bunnell *et al.* in β-actin mice knock-outs (Almuzzaini *et al*., 2006; Bunnell *et al*., 2011).

Cytosolic actins are also localized in the mitotic spindle apparatus, centriolar region, and kinetochore, where actomyosin force accounts for chromosomal movement during mitosis, and for forming and closing the contractile ring during cytokinesis. Our results show that VSMC proliferation rate is unaffected by the addition of CL compared to the control sample. Even though we suggest that calponin1 reduction by CL affects β-actin polymerization, which is involved in both migration and proliferation, we propose that the effect of CL on migration is more significant than on proliferation because β-actin is the only actin involved in migration. In contrast, calponin1 has no effect on the cell-cycle functions performed by β-actin monomers. Since calponin1 is involved in β-actin polymerization, which is involved in cytokinesis and is potentially affected by β-actin reduction, we propose that γ-actin compensates for the loss of β-actin polymerization since γ-actin polymerization is independent of calponin1. This provides an explanation to the observed normal cell division.

We conclude that CL reduces calponin1 protein level, significantly restricting VSMC migration, but has no significant effect on VSMC proliferation or contraction. We also hypothesize that CL suppresses β-actin polymerization, which is required for VSMC migration. To confirm this, β-actin polymerization should be tested. γ-actin should also be quantified to understand whether it

compensates for the loss of β-actin polymerization. The non-significant results with the observed trends should be tested by increasing CL dosages or increasing the incubation time. Contractibility experiments should be replicated more to increase reliability. Should there be any pattern observed in high-potassium or endotheline-1 contractions, these pathways will be explored, starting by quantifying the key players involved in each contraction pathway.

# III

# Physiological Levels of Cardiolipin Acutely Affect Mitochondrial Respiration in Vascular Smooth Muscle Cells

Deema Galambo and Andreas Bergdahl

# Abstract

Cardiolipin (CL) is a phospholipid molecule found in the inner mitochondrial membrane. Normally, cardiolipin associates with and activates the mitochondrial respiratory complexes. CL also functions as a proton trap within the mitochondrial membranes. During myocardial infarction, CL gets released and becomes free to affect neighbouring cells. We previously showed that physiological concentrations of CL affect vascular epithelial cells migration and their involvement in angiogenesis, but no study was made on the effects of CL on vascular smooth muscle cells (VSMCs). VSMCs dedifferentiate and migrate during vascular injury and are key players in atherosclerosis development. Due to the central role that VSMCs play during atherogenesis and because CL is vital to cellular life, we hypothesize that abnormalities in CL concentrations may have significant implications on VSMC mitochondrial dysfunction and hence in cellular adaptations. In our study, we examined the acute effect of 1 μM and 10 μM CL on VSMC mitochondrial respiration using high-resolution respirometry technique in conjunction with the multiple substrate-uncoupler-inhibitor titration protocol. We found that CL significantly lowers VSMC respiration of complex I and complex III, which could potentially affect other functional aspects of VSMCs.

# Introduction

Cardiolipin (CL) is a highly conserved (Mileykovskaya and Dowhan, 2009; Balasubramanian *et al*., 2015), unique phospholipid (Pangborn, 1942) molecule composed of four fatty acid side chains and three glycerol units found almost exclusively in the inner mitochondrial membrane (Schlame and Haldar, 1993; Hatch, 1998). Within these organelles, CL provides structural support (Gonzalvez and Gottlieb, 2007) and acts as a proton trap (Haines and Dencher, 2002) but has also been shown to promote mitophagy (Chu *et al*., 2013) by serving as an external signal for damaged mitochondria (Smith *et al*., 2011; Balasubramanian *et al*., 2015). CL is also involved in programmed cell death through anchoring of cytochrome c which, when released, triggers downstream apoptotic cascades (McMillin and Dowhan, 2002; Tuominen *et al*., 2002). Interestingly, CL is the only phospholipid molecule (De Windt *et al*., 1998; Paradies *et al*., 1999; Lesnefsky, 2001) that is significantly released during cardiomyocyte perforation after the myocardial infarction occurrence (Carnevale and Bergdahl, 2015), consequently affecting neighboring cells (Beauregard *et al.* 1980).

Myocardial infarctions often originate from vascular occlusion involving the transition of the normally contractile vascular smooth muscle cells (VSMCs) to a synthetic, dedifferentiated phenotype, accompanied by characteristic changes in protein composition such as decreases in contractile filaments (Owens, 1995; Bergdahl *et al.,* 2003; Majesky, 2007). This distinct plasticity is essential during the early stages of atherogenesis, and may confer a survival advantage as it provides means for the VSMCs to respond to altered conditions in its surroundings. Although a number of transcriptional networks that facilitate VSMC dedifferentiation have been mapped, the master regulators of smooth muscle cell phenotype shift remain elusive. There are no, or very few, studies describing the significance of CL in this process.

CL is vital to cellular life and it is thus natural to imagine that this substance could also play an important role in cellular dedifferentiation and functions. Since CL is an important phospholipid located in the mitochondria and is essential in maintaining mitochondrial function, it is likely that abnormalities in CL concentration may have significant implications for mitochondrial dysfunction and hence in cellular adaptations.

The primary function of mitochondria is to produce ATP through the process of oxidative phosphorylation, conducted by complexes I–IV and the ATP synthase (complex V) (Duchen 2004). This capacity relies heavily on substrate availability to produce energy as mitochondria utilize molecules derived from glucose and fatty acid oxidation. A key function of the mitochondria is to maintain the proton gradient created by the passage of electrons along the complexes (Duchen, 2004; Irwin *et al*., 2013). Energy stored by shunting these $H^+$ to the outer membrane fuels ATP synthesis through complex V (Schultz and Chan, 2001; Irwin *et al*., 2013). In the normal situation, CL activates the respiratory complexes, as well as it functions as a proton trap within the mitochondrial membranes to concentrate the proton pool and to minimize the changes in pH in the mitochondrial intermembrane space (Carnevale and Bergdahl, 2015).

Despite its seemingly central role in cellular function, little is known about CL's effects on VSMC mitochondrial respiration. The aim of this study was thus to investigate whether physiological concentrations of CL downregulates mitochondrial respiration rates and ATP production. We hypothesize that an increase in CL concentration, in ranges normally seen following cardiac ischemia, will acutely reduce the oxidative phosphorylation in VSMCs from fresh aortic mouse tissue.

# Methods

### *Animal Care*

Male C57Bl/6 mice, 7-8 months old were obtained from our Concordia University breeding colony and randomly assigned to either a control or two CL groups (1 μM and 10 μM). The mice were housed in a thermo-neutral environment (22°C), on a 12:12 h photoperiod, and were provided access to standard dry laboratory chow as well as water ad libitum. All procedures were approved by the Animal Ethics Committee of Concordia University (#30000259) and were conducted in accordance with guidelines of the Canadian Council on Animal Care.

### *Experimental protocol*

The animals were euthanized by $CO_2$ according to the approved animal ethics protocol after which the aorta was removed and immediately placed in an ice-cold buffer solution (BIOPS) containing (in mM): $CaK_2EGTA$ 2.77, $K_2EGTA$ 7.23, $Na_2ATP$ 5.77, $MgCl_2 \cdot 6H_2O$ 6.56, Taurine

20, $Na_2$ Phosphocreatine 15, Imidazole 20, Dithiothreitol 0.5, MES 50, pH 7.1. The aortic samples were gently dissected, connective tissue and fat were removed before the vessels were separated using sharp forceps in ice-cold BIOPS buffer. Two aortas were used per condition, which provided approximately 6-8 µg of muscle tissue (wet weight) per sample. After dissection, the vessels were incubated in 2 ml BIOPS buffer containing 50 µg/ml saponin for 30 minutes. The aortas were then washed in ice-cold buffer (MiR05) for 2 x 10 min. MiR05 contains (in mM): EGTA 0.5, $MgCl_2 \cdot 6H_2O$ 3.0, K-lactonionate 60, Taurine 20, $KH_2PO_4$ 10, HEPES 20, Sucrose 110, BSA 1g/l, pH 7.1. Measurements of oxygen consumption were performed at 37°C using high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). The respirometric measurements were performed in the buffer MiR05 loaded in each chamber. This technique has been described in further detail elsewhere (Kuznetsov *et al.*, 2008; Larsen *et al.,* 2015).

### *Mitochondrial respiratory measurements*

To avoid potential oxygen limitation, all experiments were carried out under hyperoxygenated conditions. 1 µM and 10 µM CL were added to each oxygraph chamber, which has a total volume of 2 ml. State 2 respiration (absence of adenylates) was assessed by addition of malate (2 mM) and octanoyl carnitine (1.5 mM; $ETF_L$), state 3 respiration was achieved by adding ADP (5 mM; $ETF_P$). This was followed by the addition of glutamate (10 mM; $CI_P$) and succinate (10 mM; $CI+II_P$), thus achieving maximal coupled respiration with convergent electron input to complex I and II of the electron transport system. Thereafter oligomycin (2 µg/ml) was added to block complex V (LEAK) followed by antimycin A (2.5 µM) to inhibit complex III (ROX). Finally, ascorbate (2 mM) and TMPD (500 µM) were added to measure COX activity. In this experiment, n = 5.

### *Statistics*

Data are presented as means ± SE in all figures and tables. For all statistical evaluations, $P<0.05$ was considered significant. One-way ANOVAs for the CL concentration factor was performed. Significant main effects or interactions were further analyzed by the Tukey post hoc test. The statistical analysis was performed using the software OriginPro 2015 (OriginLab Corporation, Northampton, MA, USA).

# Results

To investigate whether physiological concentrations of CL acutely affect VSMC mitochondrial respiration, we studied the oxygen consumption rates using high-resolution respirometry.

As seen in Figure 8, there was a significant effect of CL after addition of the Complex I linked substrate malate (basal, ADP-restricted) at the $p < 0.05$ level for the conditions [$F(2, 12)$ $F = 53.54$, $p = 0.00001$]. A Tukey post-hoc test revealed significant differences between control and 1 ($p < 0.001$) as well as control and 10 µM ($p < 0.001$) CL.

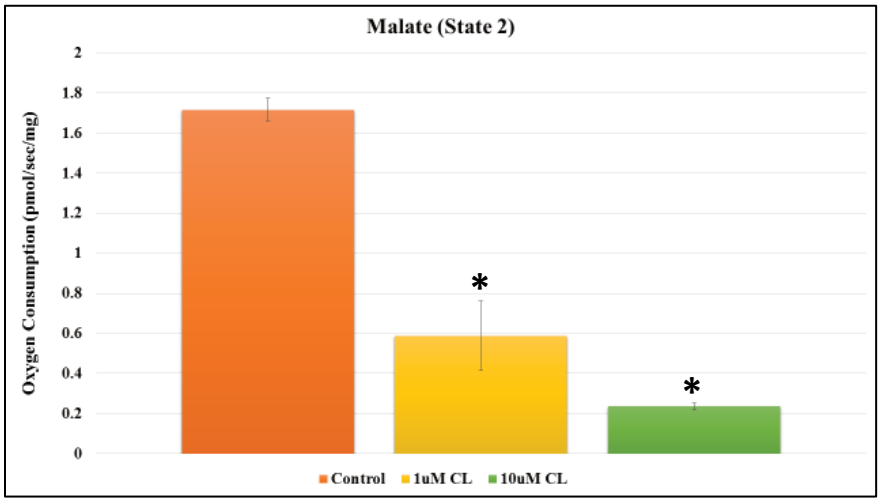


**Figure 8. Malate administration:** Mitochondrial OXPHOS capacity per mg tissue measured in permeabilized muscle fibers as the concentration of CL increases after the addition of malate. The graph depicts a significant drop in mitochondrial respiration upon malate addition (p-value = 0.000) as the concentration of CL increased. The chart presents the average data of n = 5. Standard Error Mean measurements were used to calculate the error bars.

Figure 9 demonstrates a significant effect of CL on lipid OXPHOS capacity induced by octanoylcarnitine at the $p < 0.05$ level [$F(2, 12)$ $F = 19.96$, $p = 0.0002$] with a Tukey post-hoc test proving significant differences between control and 1 ($p < 0.01$) as well as control and 10 µM ($p < 0.001$) CL.

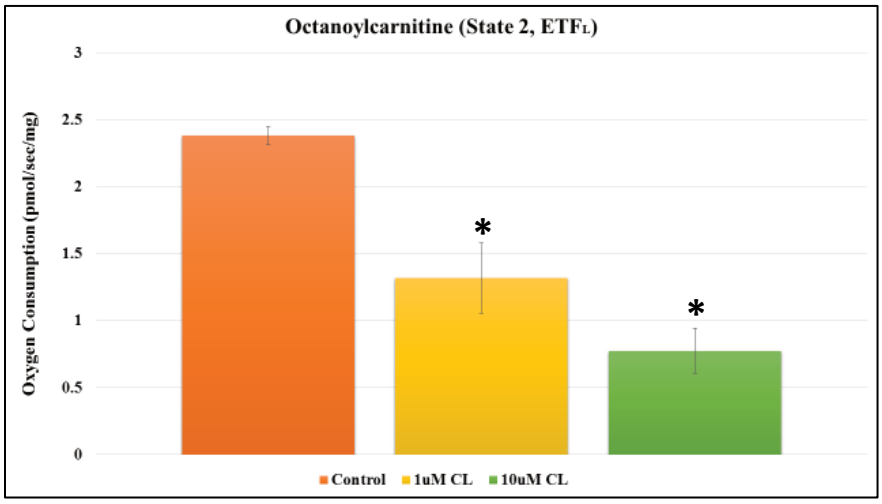


**Figure 9. Octanoylcarnitine administration:** Mitochondrial OXPHOS capacity per mg tissue measured in permeabilized muscle fibers as the concentration of CL increases after the addition of octanoylcarnitine. The graph depicts a significant drop in mitochondrial respiration upon octanoylcarnitine addition (p-value = 0.000) as the concentration of CL increased. The chart presents the average data of n = 5. Standard Error Mean measurements were used to calculate the error bars.

Further stimulating complex I respiration by the addition of glutamate, but now in an ADP-stimulated environment, decreases significantly after addition of CL (Figure 10) [$F(2, 12)$ $F = 9.55$, $p = 0.003$]. Tukey post-hoc test revealed significant difference between control and 10 ($p < 0.05$) as well as 1 and 10 µM ($p < 0.01$) CL.

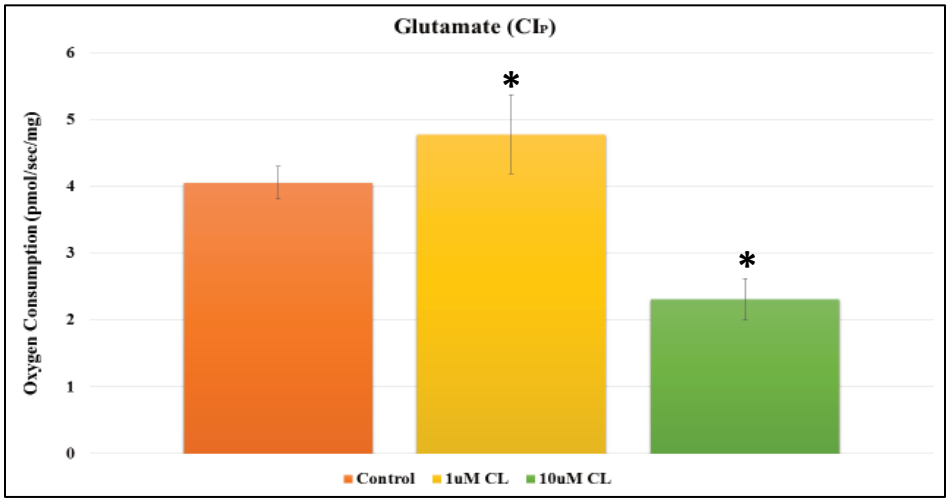


**Figure 10. Glutamate administration:** Mitochondrial OXPHOS capacity per mg tissue measured in permeabilized muscle fibers as the concentration of CL increases after the addition of glutamate in ADP-stimulated environment. The graph depicts a significant drop in mitochondrial respiration upon glutamate addition (p-value = 0.003) as the concentration of CL increased. The chart presents the average data of n = 5. Standard Error Mean measurements were used to calculate the error bars.

The change in respiration among the three samples after the addition of succinate and oligomycin, complex II and V specific substrates, was not significant, even though a decreasing pattern of respiration was observed (data not shown).

The residual oxygen consumption (ROX) demonstrated by addition of antimycin, which inhibits complex III activity, was significantly downregulated after addition of CL as seen in Figure 11 [$F(2, 12)$ $F = 13.56$, $p = 0.0008$]. Using a post-hoc test (Tukey) it was determined that there was a significant difference between control and 10 ($p < 0.01$) as well as 1 and 10 µM ($p < 0.001$) CL. The addition of ascorbate + TMPD, which stimulates complex IV, revealed no significant difference (data not shown) in oxygen consumption between control and CL-treated samples.

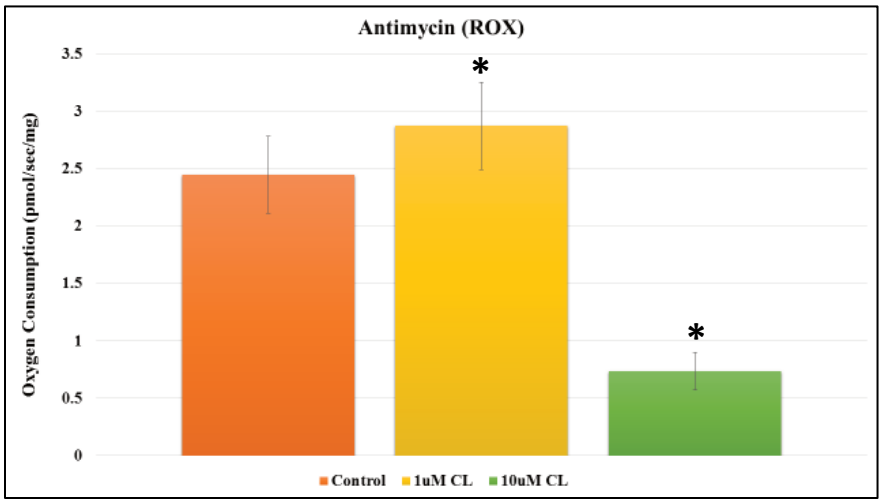


**Figure 11. Antimycin administration (Residual Oxygen Consumption):** Mitochondrial OXPHOS capacity per mg tissue measured in permeabilized muscle fibers as the concentration of CL increases after the addition of antimycin in the ADP-stimulated environment. The graph depicts a significant drop in mitochondrial respiration upon antimycin addition (p-value = 0.001) as the concentration of CL increased. The chart presents the average data of n = 5. Standard Error Mean measurements were used to calculate the error bars.

# Discussion

CL has been shown to have pleiotropic roles in mitochondrial function including interactions with a number of inner mitochondrial membrane proteins, enzymes and metabolite carriers (Houtkooper *et al.,* 2008; Paradies *et al.,* 2009). This ubiquitous and intimate association between CL and energy-transducing membranes suggests an important role of CL in mitochondrial bioenergetics processes. Consequently, the ablation of CL causes abnormalities in mitochondrial structure and function, such as disorganized cristae and dissociated supercomplexes along with the expected defects in membrane potential and oxidative

phosphorylation. An example of this is Barth syndrome, caused by a mutation in tafazzin, the enzyme responsible for CL remodeling. The biochemical consequences of tafazzin deficiency include a decrease in CL and an altered composition of CL molecular species (Schlame and Ren, 2006). The clinical presentation of Barth syndrome includes cardiomyopathy, skeletal myopathy with muscle wasting and growth delay (Barth *et al.,* 1983; Kelley *et al.,* 1991).

Hamsten *et al.* (1986) observed an increased number of patients with elevated anti-CL antibody levels in a group of young patients with myocardial infarction. This led us to study the acute effects of adding physiological concentrations of CL on VSMC mitochondria. CL is required for optimal activity of complex I-V (Fry and Green, 1981; Eble *et al.,* 1990; Robinson, 1993). The currently favored view of the mitochondrial electron transport chain is the 'random collision' model, first proposed by Hackenbrock *et al.* (1986). According to this model, all components of the electron transport chain are distinct entities that can diffuse individually in the mitochondrial membrane. The electron transport depends on the random transient encounter of the four individual proteins of the complexes and the two small mobile electron carriers, CoQ and cytochrome c. CL seems to participate in the structural organization and stabilization of the respiratory chain complexes (Shägger, 2002). Such supercomplex organization of electron transport chain would likely increase the efficiency of electron/proton flux and hence, that of ATP synthesis, while also minimizing the generation of potentially toxic reactive oxygen species, that have been proposed to be involved in the pathogenesis of cardiovascular diseases.

In our study, we examined the effect of 1 μM and 10 μM CL on VSMC mitochondrial respiration using high-resolution respirometry technique in conjunction with the multiple substrate-uncoupler-inhibitor titration protocol. This allowed us to understand the effect of CL on each complex.

Our results indicate that CL significantly suppresses complex I mitochondrial respiration since oxygen consumption had significantly dropped in a dose-dependent manner as the concentration of CL increased after the addition of complex I inducer (Figure 8 and Figure 10).

Since we used a sequential substrate addition protocol, we were able to quantify the change in respiration contributed by complex II. Because respiration in the three groups have increased approximately by the same amount upon the addition of complex II inducer (data not shown), we concluded that complex II activity was unaffected by CL addition.

To understand whether the drop in respiration is associated to glycolysis or β-oxidation, we titrated a β-oxidation inducer. The overall respiration of the samples increased approximately by the same amount, and the respiration ratio of lipid coupling was not significantly increased (comparing Figure 8 and Figure 9 data). This indicates that β-oxidation was not disrupted by CL. CL associates with pyruvate transporters, which is produced during glycolysis (Chicco and Sparagna, 2006; Paradies *et al*., 2014). Thus, we hypothesize that a disruption in CL level impacts the function of the transporter into the mitochondria, consequently restricting energy production through glycolysis.

When complex III was inhibited, respiration has significantly dropped, indicating that complex III is tightly associated with CL (Figure 11), while no significant drop in respiration was observed by complex IV and complex V.

Previous histological studies demonstrated that CL associated to complexes I-V. However, our results indicate that acute addition of physiological concentrations of CL significantly impacts mitochondrial respiration, specifically complex I and III. We hypothesize that this could be due to the affinity or the proximity of CL's binding to the proton channels. CL could also hinder binding of the citric acidic cycle coenzymes. CL has been shown to also associate with cytochrome c. Normally, cytochrome c functions as an electron shuttle between respiratory complexes III and IV in mitochondria. CL is proposed to disrupt this electron transfer function, which could lower cellular respiration.

All in all, the drop of mitochondrial respiration due to CL, which is released upon myocardial infarction, could physiologically translate to lowering blood pump demand by the heart as oxygen required by cells decreases due to the decrease in their cellular demand. It has also been shown that when VSMCs were incubated with physiological concentrations of CL for 48 hours, the level of calponin significantly decreased (unpublished data). Calponin is a protein involved in VSMC migration through actin polymerization, which requires ATP. A drop in calponin lowered VSMC migration, and is proposed to lower actin polymerization rate, and thus the need for ATP drops. We hypothesize that VSMCs adjust to the drop in basal respiration by downregulating ATP-demanding processes, such as migration, which plays a vital role in atherosclerosis development.

# IV

# Concluding Remarks

# Conclusion

The objective of this thesis was to understand the effects physiological concentrations of CL on VSMCs phenotypic switch, mitochondrial respiration, and functions, such as migration, proliferation, and contraction. We found that CL administration significantly affected the overall mitochondrial respiration rate, specifically complex I and III. CL also significantly affected only calponin out of the other tested markers, suggesting that CL has a specific effect on calponin. Because CL did not affect other dedifferentiation markers, we suggest that it is unlikely that CL stimulates VSMC dedifferentiation.

Because CL is associated with α- actin polymerization, which is involved in aortic contraction, and β-actin polymerization, which is involved in migration and proliferation. This led us to explore the effect of CL on these functions, and found that contraction, which is α- actin dependent, was unaffected by calponin reduction, proposing a potential protein that could be involved in α- actin polymerization. We found a significant restriction in VSMC migration upon the administration of CL. Since β-actin is the only actin involved in migration, we proposed that β-actin polymerization could be affected by calponin's drop, which in turn impairs VSMC mobility. In contrast, we proposed that CL administration would not have a significant impact on VSMC proliferation since β-actin is not the only actin involved in proliferation, and that γ-actin could compensate for its function. Our data indeed demonstrates that addition of physiological concentrations of CL has no significant effect on VSMC proliferation.

All in all, because VSMC migration determines the beginning of the irreversible stages in atherosclerosis development, CL is proposed to potentially arrest atherosclerosis development as it restricts VSMC migration. Moreover, cardiolipin lowered VSMC mitochondrial respiration, which physiologically could translate to lowering blood pump demand by the heart as the oxygen demand by cells decreases after a myocardial infarction. Thus, we hypothesize that CL could act as a defense mechanism against and a homeostasis mechanism after the occurrence of a second myocardial infarction.

# V

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# Appendix

# Cardiolipin Review

## What is Cardiolipin?

Cardiolipin (CL) is a phospholipid that was isolated in 1942 by Mary Pangborn from bovine cardiac muscle, from which CL derived its name (Chicco and Sparagna, 2006; Houtkooper and Vaz, 2008). CL has a diphosphatidylglycerol head, which accounts for two phosphates and three glycerol molecules (Haines and Dencher, 2002; Zhong *et al*., 2014). Unlike most phospholipids, CL has four fatty acyl chains that hydrophobically interact with each others (Lewis and Mcelhaney 2009). The presence of four fatty acyl chains is primordially preserved, and account for CL's conical structure and phase behavior (Zhong *et al*., 2014; Balasubramanian *et al*., 2015; Kooijman *et al*., 2017). CL forms a bilayer with negative membrane curvature (Ren *et al*., 2014) at physiological pH, while it forms a hexagonal HII phase arrangement at pH lower than ≈2.8 (Ren *et al*., 2014; Kooijman *et al*., 2017).

There is controversy to whether CL carries one or two negative charges. CL is said to have a $PK_1$ value below 4.0, and $PK_2$ value above 8.0 (Haines and Dencher, 2002). When the first phosphate group is deprotonated, the free hydroxyl group of the central glycerol molecule forms a hydrogen bond with the oxygen atom of the other phosphate groups, stabilizing the molecule (Haines and Dencher, 2002). This results in a higher $PK_2$ value, and implies that CL has one negative charge at physiological pH (Haines and Dencher, 2002; Kooijman *et al*., 2017). This notion has been challenged. Recent studies show that both CL PK values are ≈2.5, meaning that the phosphate groups give CL two negative charges at physiological pH (Tyurina *et al*., 2006; Paradies *et al*., 2014; Kooijman *et al*., 2017).

## Cardiolipin biosynthesis

In eukaryotic cells, CL resides in the inner mitochondrial membrane (Chicco and Sparagna, 2006; Zhong *et al*., 2014). It makes up 10-20% of the phospholipids present in the mitochondrial inner membrane, and is the only phospholipid that is synthesized there (Haines and Dencher, 2002; Paradies *et al*., 2014). CL is biosynthesized in four steps in the inner mitochondrial membrane inner leaflet (Chicco and Sparagna, 2006; Paradies *et al*., 2014).

On the outer leaflet of the mitochondrial outer membrane, phosphatidic acid is made (Chakraborty *et al*., 1999), which is the first precursor involved in CL biosynthesis (Ren *et al*., 2014). Since CL is biosynthesized in the inner leaflet of the inner mitochondrial membrane, phosphatidic acid has to be transported across the membranes (Ren *et al*., 2014). This transportation is mediated by Ups1 (in yeast) (Connerth *et al*., 2012).

In the mitochondrial inner membrane, phosphatidic acid is condensed with CTP (Cytidine triphosphate Tam41 in yeast) (Petit *et al*., 1994), mediated by CDS (CDP-DG synthase) (Tamura *et al*., 2010), to form cytidinediphosphate-diacylglycerol (CDP-DAG). PGP synthase (PGPS Pgs1 in yeast) (Chang *et al*., 1998) mediates the conversion of CDP-DAG to PG-P (Phosphatidylglycerol-phosphate) (Ren *et al*., 2014). PGPP (PGP phosphatase; Gep4 in fungi (including yeast) and some plants (Osman *et al*., 2010), and PTPMT1 in mammals) (Zhang *et al*., 2011) dephosphorylates PG-P into PG (Phosphatidylglycerol) (Chicco and Sparagna, 2006; Ren *et al*., 2014). Finally, CL is assembles by condensing phosphatidylglycerol and cytidinediphosphate-diacylglycerol molecules, mediated by CL synthase (Crd1 in yeast) (Jiang *et al*., 1997; Chang *et al*., 1998; Tuller *et al*., 1998; Chicco and Sparagna, 2006).

This biosynthesis process yields asymmetrical and saturated immature CL that is variable in length (Chicco and Sparagna, 2006). The final, mature CL structure is achieved though remodeling process, which is not yet completely understood, forming a homogeneous and unsaturated mature molecule (Chicco and Sparagna, 2006; Claypool and Koehler, 2012).

## Cardiolipin remodeling

CL fatty acyl chain composition vary among organisms and tissues to fit to their biological needs, even though little is known about the variable CL species (Chicco and Sparagna, 2006; Claypool and Koehler, 2012). In eukaryotes, CL mostly consists of linoleic acid (18:2) acyl chains, suggesting the biological significance of the 18:2 CL across species (Chicco and Sparagna, 2006; Zhong *et al*., 2014). The 18:2 CL also makes up 70-95% of CL found in a healthy mammalian heart (Lesnefsky *et al*., 2001; Chicco and Sparagna, 2006; Zhong *et al*., 2014). To generate functional-specific, symmetrical, mature CL species, the CL remodeling process is needed, which is incompletely understood (Claypool and Koehler, 2012). It remains unclear whether CL remodeling process also repairs damaged CL molecules (Claypool and

Koehler, 2012). It has been argued that CL remodeling occurs exclusively to newly synthesized CL molecules, and so cannot be involved in fixing damaged CL (Xu and Schlame, 2014), which other studies has supported that it can fix oxidatively damaged CL (Claypool and Koehler, 2012).

The remodeling process starts by deacylating a fatty acyl chain from the nascent, immature CL through phospholipases (Cld1p in yeast, $iPLA_{2\gamma}$ in mammals), which is activated by reactive oxygen species (ROS) (Chicco and Sparagna, 2006; Claypool and Koehler, 2012; Ren *et al*., 2014). This forms a monolysocardiolipin molecule, which is a CL with three acyl chains (Claypool and Koehler, 2012). A deletion in the Cld1p-coding gene disrupts CL remodeling, altering the CL-acyl composition and favoring saturated fatty acyl chains over the 18:2 composition (Beranek *et al*., 2009; Claypool and Koehler, 2012). iPLA2γ mutations reduce total CL content and disrupts its molecular structure (Claypool and Koehler, 2012). $iPLA_{2\gamma}$-mutated mice experience a compromised cold-tolerance ability, and reduced bioenergetics functions (Claypool and Koehler, 2012). $iPLA_{2\gamma}$ deletions do not display the complete loss-of-CL, lethal phenotype observed in PGPP deletions (Claypool and Koehler, 2012). $iPLA_{2\gamma}$-deleted mice had healthy and mature CL molecules, suggesting that $iPLA_{2\gamma}$ may not be the only enzyme responsible for the remodeling-initiation in mammals (Claypool and Koehler, 2012). This indicates that $iPLA_{2\gamma}$ is not a Cld1p ortholog, and that its function is complemented by another enzyme (Ren *et al*., 2013).

Other phospholipases can also deacylate CL into monolysocardiolipin. These include, iPLA1β, iPLA1γ, and cPLA2 (Ren *et al*., 2014). iPLA1β (found in mice, drosophila, and humans) resides in the mitochondria (Gadd *et al*., 2006; Seleznev *et al*., 2006; Malhotra *et al*., 2009). iPLA1β depletes CL and increases monolysocardiolipin levels in the absence of re-acylation enzymes, confirming its deacylating role in CL (Malhotra *et al*., 2009; Hsu *et al*., 2013). iPLA1β is also associated with repairing peroxidized CL and inhibiting apoptosis (Seleznev *et al*., 2006; Zhao *et al*., 2010). This further supports the role of iPLA1β in CL remodeling and maturation. iPLA1γ is another phospholipase found in mitochondria and peroxisomes (Ren *et al*., 2014). iPLA1γ – diminished hippocampi is associated with decreased monolysocardiolipin levels, and increased immature CL levels (Mancuso *et al*., 2009; Zachman *et al*., 2010). This confirms its role as a CL deacylator. Cytosolic PLA2 (cPLA2) hydrolyzes CL present on the mitochondrial surface

(Buckland *et al*., 1998; Hsu *et al*., 2013; Ren *et al*., 2014). This regulates externalized-CL, and could act as a defense mechanism against mitophagy and apoptosis (explained later).

Following CL hydrolysis, CL is reacylated by reattaching a fatty acyl chain to the monolysocardiolipin molecule, restoring the tetra-acyl chain structure of CL (Claypool and Koehler, 2012). Re-acylation can be performed by three different enzymes, collectively referred to as monolysocardiolipin remodelers. These include: Tafazzin, monolysocardiolipin acyltransferase 1 (MLCLAT1), and acyl-CoA:lysocardiolipin acyltransferase-1 (ALCAT1), the first being the most understood. These remodeler enzymes are located in distinct sites, but it remains unknown whether these monolysocardiolipin remodelers are CL-species or -location specific (Claypool and Koehler, 2012).

Tafazzin is an integral transcyclase, present in both mammals and yeast (Xu *et al*., 2006; Claypool and Koehler, 2012). It is located in the inner and the outer mitochondrial membranes in the leaflets facing the intermembrane space (Claypool and Koehler, 2012). Tafazzin finalizes CL remodeling by non-specifically translocating a fatty acyl chain from a neighboring phospholipid molecule to the monolysocardiolipin, until a stable, homogeneous CL is formed (Claypool and Koehler, 2012). This is challenged by a series of experiments demonstrating that tafazzin has high acyl-specificity transfer that is based on the lipid phase state and properties (Schlame *et al*., 2012). Tafazzin is argued to mediate the de-attachment and re-attachment of acyl chains needlessly of phosphlipases (Ren *et al*., 2014).

MLCLAT1 is another monolysocardiolipin remodelers found in mammalian mitochondria in the matrix-facing leaflet of the inner membrane (Claypool and Koehler, 2012). MLCLAT1 has acyl chain and lysolipid specificity required to attain a mature, tetralineoyl-CL (Claypool and Koehler, 2012). MLCLAT1-knockout HeLa cells have reduced linoleate CL molecules, while its upregulation increased the linoleate content in cardiolipin (Claypool and Koehler, 2012).

ALCAT1 is present in mammals, and is localized in the mitochondria associated membranes of the endoplasmic reticulum (Claypool and Koehler, 2012). ALCAT1 reacylates monolysocardiolipin with CoA-bound long, unsaturated acyl chains (Claypool and Koehler, 2012). Upregulated ALCAT1 gene has decreased mature and total CL pool (Claypool and Koehler, 2012). This causes proton leakage, which disrupting mitochondrial respiration and the mitochondrial inner membrane potential (Claypool and Koehler, 2012). ALCAT1

overexpression also impaired mitochondrial fusion mechanism, in which CL plays an essential role. It increased ROS production and polyunsaturated-containing CL levels that are associated with mitochondrial dysfunction and mtDNA instability (Claypool and Koehler, 2012). ALCAT1 knockdown rodents had increased linoleate, mature CL and the total CL pool (Claypool and Koehler, 2012). This suggests that, unlike tafazzin and MLCLAT1, ALCAT1 acylates monolysocardiolipin to impair CL molecules (Claypool and Koehler, 2012). The relevance of this remains unknown, but it is possible that ALCAT1 promotes apoptosis when CL is externalized.

# Cardiolipin Translocation and Release

CL biosynthesis requires phosphatidic acid translocation from the outer to the inner membrane. CL translocation from the inner to the outer mitochondrial membrane is needed to relocate remodeled CL, and to mediate cellular processes like mitophagy or apoptosis, depending on the degree of the mitochondrial damage (Heit *et al*., 2011; Chu *et al*., 2013). Several translocating proteins have been identified, even though CL translocation mechanisms between and within membranes remain unclear (Connerth *et al*., 2012; Ren *et al*., 2014).

***Intramembrane translocation***

*ADP/ATP carriers*

Intramembrane translocation of CL from one leaflet to the other in the inner membrane remains unclear (Ren *et al*., 2014). It has been proposed that CL might flip from one leaflet to the other by interacting with integral proteins such as the ADP/ATP carriers as it is the most abundant protein carrier within the mitochondrial inner membrane (Klingenberg, 2009; Ren *et al*., 2014).

***Intermembrane translocation:***

*UPS1*

Ups1 is a conserved intermembrane space protein found in yeast (Connerth *et al*., 2012). During the early stages of CL biosynthesis, Ups1 shuttles phosphatidic acid from the outer mitochondrial membrane to the inner mitochondrial membrane via a lysosome, but cannot translocate CL or other negatively charged phospholipids (Connerth *et al*., 2012). Phosphatidic acid-bound Ups1 interacts with a stabilizing protein called Mdm35. This Ups1-Mdm35 complex recognizes

negatively charged phospholipids on the recipient membranes, which triggers Ups1-Mdm35 dissociation, releasing phosphatidic acid into the recipient, inner membrane, where CL biosynthesis takes place (Connerth *et al*., 2012; Ren *et al*., 2014).

*Pro-mitophagy stimuli*

Pro-mitophagy stimuli, including: rotenone, 6-hydroxydopamine, and staurosporine, trigger CL externalization from the inner mitochondrial membrane to the outer mitochondrial membrane in SH-SY5Y cell-line and in primary cortical neurons (Chu *et al*, 2013).

*MINOS/MICOS/MitOS*

MINOS/MICOS/MitOS is a large hetero-oligomeric protein complex and is also known to connect the inner mitochondrial membrane to the outer mitochondrial membrane (van der Laan *et al*., 2012), and to bind CL (Hoppins *et al*., 2011; Weber *et al*., 2013; Ren *et al*., 2014). Mutations in this complex did not affect the distribution of phospholipids in the mitochondrial membrane (Harner *et al*., 2011), so it remains unclear whether the complex is involved in CL translocation (Ren *et al*., 2014).

*ATAD3*

ATPase family AAA domain-containing protein 3 (ATAD3) is an ATPase-bound protein family that is also present at the inner-outer membrane contact sites (Li and Rousseau, 2012) and so is important to sustain the mitochondrial network (Hoffmann *et al*., 2009; Gilquin *et al*., 2010). ATAD3 is involved in cholesterol transfer (Gilquin *et al*., 2010; Issop *et al*., 2013), but it remains unclear whether this protein translocates CL (Connerth *et al*., 2012; Ren *et al*., 2014).

*ATP8B1*

ATP8B1 is a CL pump protein conserved in humans, mice, and rats, and is present in apical epithelial membranes (Ray *et al*., 2010). Atp8b1 binds and translocates CL from the outer to the inner leaflet of membrane bilayers and is important for maintain a healthy alveolar surfactant in the lungs (Ray *et al*., 2010). Atp8b1 mutations result in increased CL concentrations in the lung's fluid, which promotes pneumonia (Ray *et al*., 2010). This signifies the important role of Atp9b1 as a CL transporter.

*NDPK-D and MtCK*

Nucleoside diphosphate kinase D (NDPK-D) and mitochondrial creatine kinases (MtCK) are kinases that physically link the inner and the outer mitochondrial membranes together (Eder *et al*., 2000; Schlattner and Wallimann, 2000; Tokarska-Schlattner *et al*., 2008; Schlattner *et al*., 2009; Ren *et al*., 2014). These two kinases have high affinity to CL, and promote lipid intermembrane transfer (Epand *et al*., 2007). Overexpression of either NDPK-D or MtCK kinases have increased CL content in the outer mitochondrial membrane, indicating that NDPK-D and MtCK are involved in CL transfer from the inner to the outer membrane, and that they are complementary to one another (Lacombe *et al*., 2009; Schlattner *et al*., 2009; Schlattner *et al*., 2013).

*PLS3*

Phospholipid scramblase-3 (PLS3) is a mitochondrial enzyme found in mammals (Chu *et al*, 2013; Inuzuka *et al*., 2013), and is linked to cell signaling, injury, and apoptosis (Liu *et al*., 2003; Gonzalvez and Gottlieb, 2007; Sahu *et al*., 2007; Van *et al*., 2007; Bevers and Williamson, 2010; Chu *et al*, 2013). PLS3 is involved in CL translocation from the inner mitochondrial membrane to the outer mitochondrial membrane (Liu *et al*., 2003; Van *et al*., 2007; Chu *et al*., 2013), even though it is not the actual vehicle that translocates CL (Ren *et al*., 2014). Instead, integral outer proteins that bind CL are thought to directly translocate CL. These proteins include the Bcl-2 family or VDAC (Rostovtseva and Bezrukov, 2008; Raemy and Martinou, 2014).

***CL translocation beyond mitochondria***

CL has been detected in peroxisomes and the plasma membrane, indicating that CL is translocated from the mitochondrial surface to other organelles (Zinser *et al*., 1991; Sorice *et al*., 2000; Sorice *et al*., 2004; Wriessnegger *et al*., 2007; Ren *et al*., 2014). It has been shown that mitochondria-derived vesicles mediate CL translocation from mitochondria to peroxisomes (Neuspiel *et al*., 2008).

*Myocardial impairments*

CL loss appear to exceed the loss of other phospholipids from cardiomyocytes during myocardial ischemia or myocardial infarction (Chicco and Sparagna, 2006). During these myocardial defects, it also appeared that CL was selectively lost from the subsarcolemmal mitochondria,

which are located beneath the interfibrillar and plasma membrane, but not from the interfibers' (Rossen *et al*., 1994)), which are located between myofibrils (Lesnefsky *et al*., 2001; Chicco and Sparagna, 2006). Myocardial infarction and ischemia trigger sarcolemma tear, necrosis, and apoptosis, all of which releases various intracellular components, including CL-positive membrane fragments (Carnevale and Bergdahl, 2015). Released CL can affect neighboring cells, and is marked by an elevation in anti-CL antibodies, which recognizes peroxidized CL, in patients' bodies (Zhong *et al*., 2014). Cells were also found to release CL-containing mitochondrial fragments in different pathologies, such as: stress, acute trauma, necrosis, and neutrophil activation (Hamsten *et al.* (1986; Balasubramanian *et al*., 2015).

# Cardiolipin regulation

### *Regulating CL Biosynthesis*

The amount of CL present in mitochondria inner membrane is regulated (Connerth *et al*., 2012). When CL and/or phosphatidylglycerol are present in high physiological concentrations, they impair the Ups1-Mdm35 dissociation mechanism that releases phosphatidic acid to the recipient membrane. This prevents excessive CL biosynthesis and accumulation (Connerth *et al*., 2012).

### *Limiting CL externalization*

MitoPLD is a mitochondrial phospholipase D molecule that resides on the mitochondrial outer membrane. MitoPLD degrades CL into phosphatidic acid, regulating the amount of CL exiting the mitochondria (Claypool and Koehler, 2012). This prevents provoking an autoimmune response, as CL release has been shown to provoke of anti-CL antibodies (Claypool and Koehler, 2012). It could also be a regulatory mechanism to inhibit apoptosis and mitophagy.

# Roles of Cardiolipin

CL is involved in various cellular processes, such as: immunity, mitophagy, apoptosis, supports cristae, cellular respiration,… (Chicco and Sparagna, 2006; Chu *et al*, 2013; Balasubramanian *et al*., 2015).

*Immunity (Bacterial defense mechanism)*

CL is externalized and co-presented with Toll-like receptor 4 and lipopolysaccharide in gram negative bacteria when invading a host (Balasubramanian *et al*., 2015). This inhibits the host's ability to respond to the bacterial Toll-like receptor and produce cytokines against it (Balasubramanian *et al*., 2015). Thus, CL acts as a bacterial defense mechanism (Balasubramanian *et al*., 2015). Fortunately, since CD36 is not involved in this protection mechanism, the host macrophages can fight these invasive bacteria through their CD36-dependant mechanism (Balasubramanian *et al*., 2015).

*Immunity (Macrophage-dependent cell death)*

Macrophages recognize and engulf apoptotic cells that display the universal, extracellular, self-destruction signal, aminophospholipid phosphatidylserine (Balasubramanian *et al*., 2015). Aminophospholipid phosphatidylserine is found on the membranes of organelles and cells (Balasubramanian *et al*., 2015). This signal is absent from mitochondrial membrane due to the presence of phosphatidylserine decarboxylase, which degrades aminophospholipid phosphatidylserine (Balasubramanian *et al*., 2015). To overcome this, mitochondrial membrane uses CL as a macrophage-degradation signals (Claypool and Koehler, 2012; Balasubramanian *et al*., 2015).

Intracellularly, CL can trigger mitophagy when displayed on the surface of damaged mitochondria (explained later) (Balasubramanian *et al*., 2015). Extracellularly, CL is externalized and sometimes released along with mitochondrial fragments from cells that have experienced trauma or other pathophysiologies (Balasubramanian *et al*., 2015). Externalized CL are then recognized by the macrophages' surface CD36 receptors, triggering the engulfment of the CL-presenting cell (Balasubramanian *et al*., 2015). CD1 receptors of antigen-presenting cells can also bind to CL and present it to immune effector cells, such as the T cells (Claypool and Koehler, 2012). These indicate that CL could either be secreted on the cell's surface through secretory pathways, or that an autophagosome have degraded a mitochondria and then presented it to an antigen-presenting cell (Claypool and Koehler, 2012). Under normal conditions, externalized mitochondrial CL is exposed to CD1 in scarce amounts below the threshold that triggers an immune reaction (Claypool and Koehler, 2012).

The notion that CL is recognized by the immune system is further supported by the increased levels of anti-cardiolipin antibodies in patients with anti-phospholipid syndrome (Claypool and Koehler, 2012). Whether these anti-cardiolipin antibodies are produced against mitochondrial CL or pathogenic CL remains unclear (Claypool and Koehler, 2012).

External CL destruction signals seem to be fulfilling the same role as the external aminophospholipid phosphatidylserine signal, but the mechanisms by which each triggers self-clearance are remarkably different (Balasubramanian *et al*., 2015). CL's function as an internal mitochondrial self-destruction signal predominates its function as an extracellular signal, as aminophospholipid phosphatidylserine is the main externalized signal for self-destruction (Balasubramanian *et al*., 2015).

### *Mitophagy*

Mitochondria is a fundamental organelle in eukaryotic cells (Zhong *et al*., 2014). Mitochondrial respiration generates some ROS, whereas mitochondrial dysfunction overproduces it (Zhong *et al*., 2014). ROS signals for automated cell death, and so is associated with neurodegenerative diseases, aging, and other pathologies (Chu *et al*, 2013; Zhong *et al*., 2014). To avoid this, eukaryotic cells have established a process to degrade the defected mitochondria in order to preserve the cell's viability (Zhong *et al*., 2014). This process is known as mitophagy, which remains incompletely understood (Chu *et al*, 2013; Zhong *et al*., 2014).

In cortical neurons and SH-SY5Y cell line, an essential provoker of mitophagy is externalized CL from the mitochondrial inner membrane to the mitochondrial outer membrane (Chu *et al*, 2013). Different cells have different thresholds of externalized CL to induce mitophagy, but they seem to agree in the concept (Chu *et al*, 2013). CL externalization is stimulated as a response to several pro-mitophagy factors, such as: rotenone, 6-hydroxydopamine, and staurosporine (Chu *et al*, 2013). These cause CL translocation from the inner mitochondrial membrane to the outer mitochondrial membrane via PLS3 (Chu *et al*, 2013). CL externalization is compromised upon PLS3 deletion, regardless of the rotenone level (Chu *et al*, 2013).

Upon CL externalization, CL binds and is recognized by autophagosomes through microtubule-associated-protein-1 light chain 3 (LC3) (Chu *et al*, 2013). LC3 is an autophagy protein that covalently bridges CL-containing cargo to the autophagosome, mediating mitophagy (Chu *et al*,

2013). Rotenone was found to increase the presence and the mitochondrial co-localization of LC3 (Chu *et al*, 2013). The cortical rotenone treatment had increased the percentage of only CL (but not other phospholipids) in the mitochondrial outer membrane by ten-folds, relative to the external CL basal amount, 0.8%. This shows that CL is being externalized (Chu *et al*, 2013).

In contrast to the well-studied lipids that becomes oxidized upon apoptosis, CL molecules were not peroxidized upon mitophagy, suggesting mitophagy occurs to prevent the accumulation of pro-apoptotic signals, even though this notion was rejected by some studies (Chu *et al*, 2013; Zhong *et al*., 2014).

*Apoptosis*

In contrast to mitophagy, whose occurrence is debated on whether CL gets peroxidizes, ROS oxidizes polyunsaturated CL acyl chains during apoptosis (Paradies *et al*., 2014; Zhong *et al*., 2014). CL oxidation is related to mitochondrial dysfunction, apoptosis regulation, and several pathologies (Zhong *et al*., 2014). Here we discuss CL-triggered apoptosis, and CL's link to cytochrome c, a positively charged molecule (Kooijman *et al*., 2017). Cytochrome c is a mitochondrial protein involved in electron transfer from complex III to complex IV in the electron transport chain (Paradies *et al*., 2014; Ren *et al*., 2014;). Cytochrome c plays an instrumental role in triggering apoptosis when it binds to CL (Chicco and Sparagna, 2006; Paradies *et al*., 2014).

CL primarily resides in the inner leaflet of the mitochondrial inner membrane, but is peroxidized and translocated to the outer leaflet upon mitochondrial injuries and ROS production (Belikova *et al*., 2009; Paradies *et al*., 2014). The mechanism for this and the relevance remains unclear (Gonzalvez and Gottlieb, 2007; Ren *et al*., 2014; Paradies *et al*., 2014). In the outer leaflet, CL binds to cytochrome c (Ren *et al*., 2014), which is a dormant peroxidase (Kooijman *et al*., 2017). Cytochrome c is anchored to the inner membrane through two CL binding sites, the A-site and the C-site (Paradies *et al*., 2014). The A-site binds to CL through electrostatic interactions (Chicco and Sparagna, 2006; Kapralov *et al*., 2007; Muenzner *et al*., 2013; Paradies *et al*., 2014), while the C-site binds to CL through hydrogen and hydrophobic interactions (Paradies *et al*., 2014). The latter induces partial unfolding of cytochrome Met-80 residue with its associated heme-iron (Paradies *et al*., 2014).

Peroxidized CL introduces conformational changes in cytochrome c and destabilize the tertiary structure of its heme group (Paradies *et al*., 2014; Ren *et al*., 2014; Kooijman *et al*., 2017). This CL-cytochrome c complex becomes an activated pro-apoptotic peroxidase complex (Kooijman *et al*., 2017), which further oxidizes CL (Kagan *et al*., 2005; Paradies *et al*., 2014; Ren *et al*., 2014). Oxidized CL in turn enhances the oxidation ability of the peroxidase, which further oxidizes polyunsaturated in a continuous positive-feedback loop (Kagan *et al*., 2005; Tyurina *et al*., 2006; Paradies *et al*., 2014; Ren *et al*., 2014). The unfolded cytochrome c escapes to the cytosol, where it activates cellular pathways that orchestrates apoptosis (Kagan *et al*., 2005; Chicco and Sparagna, 2006; Paradies *et al*., 2014). The release of cytochrome c to the cytosol has been linked to permeablizing the mitochondrial membrane, which facilitates the release of the apoptotic factors from the mitochondria, but this remains unclear (Belikova *et al*., 2007; Paradies *et al*., 2009; Paradies *et al*., 2014**;** Raemy and Martinou, 2014).

The oxidation reaction of CL with cytochrome c produces hydroperoxy octadecadienoic acid-CL (HpODE-CL), which gets reduced, forming hydroxyoctadienoic acid (HODE-CL) and keto-octadecadienoic acid (KODE-CL) (Zhong *et al*., 2014). Further intra-molecular reactions occur, in vivo and in vitro, to form other reactive, electrophilic aldehyde species such as, epoxyalcohol-aldehyde-CL (EAA-CL), 4-Hydroxy-2-nonenal (4- HNE), and 4-oxo-2-nonenal (4-ONE) – the mechanisms by which these molecules are formed remain ununderstood (Zhong *et al*., 2014). These electrophilic molecules covalently modify the structure of nucleophilic segments in a protein, which changes the function of the protein (Zhong *et al*., 2014). EAA-CL can only target proteins at the local environment of its generation, whereas 4- HNE and 4-ONE can diffuse and affect proteins that are further from these molecules' generation site (Zhong *et al*., 2014). The involvement of these molecules in apoptosis remains unknown (Zhong *et al*., 2014).

4-HNE has been associated to cell death and was found in atherosclerotic mice (Zhong *et al*., 2014). It is known that 4-HNE suppresses contractile function, while it enhances ROS production. 4-HNE is also known to be involved in atherosclerotic pathways and in other cardiovascular diseases (Zhong *et al*., 2014). In cases of endothelial dysfunction and atherosclerosis, anti-oxidized CL antibodies are released (Zhong *et al*., 2014). These antibodies are thought to have been formed as a result of the covalent modifications caused by the lipid electrophiles (Zhong *et al*., 2014).

Several strategies have been studied to inhibit CL oxidation, which relates to atherosclerosis and other diseases (Zhong *et al*., 2014). Inhibiting the cytochrome c-CL peroxide complex inhibited apoptosis, and overexpressing lipid-reducing factor have lowered liver apoptosis, cytochrome c release, and CL oxidation (Zhong *et al*., 2014). Acet-aminophen (ApAP) is a peroxidase inhibitor. It has been shown that ApAP inhibit the formation of peroxidized CL and its by-products (EAA-CL and HODE-CL) in dose-dependent manner (Zhong *et al*., 2014). Recent studies show that ApAP protects the kidney against oxidative damage (Zhong *et al*., 2014).

In addition to the role of CL in apoptosis through cytochrome c, CL is also involved in other pathways that lead to apoptosis (Paradies *et al*., 2014). CL on the outer mitochondrial membrane anchors and activates recruited caspase-8 proteins (Paradies *et al*., 2014). Caspase-8 activation truncates Bid protein, which belongs to the pro-apoptotic Bcl-2 family, into t-Bid (Gonzalvez and Gottlieb, 2007; Paradies *et al*., 2014). t-Bid is then translocated to the outer mitochondrial membrane, where it allows the binding of Bax oligomers, another pro-apoptotic protein that promotes mitochondrial outer membrane permeablization (Gonzalvez and Gottlieb, 2007; Paradies *et al*., 2014). t-Bid also interacts with mitochondria to remodels the cristae and further promote cytochrome c release, stimulating mitochondrial fragmentation and apoptosis (Gonzalvez and Gottlieb, 2007; Paradies *et al*., 2014).

It has been shown that under the sub-lethal threshold, ROS levels are enough to trigger CL translocation, but not CL peroxidation – a characteristic observed in mitophagy, despite the debate (Chu *et al*., 2013). Thus, unoxidized CL is translocated from the inner to the outer leaflet of the mitochondrial inner membrane, rather than oxidized CL (Chu *et al*., 2013; Ren *et al*., 2014). Through this, mitophagy was initiate, but not apoptosis in sub-lethal mitochondrial injuries, preserving the cell's viability (Chu *et al*., 2013; Ren *et al*., 2014).

*Supports cristae*

The four fatty acyl chains of CL accounts form its conical structure and phase behavior (Zhong *et al*., 2014; Kooijman *et al*., 2017). CL forms a bilayer with negative membrane curvature at physiological pH, while it forms a hexagonal HII phase arrangement (Ren *et al*., 2014; Kooijman *et al*., 2017) at pH lower than ≈2.8 or at NaCl concentrations above 1.6 M at a neutral pH (Kooijman *et al*., 2017). These critical values depend on the length and saturation levels of the specific CL acyl chains; for example, long, unsaturated CL can switch from bilayer lamellar to

the HII phase at NaCl concentrations around 1-3 M, while short, saturated CL exhibit bilayer to HII phase switch at NaCl concentrations close to 6 M (Lewis and Mcelhaney, 2009).

The HII phase structure of CL induces a negative curvature strain, which promotes membrane bending – an important feature of the cristae (Jouhet, 2013). These bents also form the contact sites between the inner and the outer mitochondrial membrane (Jouhet, 2013). HII phase structure is also involved in the sarcoplasmic calcium pumps and in other enzymes' activities and anchoring (Jouhet, 2013). This structure also promotes for membrane stalk formation, regulating membrane fusion and fission (Jouhet, 2013).

*CL & bioenergetics (Stabilize enzymes and respiratory chain complexes)*

CL is negatively charged and have long hydrophobic tail, allowing it to form strong ionic and hydrophobic interactions with neighboring molecules (Lewis and Mcelhaney 2009; Paradies *et al*., 2014; Ren *et al*., 2014). CL tightly binds to the CL-affinity sites on the mitochondrial respiratory complexes, I-V, via hydrophobic and ionic bonds (Eble *et al*., 1989; Lesnefsky *et al*., 2001; Paradies *et al*., 2014).

Complex I, also known as NADH-ubiquinone oxidoreductase, is involved in electron transport from NADH to ubiquinone (Paradies *et al*., 2014). Complex I associates with CL (Chicco and Sparagna, 2006; Paradies *et al*., 2014). Complex I was inactivated upon CL loss in bovine heart, and was only restored upon exogenous addition of CL. 22% of CL loss have resulted in diminishing 15% of complex I activity in rat brains (Chicco and Sparagna, 2006; Paradies *et al*., 2014). CL oxidation by ROS have also been shown to inactivate complex I (Paradies *et al*., 2014). This effect was encountered upon the addition of exogenous CL (Paradies *et al*., 2014). We found that acute administration of 1 μM CL have significantly decreased the mitochondrial respiration carried by complex I. These results were intensified upon the administration of 10 μM CL, suggesting a dose-effect (unpublished data).

Complex II, also known as succinate dehydrogenase, oxidized succinate from the krebs cycle and provides electrons to the electron transport chain (Paradies *et al*., 2014). The presence of CL in the phospholipid environment of complex II is required for the stability and enzymatic activity for the complex (Paradies *et al*., 2014). CL presence around complex II seems to restrict ROS production, even though this remains unclear (Paradies *et al*., 2014).

CL stabilizes the quaternary structure of complex III, also known as ubiquinol-cytochrome c oxido-reductase (Haines and Dencher, 2002; Chicco and Sparagna, 2006; Paradies *et al*., 2014). Multiple CL molecules bind to complex III to stabilize and maintain its enzymatic activity (Haines and Dencher, 2002; Paradies *et al*., 2014). In the absence of CL, the complex was destabilized and its enzymatic activity was significantly diminished (Chicco and Sparagna, 2006; Paradies *et al*., 2014). CL oxidation by ROS also been inactivates complex III (Paradies *et al*., 2014). Complex III full activity and stability was rescued only upon the administration of CL, signifying the importance of CL and its association to complex III (Paradies *et al*., 2014).

Complex IV, also known as cytochrome c oxidase, tightly binds to three CL molecules through CL-specific binding sites (Paradies *et al*., 2014). Complex IV was also found to be stabilized by and associated with unsaturated CL molecules for its optimal enzymatic activity (Haines and Dencher, 2002; Chicco and Sparagna, 2006; Paradies *et al*., 2014). Absence of CL has been shown to destabilize complex IV and reduced its activity (Paradies *et al*., 2014). CL peroxidation by ROS also inactivates complex IV (Paradies *et al*., 2014). Its activity was rescued upon the addition of exogenous CL (Paradies *et al*., 2014).

Complex V, also referred to as $F_0F_1$ ATP-synthase, uses proton gradient-derived energy to phosphorylate ADP into ATP (Paradies *et al*., 2014). In complex V, at least four sites were found to bind exclusively to CL with high affinity (Chicco and Sparagna, 2006; Paradies *et al*., 2014). Even though the exact contribution of CL to complex V is not yet well studied, but it is evident that CL supports the enzymatic activity of complex V (Haines and Dencher, 2002). The removal of phospholipids surrounding complex V have significantly diminished its activity, and the restoration of complex V activity was more effective upon the addition of CL compared to other phospholipids (Paradies *et al*., 2014).

It has also been shown that CL is associated with numerous other proteins and that it is required for their optimal enzymatic activities (Paradies *et al*., 2014). These proteins include: mitochondrial glycerol-3-phosphate dehydrogenase (Eble *et al*., 1989; Chicco and Sparagna, 2006), the phosphate transporter (Chicco and Sparagna, 2006; Paradies *et al*., 2014), ADP/ATP carrier protein (Beyer and Klingenberg, 1985; Chicco and Sparagna, 2006; Haines and Dencher, 2002; Paradies *et al*., 2014; Ren *et al*., 2014), mitochondrial creatine kinase, carbamoyl phosphate synthetase I, carnitine/acylcarnitine carrier, nucleoside diphosphate kinase, phosphate

transporter, pyruvate transporter, tricarboxylate carrier (Chicco and Sparagna, 2006; Paradies *et al*., 2014). The association strength between CL and these proteins as well as the binding sites have not yet been determined (Eble *et al*., 1989; Chicco and Sparagna, 2006; Paradies *et al*., 2014).

### *Proton trap: involved in energy metabolism*

The exclusive presence of CL in oxidative phosphorylation molecules, in mitochondria and bacteria, suggest its importance in generating ATP (Haines and Dencher, 2002; Claypool and Koehler, 2012). Indeed, CL has been reported to serve as a proton trap (Haines and Dencher, 2002).

The central glycerol molecule in the CL head group has a free hydroxyl group, which forms an internal hydrogen bond with a negatively charged oxygen from one of the two phosphate groups. This binding creates a tight bicyclic structure that can trap one proton (Haines and Dencher, 2002). Mobile CL head group domain laterally shuttles the proton it had trapped from complex I, III, and IV to ATP synthase (Haines and Dencher, 2002; Paradies *et al*., 2014). CL head group domain also buffers the change in the acidity across the inner membrane while pumping the protons from the matrix to the intermembrane space as CL has a pK2 value above physiological conditions (Haines and Dencher, 2002). This allows for the change in potential across the inner membrane while buffering the change in pH across the inner membrane (Haines and Dencher, 2002). Recent work argues that CL PK values are below physiological pH (Kooijman *et al*., 2017). So CL could probably trap two H+ instead of one.

### *Mitochondrial permeability transition pore*

Oxidative stress and elevated levels of intra-mitochondrial $Ca^{2+}$ triggers sudden increase in the permeability of the inner mitochondrial membrane through the formation of mitochondrial permeability transition pore (MPTP), which consists of multiple proteins including the ADP/ATP carriers (Paradies *et al*., 2014). These pores increase the permeability of solutes below 1.5 kDa, and diminish the ion gradient across the inner membrane. This affects the inner membrane depolarization, and so the oxidative phosphorylation efficiencies, affecting the cell's bioenergetics and viability (Paradies *et al*., 2014).

Exogenous addition of oxidized CL has been shown to induce the opening of the MPTP by enhancing $Ca^{2+}$-sensitivity in the mitochondria (Paradies *et al*., 2014). The mechanism is not very understood, but it was proposed that oxidized CL interacts with MPTP, inducing their opening (Paradies *et al*., 2014). Three CL bind to each ADP/ATP carrier protein, and is required for the carrier's stability and function (Paradies *et al*., 2014). When CL is oxidized in the presence of $Ca^{2+}$, ADP/ATP carriers are destabilized and their conformation is changed (Paradies *et al*., 2014). This favors MPTP opening and cytochrome c release, which triggers apoptosis and promots aging, myocardial ischemia, as well as other diseases (Paradies *et al*., 2014).

## Cardiolipin-Associated Diseases

Given the importance of CL in the mitochondria and the impact it has on the cell, it comes to no surprise that an alteration in CL composition or abundance disturbs cellular metabolism, and is related to many dysfunctions and diseases (Chicco and Sparagna, 2006; Paradies *et al*., 2014). There are three main pathological alterations of CL that are observed in disease conditions: Loss of CL content, change in CL composition, and CL peroxidation (Chicco and Sparagna, 2006) – all of which are associated with mitochondrial dysfunction and pathologies (Paradies *et al*., 2014).

Several ways can reduce CL content in the mitochondria, including: stimulation of CL degradation process, upregulation of ALCAT1 activity, disruption of the Ups1-Mdm35 dissociation mechanism, impaired CL-biosynthesis enzymes, and reduction in CL-biosynthesis precursors (Chicco and Sparagna, 2006; Claypool and Koehler, 2012).

It is not exactly known why does CL acyl content varies among different cells and organisms, but it seems that this variation offers an important biological need to its respective cell (Claypool and Koehler, 2012). In the heart, CL acyl chains are primarily composed of linoleic acid (18:2), and an alteration in the composition of these chains seem to compromise the function of CL (Chicco and Sparagna, 2006; Zhong *et al*., 2014). A decrease in linoleic acid  CL bioavailability can be due to a dysfunction of the CL remodeling enzymes (Chicco and Sparagna, 2006). A decrease in the dietary intake of linoleic acid could also account for a decrease in linoleic acid CL bioavailability, affecting CL function in the heart (Chicco and Sparagna, 2006).

Due to the polyunsaturated acyl chains in CL, CL is susceptible to peroxidation by ROS (Chicco and Sparagna, 2006). Peroxidation affects CL integrity and functions, making it more susceptible to degradation by phospholipases, which decreases the CL pool and triggers cell apoptosis (Chicco and Sparagna, 2006; Claypool and Koehler, 2012; Zhong *et al*., 2014).

### *Barth syndrome*

Barth syndrome is an X-linked disease, and is characterized by a myopathic cardiac and skeletal mitochondria that can be lethal (Chicco and Sparagna, 2006; Claypool and Koehler, 2012). Barth syndrome is associated with a mutation in the tafazzin gene, which is essential for CL remodeling and maturation (Chicco and Sparagna, 2006). This causes Barth syndrome patients to have reduced amounts of the mature tetra-linoleoyl CL species, and have high monolysocardiolipin and immature CL levels relative to the total CL pool, which is also reduced (Chicco and Sparagna, 2006; Claypool and Koehler, 2012). Dysfunctional CL results in an abnormal mitochondrial ultrastructure (Claypool and Koehler, 2012). Additionally, yeast cells with tafazzin deletion have accumulated oxidatively damaged proteins, which indicates an increase in ROS production by the mitochondria (Claypool and Koehler, 2012). However, it remains under debate to whether CL dysfunction causes excessive ROS production or vice versa (Claypool and Koehler, 2012). It is possible that decreasing unsaturated CL is a defense mechanism against cellular apoptosis since polyunsaturated CL are more prone to peroxidation by ROS.

Exogenous addition of 18:2 CL to fibroblasts from Barth syndrome patients have rescued the CL pool in a dose and time dependent manner, proposing a potential treatment for Barth syndrome (Chicco and Sparagna, 2006). Mitochondrial function was not tested upon the administration of CL, so further research is required (Chicco and Sparagna, 2006).

### *Ischemia and Reperfusion*

It is well-established that ischemia and reperfusion (I/R) causes oxidative injury by ROS, which leads to phospholipid peroxidation (Chicco and Sparagna, 2006). Peroxidized phospholipids are hard to detect, and are more susceptible to hydrolysis by phospholipases, eventually this causes their loss (Chicco and Sparagna, 2006). I/R results in phospholipids loss from cardiomyocytes (Chicco and Sparagna, 2006). Interestingly, CL loss appear to exceed the loss of other

phospholipids from these cardiomyocytes (Lesnefsky *et al*., 2001; Chicco and Sparagna, 2006; Paradies *et al*., 2014). CL was lost from the mitochondria of the subsarcolemma, which are located beneath the interfibrillar and plasma membrane, but not from the interfibers, which are located between the myofibrils (Lesnefsky *et al*., 2001; Chicco and Sparagna, 2006). CL loss had a drastic effect on mitochondrial respiration (Lesnefsky *et al*., 2001; Chicco and Sparagna, 2006), especially on the function of complex IV – even though complex VI abundance remained unchanged (Chicco and Sparagna, 2006). Restricting mitochondrial ROS production in heart and skeletal muscles have restored CL levels after I/R, offering a potential therapeutic (Chicco and Sparagna, 2006).

The CL tetra-linoleoyl composition was unaffected by I/R (Lesnefsky *et al*., 2001; Chicco and Sparagna, 2006). CL biosynthesis was also affected by I/R, as CDP-DAG formation was interrupted (Chicco and Sparagna, 2006).

### *Heart Failure*

A loss in CL pool, 18:2 CL content, and a decrease in CL biosynthesis were observed during heart failure (Chicco and Sparagna, 2006; Sparagna and Lesnefsky, 2009). Data has suggested that a change in CL composition occurred as a response to heart failure in both, interfibrillar and subsarcolemmal mitochondrial (Chicco and Sparagna, 2006). Further, a group of scientists found that CL tetra-linoleoyl content and oxidative phosphorylation activity had decreased a few months before developing heart failure in rats. This raises the question to whether loss of 18:2 CL content is a consequence or a cause of heart failure. Contrary, hypertension seemed to increase the total CL content relative to other phospholipids in rats (Chicco and Sparagna, 2006).

### *Atherosclerosis*

CL has been found to affect aortic cells such as endothelial cells (Carnevale and Bergdahl, 2015) and vascular smooth muscle cells. Endothelial cells are involved in a physiological process called angiogenesis, which forms new capillaries from pre-existing blood vessels. Angiogenesis is important during ischemic conditions to promote healing (Carmeliet, 2003). This process was significantly suppressed by the addition of physiological CL concentrations in a dose-dependent manner (Cernevale and Bergdahl, 2015). This indicates that CL has anti-proliferative properties

on vascular endothelial cells, and that CL disrupts endothelial cells migration and degradation of the basement membrane (Carnevale and Bergdahl, 2015).

While the main function of vascular smooth muscle cells is to contract in the blood vessels, they possess the ability to switch into a proliferative, migratory phenotype that contributes to atherosclerosis development as a response to environmental cues. This phenotypic switching from the contractile phase to the migratory and proliferative phase is a hallmark in atherosclerosis progression and persistence. Recent studies have shown that the migration of vascular smooth muscle cells was hindered in the presence of CL, while no effect was observed in their proliferation in the presence of CL (unpublished data). This indicates that CL affects the early stages of the vascular smooth muscle cells phenotypic switching since migration occurs at an early stage compared to proliferation (Louis and Zahradka, 2010). Taken together, it seems that CL could potentially play a protective role against atherosclerosis development since it diminishes vascular smooth muscle cells migration, preventing the advancement of the disease. CL could also play a role in energy preservation, since it blocks angiogenesis, a process that would have little significance in the absence of atherosclerosis that contributes to ischemia.

### *Thyroidism*

Thyroid hormones promote mitochondrial respiration, and lipid metabolism (Chicco and Sparagna, 2006). They also stimulate CL synthase, PG synthase, and MLCLAT (Chicco and Sparagna, 2006). Thus, hyperthyroidism increases the total CL pool content and the activity of the oxidative phosphorylation complexes, whereas CL content and complexes activity were reduced in hypothyroidism (Chicco and Sparagna, 2006). Whether an alteration in thyroid activity affects CL tetra-linoleoyl content remains on debate (Chicco and Sparagna, 2006).

CL content and the complexes activities were replenished in hypothyrodism-induced rat models upon the administration of thyroid hormone (Chicco and Sparagna, 2006). This supports the importance of the thyroid hormone for CL metabolism and mitochondrial bioenergetics, and provides potential therapeutics for hypothyroidism (Chicco and Sparagna, 2006).

### *Neurodegenerative Disease*

Free radical stress in rats initiate phospholipid peroxidation. Phospholipid peroxidation results in CL loss in aging brains, and is associated with mitochondrial dysfunction and neuronal loss in

the substantia nigra. This neuronal loss contributes to neurological diseases such as Parkinson's disease (Chicco and Sparagna, 2006). Parkinson's disease is associated to mutations in the α-synuclein-coding gene (Chicco and Sparagna, 2006), and the lack of α-synuclein has been associated with 22% reduction of the total CL pool in brain, a reduction in CL containing polyunsaturated acyl chains, reduction in a CL-biosynthesis precursor, and an increase in CL containing saturated acyl chains. No change was observed in the brain content for other phospholipids (Chicco and Sparagna, 2006). Further, complex I and III activities were reduced by 15% as a response to these changes in CL content and composition (Chicco and Sparagna, 2006).

### *Diabetes*

CL studies on diabetic rat models is inconclusive (Chicco and Sparagna, 2006). In type I diabetic rat models, CL content seemed to have decreased in the brain, unaffected in the heart, and increased in the liver (Chicco and Sparagna, 2006). CL biosynthesis and remodeling enzymes were unaffected in the heart (Chicco and Sparagna, 2006). So, although it seems that diabetes might not have an effect on CL in the heart, other organs that are influenced by diabetes should be investigated for their CL content and composition (Chicco and Sparagna, 2006).

### *Lung injury in pneumonia*

CL is present in alveolar surfactant, and it covers up to 1-2% of its surface. Low amounts of CL are found in healthy lungs' fluid, while CL level is high in injured and pneumonic lungs (Ray *et al*., 2010). Increased CL concentration has been suggested to be directly linked to ATP8b1 mutations, which binds and translocates CL from the outer to the inner leaflet of membrane bilayers (Ray *et al*., 2010). In the lung fluid, CL decreases epithelial cell viability (Ray *et al*., 2010). CL also inhibits the surfactant even at low concentrations (~2 mol%) by impairing the major surfactant phospholipid, dipalmitoylphosphatidylcholine, disrupting the lung structure and function (Ray *et al*., 2010). This leads to high surface-tension pulmonary edema and makes the lungs more prone to infections by bacteria (Ray *et al*., 2010). It has also been proposed that bacteria might degrade Atp8b1 transporters, disrupt the catalytic function of Atp8b1, or mask the CL-binding sites of Atp8b1 (Ray *et al*., 2010).

### *Aging*

Aging relates to hydrogen peroxide production in rat hearts (Chicco and Sparagna, 2006). This oxidative stress causes CL peroxidation and a decline in the CL content, contributing to the decrease in mitochondrial activity and dysfunction observed in aging (Chicco and Sparagna, 2006). Loss of 18:2 CL and an increase in other polyunsaturated acyl chains were also observed in aging rodent heart and liver (Chicco and Sparagna, 2006). This makes CL more susceptible to peroxidation and hydrolysis by phospholipases (Chicco and Sparagna, 2006). In age-dependent CL loss experiments, it was revealed that CL loss correlates with a reduction in the activity of adenine nucleotide transporters, pyruvate carrier, phosphate transporter, carnitine-acylcarnitine transporter, and complex IV (Chicco and Sparagna, 2006).

### *Dietary 18:2 Deficiency*

Dietary fatty acids was found to influence CL acyl chain composition in the heart, kidney, liver, and brain within hours of consuming the fatty acids (Chicco and Sparagna, 2006). 18:2 CL content and mitochondrial respiration in the heart were drastically affected proceeding an 18:2-deficient intake for a month, while 18:2-CoA supplements have restored CL biosynthesis and remodeling (Chicco and Sparagna, 2006). Altering dietary fatty acid content provides a tool to study the influence of various CL compositions on mitochondrial proteins and activity (Chicco and Sparagna, 2006).

### *Chronic Ethanol Consumption*

Chronic ethanol consumption has been shown to reduce tetra-lineoyl CL level in liver mitochondria (Chicco and Sparagna, 2006). This might be through disturbing CL remodeling or CL precursors availability, even though the mechanism has not yet been confirmed, and further investigation is required (Chicco and Sparagna, 2006).