

**Choline Transport and Metabolism in Genetically Deficient and Chronic
Disease States**

by

Laila Cigana Schenkel

**A Thesis
presented to
The University of Guelph**

**In partial fulfilment of requirements
for the degree of
Doctor of Philosophy
in
Human Health and Nutritional Sciences**

Guelph, Ontario, Canada

© Laila Cigana Schenkel, August, 2014

ABSTRACT

CHOLINE TRANSPORT AND METABOLISM IN GENETICALLY DEFICIENT AND CHRONIC DISEASE STATES

Laila Cigana Schenkel

University of Guelph, 2014

Advisor:

Marica Bakovic

Choline is required for the biosynthesis of phosphatidylcholine (PC) by the CDP-choline Kennedy pathway and of betaine. Choline also plays a role in lipid metabolism and hepatic and muscle function. The availability of intracellular choline is regulated by the choline transporter CTL1/SLC44A1 at the plasma membrane. This thesis aims to elucidate the effect of metabolic altered states, such as lipid overload, choline deficiency and CDP:phosphoethanolamine cytidyltransferase (Pcyt2) genetic modified models, on the choline transport and metabolism.

First, we investigated the effect of high fatty acid supply in C2C12 muscle cells. Palmitic acid (PAM) reduced total and plasma membrane CTL1/SLC44A1 protein by activating lysosomal degradation, and limited the choline uptake while increasing diacylglycerol (DAG) and triacylglycerol (TAG) synthesis. Oleic acid (OLA) maintained total and plasma membrane CTL1/SLC44A1, increasing PC and TAG synthesis more than PAM, which offers a protection mechanism from the excess of intracellular DAG and autophagy.

We next characterized the choline metabolic defect in fibroblast cells isolated from a Postural Tachycardia Syndrome (POTS) patient, who had low plasma choline. In the POTS fibroblasts, the CTL1/SLC44A1 expression and choline uptake were decreased. PC synthesis and the phospholipid pool were reduced these cells compared to control. Triacylglycerol formation

increased 50% in POTS, which is a feature of choline deficiency. The characteristics of the POTS fibroblasts represent a model of choline transport dysfunction.

The role of choline in protecting against lipid accumulation and metabolic disease development was elucidated by choline supplementation of Pcyt2^{+/-} mice. In Pcyt2^{+/-} mice the CDP-ethanolamine pathway is downregulated and the TAG formation increased, resulting in adult-onset obesity and liver steatosis. Choline supplementation reverses the Pcyt2^{+/-} phenotype by facilitating the metabolic flux through the CDP-choline pathway, reducing TAG synthesis and increasing expression of genes involved in TAG and DAG degradation.

The cross-regulation between CDP-choline and CDP-ethanolamine pathways was also demonstrated in Pcyt2 siRNA knock-down human fibroblasts (KD5), where the rate of choline uptake and PC synthesis were higher. Altogether, this thesis established the metabolic links between CDP-choline and CDP-ethanolamine pathways and provided a mechanism of how choline could be an important modifier of lipid metabolism under various conditions.

ACKNOWLEDGEMENTS

First I would like to thank my advisor Dr Marica Bakovic for the opportunity to work in her Lab and for her guidance, patience and friendship. I appreciate your support and willingness to teach over the years.

I further like to thank my advisory committee, Dr Graham Holloway and Dr Fred Brauer for their guidance during this process.

The collaboration of various laboratories made this thesis possible. Thank to Linda Grocock for helping with the mice work. Thank to Dr Steven Zeisel, Dr Kerry-Ann da Costa, Dr Amy Johnson and Dr Harvey Mudd for the collaboration on the POTS project and to Genevieve Anna Tyrrell for the inspiration. Thank to Michael Leadley and the Analytical Facility for Bioactive Molecules at the Sick Kids Centre and to Audric Moses and the Women and Children's Health Research Institute at the University of Alberta in assisting with the spectroscopy analysis. Also, thank to Junzeng Zhang, Institute for Nutrisciences and Health National Research Council Canada, for the NMR analysis.

To my friends in the Bakovic lab, thank you for the help and the good times. Specifically, to Dr Ratnesh Singh, Zvezdan Pavlovic, Poulami Basu and Sugan Sivanesan.

Lastly, thank to my family, who supported my decision to move so far away to do my PhD and who always gives me strength to succeed. And thanks to destiny for showing my path and bringing my love.

TABLE OF CONTENTES

ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTES	v
LIST OF TABLES	ix
LIST OF FIGURES.....	x
ABREVIATIONS.....	xii
1 INTRODUCTION	1
1.1 Choline: an essential nutrient	1
1.2 Absorption and transport of choline.....	3
1.3 Betaine production and methylation pathway	6
1.4 The membrane phospholipids	9
1.4.1 Phospholipids in the plasma membrane.....	9
1.4.2 Mitochondrial phospholipids and their role in bioenergetics	10
1.4.3 The Kennedy pathway and the biosynthesis of the major membrane phospholipids in the ER	13
1.4.4 The biosynthesis of mitochondrial phospholipids	18
1.4.5 Remodeling of phospholipids and the role of dietary lipids	20
1.4.6 Degradation of phospholipids	23
2 Rationale for the studies.....	26
3 Palmitic acid and oleic acid differentially regulate choline transporter-like 1 levels and glycerolipid metabolism in skeletal muscle cells	29
3.1 Introduction	30
3.2 Materials and Methods	32
3.2.1 Cell culture and treatments	32
3.2.2 Cell viability.....	32
3.2.3 RT-PCR.....	33
3.2.4 Cell lysates and subcellular fractionation	34
3.2.5 Immunoblotting.....	34
3.2.6 Assays for protein degradation and autophagy	36
3.2.7 Choline uptake	36
3.2.8 Radiolabeling of the CDP-choline pathway with [³ H-methyl]choline	37
3.2.9 Choline efflux	38
3.2.10 Radiolabeling of DAG and TAG with [³ H]glycerol	38

3.2.11	Measurement of total lipids	39
3.2.12	Mitochondrial potential.....	39
3.2.13	Statistical analysis.....	40
3.3	Results	40
3.3.1	Differential effects of OLA and PAM on cell growth and CTL1/SLC44A1 protein content and localization	40
3.3.2	Mechanisms for reduction of total CTL1/SLC44A1 protein content by PAM	45
3.3.3	OLA and PAM alter the cell surface choline uptake differently.....	46
3.3.4	OLA and PAM both reduce the mitochondrial choline uptake	48
3.3.5	OLA stimulates the CDP-choline pathway but not PC levels	48
3.3.6	PAM and OLA differently modulate TAG and DAG synthesis and content	51
3.3.7	Lipid content in untreated and treated myotubes	52
3.4	Discussion	54
4	Mechanism of choline deficiency and membrane alteration in postural tachycardia syndrome primary skin fibroblasts.....	60
4.1	Introduction	61
4.2	Materials and methods	63
4.2.1	Blood metabolites	63
4.2.2	Isolation and maintenance of primary POTS cells	64
4.2.3	Cell treatments with excess choline.....	65
4.2.4	Immunoblotting.....	65
4.2.5	RNA isolation and RT-PCR.....	66
4.2.6	Isolation of mitochondria.....	66
4.2.7	Choline uptake in whole cells	67
4.2.8	Mitochondrial membrane potential and mtDNA content	67
4.2.9	Pulse and pulse-chase radiolabeling of the CDP-choline pathway.....	68
4.2.10	Determination of total glycerolipid pools.....	69
4.2.11	Lipidomic analysis	69
4.2.12	Statistical analysis	70
4.3	Results	70
4.3.1	Blood metabolites are altered in POTS patient.....	70
4.3.2	CTL1/SLC44A1 transporter and choline transport are reduced in POTS	72
4.3.3	Phospholipid and triglyceride pools were modified in POTS cells	74
4.3.4	PC synthesis is reduced in POTS cells	78
4.3.5	PC degradation was increased in POTS cells	79

4.3.6	Lipidomic analysis	81
4.3.7	Mitochondrial function is impaired in POTS cells	86
4.4	Discussion	88
5	Choline supplementation restores substrate balance and alleviates complications of Pcyt2 deficiency	94
5.1	Introduction	95
5.2	Methods	98
5.2.1	Animals	98
5.2.2	Genotyping	98
5.2.3	Choline treatments	99
5.2.4	In-vivo radiolabeling of the CDP-choline pathway	100
5.2.5	Immunoblotting	100
5.2.6	Gene regulation by choline treatment	101
5.2.7	Measurement of mitochondrial membrane potential	101
5.2.8	Plasma metabolomic analysis	102
5.2.9	Statistical analysis	103
5.3	Results	104
5.3.1	Choline supplementation prevents weight gain and alleviates liver steatosis	104
5.3.2	Choline upregulates CDP-choline pathway for PC synthesis	105
5.3.3	Choline does not modify expression of most phospholipid genes	107
5.3.4	Choline increases expression of triglyceride and fatty acid metabolic genes	109
5.3.5	Amino acid and acylcarnitine profiling	112
5.3.6	Metabolomic analysis by NMR	114
5.4	Discussion	119
5.4.1	Metabolic changes by choline supplementation	120
5.4.2	Metabolomic analysis	121
5.5	Conclusion	124
6	Characterization of the CDP-choline pathway in Pcyt2 deficient cells	125
6.1	Introduction	126
6.2	Methods	127
6.2.1	Pcyt2 Knock-down cells	127
6.2.2	Radiolabeling of the CDP-choline pathway with [³ H-methyl]choline	128
6.2.3	Pool sizes of TAG, PC and PE	129
6.2.4	Choline uptake	129
6.2.5	Expression of genes in the PC and PS metabolic pathway	129

6.3	Results	130
6.3.1	Choline uptake and the CDP-choline pathway are up-regulated in Pcyt2 knock-down cells	130
6.3.2	Pool of glycerolipids is maintained in KD5 cells	133
6.3.3	Expression of genes in the choline pathways	134
6.4	Discussion	137
7	Integrative discussion.....	139
7.1	Regulation of choline transport and metabolism with fatty acids.....	139
7.2	Choline transporter-like 1 protein in the mitochondria.....	141
7.3	Consequences of choline transporter deficiency.....	143
7.4	Choline treatment and regulation of lipid metabolism.....	144
7.5	Integration of CDP-choline and CDP-ethanolamine pathways.....	145
7.6	Conclusion and future directions	147
	REFERENCES	150

LIST OF TABLES

Table 4. 1: Metabolites measured from the POTS patient.....	92
Table 4. 2: Relative amount of individual fatty acids identified by LC/MS/MS in POTS and control fibroblasts..	93
Table 5. 1: Primer sequences, fragment size and melting temperature (TM) for reverse transcription polymerase chain reaction (RT-PCR)	99
Table 5. 2: Plasma concentration of metabolites identified by ¹ H-NMR.....	119
Table 6. 1: Genes and respective primers used for RT-PCR in KD5 and MCH58 cells	130

LIST OF FIGURES

Figure 1. 1: Betaine-homocysteine-methyltransferase (BHMT) metabolic pathway.....	8
Figure 1. 2: Structural formulas of key phospholipids.	17
Figure 1. 3: Pathways for the synthesis of phospholipids and their interconnection.....	18
Figure 1. 4: The major catabolic pathways for PC.	25
Figure 3. 1: PAM and OLA treatments did not reduce the cell viability and CTL1/SLC44A1 mRNA levels.....	42
Figure 3. 2: : FA treatments differently affected the CTL1/SLC44A1 protein content and distribution.	44
Figure 3. 3: Mechanism of the CTL1/SLC44A1 protein degradation by PAM.	46
Figure 3. 4: Different effects of PAM and OLA on choline uptake..	47
Figure 3. 5: The effect of fatty acid treatments on the rate of PC synthesis and degradation..	50
Figure 3. 6: The effect of FA on TAG and DAG turnover.	52
Figure 3. 7: Total glycerolipid content after PAM and OLA treatments.....	54
Figure 4. 1: CTL1 protein and mRNA expression are down-regulated in POTS fibroblasts.....	73
Figure 4. 2: Choline uptake is affected in POTS..	74
Figure 4. 3: Phospholipid content is altered in POTS.....	76
Figure 4. 4: TAG and DAG are altered in POTS.....	77
Figure 4. 5: : PC synthesis is downregulated in POTS fibroblasts.	79
Figure 4. 6: The degradation of PC and Kennedy pathway intermediates is faster in POTS.....	80
Figure 4. 7: Glycerolipid molecular species composition analysed by LC/MS/MS.....	86
Figure 4. 8: Mitochondrial capacity is reduced in POTS fibroblasts.....	87
Figure 5. 1: Choline reduces weight gain in Pcyt2 HET mice.....	104

Figure 5. 2: In-vivo labeling of male mice with [C14]-Oleic acid and [Methyl-3H] choline chloride..	106
Figure 5. 3 mRNA expression of phospholipid metabolism genes analyzed by RT-PCR.	108
Figure 5. 4: HET mice had lower CTL1 protein content.	109
Figure 5. 5: mRNA expression of lipid metabolism genes analyzed by RT-PCR.....	110
Figure 5. 6: Mitochondrial potential was increased in HET+choline mice..	111
Figure 5. 7: Amino acid and acylcarnitine analysis in blood.....	114
Figure 5. 8 Metabolomic analysis by ¹ H-NMR spectra.....	118
Figure 6. 1: Choline uptake is increased in KD5 cells.....	131
Figure 6. 2: PC synthesis is upregulated in KD5 cells.....	132
Figure 6. 3: CDP-choline intermediates content in Pcyt2 Knock-down cells..	133
Figure 6. 4 : Pool of glycerolipids in Pcyt2 Knock-down cells.	134
Figure 6. 5: mRNA expression of phospholipid metabolic genes analyzed by RT-PCR.....	136

ABREVIATIONS

AdoHcy	S-adenosylhomocysteine
AdoMet	S-adenosylmethionine
BHMT	Betaine homocysteine methyltransferase
CDP-choline	Cytidine 5'-diphosphocholine
CCT/Pcyt1	CTP:phosphocholine cytidyltransferase
CL	Cardiolipin
CTL1/SLC44A1	Choline transporter-like protein 1
Ctrl	Control
DAG	Diacylglycerol
ECT/Pcyt2	CDP:phosphoethanolamine cytidyltransferases
FA	Fatty acid(s)
MM	Mitochondrial membrane
OLA	Oleic acid
PA	Phosphatidic acid
PAM	Palmitic acid
PM	Plasma membrane
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine-N-methyltransferase
PI	Phosphatidylinositol
PLD	Phospholipase D

POTS	Postural Tachycardia Syndrome
PS	Phosphatidylserine
SM	Sphingomyelin
TAG	Triacylglycerol
tHcy	Homocysteine

PUBLICATIONS

This thesis is based on the following publications

Schenkel LC, Bakovic M (2013). Formation and Regulation of Mitochondrial Membranes. *International Journal of Cell Biology* 2014:709828. DOI: 10.1155/2014/709828.

Schenkel LC, Bakovic M (2014). Palmitic acid and oleic acid differentially regulate choline transporter-like 1 levels and glycerolipid metabolism in skeletal muscle cells. *Lipids* DOI: 10.1007/s11745-014-3925-4.

Schenkel LC, Singh RK, Michel V, Zeisel SH, da Costa KA, Johnson AR, Mudd HS and Bakovic M (2014). Mechanism of choline deficiency and membrane alteration in postural tachycardia syndrome primary skin fibroblasts. Submitted to *The FASEB Journal*.

Schenkel LC, Sivanesana S, Wuyts B, Zhang J, Bakovic M (2014). Choline supplementation restores substrate balance and alleviates complications of Pcyt2 deficiency. Submitted to *J Nutr Biochem*.

1 INTRODUCTION

1.1 Choline: an essential nutrient

Choline is a quaternary amine (trimethyl- β -hydroxy-ethylammonium) that was discovered in 1860. Its significance as a nutrient was identified a half-century later, during studies related to the discovery of insulin by Banting and Best at the University of Toronto in 1921–1922 [1]. In this study, they used depancreatized dogs, which eventually developed fatty liver and died [2]. They observed that fatty liver could be corrected in these dogs by feeding with raw pancreas or lecithin (phosphatidylcholine). Later, Best and Huntsman (1932) confirmed choline as the factor that prevented the fatty liver and death of depancreatized dogs [3].

Choline is now considered an essential nutrient (needs can not be met by endogenous biosynthesis) for both animals and humans [3, 4]. The need for choline has been evidenced by several models. The phosphatidylethanolamine-N-methyltransferase Knock out (PEMT^{-/-}) mouse model, which lacks an important pathway for phosphatidylcholine (PC) synthesis, when fed a choline deficient diet, develop steatosis, steatohepatitis and die from liver failure after 3 days [5]. Healthy male humans fed a choline-deficient diet, with normal folate and vitamin B12 status, also develop liver damage [6]. A second human study showed that almost all men and post-menopausal women develop fatty liver, liver and/or muscle damage when fed a low choline diet and this was resolved when choline was restored in the diet [7].

Choline is found in a wide variety of foods, including liver, eggs, and wheat germ. Daily requirements for choline in humans are 550mg for men and 425mg for women. Based on

experimental data in humans, this amount was found to prevent liver damage and fatty liver associated with choline-deficient diets [8]. Recent studies reported that healthy men and women consumed amounts of choline that are at or slightly higher than the current daily requirements [9]. However, some individual subjects, especially women in reproductive age and pregnant and lactant women consumed slightly less [10, 11]. Although the majority of choline is obtained from diet, choline can be obtained endogenously by the *de novo* synthesis of phosphatidylcholine (PC) catalysed by phosphatidylethanolamine-N-methyltransferase (PEMT). However, a study by Zeisel showed that humans deprived of choline appear not to be able to make up this dietary deficit by the *de novo* synthetic pathway; therefore, choline is indeed an essential nutrient for humans [12].

Choline is metabolically involved in four pathways: 1) it is oxidized to betaine, a methyl group donor; 2) phosphorylated to phosphocholine, the first step of PC synthesis; 3) acetylated to the neurotransmitter acetylcholine and 4) participates in a base-exchange reaction that substitute the head groups of serine or ethanolamine for choline on endogenous phospholipids. The majority of choline (90%) is incorporated into PC through the Kennedy pathway. The PC Kennedy pathway and the choline-betaine pathway will be further elaborated in the following sections.

Besides being involved in these different physiologic roles, as a nutrient, choline has a significant impact in lipid transport, generation of second messengers and cell membrane signal transduction [13, 14]. During choline deficiency large amount of triacylglycerol (TAG) accumulates in the liver. In order to be exported from the liver, TAG is packed with PC into very low density lipoproteins (VLDL) [15]. Thus, choline supply for PC synthesis is required for VLDL secretion and choline (PC) deficiency reduces the plasma levels of VLDL [16]. In humans, inadequate intake of choline can result in fatty liver and liver damage [12, 17]. Plasma

choline concentrations are decreased in malnourished patients, at the same time that they develop liver dysfunction [18, 19]. When these patients were treated with an amino acid-glucose solution lacking choline, plasma choline concentrations in these patients declined further. However, when patients were treated with PC containing-lipid emulsion, their plasma choline concentrations rose slightly. Thus, conditions that enhance hepatic triglyceride synthesis (such as high-calorie total parenteral nutrition solutions) increase the requirement for liver PC [20] and in the case of malnourished patients choline stores are quickly depleted, which might cause hepatic dysfunction [12].

1.2 Absorption and transport of choline

The extent to which dietary choline is bioavailable depends upon the efficiency of its absorption from the intestine. Free choline from the diet is absorbed from the small intestine through transporter proteins. However, the majority of choline in the diet is available as phosphatidylcholine [4]. PC from the diet is mainly hydrolysed by pancreatic phospholipase A2 present in intestinal mucosas. Phospholipase A2 hydrolyses PC at the β -fatty acid moiety generating lysophosphatidylcholine (LPC) and free fatty acid [21, 22]. Within the gut mucosal cells, LPC can be further deacylated to form glycerophosphocholine (GPC), or it can be acylated to reconstitute PC [21, 22]. The PC enters the lymphatic circulation, and then enters the blood. Many tissues possess enzymes that are capable of degrading PC and LPC, releasing free-choline [23]. Thus, both blood choline and fatty acids increase after PC absorption from diet.

The transport of choline is an important aspect of choline metabolism [24]. Impaired choline transport has been linked to the pathogenesis of multiple diseases, including neurological,

muscular, and immunological disorders [25], as well as breast and ovarian cancer [26, 27]. As a positively charged quaternary amine, choline requires a protein-mediated mechanism to pass the plasma membrane. Using kinetic methods, different types of transport for choline have been established: a facilitated diffusion in red cells [28] and blood-brain barrier [29] and active transport systems in other tissues [30]. Three protein-mediated systems have been described for choline transport. First, the high-affinity choline transporter 1 (CHT1) provides choline for the synthesis of acetylcholine in cholinergic neurons [31]. Second, the low-affinity organic cation transporters (OCTs), which comprise 5 members (OCT 1-3, OCTN1 and OCTN2), transports endogenous organic cations and exogenous drugs. OCT1 and OCT2 are choline transporters in the liver, brain, kidney and intestine [31]. Third, the choline-specific transporter-like proteins comprise five genes (CTL1-CTL5). CTL1, now officially known as SLC44A1, is the main member of this family and was characterized as a medium affinity, specific, choline transporter that is ubiquitously expressed.

CTL1 was first cloned in 2000 by O'Regan et al. in *Torpedo marmorata* (fish) and rat [32]. The human form of CTL1 (hCTL1) was first cloned and identified as the antigen CDw92 located at chromosome 9q31.2 [33, 34]. Mouse CTL1 (mCTL1) was cloned and fully characterized by our group. mCTL1 gene is located at chromosome 4B2 [35].

The CTL1 membrane topology first established by our group showed that CTL1 incorporates into membranes anchored by N-terminus intracellularly and C-terminus extracellularly with 9 trans-membrane domains [36]. The removal of CTL1 from the membrane is regulated by protein kinase C (PKC)-mediated phosphorylation [37].

The functionality of CTL1 protein as a choline transporter has been characterized in several cell models. CTL1 was identified as a Na⁺-independent, saturable choline transport system in

primary rat astrocytes with an intermediate affinity for choline ($K_m = 37.7 \mu\text{M}$). Choline uptake in these cells was inhibited by HC-3, and to a lesser extent by OCT inhibitors [38]. In addition, knock-down of CTL1 impaired choline uptake, providing evidence for the main role of CTL1 in choline transport in rat astrocytes [38]. Overexpression of mCTL1 cDNA in Cos-7 cells increased the choline uptake into the cells [35]. hCTL1 was identified as the major choline transporter in human monocytes, and the transport of choline was regulated by trafficking of CTL1 to and from the plasma membrane [37].

The gene regulation of CTL1 has been studied by our group [33]. CTL1 expression was down-regulated during choline deficiency in mouse skeletal muscle cells (C2C12), resulting in intracellular TAG accumulation caused by a shifted balance of diacylglycerol (DAG) towards TAG synthesis instead of PC synthesis [39]. On the other hand, the increase in intracellular choline by an inhibitor of Hsp90, a molecular chaperone responsible for the folding and stability of multiple proteins, increased the CTL1 expression and PC turnover [40].

Although the major role of choline transporter is the uptake of choline from the extracellular space, choline transporters are also important for mitochondrial choline uptake for oxidation to betaine in liver and for choline storage. In the outer mitochondrial membrane, CTL1 was characterized as a choline transporter and it regulates the choline uptake into mitochondria [36]. CTL1 was identified in the membrane of mouse liver mitochondria, but also in mitochondria of non-betaine-producing C2C12 cells, mouse heart and MCF7 human breast epithelial cells, showing the presence of mitochondrial choline transport which is independent of betaine synthesis. CTL1 mediated choline transport into and out of mitochondria may provide a means of balancing the intracellular choline [36]. In addition, mitochondrial CTL1 and mitochondrial

choline transport were downregulated in choline-deficiency, indicating an adaptation of this transport system to reduce the pool of mitochondrial choline during choline deficiency [41].

A transporter in the inner mitochondrial membrane was also identified and has been shown to participate in choline oxidation to betaine; however its identity is still unknown [42]. CTL2 is also a choline transporter likely present in the mitochondria, but this is not firmly established [43].

1.3 Betaine production and methylation pathway

In the liver and kidney mitochondria, choline is oxidized to betaine in a two-step reaction catalyzed by choline dehydrogenase on the inner side of the inner mitochondrial membrane and by betaine aldehyde dehydrogenase in the mitochondrial matrix [44]. The availability of choline in the mitochondria, and thus the rate of choline oxidation, is controlled by the choline transport through mitochondrial membranes. Betaine, similar to methyltetrahydrofolate, is a methyl group donor that methylates homocysteine to methionine in a reaction catalysed by betaine-homocysteine-methyltransferase (BHMT) (Figure 1.1). As it donates a methyl group to homocysteine, betaine is converted into dimethylglycine (DMG). The transfer of adenosine to methionine then yields S-adenosylmethionine (AdoMet/SAM). AdoMet is the major methyl group (CH_3) donor in the cell. It methylates DNA by various DNA methyltransferases, and PE by PEMT to form PC.

Choline, betaine and folate are interchangeable sources of methyl groups. In individuals with folate deficiency, betaine is an important donor of methyl groups and a strong determinant of plasma homocysteine levels [45]. Elevated plasma homocysteine concentration, caused by

decreased methylation of homocysteine, is correlated with an increased risk of atherosclerotic vascular disease [46]. As expected, choline deficiency then could increase plasma homocysteine [46]. A choline deficient (CD) diet lowered methionine formation in animal livers by 20-25% and reduced liver AdoMet by 60% [47]. The effect of choline deficiency on lowering AdoMet is probably mediated by low choline oxidation into betaine as well as by increased AdoMet utilization in the PE methylation pathway to produce more choline via PC [48].

DNA methylation in the CpG islands is one of the epigenetic mechanisms to regulate gene expression. Choline deficient diet has been shown to alter global and gene-specific methylation in fetal brain [49, 50]. Animal models of diet-induced obesity accumulate fat in the liver associated with alterations in epigenetic profile. Dietary methyl-donor supplementation (including betaine, choline, vitamin B12 and folic acid) prevents diet-induced liver fat accumulation by affecting DNA methylation and gene expression [51]. Betaine supplementation in ApoE^{-/-} mice could reduce hepatic TAG accumulation and improved oxidative capacity by upregulating PPAR α gene expression, as a result of hypomethylation of its promoter [52]. Thus, choline and betaine may also ameliorate metabolic disease by regulating DNA methylation and specific gene expression through stimulation of BHMT pathway to produce AdoMet.

Betaine and choline are considered lipotropic compounds that can influence lipid metabolism in animals. It was suggested that betaine can improve energy metabolism probably by supporting the synthesis of carnitine, which is necessary for the transport of long-chain fatty acids to the mitochondria for oxidation [53]. Betaine has also a choline-sparing effect: betaine may decrease the demand for choline, thus increasing choline availability for lipid metabolism [54]. In a study in overweight men, plasma betaine and betaine to choline ratio was negatively associated with

metabolic stress, suggesting that betaine supplementation could ameliorate fatty liver and metabolic disease [55].

Besides its role as a methyl group donor, betaine is an important organic osmolyte that helps to maintain cell volume and hydration of cellular proteins in the kidney [56, 57]. High sodium chloride intake decreased the expression of BHMT in kidney and liver [58]. Thus osmoregulation of BHMT may control the portioning of betaine between its use as methyl donor and its accumulation as osmoprotectant [59]. However, betaine synthesis from choline is not affected by hypertonicity, but seems to be controlled by choline transport into the mitochondria [60, 61].

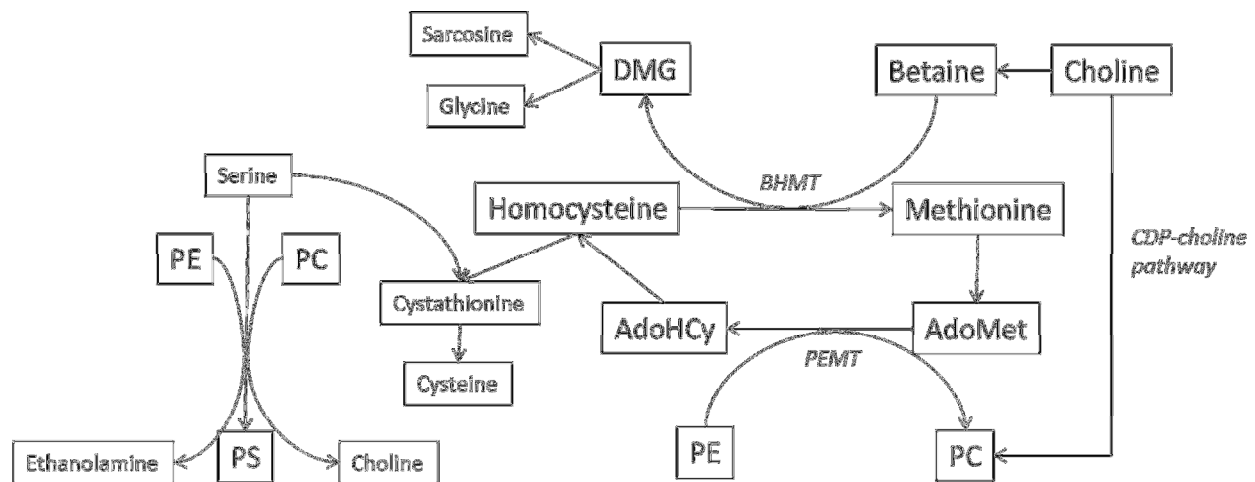


Figure 1. 1: Metabolic links between betaine-homocysteine-methyltransferase (BHMT) pathway, choline and phospholipids metabolism. Choline enters the CDP-choline pathway to synthesize phosphatidylcholine (PC) or is oxidised to betaine, which donates methyl group to homocysteine to produce methionine and yield dimethylglycine (DMG). Methionine generates AdoMet, which donates 3 methyl groups for PEMT reaction to produce PC from phosphatidylethanolamine (PE). Serine is a precursor for phosphatidylserine (PS) synthesis by base-exchange reaction with PC and PE, but also participates on the synthesis of cystathionine, a metabolite of homocysteine.

1.4 The membrane phospholipids

The next sections are adapted from the review published in the International Journal of Cell Biology: “Formation and Regulation of Mitochondrial Membranes”

1.4.1 Phospholipids in the plasma membrane

The matrix of cellular membranes is formed by polar lipids, which consist of a hydrophobic and a hydrophilic portion. The amphipathic property of lipids enables cells to segregate their internal constituents from the external environment [62]. The same principle is applied within the cells to produce organelles. In addition to the barrier function, lipids provide membranes with the potential for tubulation, protein binding, fission and fusion, and also can act as second messengers. Altogether, these characteristics show that membrane lipids are essential for cell division, biological reproduction, signal transduction and intracellular membrane trafficking [62].

As one of the major classes of lipids in the lipid bilayer of cell and organelle membranes, phospholipids (PL) are traditionally known for maintaining both the structural integrity of a cell and spatial separation of subcellular compartments. More recent research have shown that phospholipid metabolism is involved in a wide range of functions, including the generation of signaling molecules and second messengers [63]. The major classes of phospholipids found in the membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA). PC accounts for >50% of the phospholipids in most eukaryotic membranes [62]. The plasma membrane also

contains high amounts of sterols and sphingolipids (sphingomyelin-SM), which give resistance to mechanical stress [62]. Phospholipids are distributed asymmetrically across the bilayer leaflet resulting in an enrichment of PC and SM in the outer leaflet and of PE and PS in the cytoplasmic leaflet. The lipid asymmetry provides the two sides of the plasma membrane with different biophysical properties and influences numerous cellular functions, such as cell fusion and programmed cell clearance [64].

The structure of all phospholipids, except sphingomyelin, contains a diglyceride (two hydrocarbon chain), a phosphate group, and a simple organic molecule as a head group (Figure 1.2). Each phospholipid class consist of many molecular species that have the same head group but differ in the acyl chain [65]. The length of the hydrocarbon chain varies from 14 to 24 carbons, with the one in *sn-1* being typically a saturated or monounsaturated alkyl and the one in *sn-2* a polyunsaturated alkyl [65].

Mammalian cells must maintain the concentration of their phospholipids within narrow limits. An alteration in the phospholipid composition by genetic disruption of key enzymes in the PL synthesis can compromise vital functions in mice [66]. In addition, changes in membrane fluidity, caused by alteration in the lipid composition, have been associated with liver disease [67]. Owen *et al.* demonstrated that abnormal lipoproteins in patients with liver disease are associated with change in erythrocyte membrane PC and cholesterol composition. Therefore, it is important to have equilibrium in the synthesis, degradation and trafficking of phospholipids to maintain homeostasis of the membranes and cell function.

1.4.2 Mitochondrial phospholipids and their role in bioenergetics

Mitochondria have a structure distinct from that of other organelles since they contain two membranes: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), which separates the intermembrane space (IMS) from the matrix. The composition of the mitochondrial membranes is similar to that of other membranes, however. The major phospholipids in the mitochondrial membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA), as in plasma membrane; and phosphatidylglycerol (PG) and cardiolipin (CL), exclusively components of mitochondrial membrane (Figure 1.2). PC and PE are the most abundant phospholipids, comprising 40 and 30% of total mitochondrial phospholipids, respectively. PA and PS comprise 5% of the total mitochondrial phospholipids [68, 69]. Unlike plasma membrane, mitochondrial membranes contain high levels of cardiolipin (~15% of total phospholipids), and low levels of sphingolipids and cholesterol [70, 71].

The phospholipid cardiolipin not only has a role in maintaining membrane potential and architecture of the inner mitochondrial membrane, but also provides essential structural and functional support to several proteins involved in mitochondrial respiration. The unique structure of CL (Figure 1.2), which contains three glycerol backbones and four fatty acyl chains, makes it highly important for optimal function of the mitochondria [72]. The incorporation of four linoleic acid side chains in the same CL molecule renders it the primary target of the attack by free radicals, causing lipid peroxidation. Mitochondrial CL peroxidation appears to be an early event preceding the intrinsic apoptotic cell death [73]. Because its role in apoptosis and mitochondrial function, CL levels and CL peroxidation has been implicated in several human diseases such as atherosclerosis [74], cancer [75], Barth syndrome [76] as well as neurodegenerative disorders including Alzheimer's [77] and Parkinson's disease [78].

A peculiarity of the IMM is that the amount of membrane-associated proteins is high. Some studies estimate the protein:lipid ratio of the inner membrane to be as high as 3:1 [79]. Among the IMM proteins, a significant portion of proteins comprises the oxidative phosphorylation system, which contains five multiprotein complexes. The importance of this system relies on its role in providing the cellular ATP molecules necessary for cell survival. The activity and stability of the oxidative phosphorylation proteins are affected by their interaction with phospholipids in the IMM. For example, lack of cardiolipin in IMM was found to destabilize the complex III and IV of the oxidative phosphorylation system [80, 81], illustrating the importance of CL for mitochondrial respiration.

In addition to having a role of phospholipids in regulating protein import and activity, CL and PE are important for tubular mitochondrial morphology and membrane fusion [82-86]. Mitochondrial morphology depends on a balance between fusion and fission events. Mitochondrial fusion requires hydrolysis of CL to generate PA, a fusogenic lipid, by Phospholipase D enzyme [84]. PE, another fusogenic phospholipid, is also required for the mitochondrial fusion. Disruption of the PE synthesis through PS decarboxylation pathway cause mitochondria fusion defects [83]. In addition, mitochondrial morphology was found to be altered by a decrease in mitochondrial content of PE in yeast [85] and mammalian cells [83]. Joshi and colleagues have shown that CL and PE have overlapping functions on the mitochondrial fusion and that disruption of both PE and CL in yeast causes loss of mitochondrial membrane potential and fragmentation of mitochondria [85].

The phospholipid PA has important functions as a lipid anchor to recruit proteins involved in trafficking [87], lipid signaling [88] and fusion [89] to membrane surfaces. The regulation of mitochondria fusion and fission by PA was found to enable mitochondria to alter their

morphology, to increase the efficiency of energy production and to mark unhealthy mitochondria for autophagy [90]. In addition, PA can serve as substrate for production of the signaling lipids diacylglycerol (DAG) and lysophosphatidic acid. Mutations of Lipin-1, the PA phosphatase that generates DAG from PA, has been associated with metabolic and neurological disease [91].

Because of their broad role in the mitochondrial function, phospholipid alteration has been associated with several diseases [92]. For example, cancer cells, which are characterized by alterations in bioenergetics and apoptosis, have altered mitochondrial phospholipid composition [93]. Cardiac pathologies result in a reduction of tetralinoleyl-CL, with consequent increase in hydrogen peroxide by the respiratory chain and apoptosis [94, 95]. Metabolic diseases, such as diabetes and non-alcoholic fatty liver disease, have also been associated with decrease in mitochondrial CL and alteration of CL acyl chain remodeling [96, 97]

This evidence demonstrates the importance of the mitochondrial membrane phospholipids for the IMM architecture, the apoptosis, the activity of respiratory proteins and the transport of proteins into the mitochondria.

1.4.3 The Kennedy pathway and the biosynthesis of the major membrane phospholipids in the ER

Two major membrane bilayer phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are produced from choline and ethanolamine and the common lipid intermediate diacylglycerol (DAG) in the process known as the *de novo* CDP-choline and CDP-ethanolamine Kennedy pathway [98, 99] (Figure 1.3). Both branches of the pathway depend on the availability of the extracellular substrates, choline and ethanolamine, and on their

entrance into the cells. Those substrates are polar molecules and have to be actively transported into the cells. The transport system for ethanolamine has not been extensively studied and remains poorly understood. Choline transport for PC synthesis is known to be mediated by a group of ubiquitous solute carriers of the SLC44A family, mainly by the member A1, also known as the choline transporter-like protein 1 (CTL1) and as the cell surface antigen CDw92 [31]. Immediately after entering the cells, choline and ethanolamine are phosphorylated by choline and ethanolamine kinases, of which CK α and - β and EK α and - β were identified and fully characterized in mammalian systems [100]. The kinase products, phosphocholine and phosphoethanolamine, are then coupled with CTP by the specific and the regulatory pathway enzymes CDP:phosphocholine and CDP:phosphoethanolamine cytidylyltransferases (CCT/Pcyt1 and ECT/Pcyt2) to yield CDP-choline and CDP-ethanolamine, respectively, and to release inorganic pyrophosphate. In the final steps, the CDP-choline and the CDP-ethanolamine derivatives are condensed with diacylglycerol, catalysed by multiple DAG:choline- and DAG:ethanolamine phosphotransferases (CPT, EPT, CEPT), to release CDP and to produce the bilayer forming phospholipids PC and PE at the endoplasmic reticulum (ER).

The major regulatory points for the PC and PE synthesis through the Kennedy pathway are the slowest (rate-limiting) reactions governed by Pcyt1 and Pcyt2, respectively, and as in other metabolic pathways by the substrate (choline/ethanolamine and DAG) availabilities. The regulation of Pcyt1 and Pcyt2 are extensively studied and have historically being considered similar, but evidence are emerging that they are completely distinct and differently regulated [63, 101-105].

The rate of PC synthesis is predominantly regulated by Pcyt1 activity [106]. Pcyt1 is activated by association with membranes and by phosphorylation [107]. The translocation of Pcyt1 into cell

membranes was found to be regulated by fatty acids (FA) [108-111]. FA as well as anionic phospholipids, were found to induce the Pcyt1 binding to membranes, because of their negative charged headgroup [111]. Thus, changes in the lipid composition of the membrane can activate Pcyt1, which leads to stimulation of PC synthesis.

As another regulatory step for PC synthesis, the availability of intracellular choline is regulated by protein-mediated transport to pass choline through the plasma membrane. The CTL1 transports choline in various tissues [33, 35, 37] and it is considered to be the predominant choline transporter in nonneuronal cells [31, 33, 37, 112]. The availability of choline regulates PC synthesis together with modifying choline transport and DAG/TAG homeostasis [41]. In choline deficient cells, PC synthesis from choline was blunted, which led to an increased availability of DAG and FA for TAG synthesis [41].

The *de novo* synthesis of PE could be limited by the availability of substrates ethanolamine [113, 114] and DAG [115] or the enzymes EK [116] and Pcyt2 [105]. Based on numerous radiolabeling studies and on animal models, Pcyt2 is the main regulatory enzyme in the CDP-ethanolamine pathway. Heterozygous mice for the Pcyt2 gene (Pcyt2^{+/-}) have reduced formation of CDP-ethanolamine, which limits the PE synthesis and increases the availability of DAG for TAG synthesis [117]. On the other hand, the overexpression of Pcyt2 could not accelerate PE synthesis when DAG availability for the last step in the pathway was limiting the PE formation [115], whereas EK overexpression accelerated PE synthesis in Cos-7 cells [116]. At low levels of ethanolamine, Pcyt2 limits the reaction rate, whereas at high levels of ethanolamine, EK limits the rate [118]. In ethanolamine deficient media, cells adapt the mitochondrial PS decarboxylation pathway to produce additional PE from serine (see section 4) [119]. However this is not the case

in normal metabolism. Animal studies indicate that CDP-ethanolamine pathway is the major contributing pathway for the synthesis of PE [105].

In the liver, an alternative pathway utilizes PE to produce more PC and choline in a three-step methylation of PE by S-adenosylmethionine (AdoMet) catalysed by phosphatidylethanolamine-N-methyltransferase (PEMT). The PEMT pathway accounts for ~30% of the hepatic PC synthesis and is coregulated with the CDP-choline pathway [120]. It is not known how the CDP-ethanolamine pathway, which only makes PE *de novo*, is linked with the PE used in the PEMT pathway. However, in the PEMT knock-out mice the CDP-choline pathway is upregulated, whereas during the PEMT overexpression in hepatoma cells it is downregulated, showing a strong link of PEMT pathway with the liver PC metabolism [121, 122]. As a backup liver pathway, PEMT supplies PC for the very low-density lipoprotein (VLDL) assembly and for the bile production, and is a source of choline for the betaine synthesis in mitochondria [123]. The liver methylation of PE also provides PC for the PS synthesis by the base-exchange reaction catalysed by PS synthase. The newly formed PS then could be transformed into mitochondrial PE by PS decarboxylase (PSD), forming a specific liver PE cycle [124] (Figure 1.3). The cycle PE-PC-PS may be involved in the maintenance of phospholipid levels when CDP-choline or CDP-ethanolamine pathways are impaired.

In mammals, PS could be only made from the pre-existing phospholipids (PC and PE) and L-serine, catalysed by PS synthases 1 and 2 (PSS1 and PSS2) in the mitochondria-associated membranes (MAM), a subfraction of the ER. MAM have been proposed to be a distinct domain of the ER that comes into close contact with OMM and thereby mediates the import of newly-synthesized PS into mitochondria to be decarboxylated into PE. The PS decarboxylation by PSD to form mitochondrial PE is a key function of PS.

The synthesis of PS is regulated by phosphorylation of PS synthase in yeast and mammalian cells [125, 126]. The major mechanism for regulating PS synthesis in mammalian cells is a feed-back mechanism whereby the activity of PS synthase 1 and 2 is regulated by the end-product, PS [124, 127, 128]. In addition, PSS1 and PSS2 differentially modulate phospholipid metabolism. Over-expression of PSS1 in hepatoma cells decreases the rate of PE synthesis *via* the CDP-ethanolamine pathway [129], whereas overexpression of PSS2 does not [130].

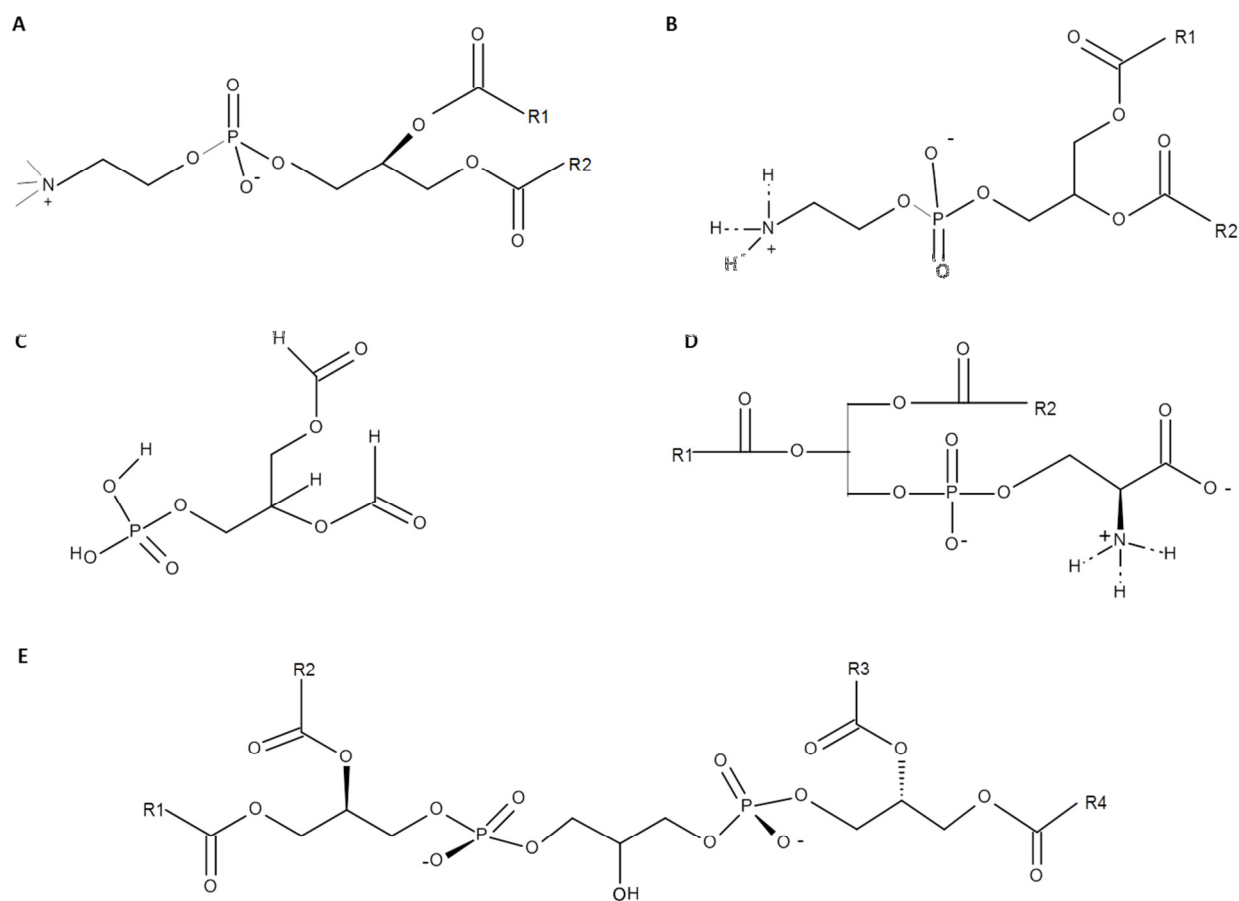


Figure 1. 2: Structural formulas of key phospholipids. (A) phosphatidylcholine – PC; (B) phosphatidylethanolamine – PE; (C) phosphatidic acid –PA; (D) phosphatidylserine – PS; (E) cardiolipin – CL.

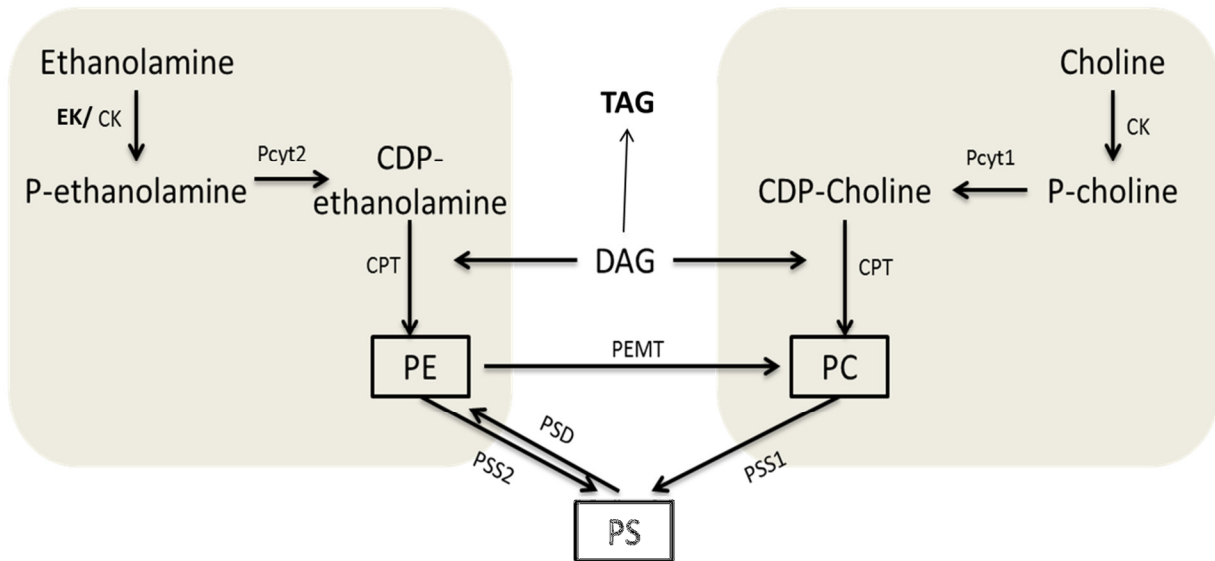


Figure 1. 3: Pathways for the synthesis of phospholipids and their interconnection. The two branches of the Kennedy pathway are represented in grey. The key metabolites and enzymes catalysing the respective reactions are indicated. Abbreviations are as indicated in the main text.

1.4.4 The biosynthesis of mitochondrial phospholipids

The maintenance of the mitochondrial bilayer and of a defined composition of mitochondrial phospholipids relies on the organelle capacity to synthesize CL, PE, PG and PA *in situ*, and on the external supply of PC and PS, which are exclusively synthesized in the ER and MAMs and must be imported into the mitochondria [70].

PE is made in mitochondria by decarboxylation of phosphatidylserine. This reaction is catalysed by the inner mitochondrial enzyme PSD. Even though PE produced by the CDP-ethanolamine pathway can be imported to IMM, the PS decarboxylation provides the majority of mitochondrial

PE [131]. PSD knock-out mice have mitochondrial dysfunction and die in the embryonic phase [83]. In addition, the mitochondrial PE deficiency resulting from siRNA silencing of PSD in CHO cells alters mitochondrial morphology and affects the ATP production, oxygen consumption and the activity of the electron-transport components [132].

Cardiolipin, an utmost important phospholipid in the mitochondrial membrane, is synthesized via condensation of phosphatidylglycerol (PG) and CDP-diacylglycerol catalysed by cardiolipin synthase (CLS) in the matrix side of the inner mitochondrial membrane (Figure 2). PG is produced by a two-step reaction: glycerol-3-phosphate and CDP-diacylglycerol condense to yield phosphatidylglycerol phosphate, which is then dephosphorylated to PG. Lack of CLS reduces the activity of the oxidative phosphorylation system and the import of proteins into mitochondria in yeast [133], but the fundamental functions in mitochondria remain intact [134]. The regulation of CL synthesis was found to involve multiple mechanisms, such as factors that affect mitochondrial biogenesis and matrix pH and respiration [135]. Recently, Tam41 (translocator and maintenance protein 41), a component of the mitochondrial translocator system, was found to regulate CL synthesis [136]. *Tam41* mutant cells showed an almost complete absence of CL and PG, suggesting that regulation may occur at the level of CDP-diacylglycerol (CDP-DAG) synthase. This study demonstrates that Tam41 is primarily required for PG and CL biosynthesis, and that defects in protein import in Tam41 deficient cells are a consequence of the loss of PG and CL [136].

Finally, the phospholipid phosphatidic acid represents a branch-point for the synthesis of all phospholipids [137] (Figure 2). PA may be converted to CDP-DAG by the CDP-DAG synthase for the synthesis of PG, PS and CL in a reaction catalysed by CTP:PA cytidyltransferase [138]. Alternatively, PA may be converted to DAG, which is a substrate for the synthesis of PE and PC,

in a reaction catalysed by PA phosphatases such as Lipin-1 [91, 139]. The PA phosphatases are among the most highly regulated enzymes in lipid metabolism. Their activity is governed by phosphorylation, association with membranes and modulation by components of lipid metabolism, such as CL and CDP-DAG [140, 141].

The synthesis of PA occurs by two acylation steps; first glycerol-3-phosphate is converted to 1-acylglycerol-3-phosphate (lysophosphatidic acid) by glycerol-3-phosphate acyltransferase. The 1-acyl-sn-glycerol-3-phosphate product is then acylated to 1,2-diacyl-sn-glycerol-3-phosphate (phosphatidic acid) by 1-acyl-sn-glycerol-3-phosphate acyltransferase [142]. Studies have indicated that both lysophosphatidic acid and phosphatidic acid are synthesized on the outer surface of the mitochondrial outer membrane and then phosphatidic acid moves to the inner mitochondrial membrane where it serves as a precursor for cardiolipin biosynthesis [143]. PA can also be generated *via* phosphorylation of DAG through the action of a large family of DAG kinases (DAGK) or by hydrolysis of the phospholipids PC and CL by phospholipase D. In turn, PA can be reversibly metabolized to lysophosphatidic acid by phospholipase A2 or dephosphorylated to DAG by Lipin-1.

1.4.5 Remodeling of phospholipids and the role of dietary lipids

After the *de novo* synthesis of phospholipids, many of them undergo acyl chain remodeling, known as Lands' cycle. The variation in chain length and degree of unsaturation of fatty acids present within phospholipids contributes to the diversity of plasma and mitochondrial membrane phospholipids, which is important for the biophysical properties of the membranes [144, 145]. In mammalian cells, phospholipid composition differs among cell types, organelles, and inner/outer

membranes. These differences are known to play important roles in various functions, including signal transduction, vesicle trafficking, and membrane fluidity [62]. Conventionally acyl chain remodeling involves the enzymes phospholipases A (PLAs), which catalyse the removal of an acyl chain from the glycerol moiety; and transacylases, which mediate reacylation with different FA [144, 146].

Phospholipid remodeling is important for the production of polyunsaturated fatty acids (PUFA)-containing phospholipids, which are major sources of FA-derived lipid mediators and endocannabinoids [147-150]. Moreover, PC remodeling produces the majority of dipalmitoyl-PC, the main lipid component of pulmonary surfactant [151]. The fatty acid composition of PS and PI contributes to their cellular functions as signaling molecules [152, 153], and changes in the fatty acyl-chain of PS and PI seems to be controlled by remodeling [154]. In addition, activation of enzymes in the inner mitochondrial membrane requires acyl chain remodeling. For example, impairment of CL remodeling may interfere with assembly and stability of the respiratory chain proteins [145].

The fatty acid composition of the diet can modify the acyl-chain remodeling of phospholipids and the mitochondrial function. In one study, mice exposure to rapeseed oil-rich diet showed altered mitochondrial membrane phospholipid composition in terms of both the type of acyl chains within the phospholipids and the proportions of phospholipid classes. As a result of altered mitochondrial membrane phospholipid composition these mice showed an altered hepatic mitochondrial bioenergetics [155]. Guderley and collaborators showed that in trout mitochondria the changes in membrane phospholipid characteristics, including the acyl-chain composition, follow the patterns of fatty acids present in the diet. In this same study, the respiratory capacity of the red muscle mitochondria was altered by the different diets, and was found to be higher in

the diet rich in polyunsaturated lipids [156]. Youth male subjects supplemented with fish oil (omega-3) also showed alterations in mitochondrial membrane structure together with improvements in ADP sensitivity [157]. Several other studies in mammals have demonstrated that dietary changes modify the FA composition of the major mitochondrial phospholipids [158-160] and, in particular, the molecular species associated with mitochondrial cardiolipin [109]. Following a diet deficient in linoleic acid (C18:2), an essential fatty acid, the rat heart showed a significantly decrease in tetralinoleoyl CL, which affected mitochondrial oxygen consumption [159, 161]. Conversely, dietary supplementation with linoleic acid restored tetralinoleoyl CL in cultured fibroblasts from Barth syndrome patients and elevated CL level [162].

The n-3 PUFA content of the plasma membrane of different mouse tissue has the greatest sensitivity to changes in dietary fatty acids [163]. The fact that both n-6 and n-3 PUFA classes cannot be synthesised *de novo* by higher animals suggests that the fatty acid composition of membrane phospholipids may be strongly influenced by the abundance of n-6 and n-3 PUFAs in the diet. Several studies have shown that increasing the PUFA content of the diet increases the metabolic rate [164-166]. For example, treatment of rats with n-3 PUFA decreased proton leakage from the respiratory chain and this was related to incorporation of the PUFAs into mitochondrial membrane PC, PE and CL [167]. Treatment with docosahexaenoic acid (DHA), but not eicosapentaenoic acid (EPA), profoundly alters fatty acid composition of mitochondrial phospholipids in rat hearts, decreasing arachidonic acid and increasing DHA. The change in mitochondrial phospholipids by DHA delays the mitochondrial permeability transition pore (MPTP) opening, which is associated with apoptosis and myocardial damage during ischemia [127]. DHA acyl chain incorporation into cardiolipin was also found to regulate mitochondrial lipid-protein clustering, which alters several aspects of mitochondrial function [79]. Taken

collectively, these results suggest that increasing the level of PUFA in the diet changes the composition of mitochondrial membrane phospholipids and consequently may alter mitochondrial capacity.

The response to dietary fatty acids also varies according to the phospholipid class. PC was found to be more responsive to variation in dietary MUFA content than the other phospholipid classes, whereas PE was more responsive than PC to both dietary n-6 PUFA and the n-3 PUFA [163]. Interestingly, *Pcyt2* deficient mice, which have a decreased rate of PE synthesis, is PUFA deficient and has specifically modified FA composition in PE and in TAG [117]. Therefore, change in the phospholipid fatty acyl chain composition relies not only on the dietary fatty acid profile but also on the classes of phospholipids affected.

Taken together, these studies demonstrated that disturbance of enzymes involved in phospholipid remodeling, as well as changes in the dietary fatty acids may alter the phospholipid composition of the membranes. Dietary interventions that are able to influence mitochondrial membrane phospholipids, hence modifying its physical properties, respiration, and other processes such as MPTP, are emerging as novel therapeutic strategies [155]. Therapies addressed to mitochondrial phospholipids have been suggested as potentially useful to treat pathologies, such as cancer, cardiovascular and neurodegenerative diseases, obesity and metabolic disorders [155].

1.4.6 Degradation of phospholipids

Many phospholipids have a rapid turnover, indicating that their degradation plays an important role in membrane homeostasis. The phospholipid degradation is mainly catalysed by non-lysosomal phospholipases. These phospholipases are divided into three classes based on the

bound they cleave: Phospholipases A (PLAs), phospholipases C (PLCs) and phospholipases D (PLD).

PLAs release the fatty acid in the *sn*-1 and *sn*-2 position of the glycerol moiety generating lysophospholipid and free FA. The lysophospholipid is reacylated to generate new phospholipid, the mechanism known as FA remodeling, or it is degraded by a lysophospholipase. Several studies suggest that PLAs mediate much of the phospholipids turnover in cultured cells [168, 169]. The PLA2 family, which hydrolyses FA bound at the *sn*-2 position of phospholipid, has been related to turnover and remodeling of phospholipids as well as to membrane homeostasis [170, 171]. iPLA2 γ , a component of the Ca²⁺-independent PLA2 family, is preferentially distributed in the mitochondria. Because of the association of iPLA2 γ with mitochondrial membranes and its role in cardiolipin remodeling, this enzyme may be involved in integrating lipid and energy metabolism. Mice null for iPLA2 γ display abnormal mitochondrial function with a dramatic decrease in oxygen consumption [172]. Thus, iPLA2 γ is essential for maintaining bioenergetic mitochondrial function through regulation of mitochondrial membrane phospholipid homeostasis.

Phospholipases C hydrolyse the bond between glycerol backbone and phosphate to yield DAG and phosphorylated head group. PLCs are generally involved in generation of second messengers for signalling events [173], but they also play a role in the turnover of PC and PE [174, 175].

Phospholipases D hydrolyse the bound between phosphate and the head group to yield PA and free head group. Both classical Phospholipase D (PLD) family members found on many cytoplasmic membrane surfaces and MitoPLD, the PLD family member found on the mitochondrial surface, can generate PA *via* hydrolysis of the phospholipids PC and CL [26]. MitoPLD-generated PA regulates mitochondrial shape through facilitating mitochondrial

fusion [176]. Mitochondrial fusion and fission are very important in maintaining mitochondrial and cellular function, and morphological changes of the mitochondria are linked to cell apoptosis and neurodegenerative disease [176]. For example, in brains of Alzheimer's disease patients, PLD1 is up-regulated in the mitochondrial membrane, which affects the composition of mitochondrial membrane phospholipids [177].

The involvement of phospholipases in phospholipid homeostasis is also demonstrated by PLD-like enzymes that catalyse base-exchange reactions. PS synthases (PSS), a PLD-like enzyme, catalyse the exchange of the head group of PE or PC for serine or the exchange of the serine head group of PS for choline or ethanolamine. Thus PLD and PLD-like enzymes could play an important role in different phospholipid homeostasis [131].

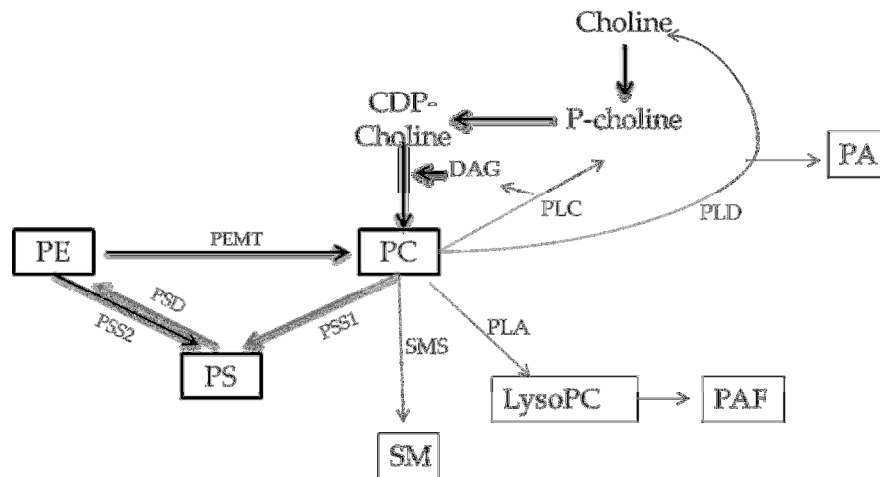


Figure 1. 4: The major catabolic pathways for PC. PC can be metabolized to generate other phospholipids or signaling molecules. PC can be metabolised to PS, which can then generate PE, forming a specific liver PC-PS-PE cycle. PC can generate sphingomyelin (SM) catalysed by sphingomyelin synthase (SMS), and lysoPC, catalysed by PLA, which can generate platelet activator factor (PAF). Also PLC can catabolise PC to P-Choline and DAG, and PLD can catabolise PC to free choline and PA.

2 Rationale for the studies

The interactions between the nutrient choline and lipid metabolism have not been very well investigated. There is also little information on how fatty acids and choline interrelate, as well as how choline and lipid metabolism are affected in metabolic disorders. In addition, the cross-regulation of CDP-choline and CDP-ethanolamine to maintain membrane homeostasis has not been investigated. As the first step in choline metabolism is the cellular uptake, one of the goals of this thesis was to understand the mechanisms of choline transport and the role of the choline specific transporter CTL1/SLC44A1 under different metabolic conditions.

It was hypothesized that:

I- Fatty acids affect choline uptake and metabolism of lipids

Development of insulin resistance, metabolic syndrome and type-2 diabetes is characterized by increased quantities of circulating fatty acids [178]. Choline deficiency results in accumulation of TAG in muscle cells due to reduced use of DAG and FA for PC synthesis through the CDP-choline pathway [41]. We postulated that choline metabolism and CTL1/SLC44A1 function are coupled with FA metabolism, particularly because both are critical for the formation of glycerolipids (PC, DAG, TAG) homeostasis. *The primary objective was to analyse the effects of excessive fatty acid supply, as observed in insulin resistance and metabolic syndrome states, on choline transport and PC synthesis in skeletal muscle cells.*

II- Disfunctional CTL1/SLC44A1 and related choline deficiency in postural tachycardia syndrome (POTS) primary skin fibroblasts

A patient diagnosed with postural tachycardia syndrome (POTS) showed very low choline and betaine, but normal PC, in the blood. Our group postulated that the defect has to be at the

transport level not at the PC synthesis level. We studied the mechanisms for this hypothesis at the cellular level in fibroblasts cells isolated from skin biopsies of POTS patient. CTL1/SLC44A1 was indeed found to be down-regulated. *The objective here was to further establish the mechanism of CTL1/SLC44A1 deficiency.* It was hypothesized that the PC homeostasis will be different, although the total PC was not affected in POTS. It was, however, expected to find alterations on the membrane composition and mitochondrial function as a consequence of the modified choline transport and metabolism.

III- Choline supplementation alleviates complications of Pcyt2 deficiency

Our lab has developed knock-out mice for the Pcyt2 gene (Pcyt2^{-/-}). Pcyt2 is the rate-regulatory enzyme of the CDP-ethanolamine pathway for PE synthesis. Pcyt2^{+/-} mice have reduced flux through this pathway (synthesis of PE), but PE remains unchanged because of reduced degradation. These mice, however, accumulate TAG and develop adult-onset metabolic disease phenotype, including obesity, fatty liver and insulin resistance [117]. Initial studies by our Lab showed that choline supplementation of Pcyt2^{+/-} mice could reduce liver steatosis and altered the expression of several lipid metabolic genes [179]. *Our objective was to further determine the mechanism by which choline supplementation could reverse the Pcyt2^{+/-} phenotype.* Because choline is a substrate for the CDP-choline pathway, our hypothesis was that choline would stimulate the CDP-choline pathway for PC synthesis, and therefore, increase DAG utilization and reduce TAG levels.

IV- The CDP-choline pathway compensates for the disruption in the CDP-ethanolamine pathway in human fibroblasts

The cross-regulation of the Kennedy pathway for PC and PE synthesis was established in Pcyt2 deficient models. *The objective here was to further investigate the CDP-choline pathway in*

fibroblast cell culture deficient in the CDP-ethanolamine pathway (human siRNA Pcyt2). It was expected that the choline uptake, metabolism to PC and PC-PS-PE reactions would be up-regulated in the Pcyt2 knock-down cells in order to maintain PE levels and phospholipid homeostasis.

3 Palmitic acid and oleic acid differentially regulate choline transporter-like 1 levels and glycerolipid metabolism in skeletal muscle cells ¹

Abstract

Choline is an essential nutrient required for the biosynthesis of membrane lipid phosphatidylcholine (PC). Here we elucidate the mechanism of how palmitic acid (PAM) and oleic acid (OLA) regulate choline transporter-like protein 1 (CTL1/SLC44A1) expression. We evaluated the mechanism of extracellular and intracellular transport of choline, and their contribution to PC and other glycerolipid-diacylglycerol (DAG) and triacylglycerol (TAG) homeostasis in differentiated skeletal muscle cells. PAM reduces total and plasma membrane CTL1/SLC44A1 protein by lysosomal degradation, and limits the choline uptake while increasing DAG and TAG synthesis. OLA maintains total and plasma membrane CTL1/SLC44A1, but increases PC synthesis more than PAM. OLA does not increase the rate of DAG synthesis, but does increase TAG content. Thus, the CTL1/SLC44A1 presence at the plasma membrane regulates choline requirements in accordance with the type of fatty acid. The increased PC and TAG turnover by OLA stimulates cell growth and offers a specific protection mechanism from the excess of intracellular DAG and autophagy. This protection was present after OLA treatments, but not after PAM treatments. The mitochondrial choline uptake was reduced by both FA; however, the regulation is complex and guided not only by the presence of the mitochondrial CTL1/SLC44A1 protein but also by the membrane potential and general mitochondrial function.

¹ Schenkel LC, Bakovic M. Palmitic Acid and Oleic Acid Differentially Regulate Choline Transporter-Like 1 Levels and Glycerolipid Metabolism in Skeletal Muscle Cells. *Lipids*. 2014 DOI 10.1007/s11745-014-3925-4

3.1 Introduction

In the muscle, choline is exclusively consumed for *de novo* phosphatidylcholine (PC) synthesis by the CDP-choline (Kennedy) pathway [31]. In this pathway, the extracellular choline passes the plasma membranes (PM) by a transport system typically mediated by choline transporter-like protein 1 (CTL1/SLC44A1) [31, 35, 112]. Immediately after entering the cell, choline is phosphorylated by choline kinase ($CK\alpha/\beta$) to phosphocholine (P-Cho). The produced P-Cho is then coupled with CTP by CTP:phosphocholine cytidyltransferase ($CCT\alpha/\beta$) to yield CDP-choline (CDP-Cho) and inorganic phosphate. The final reaction of CDP-choline with diacylglycerol (DAG) is catalyzed by DAG: choline phosphotransferase (CPT/CEPT) to produce PC.

CTL1/SLC44A1 is a ubiquitous choline transporter abundantly expressed in human and mouse skeletal muscle [33, 35, 112]. CTL1/SLC44A1 was initially discovered as a plasma membrane transporter with the specific function of supplying choline for phospholipid synthesis [32, 35]. A recent study by our group identified CTL1/SLC44A1-mediated choline transport in mitochondria of different cell types as a way to balance the subcellular pools of choline, provide choline for betaine production, or both [36]. The importance of the plasma membrane CTL1/SLC44A1 in regulating the cellular uptake of choline has been well established [36, 37, 180, 181]. This form of choline transport plays a critical role in the synthesis of the lung surfactant PC in alveolar cells [181, 182]. In human keratinocytes, CTL1/SLC44A1 is responsible for choline uptake, and it regulates phospholipid metabolism and keratinocyte proliferation [183]. Additionally, as a major choline transporter in human monocytes, CTL1/SLC44A1 has been found to be regulated by trafficking from intracellular stores to the cell surface during the monocytes differentiation to macrophages [37].

In the skeletal muscle, choline deficiency down-regulates expression of the CTL1/SLC44A1 choline transporter and the CDP-choline pathway resulting in redirection of FA and DAG towards triacylglycerol (TAG) formation and in lipid droplet accumulation. The mechanism for the increased muscle TAG accumulation in choline deficiency is different from liver choline deficiency, because muscle cells do not produce betaine, or secrete lipoproteins, or have the supplementary phosphatidylethanolamine methylation (PEMT) pathway for PC production [39]. In muscle cells, choline deficiency limits PC synthesis by the Kennedy pathway and the unused substrates DAG and FA consequently accumulate as TAG lipid droplets [36, 39]. We postulated that CTL1/SLC44A1 might be coupled with FA metabolism, particularly since the intracellular choline availability seems critical for all glycerolipids (PC, DAG, TAG), and depends on choline transport. The FA regulation of the choline transport by CTL1/SLC44A1 is however completely unknown.

Development of insulin resistance, metabolic syndrome and type-2 diabetes is characterized by increased quantities of circulating FA leading to deposition of TAG [178]. It was indicated that obese rodents increase plasma FA from 0.5mM (non-obese) to over 2mM. In addition, in obesity-induced elevation of circulating free-FA, palmitic acid concentration can increase more than 2 mM [184]. In the chronic states, the elevated intracellular FA and other lipid intermediates (DAG and ceramides) progressively impair the muscle glucose uptake and mitochondrial FA oxidation, which are two critical events in the pathogenesis of insulin resistance [185-187]. Previous studies demonstrated that saturated palmitic acid (PAM) induces insulin resistance in skeletal muscle cells, whereas monounsaturated oleic acid (OLA) is protective and improves insulin sensitivity [188, 189]. Here we elucidate the mechanism of how excess levels of PAM and OLA regulate CTL1/SLC44A1 mediated choline transport and glycerolipid homeostasis in

skeletal muscle cells. We show that PAM and OLA regulate choline transport and CTL1/SLC44A1 differently at the level of the plasma membrane and mitochondria. We measure cell growth, turnover and total glycerolipid content and offer new evidence implicating differences in the rates of PC synthesis by the CDP-choline pathway with different utilization of OLA and PAM, leading to differences in muscle FA and TAG content.

3.2 *Materials and Methods*

3.2.1 Cell culture and treatments

The C2C12 mouse myoblasts were maintained at 37°C in Dulbecco's Modified Eagle's Medium (DMEM, containing 4mg/L of choline) supplemented with 10% fetal bovine serum (FBS). Myoblasts were differentiated into myotubes by keeping the confluent cells in a serum-reduced media (1% FBS +DMEM) for 4 days. FA were added to media of differentiated cells as a conjugate with 2% FA-free bovine serum albumin (BSA), as described previously [184]. Differentiated C2C12 myotubes were first incubated with serum-free DMEM for 3 hours and then treated with 1.25mM and 2mM PAM or OLA (bound to 2% BSA) in 1% FBS+DMEM for 16h. The myotubes incubated in the same media but without FA served as controls.

3.2.2 Cell viability

A standard colorimetric assay was performed to measure the cell viability. Briefly, C2C12 cells were cultured in 96-well plates, differentiated for 4 days in 1% FBS DMEM and treated with different amounts of PAM and OLA for 16h as above. The tetrazolium compound MTT (3-[4, 5-

dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide), which is reduced to purple formazan in living cells, was added (0.5mg/ml) and the cells were additionally incubated for 3 h. DMSO was added to the wells to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution was determined spectrophotometrically at 570 nm, and the values for OLA and PAM treated cells were compared to untreated cells. Experiments were performed four times in triplicates (3 Ctrl, 3 OLA, 3 PAM).

3.2.3 RT-PCR

Total mRNA from PAM and OLA treated and untreated C2C12 cells were isolated with TRIzol (Invitrogen). DNase I was used to eliminate genomic DNA and cDNA was synthesized from 2µg of mRNA using Superscript II reverse transcriptase (Invitrogen). Expression of CTL1/SLC44A1 mRNA in C2C12 cells was determined by polymerase chain reaction (PCR) using the forward primer 5'-GAACGCTCTGCGAGTGGCTGC-3' and the reverse primer 5'-CGGCTTTAGCTCTCGGGCGT-3', which yield a 376 bp product. PCR conditions were 94°C for 5 min, 36 cycles of 30s at 94°C, 30s at 50°C and 1 min at 72°C, and final extension for 6 min at 72°C. Reactions were standardized by amplifying the β -actin 219bp product (forward primer 5'-CCCAAGGCCAACCGCGAGAAGAT-3' and the reverse primer 5'-GTCCCGGCCAGCCAGGTCCAG-3) under the same conditions. Experiments were performed three times in duplicates (2 Ctrl, 2 PAM, 2 OLA).

3.2.4 Cell lysates and subcellular fractionation

Control and FA treated C2C12 myotubes were harvested in a lysis buffer (10mM HEPES-KOH, 10mM KCl, 1.5mM MgCl₂) containing 20μL/mL of protease inhibitor 50x (Sigma) and 5μL/mL of NP-40 (Thermo Scientific). The cell debris was removed by centrifugation at 3000 rpm for 5 min and the supernatant used for further analysis as a total cell lysate.

Mitochondrial fractions of FA treated and control myotubes were isolated as described previously [4]. Briefly, the cells were pelleted at 500 rpm, resuspended in 4ml of a homogenization buffer (0.25M sucrose and 10mM HEPES), and homogenized with 30 strokes in a Potter Elvehjem homogenizer. The homogenate was centrifuged at 2500 rpm for 5 min to pellet the cell debris and the supernatant further centrifuged at 12,500 rpm for 20 min at 4 °C to obtain the mitochondrial fractions.

The plasma membrane fractions were isolated with the Plasma Membrane Protein Extraction Kit (Abcam-65400). Briefly, after washed with PBS and pelleted, cells were homogenized in the Buffer Mix and centrifuged at 700g for 10 min. The supernatant was centrifuged at 10,000g for 30 min at 4°C to pellet the membranes. Plasma membrane was then purified by successive centrifugations at 1000g for 5 min at 4°C with Upper Phase Solution and Lower Phase Solution from the kit.

3.2.5 Immunoblotting

Immunoblotting for CTL1/SLC44A1 in total cell lysates or plasma and mitochondrial fractions was typically performed with 25 μg of protein, determined by bicinchoninic acid assay kit (Pierce). Previous work showed that under denaturing conditions the C-terminal antibody ENS-627 for CTL1/SLC44A1 detects the predicted 72 kDa-sized band, while the N-terminal antibody

LV-58 showed a protein at 50 kDa in C2C12 [36, 190]. Under non-denaturing conditions both CTL1/SLC44A1 antibodies detect the expected 72 kDa protein [36]. Here we used the C-terminal antibody under non-denaturing conditions to detect CTL1/SLC44A1. For immunoblotting, the samples dissolved in a non-denaturing loading buffer (62mM Tris-HCl, 0.01% bromophenol-blue and 10% glycerol) were separated by polyacrylamide gel electrophoresis (PAGE) at 110V for 1.5h, and then transferred to polyvinylidenedifluoride (PVDF) membranes (Roche). Ponceau S staining and β -tubulin were used as loading controls. Previous studies have shown that Ponceau S staining of proteins is a suitable alternative to housekeeping genes as a loading control [191, 192]. It has still an advantage in that it does not rely on a single protein for normalization [192]. After staining, the PVDF membranes were blocked in 5% skim milk in PBS-T and incubated overnight at 4 °C with the C-terminal CTL1/SLC44A1 antibody produced in our laboratory (ENS-627; 1:100 in 5% skim milk in PBS-T) or anti β -tubulin (1:1000 in 5% skim milk in PBS-T; from Sigma Aldrich). Membranes were washed 4 times with PBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:10,000 in 5% skim milk in PBS-T) for 1 h. After membrane washing in PBS-T, proteins were visualized using a chemiluminescent substrate (Amersham). To test the purity of the plasma membrane and mitochondrial fractions, we performed immunoblotting with Na^+/K^+ ATPase and COX4 antibodies (1:1000 dilution; antibodies from Abcam), respectively, under denaturing conditions. Immunoblottings were performed at least three times in duplicates.

3.2.6 Assays for protein degradation and autophagy

Lysosomal or proteasomal protein degradation of CTL1/SLC44A1 was investigated in PAM treated myotubes and controls after overnight incubations with 50 μ M of the lysosomal inhibitor leupeptin and 20 μ M of the proteasomal inhibitor MG132 (Z-Leu-Leu-Leu-Al), both from Sigma–Aldrich. CTL1/SLC44A1 protein expression was analysed by Western blotting in three independent experiments. OLA treatment did not cause CTL1/SLC44A1 degradation.

The autophagy biomarker LC3 was determined in both, PAM and OLA treated cells. Total cell protein (15 μ g) was loaded into a 12% SDS-PAGE gel and run under denaturing conditions. The membrane was blocked with 5% skim milk in PBS-T and incubated with LC3 antibody (1:1000 dilution; Novus Biologicals) in BSA overnight at 4°C. The membrane was washed and incubated with the secondary antibody horseradish peroxidase-conjugated anti-rabbit IgG for 1h and protein visualized by chemiluminescent peroxidase substrate assay (Sigma-Aldrich). Experiments were repeated three times in duplicates.

3.2.7 Choline uptake

The choline uptake assay was performed as described previously [36]. PAM and OLA treated and control myotubes were washed with KRH buffer (130mM NaCl, 1.3mM KCl, 2.2mM CaCl₂, 1.2mM MgSO₄, 1.2mM KH₂PO₄, 10mM HEPES pH 7.4 and 10mM glucose) and incubated 5 min with 1 μ Ci [³H-methyl]choline (60-90 Ci/mmol; American Radiolabeled Chemicals) in KRH buffer. The labelled cells were washed in ice-cold KRH buffer containing 1mM of excess “cold” choline to stop the uptake and remove the extracellular radiolabeled choline. Cells were then lysed in 500 μ L of the ice-cold lysis buffer (10mM Tris-HCl, 1mM EDTA, 10mM NaF). The [³H-methyl]choline radioactivity was determined with liquid scintillation counting (LSC) and the

choline uptake calculated as DPM/mg/min. Protein amount was determined using a BCA protein assay (Thermo Scientific).

Crude mitochondria were isolated according to previous study [4]. Cells were incubated 10 min with RSB buffer (10mM NaCl, 1.5mM MgCl₂, 10mM Tris-HCl) and homogenized. Then MS buffer (210mM mannitol, 70mM sucrose, 5mM Tris-HCl and 1mM EDTA) was added and it was centrifuged at 2500 rpm for 10 min. Final supernatant was then centrifuged at 12,500 rpm for 20 min. For mitochondrial choline uptake, crude mitochondria were suspended in a mitochondrial uptake buffer (120μM KCl, 5 μM HEPES/KOH, 1 μM EGTA, 5 μM KH₂PO₄, 0.5 μM MgCl₂, 5 μM L-glutamate and 1.2 μM L-malate, PH 7.2) and incubated with 5nM [³H-methyl]choline solution for 5 min. Mitochondria was pelleted, washed with excess “cold” choline, centrifuged at 12,500 rpm for 5 min and resuspended in the lysis buffer. The [³H-methyl]choline was determined with LSC and the mitochondrial total protein and rate of choline uptake determined as for the whole cell lysates. Cellular and mitochondrial choline uptake experiments were performed at least three times.

3.2.8 Radiolabeling of the CDP-choline pathway with [³H-methyl]choline

For pulse experiments, FA treated and control myotubes were incubated for 3h (synthesis) and 16h (pool size) with 5μCi [³H-methyl]choline in 2ml of the 2% BSA+1% FBS+DMEM with or without FA. Cells were washed two times with PBS, scraped and total lipids extracted by Bligh-Dyer method [193]. An aliquot of cell suspension was used to measure the protein concentration. The water-soluble metabolites choline, PCho and CDP-Cho were separated using the solvent system of ethanol/water/ammonia (48:95:7) and visualized with phosphomolybdic acid in chloroform/ethanol (1:1) + 1% stannous chloride. Specific bands were scraped from TLC plates

and radioactivity determined by LSC. The radiolabelled PC was determined by LSC directly from the lipid phase or after separation with chloroform/methanol-acetic acid/water (40:12:2:0.75) and staining with 15% sulfuric acid and 0.5% K₂CrO₇. Results were repeated at least 3 times in triplicates.

3.2.9 Choline efflux

The efflux of choline was measured as the equilibrium [³H-methyl]choline released into the medium after different chase periods. Briefly, cells were incubated 16h with 5μCi [³H-methyl]choline in 2ml of the 2% BSA+1% FBS+DMEM with or without FA. Cells were then washed two times with PBS and 1mL of medium containing 1mM of excess cold-choline was added to the cells. After 2.5h and 4h incubation, aliquots (100μL) of medium were removed and radioactivity counted by LSC. The protein concentration of total cell lysates was also measured. The efflux rate was calculated in dpm/mg of protein and compared among different FA treatments. The experiment was performed in triplicates.

3.2.10 Radiolabeling of DAG and TAG with [³H]glycerol

FA treated and control myotubes were also radiolabeled with [³H]glycerol to determine TAG and DAG turnover. Pulse experiments were performed for 3h using 0.5μCi [³H]glycerol and pulse-chase experiments were performed with 2h pulse with [³H]glycerol followed by 3h chase with excess 'cold' glycerol. Total lipids were extracted by the Bligh-Dyer method [24] and the radiolabeled TAG and DAG separated in heptane/isopropyl alcohol/acetic acid (60:40:3). The

TAG and DAG bands were stained with iodine, scraped from the plates and the radioactivity determined by LSC. The experiments were performed in duplicated and at least three times.

3.2.11 Measurement of total lipids

High performance liquid chromatography (HPLC) was carried out on an Agilent 1100 instrument equipped with quaternary pump and Alltech ELSD2000 evaporative light-scattering detector, using a modified version of the method of Graeve and Janssen [194]. Briefly, 1 mg of cell homogenates from FA treated and untreated cells were extracted in the presence of internal standard (50 µg dipalmitoyl-phosphatidyl-dimethylethanolamine) using a modification of the method of Folch and Lees [195]. The extracted lipid phase was collected, dried, and resuspended in 100 µL chloroform/isooctane (1:1). Lipids were separated using a three-solvent gradient and a normal-phase HPLC column (Onyx monolithic silica Phenomenex). The amounts of phospholipids (PC, PE, PS, PI), TAG, free FA, sphingomyelin and cholesterol in mg lipid/mg protein were determined using appropriate standards.

3.2.12 Mitochondrial potential

The mitochondrial membrane potential was measured in 96 well plate using carbocyanine dye (JC-1) according to the Isolated Mitochondria Staining Kit (Sigma-Aldrich). Mitochondria were isolated as described for *Choline uptake* and 30µg of protein of the isolated mitochondria was added per well. JC-1 concentrates in the mitochondrial matrix and form red fluorescence. A dissipation of potential prevents the mitochondrial accumulation and JC-1 releases a green fluorescence. Red fluorescence was read at 590nm (emission) and 490nm (excitation) using a

Flex station 3 microplate reader. Prior to JC-1 staining, the dissipating agent valinomycin (1µg/mL) was added to control mitochondria as a negative control. Results were expressed as mean fluorescence $\pm$ SEM from 3 separate experiments.

3.2.13 Statistical analysis

All experiments were performed in duplicates and replicated at least in three independent experiments. One way-ANOVA with Dunnett post-hoc test was performed to compare means between PAM and OLA treated groups and untreated control group. A $p \leq 0.05$ at 95% confidence interval was considered as significant. Graphics are represented as mean $\pm$ SEM. All statistical tests were analysed with GraphPad Prism 4 software.

3.3 Results

3.3.1 Differential effects of OLA and PAM on cell growth and CTL1/SLC44A1 protein content and localization

Mouse C2C12 myoblasts were differentiated to myotubes by serum deprivation for 4 days and treated 16h with 1.25 and 2mM of PAM and OLA as described [184, 196]. To investigate if different treatments influence cells viability we performed the standard cell counting (not shown) and the cell-viability MTT assay (Figure 3.1A). The number of cells, analysed by the cell counting, as well as the rate of cell proliferation, analysed by the MTT test, were not affected by treatments. In fact, an increased cell proliferation was observed in treatments with OLA that appeared significant at higher OLA concentration. Thus, both methods showed that myotubes

exposed to PAM and OLA at 1.25 and 2mM were not significantly stressed by the treatments when compared to untreated cells. We found that these FA concentrations are consistent with the pathological in-vivo FA concentrations in obese animals [197], while still permitting survival and proliferation of cells in culture.

Under all conditions, treatments of myotubes with PAM and OLA did not influence the expression of CTL1/SLC44A1 mRNA when compared to untreated controls (Figure 3.1B). We next determined if the OLA and PAM modified the total CTL1/SLC44A1 protein content. The CTL1/SLC44A1 protein size is 72kDa and the protein could only be detected on native gels, under non-denaturing conditions, which is the method used here. Under standard western blotting-denaturing conditions CTL1/SLC44A1 appears as 50 and ~25kDa bands of variable intensity [36, 190]. As shown in Figures 2a and 2b, PAM treatments, but not OLA treatments, reduced total CTL1/SLC44A1 content that was dependent on PAM concentration (10% and 40% reduction by 1.25mM and 2mM, respectively). Because the cell viability was not impaired at higher concentration (2mM) of fatty acids and because the effect of PAM on CTL1/SLC44A1 expression was more pronounced at 2mM, we choose this concentration to perform the next experiments. Since CTL1/SLC44A1 can localize in the PM and the mitochondrial membrane (MM), we further analysed the CTL1/SLC44A1 protein distribution separately in the PM and in the mitochondria. As shown in Figure 3.2D, the PM CTL1/SLC44A1 was significantly reduced (~40%) after PAM treatment but not after OLA treatment, while the PM biomarker Na/K ATPase was intact (Figure 3.2C). Surprisingly, the opposite was observed in the MM, where CTL1/SLC44A1 was significantly reduced (~40%) by OLA but not by PAM (Figure 3.2E); the mitochondrial biomarker COX-IV even slightly increased by both treatments (Figure 3.2C). Considering the CTL1/SLC44A1 distribution in the PM and MM membranes, the

CTL1/SLC44A1 PM:MM ratio for PAM was 0.62 and for OLA 1.55 when compared to untreated cells (PM:MM=1:1). Therefore, the PAM treated cells had 38% less CTL1/SLC44A1 on the cell surface than in the mitochondria, whereas OLA treated cells had 55% less CTL1/SLC44A1 in the mitochondria than on the cell surface. Taken together, the results established that PAM and OLA differentially modified the total amount of CTL1/SLC44A1 protein content and its subcellular localization. PAM strongly reduced the total CTL1/SLC44A1 protein and the PM however it did not significantly affect the mitochondrial CTL1/SLC44A1 content. On the other hand, OLA maintained the total and the PM CTL1/SLC44A1 but had reduced mitochondrial CTL1/SLC44A1. Therefore, the data established distinct influences of FA on the intracellular pools and on the membrane localization of CTL1/SLC44A1.

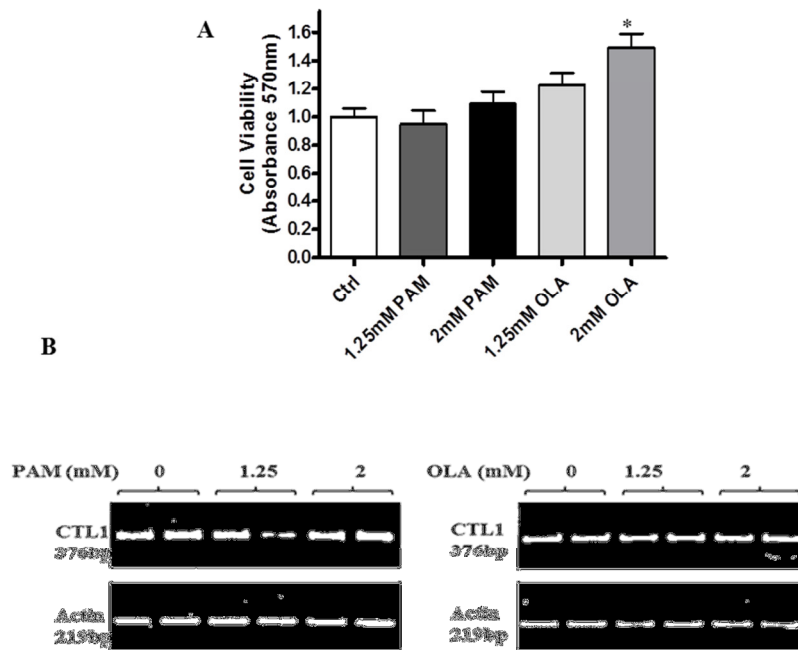


Figure 3. 1: PAM and OLA treatments did not reduce the cell viability and CTL1/SLC44A1 mRNA levels (A) C2C12 cell viability was measured after 16h treatment with different concentrations of PAM and OLA (1.25 and 2mM) and the results are expressed as absorbance at 570nm. OLA but not PAM stimulates the cell growth.

Experiment was repeated 4 times in triplicates. (B) CTL1/SLC44A1 mRNA levels in myotubes after OLA and PAM treatments determined by RT-PCR. FA treatments do not affect CTL1/SLC44A1 mRNA expression. Experiment was repeated 3 times in duplicates. The significance shown is at * $P < 0.05$ and relative to untreated controls (Ctrl).

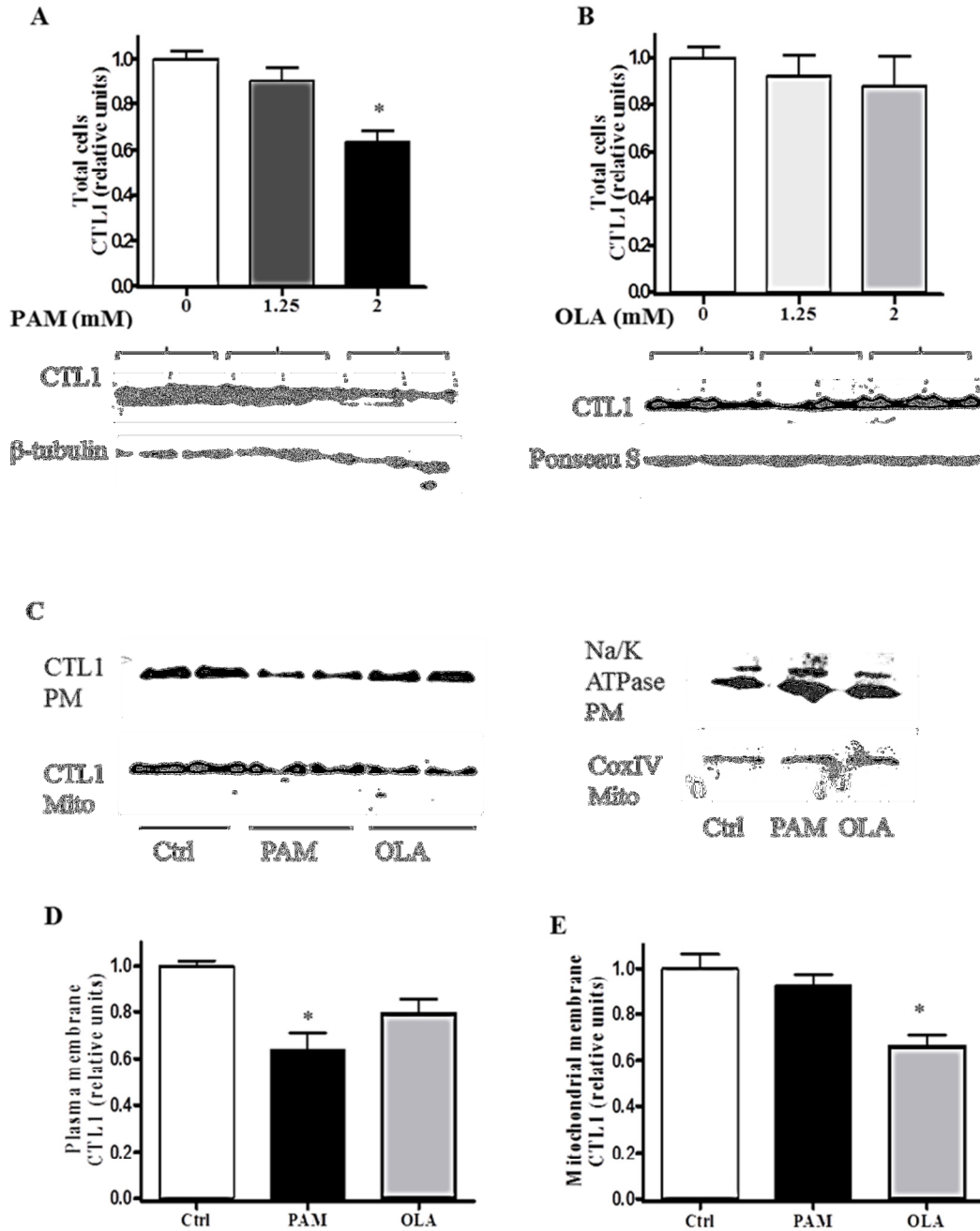


Figure 3. 2: : FA treatments differently affected the CTL1/SLC44A1 protein content and distribution. In total cell lysates, PAM (A) but not OLA (B) treatments reduce the amount of the total CTL1/SLC44A1 protein when compared with untreated cells (0mM; Ctrl). Ponceau S band at 55KDa and β -tubulin at 50KDa were used as controls. (C,D,E) PAM and OLA treatments at 2mM redistribute the CTL1/SLC44A1 protein differentially. (C) Representative figure of CTL1/SLC44A1 immunoblotting in the plasma membrane (PM) and mitochondrial membrane (Mito). Na/K ATPase and COX IV were used to test the purity of plasma membrane and mitochondrial

fractions, respectively. (D) Plasma membrane CTL1/SLC44A1 is reduced by PAM (2mM) treatments but not by OLA (2mM) treatments. (E) Mitochondrial CTL1/SLC44A1 is reduced after OLA (2mM), but not PAM (2mM) treatments. Results from at least three experiments are expressed relative to untreated controls (Ctrl=1) at *P < 0.01.

3.3.2 Mechanisms for reduction of total CTL1/SLC44A1 protein content by PAM

Since the CTL1/SLC44A1 mRNA level was not affected by PAM treatment we concluded that the reduced amount of total CTL1/SLC44A1 protein was a consequence of elevated protein degradation. To elucidate the mechanism by which the CTL1/SLC44A1 protein was degraded, PAM treated and untreated myotubes were incubated in the presence of the proteasomal inhibitor MG132 or the lysosomal inhibitor leupeptin and the CTL1/SLC44A1 protein content was determined by immunoblotting. The results (Figure 3.3A) revealed that the lysosomal inhibitor leupeptin was able to prevent the CTL1/SLC44A1 degradation in PAM treated cells, and actually to maintain its levels as in untreated cells. On the other hand, the proteosomal inhibitor MG132 did not prevent the CTL1/SLC44A1 degradation caused by PAM treatments.

To probe if the PAM, but not OLA, induced degradation of CTL1/SLC44A1 was by the autophagosomal-lysosomal pathway, we also measured the lipidation of the autophagy-specific protein LC3. Although the total LC3 protein (LC3I + LC3II) was also reduced by PAM treatments, the ratio between the lipidated and unlipidated forms (LC3II/LC3I), a measure of the autophagy level, was significantly increased by PAM (2.2 fold, $p < 0.01$), but not by OLA (Figure 3.3B). That PAM could induce the autophagy in C2C12 cells has been shown previously [27,28]. Therefore, the above data firmly demonstrated that in the PAM treated cells CTL1/SLC44A1 protein was degraded by the autophagosomal-lysosomal pathway not by the proteosomal pathway. On the other hand, OLA treatments did not have significant effect on

CTL1/SLC44A1 degradation and cell autophagy and the values remained comparable to untreated controls (Figures 3.2B, 3.3B).

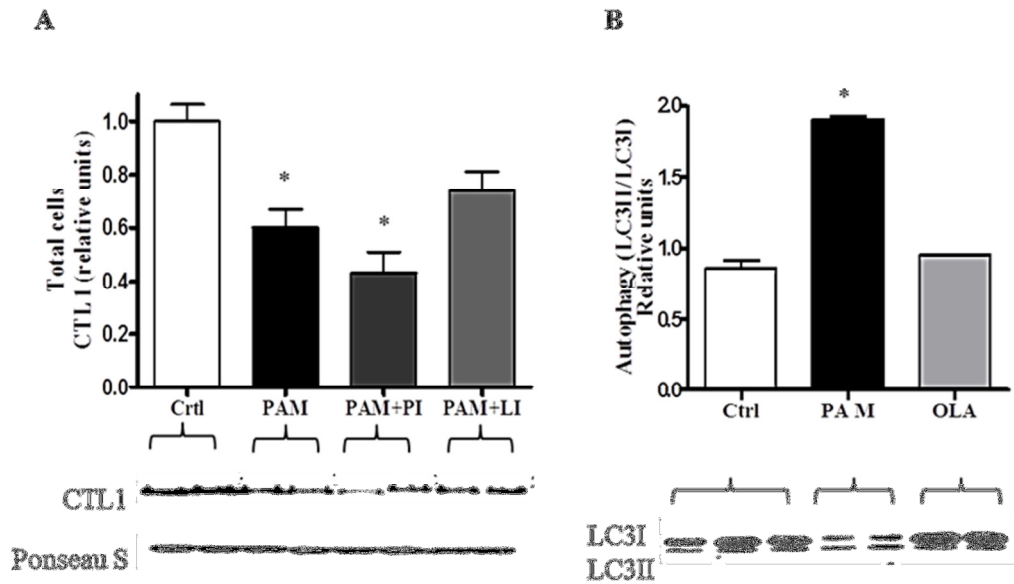


Figure 3. 3: Mechanism of the CTL1/SLC44A1 protein degradation by PAM (A) CTL1/SLC44A1 protein expression after co-incubation of PAM (2mM) treated myotubes with proteasomal (PI) or lysosomal (LI) pathway inhibitors; Lysosomal proteolysis inhibitor leupeptin prevents the CTL1/SLC44A1 degradation triggered by PAM. (B) PAM (2mM) but not OLA (2mM) treatments increase the autophagy marker LC3 lipidation (LC3II/LC3I). Results were replicated 3 times and are expressed relative to untreated controls (Ctrl=1). β -tubulin and Ponceau S were used as loading controls. *P < 0.01 compared with untreated controls-Ctrl.

3.3.3 OLA and PAM alter the cell surface choline uptake differently

After establishing differences in the CTL1/SLC44A1 total protein content and its membrane distribution, we probed if choline uptake was altered by PAM and OLA treatments. Choline uptake was determined in intact cells, to specifically measure the PM transport and in the isolated mitochondria to establish differences at this organelle only. In the intact cells, choline uptake was 50% reduced after PAM treatment (Figure 3.4A) but not after OLA treatment (Figure

3.4B) which fully agreed with the reduced CTL1/SLC44A1 content at the PM (Figure 3.2D) specifically by PAM treatments. OLA treatments on the other hand did not influence the PM CTL1/SLC44A1 content and choline transport (Figures 3.2D, 3.4B). We concluded that PAM but not OLA reduced the PM choline transport that was driven by the content of the PM CTL1/SLC44A1 transporter.

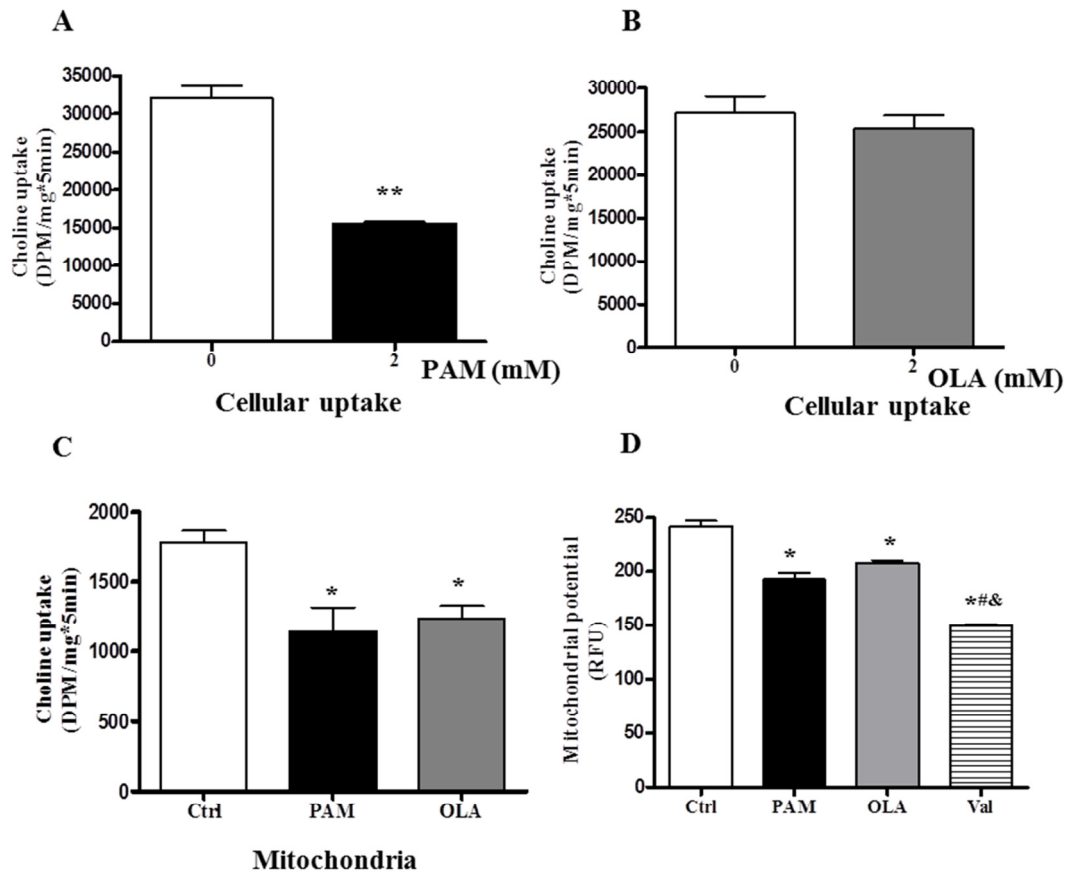


Figure 3. 4: Different effects of PAM and OLA on choline uptake. (A,B) The extracellular choline uptake is reduced with PAM (2mM) and not affected with OLA (2mM) when compared with untreated cells (Ctrl; 0mM). (C) Mitochondrial choline uptake is reduced with both, PAM and OLA at 2mM. Results from 3 different experiments are expressed as DPM/mg/5min at ** $P < 0.001$ and * $P < 0.05$ compared with Ctrl. **(D) PAM and OLA treatments reduce the mitochondrial membrane potential.** The mitochondrial potential was measured using JC-1 red fluorescence at 590nm in PAM and OLA treated and untreated cells. Valinomycin (Val) in the concentration of

1µg/mL was used as a dissipation agent. Experiment was replicated 3 times. *P<0.001 compared with Ctrl; # P<0.001 compared with PAM; &P<0.001 compared with OLA.

3.3.4 OLA and PAM both reduce the mitochondrial choline uptake

Since we also found a significantly lower mitochondrial CTL1/SLC44A1 protein after OLA but not after PAM treatments, we analysed the uptake of the radiolabeled choline in isolated mitochondria. Data in Figure 4c showed that the mitochondrial choline uptake was significantly reduced after OLA treatments as could be expected based on the obtained reduced CTL1/SLC44A1 content in the mitochondria (Figure 3.2E). Surprisingly, the mitochondrial choline uptake was also reduced after PAM treatments even though the mitochondrial CTL1/SLC44A1 protein was not significantly changed by PAM (Figure 3.4C).

Therefore, we next tested if the treatments with OLA and PAM influenced the mitochondrial function in intact cells. We measured the mitochondrial potential using the standard JC-1 staining protocol and found out that the mitochondrial potential was significantly reduced after both treatments (Figure 3.4D), showing that factors additional to CTL1/SLC44A1 are involved in regulation of the mitochondrial choline uptake.

3.3.5 OLA stimulates the CDP-choline pathway but not PC levels

Since PAM and OLA differently regulated the cell surface CTL1/SLC44A1 and choline uptake we next investigated if this effect influenced PC synthesis by the CDP-choline (Kennedy) pathway. The PC synthesis was followed by 3h pulse radiolabeling with [³H]choline in the presence of 4mg/L choline from the media (Figure 3.5). The choline intermediates of the

Kennedy pathway (choline, P-Cho and CDP-Cho) were also analyzed after 3h-pulse with [^3H]choline. We found that P-Cho labeling was decreased after both PAM and OLA treatments while CDP-Cho synthesis was significantly increased after OLA treatments (Figure 3.5A). The reduction of P-Cho with the concomitant increase in CDP-Cho demonstrated that the step regulated by CCT/Pcyt1 was up-regulated by OLA, which then contributed to the greater PC synthesis. The OLA stimulation of CCT/Pcyt1 has been extensively studied and is a well-established regulatory mechanism for the PC Kennedy pathway [16, 198, 199]. As shown in Figure 5c, the [^3H]choline incorporation into PC was significantly stimulated by OLA treatments relative to untreated cells ($p < 0.05$; 5.6 fold). On the other hand, in PAM treated cells, the incorporation of [^3H]choline into PC was only modestly increased relative to the untreated cells ($p = 0.15$; 2.4 fold) (Figure 3.5C). When we performed longer (16h) radiolabelling experiments to identify the PC pools under various FA treatments, the incorporation of [^3H]choline into PC did not vary between treatment groups and control (Figure 3.5C). Similarly, the total content of PC was not significantly altered after PAM and OLA treatments (Figure 3.7A).

PC turnover was determined by measuring the efflux of choline metabolites out of the cells after a 16h equilibrium labeling with [^3H]choline, followed by 2.5h and 4h chase with 1mM excess of cold-choline. The efflux of choline metabolites was increased by PAM treatments after 2.5 and 4h chase ($p < 0.001$; $p = 0.05$, respectively), and even more increased by OLA treatments ($p < 0.001$; $p < 0.01$, respectively; Figure 3.5D) when compared with control cells. Also, the efflux of choline was higher in OLA than PAM treatments at both time points ($p < 0.001$; $p < 0.05$, respectively). The total rate of efflux in DPM/mg*h was determined from the slopes of the curves and it was 5310 ± 378.4 , 6907 ± 1256 and 9245 ± 1686 for control, PAM and OLA cells, respectively. We

concluded that total PC content was maintained after FA treatments by elevated PC degradation, which was the most prominent after the OLA treatment.

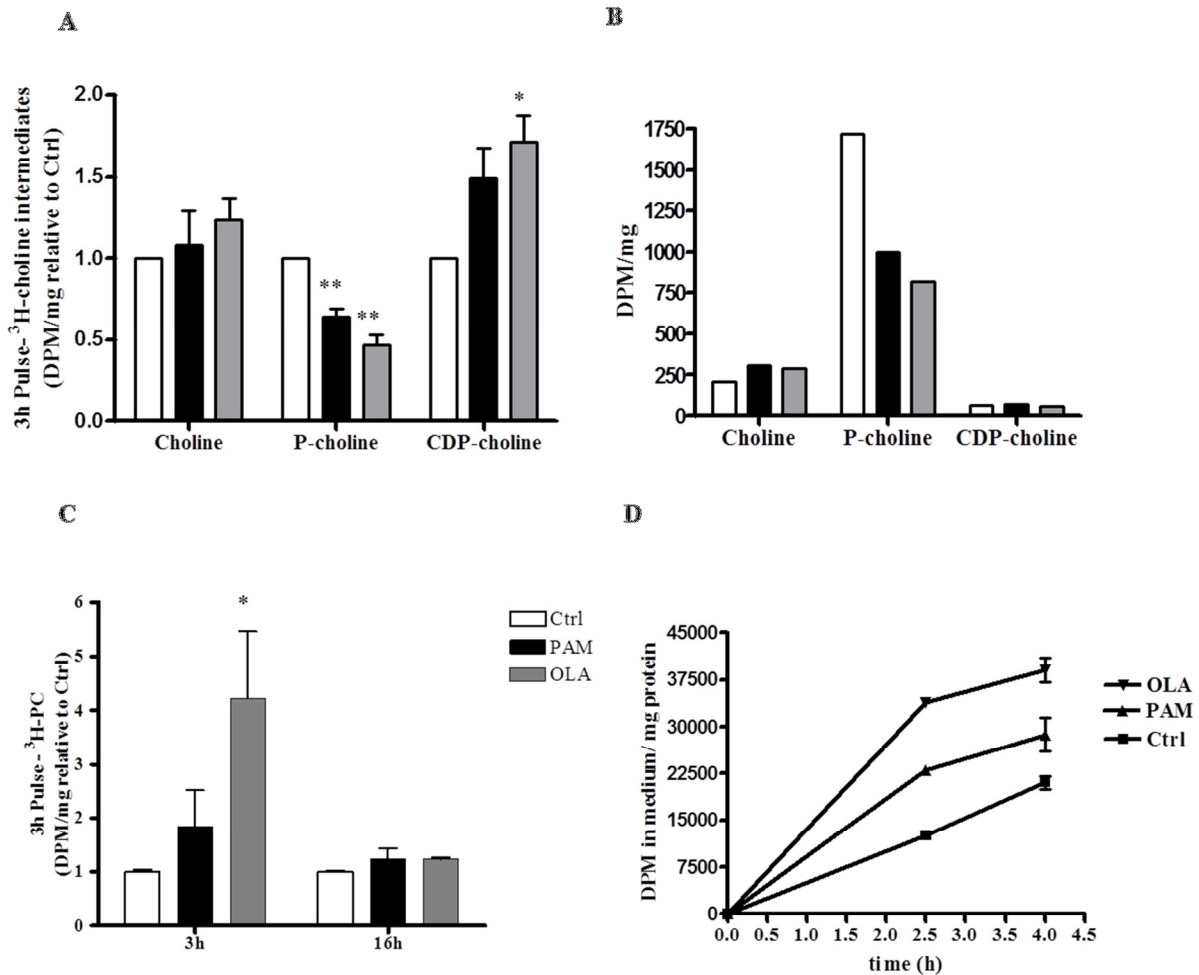


Figure 3. 5: The effect of fatty acid treatments on the rate of PC synthesis and degradation.

(A) Choline, P-choline and CDP-choline synthesis from ³H-choline after 3h-pulse. (C) PC synthesis after 3h pulse and the PC pool size after 16h of equilibrium radiolabeling. (A,C) Results from multiple experiments are expressed in dpm/mg relative to controls (Ctrl=1); for a demonstrative purpose the values for the single experiment in (B) are in dpm/mg. (D) ³H-choline

released to medium after 16h labeling followed by 2.5h and 4h chase period. Results in triplicates are expressed as media DPM/mg protein. *P < 0.05 and **P<0.01 compared with Ctrl.

3.3.6 PAM and OLA differently modulate TAG and DAG synthesis and content

In order to evaluate the effect of different FA treatments on DAG and TAG metabolism, we performed pulse and pulse-chase experiments using [^3H]glycerol (Figure 3.6). We established that TAG synthesis was not affected by PAM, but it was significantly elevated by OLA. DAG synthesis was on the other hand higher after PAM treatments and unchanged after OLA treatments (Figure 3.6A, B). The pulse-chase experiments showed that the degradation of TAG was inhibited by both treatments, however the inhibitory effect of OLA was stronger than the effect of PAM (Figure 3.6C). DAG degradation was not affected by any FA treatment (Figure 3.6C).

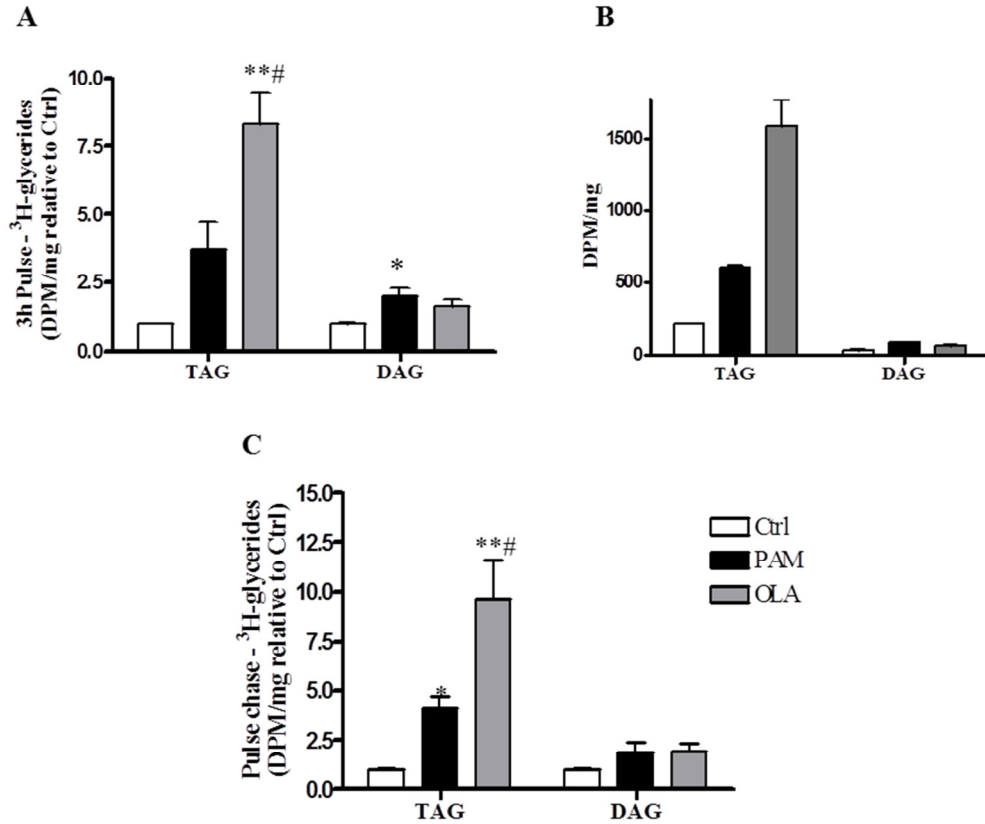


Figure 3. 6: The effect of FA on TAG and DAG turnover. (A) ³H-glycerol 3h-pulse radiolabeling showing that TAG synthesis is increased by OLA treatment, whereas DAG synthesis is increased only after PAM treatments. (B) Representative DPM/mg of 3h-pulse. (C) ³H-glycerol 3h-pulse-chase radiolabeling showing that TAG degradation is reduced after PAM and greater after OLA treatments and that DAG degradation is not altered after PAM and OLA treatments. (A,C) Results from at least three experiments are expressed as dpm/mg relative to control (Ctrl=1). *P<0.05 compared with Ctrl; **P<0.01 compared with Ctrl; #P<0.01 compared with PA.

3.3.7 Lipid content in untreated and treated myotubes

The analysis of total phospholipid content showed that PC, phosphatidylserine (PS) and sphingomyelin were not altered by PAM and OLA treatments (Figure 3.7A). In the case of PC, the amounts were not altered because the rates of PC synthesis and degradation were both

proportionally increased (Figure 3.5). Among other phospholipids, PE amount was significantly reduced (1.2 fold) with OLA while PI was significantly increased (1.3 fold) with PAM (Figure 3.7A). Some increase in PE was also observed after PAM treatments and it may be related to the increased autophagy in those cells, since LC3I is specifically lipidated with PE to form LC3II (Figure 3.3B)

We also measured TAG, free FA and cholesterol content in treated and untreated cells as shown in Figure 3.7B and 3.7C. TAG content was elevated by both FA treatments (Figure 3.7B). However, greater amounts of TAG were present after OLA treatments (275.6 ± 29 mg/mg protein) than after PAM treatments (168.9 ± 26.4 mg/mg protein), in agreement with the pulse- and pulse-chase radiolabeling data (Figure 3.6). The FA content was high in both groups and much higher in OLA (15.9 folds) than in PAM (5.5 folds) treatments (Figure 3.7C). Cholesterol levels did not change but had a tendency to be increased in PAM group and reduced in the OLA group. Therefore we concluded that PAM and OLA distributed differently in various lipids, changing the amounts and the membrane phospholipid ratio, such as between PC:PE and sphingomyelin:cholesterol ratio in those cells. The PC:PE ratio was significantly higher in OLA group (1.35 ± 0.02) compared with both PAM (1.098 ± 0.02) and Ctrl group (1.041 ± 0.03). On the other hand, sphingomyelin:cholesterol ratio was lower in PAM group (0.216 ± 0.003) than in OLA (0.236 ± 0.004) and Ctrl (0.2420 ± 0.008).

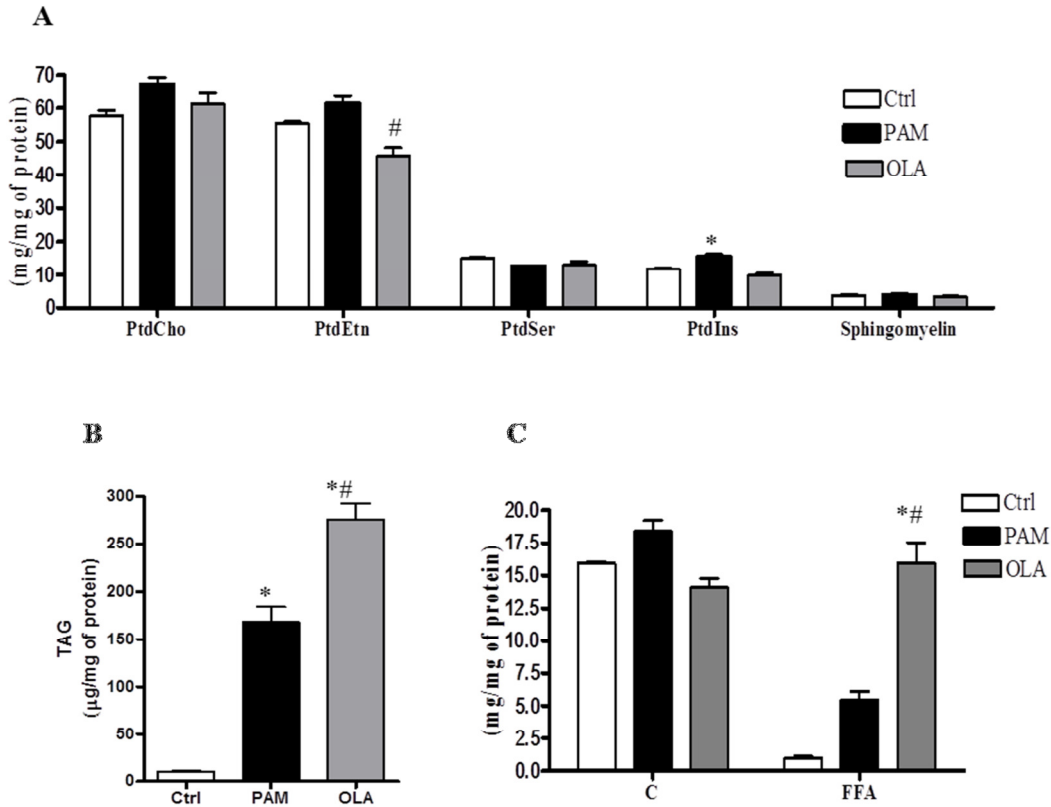


Figure 3. 7: Total glycerolipid content after PAM and OLA treatments. (A) The total amounts of PC, PE, phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SM) was analysed by HPLC. PC, PS and SM are not significantly altered by FA treatments. PE level is higher after PAM treatment compared with OLA and PI is higher in PAM group than in Ctrl and OLA (b,c) TAG, free cholesterol (C) and free FA (FFA) content were also analysed by HPLC. TAG content is higher after PAM treatment and even higher after OLA treatment. FFA content is significantly higher only after OLA treatment, and cholesterol level is not affected by FA treatments. * $p < 0.05$ compared with Ctrl; # $p < 0.05$ compared with PAM.

3.4 Discussion

The present study was the first to demonstrate that transport and metabolism of choline are differentially regulated by saturated (PAM) and unsaturated (OLA) FA in differentiated skeletal muscle cells. We established the relationship between the exposure to FA and choline transport

by specific choline transporter CTL1/SLC44A1, and their effects on intracellular homeostasis of PC and other lipids. This work may help to better understand the relationship between choline transport and FA metabolism under conditions of excess energy intake and development of muscle insulin resistance.

PAM and OLA have distinct ways of regulating energy homeostasis. PAM is associated with high intracellular content of DAG and FA, which leads to lipotoxicity and insulin resistance in the skeletal muscle [187, 188]. OLA was found to protect against lipotoxicity and increases mitochondrial FA oxidation [200]. Interestingly, when fed a high fat diet (HFD) PEMT knock-out mice, which cannot use PE to form PC in the liver, were protected against obesity (due to TAG accumulation) and insulin resistance (due to excess DAG and FA) because the upregulated *de novo* PC synthesis in the liver [201]. On the other hand, choline deficiency reduced *de novo* PC synthesis and caused accumulation of TAG in the liver and muscle cells [16, 39, 201]. Here we investigated the effect of FA on PC synthesis in muscle cells (mimicking the high fat diet) in the presence of extracellular choline. Muscle cells do not have the PEMT pathway and exclusively rely on the *de novo* Kennedy pathway for PC synthesis. We showed that as in the liver of the PEMT deficient mice fed a HFD, fatty acids could increase *de novo* PC synthesis in the muscle cells. OLA showed a higher stimulation of PC synthesis than PAM, in conjunction with an increased muscle cell proliferation. We further showed that both FA stimulated PC turnover and the efflux of choline metabolites, with OLA having a more prominent effect than PAM. In accordance, previous studies have shown that stimulation of PC synthesis by the Kennedy pathway is usually accompanied by an accelerated PC turnover, in order to maintain the membrane PC content [202]. Indeed, we found that the total content of PC did not differ between untreated and treated myotubes. As such, our study strongly suggested that in the

presence of excess of FA choline protected the muscle cells by facilitating PC synthesis and thereby reducing the accumulation of toxic lipid intermediates. We demonstrated that supply of choline is important as protection in the treatments with OLA, when choline uptake by PM CTL1/SLC44A1 was optimal to support highly stimulated PC synthesis. PAM however inhibited PM choline uptake and reduced the content of PM CTL1/SLC44A1, resulting in a limited stimulation of PC synthesis, which may reduce protection from lipotoxicity.

Treatments of muscle cells with PAM induced CTL1/SLC44A1 protein degradation through autophagy. On the contrary, OLA treatments stimulated muscle cell growth and did not cause autophagy and CTL1/SLC44A1 degradation. PAM has been shown to stimulate autophagy in C2C12 cells and other cell types, whereas OLA did not demonstrate such effect [203, 204]. PAM is also well-known for inducing damage to mitochondria, insulin resistance and cell death [189], and PAM-induced autophagy was recently identified as a pro-survival mechanism [204, 205]. We found that PAM treatments did not affect the cell viability and that the autophagy was in fact protective against PAM toxicity.

The formation of autophagic membranes involves the conjugation of LC3 to PE (lipidation) to generate LC3-II, which is one of the best characterized structural components of the autophagosomes [206]. We showed that PAM treatments induced autophagy, and increased the content of PE when compared with OLA treatments. The increase in PE levels by PAM may be a mechanism to provide more lipids for LC3 lipidation and autophagosome formation. Similar to our findings, previous study in pancreatic β -cells found an increase in PE mass after PAM treatment [207].

We also demonstrated that the mitochondrial choline transport is differently regulated in relation to the plasma membrane choline transport. The transport of choline to the mitochondria was

inhibited by both FA. Mitochondrial CTL1/SLC44A1 content was not drastically changed by PAM, but it was reduced by OLA. Since the total CTL1/SLC44A1 protein was not affected by OLA, the reduction specifically in the mitochondria indicated that OLA treatments limited the CTL1/SLC44A1 trafficking from the intracellular stores to the mitochondrial membranes. In the liver, mitochondrial CTL1/SLC44A1 is well-known to facilitate the transport of cytosolic choline for betaine synthesis. However, CTL1/SLC44A1-mediated choline transport also occurs in other cell types that do not produce betaine [36]. This ubiquitously present mitochondrial CTL1/SLC44A1 maintain the choline gradient and serve as a reservoir for choline metabolically generated from choline containing lipids such as PC and sphingomyelin. Mitochondrial membranes are abundant in PC, which is synthesised in the ER and imported into the mitochondria. Mitochondrial PC can be metabolized by phospholipase D or phosphatidylserine synthase 1 (PSS1), which both release free choline [177, 208]. CTL1/SLC44A1 may transport the newly generated choline out of mitochondria to provide free choline for PC synthesis at the ER. We showed that both PAM and OLA treatments reduce the uptake of choline into mitochondria, which offers the most plausible mechanism to preserve the cytosolic pool of choline when demands for PC synthesis increase. However, the CTL1/SLC44A1 content in the mitochondria was reduced by OLA, but not PAM, which suggests that the transport of choline into the mitochondria depends on other mechanisms besides CTL1/SLC44A1. In addition, the decrease in CTL1/SLC44A1 content by OLA may be caused by reduced PC catabolism (lower choline efflux) in the mitochondria.

We found that the mitochondrial choline uptake and the mitochondrial potential were both reduced by OLA and PAM. The regulation of choline uptake into mitochondria is complex and several factors are involved. These include changes in the electrochemical gradient, the content

and the functionality of the choline transporter CTL1/SLC44A1, and a poorly characterized inner membrane transport system [209, 210].

The lipid composition of cellular membranes is responsible for maintaining both the structural integrity and function of a cell. Disturbance of membrane homeostasis may include changes in the amount of individual phospholipids, as well as changes in the ratio of important structural lipids, such as PC:PE and sphingomyelin:cholesterol. The PC:PE ratio is positively correlated with membrane integrity and cellular and mitochondrial function [211, 212]. Here we found that PC:PE ratio is higher in OLA treated cells, suggesting an improvement in the cellular function either to compensate for the excessive FA supply or to increase mitochondria capacity to catabolise FA. Sphingomyelin:cholesterol ratio, however, was decreased in PAM treated cells. Interestingly, decrease in sphingomyelin:cholesterol ratio was found to impair glucose transport and insulin sensitivity [213], which is well known to be affected by PAM.

We demonstrated that choline transport and metabolism are differently regulated by OLA and PAM oversupply. The plasma membrane CTL1/SLC44A1 provides an efficient supply of extracellular choline; however, whether CTL1/SLC44A1 also regulates the cellular choline efflux needs to be further elucidated. PAM strongly reduced the choline uptake and CTL1/SLC44A1 content at the plasma membrane, which only offered a limited stimulation of PC synthesis when compared to OLA. The highly increased PC synthesis by OLA, however, may provide an efficient mechanism of protection from excess toxic lipid intermediates. Intracellular demands for choline utilization in the Kennedy pathway dictate the activity of the mitochondrial CTL1/SLC44A1, which became diminished when more choline was needed for PC synthesis, as shown in PAM and OLA treatments. Mitochondrial CTL1/SLC44A1 could be functionally impaired by reduction in the mitochondrial potential, as reflected by the presence of

autophagy and dysregulated mitochondria after PAM treatments [189, 214]. Further investigation is needed to identify the exact mechanisms that drive the presence and function of CTL1/SLC44A1 in the mitochondria and the role of CTL1/SLC44A1 in mitochondrial pathologies.

4 Mechanism of choline deficiency and membrane alteration in postural tachycardia syndrome primary skin fibroblasts

Abstract

Fibroblasts from a Postural Tachycardia Syndrome (POTS) patient, who presented with low plasma choline and betaine, were studied to determine the metabolic characteristics of the choline deficiency. Choline is required for the synthesis of the phospholipid phosphatidylcholine (PC) and for betaine, an important osmo-regulator. Here, choline transport, glycerolipid homeostasis, and mitochondria function were analysed in skin fibroblasts from POTS and compared with control cells. The choline transporter CTL1/SLC44A1 protein and mRNA expression were decreased 2 to 3-fold in POTS fibroblasts and choline uptake was reduced 60% ($p < 0.05$). In addition, PC synthesis via the CDP-choline pathway was reduced and PC degradation increased in these cells compared to control. There was also a >3-fold decrease in the phospholipid pool and modified fatty acid composition of PC, phosphatidylethanolamine and phosphatidylserine. Triacylglycerol formation increased 50% in POTS, which is a feature of choline deficiency. Choline deficiency also impaired mitochondria function which was observed by a reduction in oxygen consumption, mitochondria potential and glycolytic activity. When POTS cells were treated with choline, uptake increased, and the levels of glycerolipids were restored, offering an option for patient treatment. The characteristics of the POTS fibroblasts described here represent a model of choline and/or CTL1/SLC44A1 deficiency, in which glycerolipid homeostasis and mitochondrial function are impaired.

4.1 *Introduction*

Postural Tachycardia Syndrome (POTS) is characterized by orthostatic intolerance accompanied by cerebral hypoperfusion and sympathetic hyperactivity. Several studies indicate that POTS is pathophysiologically heterogeneous and frequently presents multiple chronic symptoms [215]. The cause of this condition is still unclear. Many POTS patients have hypovolemia and require volume expansion with adequate daily water and sodium intake or pharmacological therapy to promote intravascular volume expansion or peripheral vasoconstriction. Here we studied primary skin cells from a 28-year-old female diagnosed with POTS who presented with low concentrations of plasma betaine and choline. A possible connection between these observations may be the roles of betaine and glycerophosphocholine as osmolytes, which regulate blood pressure [216], as well as the role of phosphatidylcholine (PC) and its metabolites in membrane fluidity and integrity [212, 217].

Intracellular choline availability and metabolism are associated with glycerolipid homeostasis [39, 218, 219]. Choline is required for the biosynthesis of the major membrane phospholipid PC, which plays a major role in cellular proliferation, membrane fluidity and functions and lipid metabolism [220]. The majority of PC synthesis occurs via the CDP-choline or Kennedy pathway [31, 220]. The transport of choline into the cell for PC synthesis is largely regulated by the choline transport-like protein 1 (CTL1/SLC44A1) in the plasma membrane [33]. Immediately after entering the cells, choline is phosphorylated by choline kinases [100]. The kinase product phosphocholine is then coupled with CTP by the regulatory pathway enzyme CTP:phosphocholine-cytidylyltransferase (CCT/Pcyt1) to yield CDP-choline and to release inorganic pyrophosphate. In the final step, the CDP-choline derivative is condensed with diacylglycerol (DAG), catalysed by multiple DAG:choline- and DAG:ethanolamine-

phosphotransferases (CPT, EPT, CEPT), to release CDP and to produce the bilayer forming phospholipid PC at the endoplasmic reticulum. In the liver, an alternative pathway utilizes phosphatidylethanolamine (PE) to produce PC in a three-step methylation of PE by S-adenosylmethionine (AdoMet) catalysed by phosphatidylethanolamine N-methyltransferase (PEMT) [44]. The newly synthesised PC can be converted to other phospholipids: PC can be metabolized to generate phosphatidylserine (PS) by the exchange of serine for the choline head-groups of PC; PS can then be decarboxylated to PE by PS decarboxylase. In addition, PC metabolizes to glycerophosphocholine, a major osmolyte derived from choline. The PC catabolism is mainly catalysed by non-lysosomal phospholipases. These phospholipases are divided into three classes based on the bond they cleave: Phospholipase A (PLA1 and PLA2), phospholipase C (PLC) and phospholipase D (PLD). PLD hydrolyzes PC to form phosphatidic acid (PA) and choline. The latter, in turn, can re-enter the Kennedy pathway or be converted to betaine.

Choline can be oxidized to betaine in the liver and kidney mitochondria catalyzed by choline dehydrogenase and by betaine aldehyde dehydrogenase [44, 221]. The rate of choline oxidation depends on the availability of choline in the mitochondria, which is controlled by protein-mediated choline transport through mitochondrial membranes. In the outer mitochondrial membrane, CTL1/SLC44A1 is characterized as a choline transporter and regulates the choline uptake into mitochondria [36]. An inner mitochondrial membrane transporter has been shown to participate in choline oxidation to betaine; however its structural identity is still unknown [42]. Betaine is a methyl group donor that can enter the one-carbon metabolism and re-methylate homocysteine to methionine, a reaction catalysed by betaine-homocysteine-methyltransferase (BHMT). The transfer of adenosine to methionine then yields AdoMet, which is the source of

methyl groups for numerous methyltransferases, such as PEMT. Besides its role as a methyl group donor, betaine is an important organic osmolyte that helps to maintain cell volume and hydration of cellular proteins in the kidney [56, 57].

The low plasma choline observed in this POTS patient may be an indicator of altered lipid homeostasis, membrane integrity and cell function. Here we describe the results of a series of experiments designed to characterize the transport and metabolism of choline and the regulation of phospholipids in primary skin cells isolated from the POTS patient in order to investigate why plasma choline and betaine concentrations were so low.

4.2 *Materials and methods*

4.2.1 Blood metabolites

The POTS patient was fasted overnight (at least 12 hours) before blood was drawn by venipuncture into an EDTA tube, which was immediately placed on ice and spin in the cold to separate plasma for analysis, or a serum separator tube to collect serum after clotting. Choline, phosphatidylcholine, and betaine were extracted from plasma by the method of Bligh and Dyer [193]. Aqueous and organic compounds were separated, analyzed, and quantified directly by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry after the addition of internal standards labeled with stable isotopes to correct for recovery [222]. S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) in plasma were measured by HPLC with fluorescence detection after conversion into their fluorescent isoindoles [223]. These measurements were done by Dr. Conrad Wagner at Vanderbilt University, TN.

Methionine, homocysteine (tHcy), and nine other metabolites in Table 1 were measured in serum with the use of a capillary gas chromatography–mass spectrometry method previously described [224]. These measurements were done by Dr. Sally Stabler, University of Colorado. Briefly, serum samples were extracted and metabolites were isolated with the use of an anion-exchange column, derivatized with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide, and analyzed by mass spectrometry. Deuterated standards were used to correct for recovery.

4.2.2 Isolation and maintenance of primary POTS cells

Skin biopsy tissues from the POTS patient were minced into small pieces and placed in a tissue cultured flask with minimum essential medium (MEM, containing 0.001g/L of choline chloride) supplemented with 20% fetal bovine serum (FBS) and 1% antibiotic-antimycotic mixture. Flasks were kept in a humidified atmosphere at 37°C and 5% CO₂. After three weeks fibroblast cells began to grow out from the skin pieces and, once they reached confluency, cells were trypsinized and transferred to a new flask. Cells were kept in culture for approximately 10 days before passage and media was changed every 3 days. An aliquot of cells was kept frozen in liquid nitrogen. Cell lines from two unrelated healthy individuals with a similar background were purchased from the Coriell Institute's cell repositories (Catalog # GM02674, Camden, NJ, USA) and maintained under the same conditions. These fibroblasts were used as control samples for comparison with those from the POTS patient.

4.2.3 Cell treatments with excess choline

Fibroblasts from the POTS patient and controls were grown with 2.5 and 5mg/L of choline chloride (Sigma Aldrich, Oakville, ON, Canada) for 10 days, which had been added to the media described above, and compared with POTS cells and the two control cell lines grown without choline added. After treatment, cells were washed with phosphate-buffered saline (PBS), and used for protein and mRNA expression, choline uptake and metabolic studies.

4.2.4 Immunoblotting

Fibroblasts were lysed in a lysis buffer (25mM Tris, 15% glycerol, 1% Triton-100, 8mM MgCl₂, 1mM DTT, protease and phosphatase inhibitor cocktail) on ice for 5 min and scraped into 1.5mL tubes. Cell debris was removed by centrifugation at 18,500g for 2 min at 4°C. Protein concentration was determined with bicinchoninic acid (BCA; Pierce, Rockford, IL, USA). The ENS-627 antibody was previously developed by Dr. Bakovic's lab and was shown to detect the 72 kDa size CTL1/SLC44A1 protein under non-denaturing conditions [36]. Briefly, samples were mixed with non-denaturing loading buffer (62mM Tris-HCl, 0.01% bromophenol blue and 10% glycerol) and separated by polyacrylamide gel electrophoresis at 120V for 1.5 h. Proteins were transferred to polyvinylidene difluoride membranes (Roche, Indianapolis, IN, United States) and stained with Ponceau S. Membranes were blocked in 5% skim milk in TBS-T and then incubated with the CTL1/SLC44A1 antibody ENS-627 (1:100 in 5% skim milk in TBS-T) overnight at 4°C [36]. Membranes were washed with TBS-T and then incubated with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000 in 5% skim milk in TBS-T) for 1 hour. Membranes were washed in TBS-T and proteins were visualized using a

chemiluminescent substrate (Fisher Scientific, Ottawa, ON, Canada). The amount of cell β -tubulin was determined in parallel and used as a loading control.

4.2.5 RNA isolation and RT-PCR

Total RNA from POTS and control skin cells was isolated with TRIzol (Invitrogen, Life Technologies Inc., Burlington, ON, Canada). DNase I was used to eliminate genomic DNA and cDNA was then synthesized from 2 μ g of RNA using Superscript II reverse transcriptase (Invitrogen, Life Technologies Inc., Burlington, ON, Canada). Expression of CTL1/SLC44A1 mRNA was determined by PCR using the primers F/ TCAACAGCACCAACTTCTGC and R/ ACAGGAAGCAATGAGCGACT . PCR conditions were 94°C for 5 min, 32 cycles of 30s at 94°C, 30s at 55°C and 30s at 72°C, and final extension for 5 min at 72°C.

4.2.6 Isolation of mitochondria

Crude mitochondria were isolated from POTS and control cells according to standard protocols for differential centrifugation [36]. Briefly, cells were incubated 10 min with RSB buffer (10mM NaCl, 1.5mM MgCl₂, 10mM Tris-HCl) and homogenized. Then MS buffer (210mM mannitol, 70mM sucrose, 5mM Tris-HCl and 1mM EDTA) was added and the homogenate was centrifuged at 590g for 10 min at 4°C. The final supernatant was centrifuged at 14,700g for 20 min at 4°C and the resulting pellet (crude mitochondrial fraction) was resuspended in the MS buffer; 20 μ g of mitochondrial protein was analyzed for CTL1/SLC44A1 expression by immunoblotting as described above. Crude mitochondrial fractions were also used for choline uptake assays and mitochondrial membrane potential.

4.2.7 Choline uptake in whole cells

For measuring choline uptake, cells were washed with KRH buffer (130mM NaCl, 1.3mM KCl, 2.2mM CaCl₂, 1.2mM MgSO₄, 1.2mM KH₂PO₄, 10mM HEPES pH 7.4, 10mM glucose) and incubated with 1μCi methyl [3H]-choline (60-90 Ci/mmol; American Radiolabeled Chemicals) for 5 min at room temperature. Cells were then washed in ice-cold KRH buffer containing 1mM “cold” choline, to stop the uptake and remove the radiolabeled choline from the cell surface, lysed in 500μL of ice-cold lysis buffer (10mM Tris-HCl, 1mM EDTA, 10mM NaF), scraped into 1.5mL tubes and [3H]-choline was analyzed by liquid scintillation counting. The rate of choline uptake in the whole cells was calculated as DPM/5min/mg of protein. Protein was quantified using the BCA protein assay (Pierce, Rockford, IL, USA).

4.2.8 Mitochondrial membrane potential and mtDNA content

After isolating crude mitochondria as described above, mitochondrial membrane potential was measured using Isolated Mitochondria Staining Kit (Sigma-Aldrich, Oakville, ON, Canada). The method uses carbocyanine dye (JC-1), which concentrates in the mitochondrial matrix and results in red fluorescence. A dissipation of potential prevents the accumulation of JC-1 dye and results in green fluorescence. Red fluorescence was read in the Flex station 3 microplate reader fluorimeter (emission=590nm, excitation=490nm) using a kinetic method. Valinomycin was used as a dissipating agent and negative control. Results were expressed as a mean fluorescence from three independent experiments.

Mitochondrial DNA (mtDNA) content was determined relative to chromosomal DNA. Total DNA was isolated from control and POTS cells using Wizard Genomic DNA Purification Kit (Promega). PCR was performed for the nuclear β -globin gene (forward primer 5'-aactgggcatgtggagacag-3' and reverse primer 5'-ccaggccatcactaaaggca-3') and mtDNA (forward primer 5'-cgaaaggacaagagaaataagg-3 and reverse primer 5'-ctgtaaagtttaagtttatgcg-3'), using the following conditions: 94°C for 5 min, 32 cycles of 30s at 94°C, 30s at 53°C (mtDNA) or 59°C (β -globin) and 30s at 72°C, and final extension for 5 min at 72 °C. The band density was measured using ImageJ. The mtDNA/ β globin cDNA ratio was used to determine mtDNA differences in POTS and control cells.

4.2.9 Pulse and pulse-chase radiolabeling of the CDP-choline pathway

For pulse experiments, POTS and control cells were incubated for 1-3h with 5nM [3H-methyl]choline (5 μ Ci; ARC, St. Louis, MO, USA). For pulse-chase experiments, cells were pulsed for 3h with 5nM [3H-methyl]choline, washed with PBS, and then chased 1-3h with excess of unlabelled choline. At each time point, cells were washed with 2xPBS and total lipids extracted by the Bligh-Dyer method [193]. An aliquot of cell lysates was used to measure the protein concentration. The water-soluble metabolites choline (Cho), P-choline (PCho) and CDP-Choline (CDP-Cho) were separated in ethanol/water/ammonia (48:95:7) and visualized with phosphomolybdic acid in chloroform-ethanol (1:1) and 1% stannous chloride. The radiolabelled PC was determined from the lipid phase after TLC separation with chloroform/methanol-acetic acid/water (40:12:2:0.75) and staining with 15% sulfuric acid and 0.5% K₂CrO₇. Specific bands were scraped from plates and radioactivity determined by liquid scintillation counting (LSC).

4.2.10 Determination of total glycerolipid pools

For the steady-state, equilibrium radiolabeling of cell lipids, control and POTS cells were incubated 24 h with 1 μ Ci of [^{3} H]-glycerol (ARC, St. Louis, MO, USA) or [^{3} H]-oleate (ARC, St. Louis, MO, USA) in standard growth media (2 ml of 20%FBS in MEM). The total lipids were extracted by Bligh-Dyer method [193]. PE, PC and PS were separated in a TLC plate using the solvent solution of chloroform/methanol-acetic acid/water (40:12:2:0.75) and visualized by staining with 15% sulfuric acid and 0.5% K₂CrO₇. TAG and DAG were separated with heptane/isopropyl alcohol/acetic acid (60:40:3) and stained with iodine vapour. Specific lipid bands were collected and radioactivity determined by LSC.

4.2.11 Lipidomic analysis

Cell lysates (5 x 10⁵ cells/sample in 100 μ L PBS) were kept at -80°C and sent in nitrogen liquid to Lipidomic facility. Prior to experiment, cell lysates were thawed and kept at 4°C. After addition of 400 μ L of PBS, samples were spiked with a mixture of internal standards (IS) 17:0-14:1 PS, 12:0-13:0 PE, and 17:0-14:1 PC (200 ng). Samples were sonicated for 1 minute on ice (550 sonic dismembrator, Fisher). Volume was adjusted to 1 mL and lipid extraction was performed according to Bligh and Dyer [193]. Organic phases were evaporated to dryness under nitrogen and reconstituted in 500 μ L of methanol/chloroform (2:1) containing 10 mM ammonium acetate. Mass Spectrometry was performed by direct infusion of the lipid extract in to an ABSciex Qtrap5500 in both (+)ve and (–)ve ESI modes [225, 226]. Samples were infused at a constant rate of 5 μ L per minute and "T-ed" into the solvent flow of 100 μ L per minute. Data was acquired by precursor ion and neutral loss scans. Data analysis was performed with LipdView

Software (ABSciex). Results were based on the peak area and expressed as frequency (%) of each species in the total lipid identified.

4.2.12 Statistical analysis

All experiments were replicated at least in three independent experiments, except for LC/MS/MS analysis, which was performed in duplicates. One way-ANOVA was performed to compare means between POTS and 2 control cells; whereas unpaired student t-test was used to compare data between POTS and control or treated versus untreated groups. A $p \leq 0.05$ at 95% confidence interval was considered as significant. Graphics are represented as mean $\pm$ SEM. All statistical tests were performed with Graphpad Prism 4 software.

4.3 Results

4.3.1 Blood metabolites are altered in POTS patient

The patient symptoms at the age of her diagnosis included trouble maintaining normal heart rate upon standing up and with mild exercise, or even basic activities such as walking up a flight of stairs, which caused tachycardia, significant shortness of breath, and at times angina. Several episodes of pre-syncope were reported. At the time of skin biopsies, the POTS patient was on Potassium Gluconate 595mg, Wellbutrin 150mg and Loestrin 24. Analysis of serum metabolites showed that the POTS patient had very low circulating choline and betaine (Table 4.1), as observed in patients fed low choline diet [227], as well as high homocysteine (Table 4.1). These values are significantly lower than the validated control plasma which measured 9

and 48 μM for choline and betaine, respectively. In addition, when the POTS patient consumed an additional 750 mg choline per day, plasma choline and betaine concentrations did not rise appreciably as would be expected. Betaine, which is the oxidation product of choline, donates its methyl group to homocysteine, via betaine homocysteine methyltransferase (BHMT) to form methionine, which then produces the main methylation agent S-adenosyl methionine (AdoMet). In the liver, AdoMet methylates PE to PC catalysed by PEMT and this reaction produces the majority of circulating homocysteine and represents the major link between choline and methionine metabolism [44, 228]. In POTS serum, homocysteine, and its metabolite cystathione were found to be elevated; however cysteine, a metabolite of cystathione, was reduced (Table 4.1). Studies have shown that plasma concentrations of betaine and homocysteine are inversely correlated [229]. The AdoMet/AdoHcy ratio, which was not affected in the patient serum, is typically independent of fluctuations in the BHMT activity [230]. Therefore the patient metabolite profile strongly suggested that the elevated homocysteine could be reflecting deficiencies in choline and betaine. Both lack of betaine to methylate homocysteine, as well as an increase in AdoMet utilization to produce PC from PE by the PEMT pathway may increase homocysteine levels in the serum. In choline deficient state, the utilization of AdoMet to synthesise PC from PE in the liver is increased, increasing liver AdoHcy [231], which may result in increased circulating homocysteine. Because the POTS patient did not show reduced circulating PC, but lower choline, the synthesis of PC by the PEMT pathway may be increased to compensate for the lack of choline for the CDP-choline pathway. Upregulation of the hepatic PEMT pathway may also contribute to the depletion of betaine observed in the POTS serum.

4.3.2 CTL1/SLC44A1 transporter and choline transport are reduced in POTS

Since patient plasma methionine, AdoMet, and PC were normal, we postulated that a reduced choline transport for phospholipid synthesis could be causing choline and betaine deficiency. Choline transport outside of neuronal specific choline transporter CHT is largely mediated by choline transporter-like protein 1/solute carrier 44A1 (CTL1/SLC44A1) [31]. CTL1/SLC44A1 is a ubiquitous choline transporter [33] and mediates the cellular uptake of choline required for membrane phospholipid (PC and sphingomyelin) synthesis as well as mitochondrial choline uptake to store choline in most cells or to use as a source for betaine in the liver and kidney cells [36]. The expression of CTL1/SLC44A1 was reduced in POTS (Figure 4.1). While the level of control β -tubulin was unchanged, the CTL1/SLC44A1 protein was reduced ~2.5-fold in POTS compared to two controls of human skin fibroblasts (Figure 4.1A, B). Because the two control cell lines had identical levels of CTL1/SLC44A1, we decided to perform the following experiments using only one control. CTL1/SLC44A1 mRNA was reduced ~3-fold in POTS cells compared to control cells (Figure 4.1C, D). The choline uptake into the cell and into isolated mitochondria was analysed previously by Michel [190]. It was found that in POTS cells, cellular and mitochondrial choline transport was reduced. Here we confirmed the reduction on cellular choline uptake by 50% in POTS cells (Figure 4.2A). Taken together, we showed that choline uptake into the cell was reduced in the POTS cells, which was accompanied with reduction in the amount of CTL1/SLC44A1 transporter. Interestingly, choline supplementation in the cell culture medium increased the amount of choline transporter (significantly after 5mg/L; Figure 4.1B) and choline uptake (at both 2.5 and 5mg/L of choline) into deficient POTS fibroblasts (Figure 4.2B).

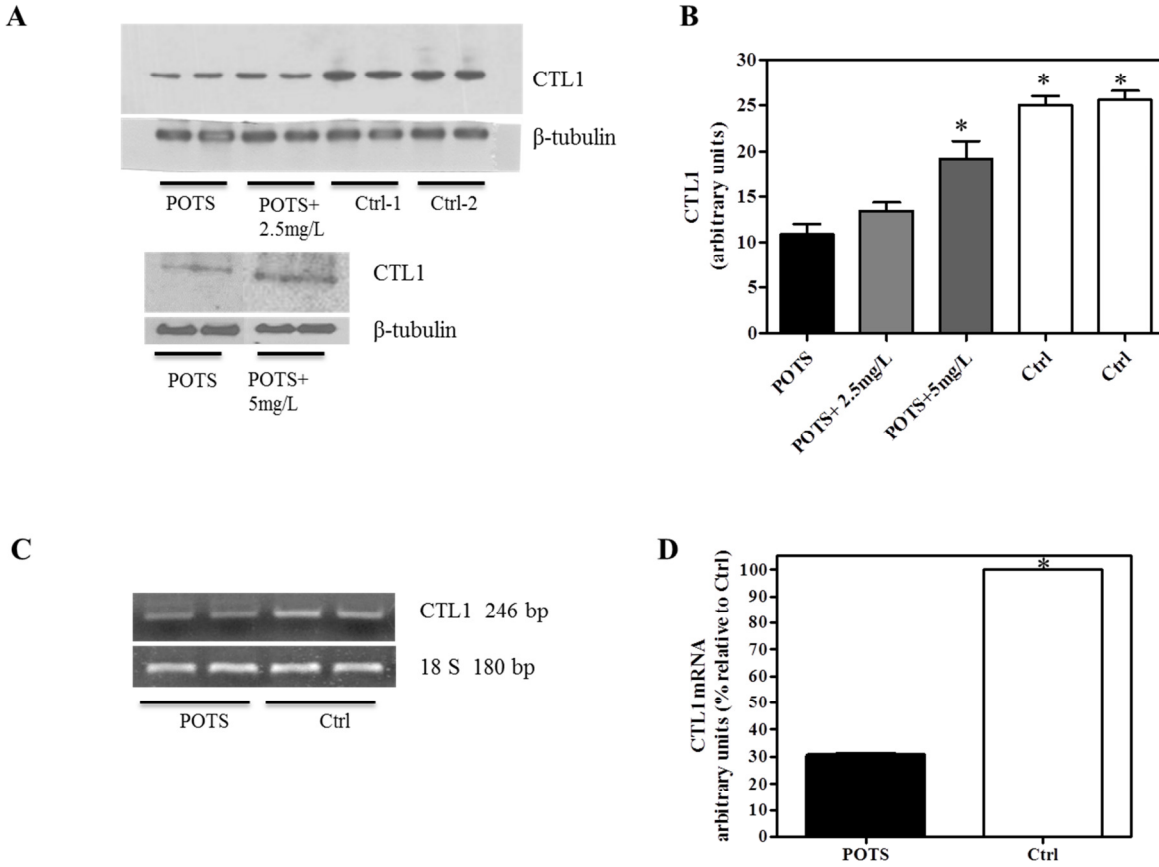


Figure 4. 1: CTL1 protein and mRNA expression are down-regulated in POTS fibroblasts. (A) Representative figure of Western blotting for CTL1 in POTS cells untreated and treated with 2.5mg/L and 5mg/L of choline and in 2 control fibroblasts. β -tubulin was used as loading control and did not differ between groups. (B) CTL1 protein expression was reduced in the patient compared to controls. Treatment with 5mg/L, but not 2.5mg/L, of choline increased significantly CTL1 content in POTS cells. (C) Representative figure of RT-PCR for CTL1 and 18S in POTS and in control fibroblasts. (D) CTL1 mRNA expression was reduced in the POTS compared to control fibroblast. The 18S loading control did not differ between groups. Intensity of CTL1 bands was quantified using ImageJ software and expressed as arbitrary units. * $p < 0.05$ compared to untreated patient.

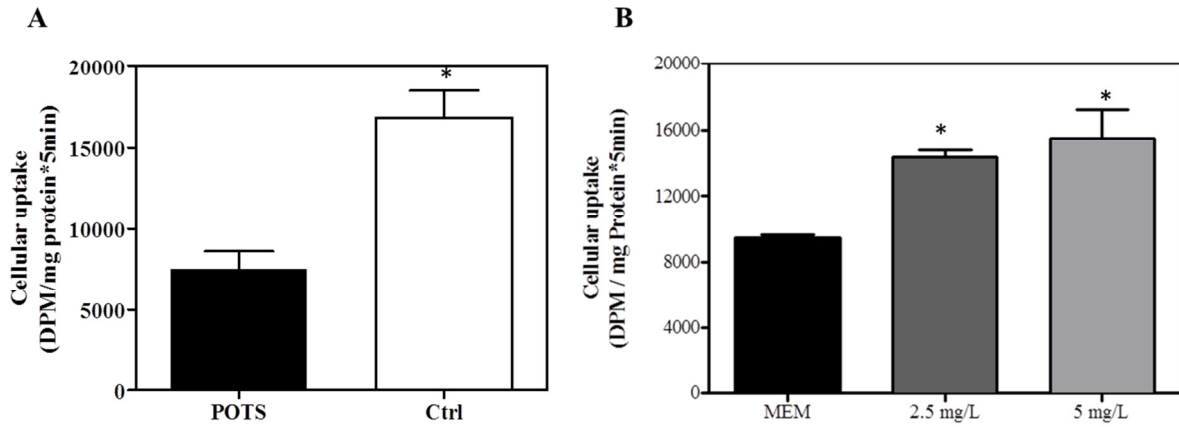


Figure 4. 2: Choline uptake is affected in POTS. Choline uptake was measured using H^3 -choline in intact cells. Choline uptake into the cell (A) was reduced in POTS compared to control fibroblasts. (B) Choline uptake into the POTS cells was increased after treatment with choline at 2.5mg/L and 5mg/L compared to untreated (MEM) cells. Results are expressed as DPM/mg protein/5min. * $p < 0.05$.

4.3.3 Phospholipid and triglyceride pools were modified in POTS cells

We next investigated if the lack of choline uptake affected glycerolipids homeostasis in POTS cells. First we analysed PE, PC and PS phospholipid pools by equilibrium (24h) radiolabelling of POTS and control cells with ^3H -glycerol. Data showed that in POTS cells the PC pool was reduced 5-fold and PE and PS pools were reduced ~3-fold (Figure 4.3A, B, C). The phospholipid pools were also analysed in the cells grown for 10 days in the presence of different amounts of choline (0, 2.5, and 5 mg/L choline). The equilibrium radiolabeling showed that 2.5 mg/L of choline supplementation of POTS cells increased the PE, PC and PS pools 1.8-, 1.5- and 1.4-fold, respectively, when compared to untreated POTS cells (Figure 4.3A, B, C). In addition, the effect of choline on POTS phospholipids was proportional to the amount of media choline, and the PE, PC and PS pools continued to increase after treatments with 5mg/L of choline (Figure 4.3D, E, F).

DAG and TAG pools in POTS cells were determined by the equilibrium radiolabeling with ^3H -oleate and ^3H -glycerol. Data showed that the DAG pool was decreased 1.5-times and TAG pool was increased 1.6-fold in POTS cells when compared to control cells (Figure 4.4A, B). POTS DAG was significantly increased only after 5mg/L of choline treatment, whereas POTS TAG decreased after both 2.5 mg/L and 5mg/L of choline treatment (Figure 4.4C, D). Interestingly, the choline treatment at 2.5mg/L increased DAG and TAG pools in control fibroblasts (Figure 4.4A, B). We concluded that glycerolipid metabolism is generally altered in POTS cells as a consequence of reduced choline availability, which directly affected PC synthesis and caused homeostatic changes in other glycerolipids. Choline supplementation was able to correct the glycerolipid imbalance in POTS cells by significantly increasing the membrane phospholipids and by reducing the elevated intracellular TAG pools.

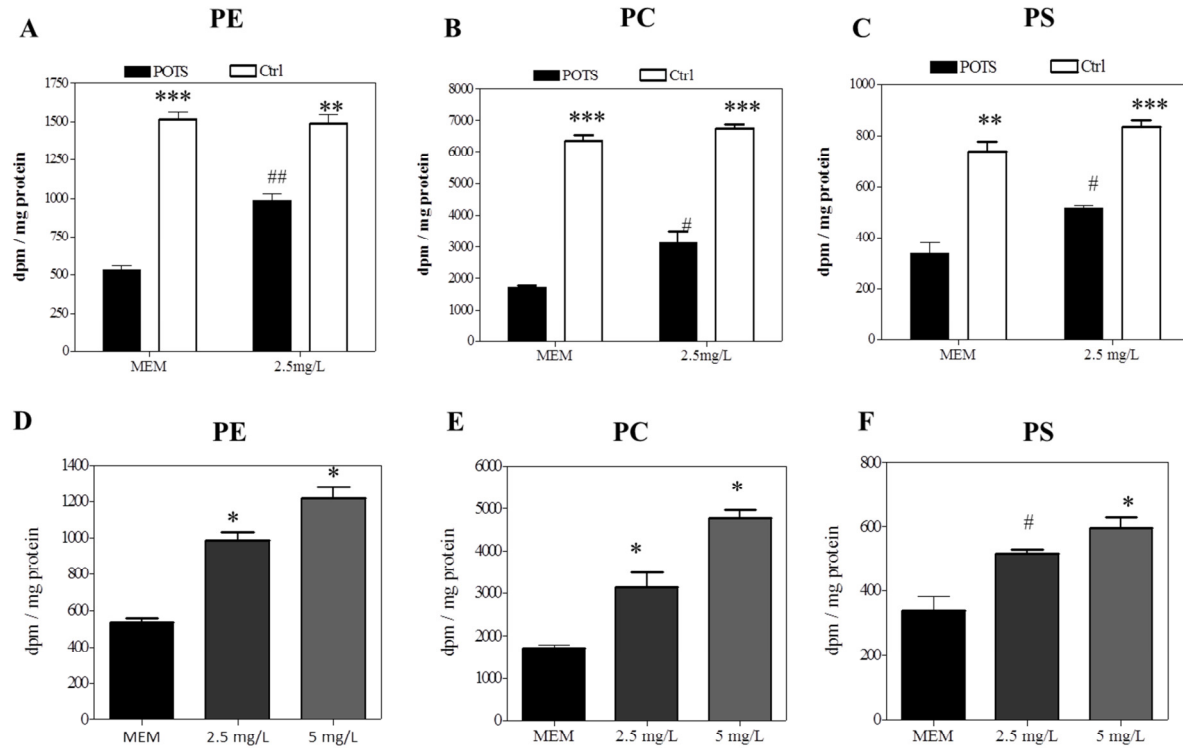


Figure 4. 3: Phospholipid content is altered in POTS. Phospholipid pool size was measured after 24h of equilibrium radiolabeling with ^3H -glycerol in POTS and control cells, untreated (MEM) or treated with 2.5mg/L of choline. (A,B,C) POTS fibroblasts had significantly lower PE (A), PC (B) and PS (C) than did controls (***) $p < 0.0001$ and ** $p < 0.005$ compared with patient). Choline treatment at 2.5mg/L increased PE (A), PC (B) and PS (C) content in POTS fibroblast (## $p < 0.001$ and # $p < 0.05$ compared with untreated POTS), and did not alter these phospholipids in control fibroblasts. (A,B,C) **Choline treatment affects phospholipid pools in a dose-dependent manner.** ^3H -glycerol equilibrium radiolabeling of POTS untreated (MEM) and treated with 2.5mg/L and 5mg/L choline. PE (D), PC (E) and PS (F) were increased after choline treatment in a dose-dependent manner. Results are expressed as DPM/mg of protein.

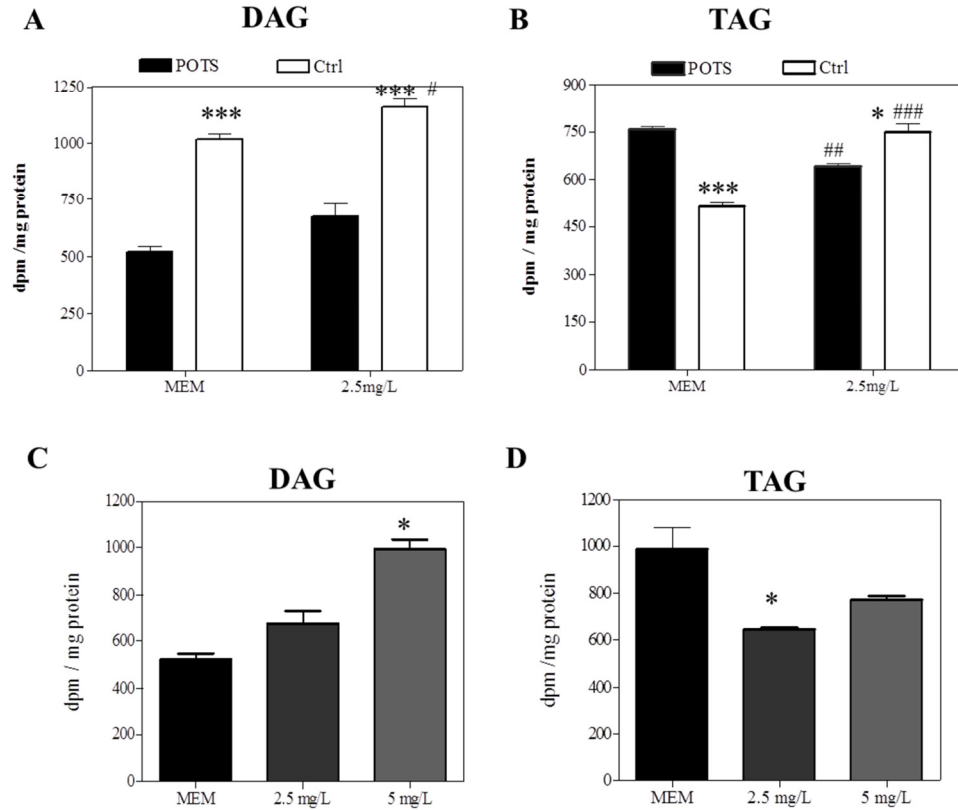


Figure 4. 4: TAG and DAG are altered in POTS. Neutral lipid pool sizes after 24h of equilibrium radiolabeling with ^3H -oleate in POTS and control cells, untreated (MEM) and treated with 2.5mg/L of choline. (A, B) DAG was lower and TAG higher in POTS compared to control fibroblasts (*** $p < 0.0001$; * $p < 0.05$). (A) Choline treatment at 2.5mg/L slightly increased DAG in control fibroblasts (# $p < 0.01$ compared with control untreated), but not in POTS (not significant; $p = 0.07$). (B) Choline treatment decreased TAG in POTS (## $p < 0.001$ compared with POTS untreated) and increased TAG in control fibroblasts (### $p < 0.0001$ compared with control untreated). Comparison between 2.5mg/L and 5mg/L choline treatment in POTS cells. DAG and TAG labelling after 24h of equilibrium radiolabeling with ^3H -oleate in POTS untreated (MEM) and treated with 2.5mg/L and 5mg/L. (C) DAG increased significantly only after 5mg/L choline treatment. (D) TAG decreased significantly after 2.5mg/L and 5mg/L of choline treatment. Results are expressed as DPM/mg of protein. * $p < 0.01$; # $p < 0.05$ compared to untreated (MEM).

4.3.4 PC synthesis is reduced in POTS cells

Since choline uptake, CTL1/SLC44A1 transporter and PC pool were all reduced in POTS cells we also investigated if the CDP-choline pathway for PC synthesis was specifically affected. To measure the flux through the CDP-choline pathway we radiolabeled POTS and two control cell lines with ^3H -choline for 1, 2 and 3h and measured the incorporation of the radiolabeled choline into various intermediates as a function of time (Figure 4.5). The radiolabeling data for the pathway intermediates Cho, P-Cho and CDP-Cho showed that the choline incorporation at all three steps of the pathway was generally reduced in POTS cells (Figure 4.5A, B, C; $p < 0.05$). The total ^3H -choline incorporation into PC was also significantly decreased in POTS cells compared to two control cells in all time points (Figure 4.5D; $p < 0.05$). The total rate of PC synthesis (area under the curve) was lower in POTS cells than in controls (POTS=7128, Ctrl1=8821, Ctrl2=8316). These results demonstrated that the flux through the Kennedy pathway and the final PC synthesis were down-regulated in POTS cells and that the reduction in all steps of the pathway was observed because of initially limited uptake of choline.

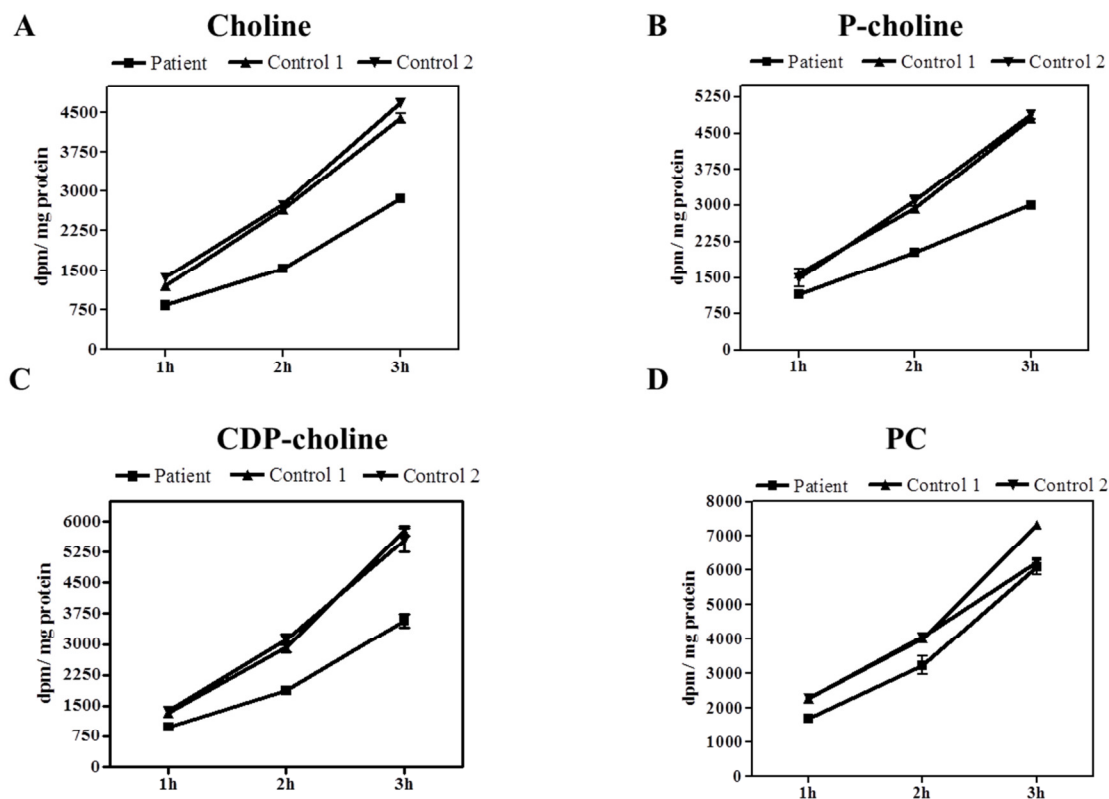


Figure 4.5: : PC synthesis is downregulated in POTS fibroblasts. Intermediates of the Kennedy pathway and PC synthesis was analysed after 1h, 2h and 3h pulse with ^3H -choline. POTS fibroblasts showed significantly reduced synthesis of choline (A), P-Choline (B), CDP-choline (C) and PC (D) compared with two control fibroblasts.

4.3.5 PC degradation was increased in POTS cells

To see if the reduced PC pool (Figure 4.3A) was also caused by increased PC turnover, we performed ^3H -choline pulse-chase radiolabeling experiments to measure PC degradation as well as degradation of the Kennedy pathway intermediates Cho, P-Cho and CDP-Cho. The cells were pre-labelled for 3h with ^3H -choline (synthesis step) and 'chased' with excess of unlabeled choline (degradation step) for different time periods. The amounts of remaining metabolites at different time points was plotted and the extent of degradation estimated from the slopes and

area under the curve as shown in Figure 4.6. ^3H -Cho and ^3H -P-Cho decays (curve slopes) were similar in POTS and two control cells; however the total choline degradation (area under the curve) was reduced (Figure 4.6A, B), in agreement with the reduced choline uptake. On the other hand, the decays of ^3H -CDP-choline and ^3H -PC were significantly faster in POTS than in control cells (Figure 4.6C, D). Therefore PC appeared to be synthesised slower and degraded faster in POTS cells than in two control cells. Both metabolic processes reduced the total PC in POTS in an attempt to provide much needed free choline.

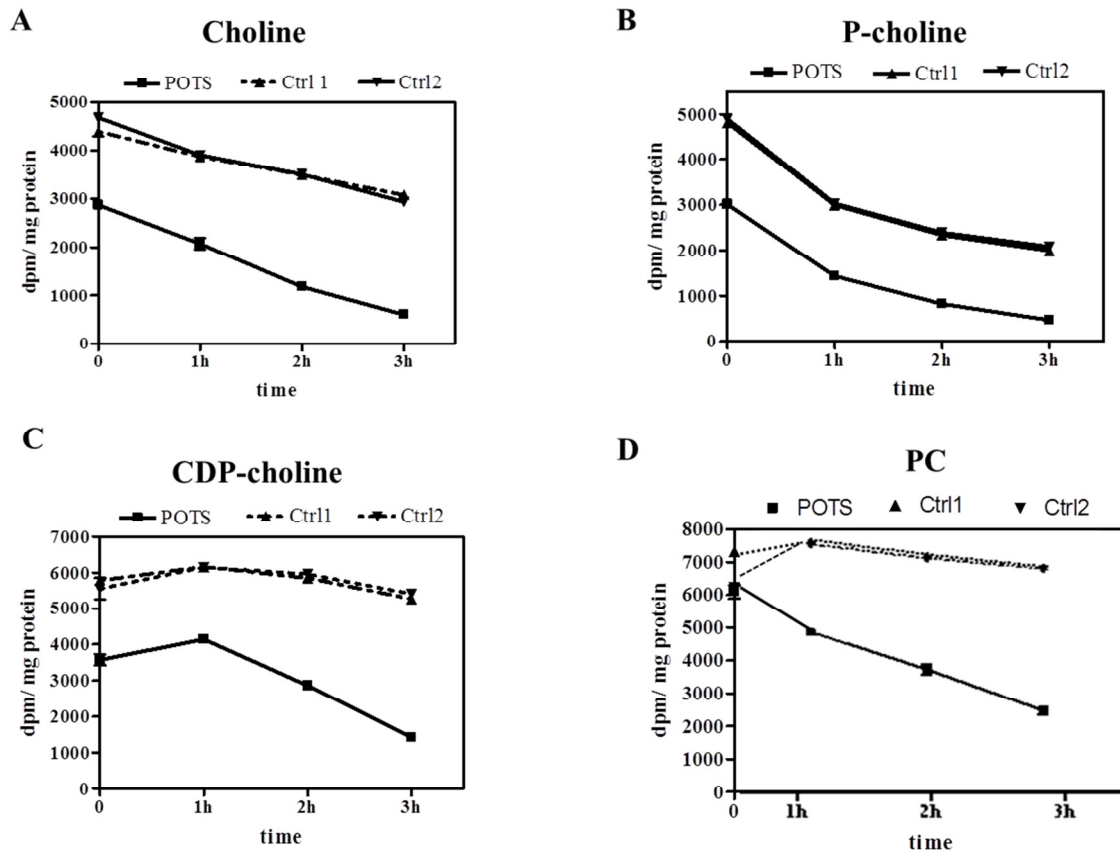
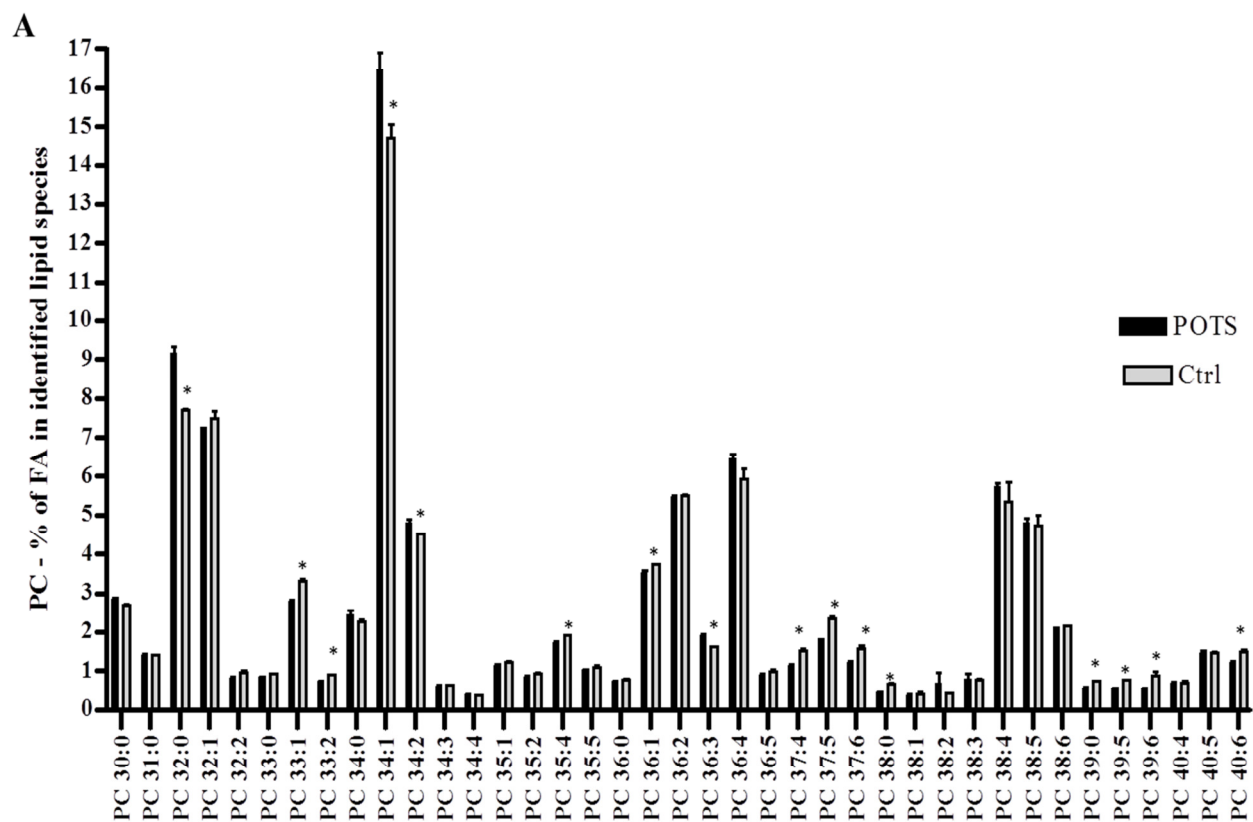


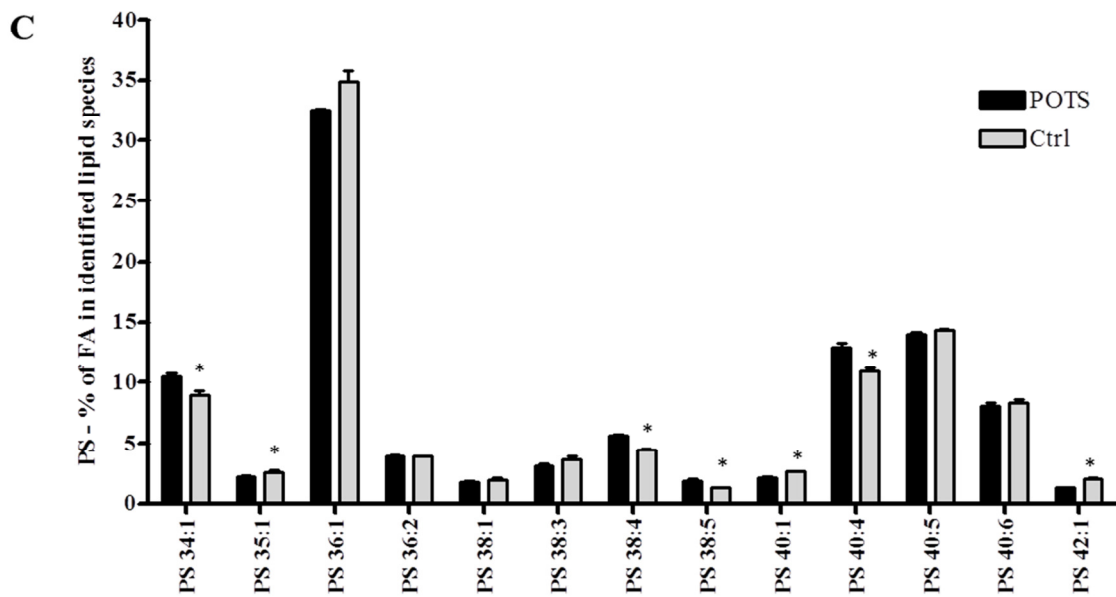
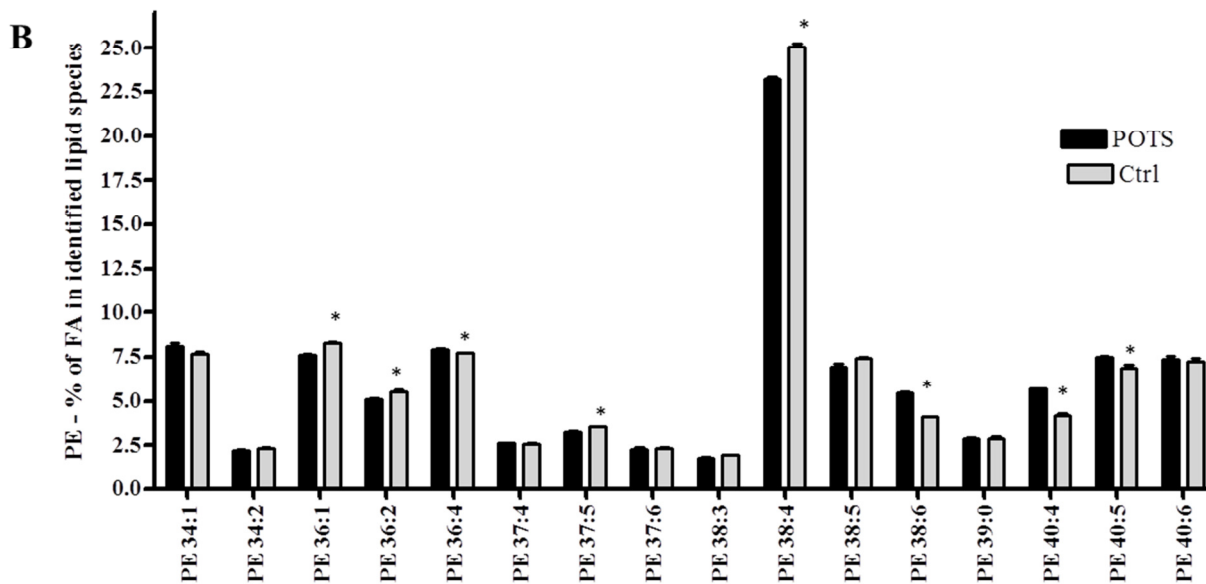
Figure 4. 6: The degradation of PC and Kennedy pathway intermediates is faster in POTS. Pulse-chase was performed by incubation with ^3H -choline for 3h and chase period of 1h, 2h and 3h. POTS fibroblasts showed faster degradation of choline (A), P-choline (B), CDP-choline (C) and PC (D) compared with two control fibroblasts.

4.3.6 Lipidomic analysis

Lipidomic analysis was performed to observe the fatty acid composition of the phospholipids (PC, PE, PS, SM-sphingomyelin and CL-cardiolipin) and neutral lipids (DAG and TAG) in POTS and control fibroblasts. A summary of the number of lipid species analyzed in the LC/MS/MS of the cell lysates are presented in Fig. 8. Results are expressed as percentage of each fatty acid in the total lipid species identified. Comparison between POTS and control cells showed that POTS had lower percentage of long-chain and very long-chain polyunsaturated (PUFA)-derived PC (37:4, 37:5, 37:6, 39:5, 39:6, 40:6), whereas the percentage of medium-chain, saturated and mono- and di-unsaturated PC (32:0, 34:1, 34:2, 36:3) were higher in the POTS (Figure 4.7A). The opposite trend was observed for PE species. The frequency of most PUFA-derived PE (36:4, 38:6, 40:4, 40:5) was higher in POTS cells, whereas the percentage of some medium-chain, saturated and mono- and/or di-unsaturated PE (36:1, 36:2) were higher in controls (Fig. 8B). PS showed similar results as PE, with higher percentage of PUFA in the POTS (Fig. 8C). A second analysis for individual fatty acid species also showed higher PS 22:4 and lower PS 16:0 in POTS (Table 4.2). These results indicate that POTS cells have relatively more PS derived from PE, and not PC, than do control cells. SM, which is synthesized from PC, showed higher frequency of medium-chain monounsaturated fatty acids in the POTS cells, similar to PC. The fatty acid profile of cardiolipin-CL was similar between POTS and Ctrl, with only CL 76:9 being higher in POTS (Figure 4.7D). Analysis of individual fatty acid in CL showed lower saturated fatty acid 16:0 and higher 20:4 and 22:4 in POTS cells (Table 4.2). The frequency of DAG species did not differ between POTS and control cells (Figure 4.7F). However, the TAG profile was highly different between groups. In general, POTS cells showed higher frequency of medium-chain and saturated fatty acid species (46:0, 48:0, 50:1) and lower frequency of long-

chain PUFA species (56:2, 56:3, 56:4, 58:6, 60:9) in the TAG fraction (Figure 4.7G). We conclude that the profile of phospholipids and TAG is altered in POTS cells compared to control, as a result of altered lipid metabolism and/or phospholipid remodeling in the choline-deficient POTS cells.





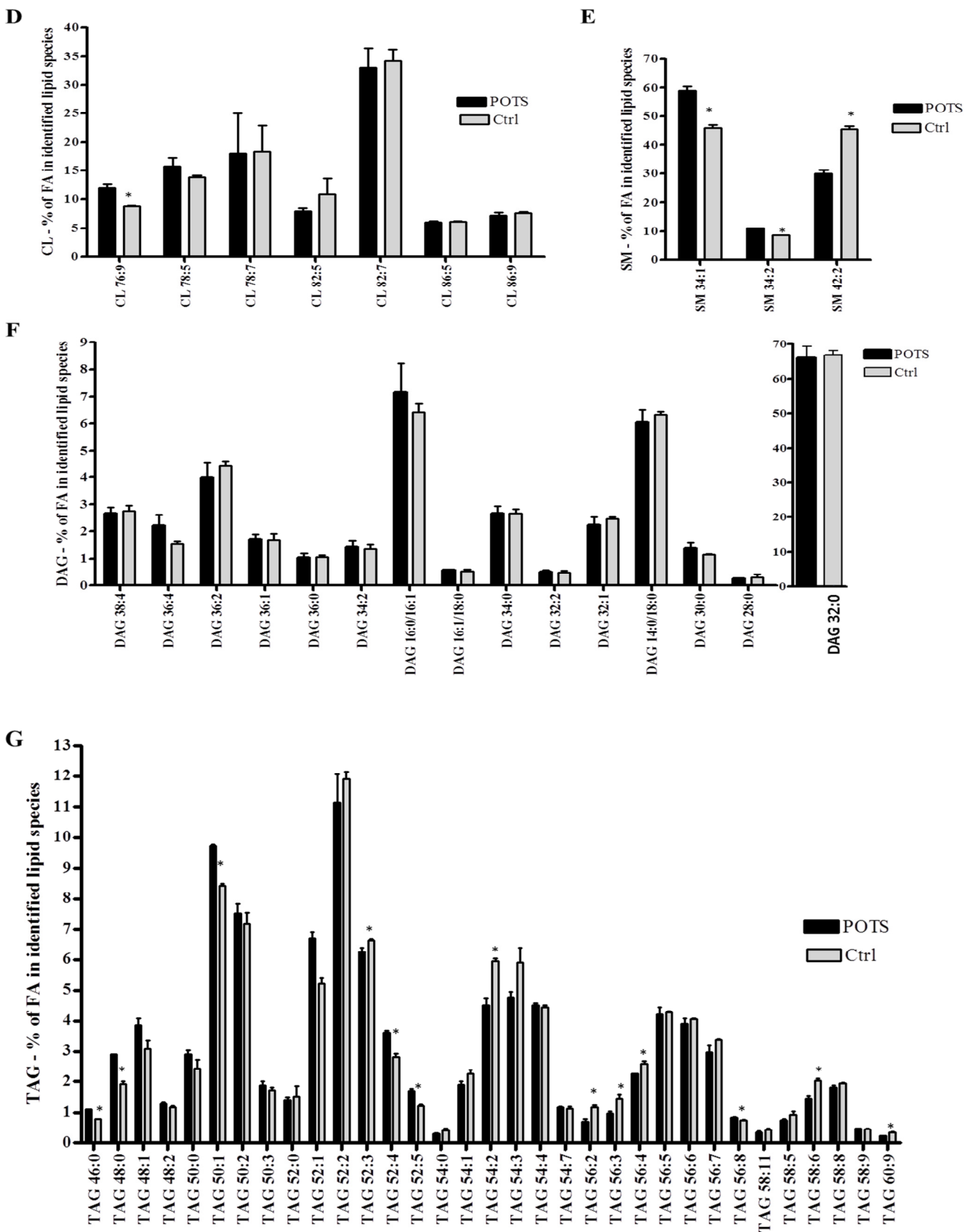


Figure 4. 7: Glycerolipid molecular species composition analysed by LC/MS/MS. The molecular species are presented with total fatty acyl chain carbon number and the total number of double bonds. (A) Phosphatidylcholine-PC species in POTS and Ctrl cells. (B and C) Phosphatidylethanolamine-PE and Phosphatidylserine-PS species in POTS and Ctrl cells. (D-G) Cardiolipin-CL, Sphingomyelin-SM, Diacylglycerol-DAG and Triacylglycerol-TAG species in POTS and control fibroblasts. Results are expressed as relative amount (%) of each species in the total lipids detected. * $p < 0.05$ compared with POTS.

4.3.7 Mitochondrial function is impaired in POTS cells

Because the mitochondrial choline uptake was significantly reduced in the POTS cells [190], we further examined the mitochondrial membrane potential of these cells. We found that the mitochondria potential was reduced 1.5-fold in the POTS cells compared to control cells (Figure 4.8A). Mitochondria potential has been related to choline uptake into the mitochondria [209] and the decrease in the potential observed here may contribute to the decrease in choline transport into the mitochondria in POTS cells. In addition, we showed that the mtDNA copy number is not altered (Figure 4.8B), so the reduction in the mitochondrial membrane potential in POTS cells could not be attributed to reduced number of mitochondria.

In collaboration with Dr Harvey Mudd (The Laboratory of Molecular Biology, National Institute of Mental Health, NIH, Bethesda, Maryland, USA) and Amy Jonhson (Department of Nutrition, University of North Carolina at Chapel Hill, Kannapolis and Chapel Hill, NC, USA), the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) was analysed in POTS cells and compared to control cells. The electron transfer rates through the electron transport chain measured by the (OCR) was almost 3 times lower in POTS fibroblasts than in control fibroblasts (Figure 4.8C). The measurement of glycolytic activity by ECAR assay showed that

baseline ECAR was 40% lower in POTS fibroblasts compared to control cells, suggesting a block in the glycolytic pathway (Figure 4.8D).

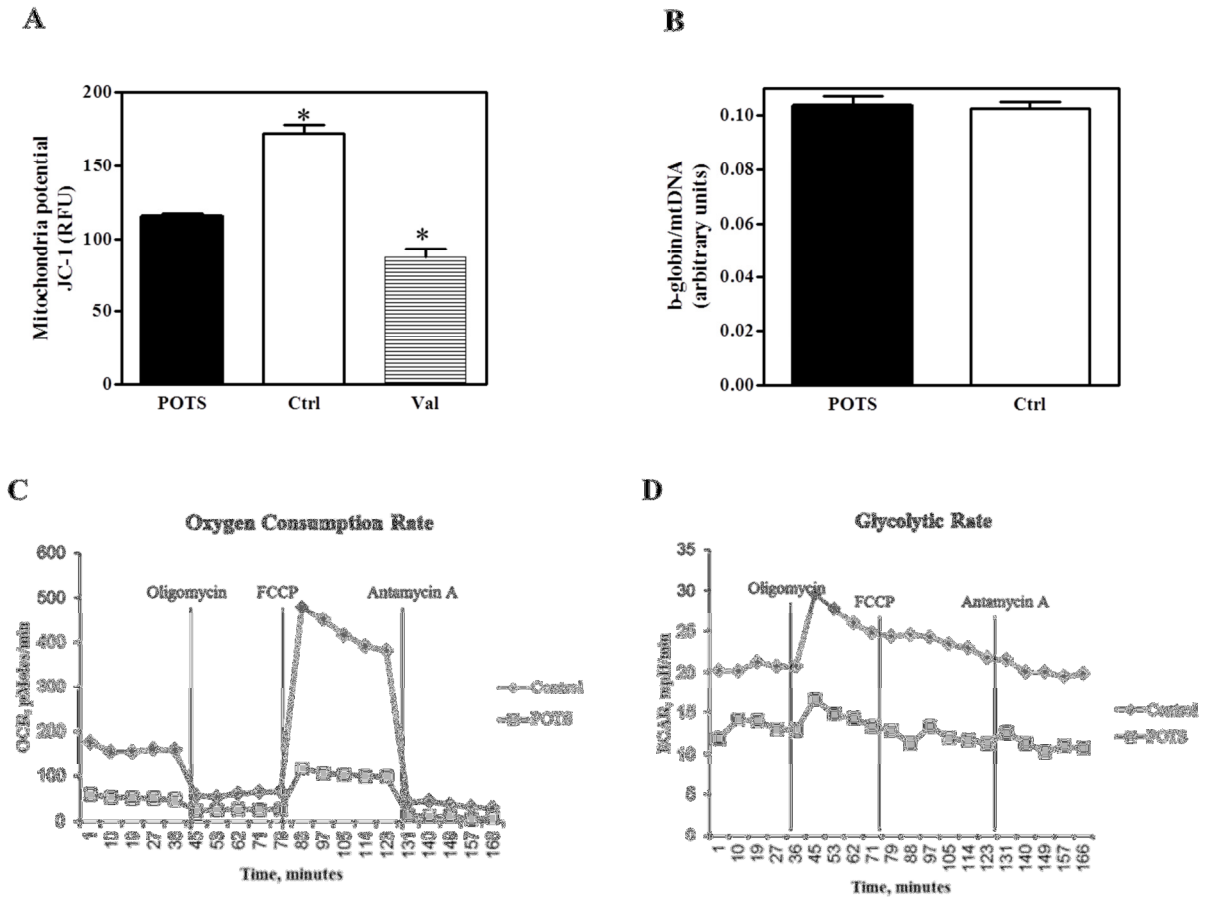


Figure 4. 8: Mitochondrial capacity is reduced in POTS fibroblasts. (A) Mitochondrial potential was reduced in POTS cells compared with control. Potential was measured using JC-1 staining and fluorescence was read at 590nm. Valinomycin (Val; 1 μ g/mL) was used as an uncoupler agent and negative control. Result is expressed as relative fluorescence units (RFU), *p< 0.01 compared with POTS. (B) mtDNA copy number relative to nuclear DNA content showed no difference in mitochondria number between POTS and control fibroblasts. (C) Oxygen consumption rate-OCR and (D) extracellular acidification rate (ECAR) results were lower in

POTS than in control fibroblasts. (E) ROS production, measured using the fluorescent probe dihydrodichlorofluorescein diacetate (DCF), was higher in POTS versus control fibroblasts.

4.4 Discussion

This study describes a disorder related to choline deficiency in a female, who was diagnosed with dysautonomia and postural orthostatic tachycardia syndrome (POTS). The analysis of patient blood showed very low amounts of choline and betaine, which suggested impairments in choline related phospholipid pathways and choline transport activity. Using cells obtained from the patient's skin biopsies we clearly established that the reduced cellular and mitochondrial choline uptake and reduced choline transporter CTL1/SLC44A1 were responsible for the observed deficiency. This is the first known case of a medical disorder related to transport-mediated choline deficiency.

Choline is required for PC synthesis through the Kennedy pathway and could regulate glycerolipid metabolism. Lack of choline decreases PC synthesis in muscle cells and, similarly to the POTS cells, the CTL1/SLC44A1 transporter was down-regulated [39]. In addition, the major phospholipid pools of PC, PE and PS were reduced in POTS cells. On the other hand, the DAG pool was decreased and the TAG pool was increased in POTS cells compared to control human fibroblasts. Previous studies with muscle cells grown in choline deficient media also showed reduced PC synthesis and accumulation of TAG [39, 232]. Dietary choline deficiency also has been shown to result in non-alcoholic fatty liver associated with TAG accumulation [233, 234] due to insufficient amounts of PC for lipid secretion [15, 235].

Because the lack of sufficient intracellular choline seems to be caused by impaired cellular and mitochondrial transport, we sought to determine the effect of choline treatment on POTS cells.

Choline uptake into the cell was increased after choline treatment in a dose-dependent manner. Also, the total CTL1 content was significantly increased after 5mg/L of choline. Choline treatment at 2.5mg/L could effectively increase phospholipid content and decrease TAG concentrations, whereas DAG levels were corrected only after treatment with 5mg/L of choline. The comparison of 2.5mg/L and 5mg/L of choline treatment showed that the increase in PC, PE, PS and DAG levels have a linear relationship with choline concentration. Therefore, choline treatment of the POTS cells could increase the choline uptake into the cell and alleviate the glycerolipid dysregulation, and therefore can be an important component of the therapy for the POTS.

We further analysed the pattern of phospholipid composition in POTS and control cells. Of the PC species identified, the frequency of those containing long-chain PUFA were lower, and the medium chain saturated and monounsaturated-containing species were higher in POTS compared to that in control fibroblasts. The content of PUFA in phospholipids contributes to the biophysical properties of the membrane phospholipids and changes in PUFA content alter cell function [236]. As a major phospholipid in the membrane, PC composition has an important role in the membrane fluidity and function. Generally, the molecular species profile of PC is determined by the species-selectivity of the biosynthesis routes as well as by PC turnover and by acyl chain remodeling [237]. Because fibroblasts cells synthesize PC only from the CDP-choline pathway, the changes in the fatty acid profile observed here must have resulted from differences in PC turnover and fatty acyl remodeling. Remodeling provides diversity in the molecular species of PC to maintain membrane homeostasis [238]. In addition, remodeling is considered the main mechanism for PUFA incorporation into PC [238]. Interestingly, during choline and, consequently, PC depletion in yeast, the remaining PC was subject to acyl-chain remodeling to

form a more saturated fatty acid composition [239]. On the other hand, PE and PS in POTS cells showed higher frequency of long-chain PUFA species and lower frequency of medium-chain fatty acid species.

The composition of the neutral lipids DAG and TAG was also analysed in POTS. The relative composition of DAG species did not differ between POTS and control cells. The frequency of TAG species, however, varies between the groups. POTS cells generally showed higher frequency of medium-chain saturated and monounsaturated derived TAG (46:0, 48:0, 50:1), and lower frequency of PUFA-derived TAG, when compared to control cells. In the POTS cells we observed an increase in the TAG pool, which is a feature of the choline deficiency state. Previous study showed that in non-alcoholic fatty liver state, which accumulates TAG in the liver, the composition of TAG changes. Similar to our findings, they found an increase in saturated and monounsaturated derived TAG, and a decrease in PUFA [240]. Interestingly, the same trend of fatty acid-derived TAG was observed in the PC. These results suggest that the TAG fatty acid species were mostly derived from PC catabolism in the POTS cells.

Our group has identified CTL1/SLC44A1 mediated choline transport in mitochondria of different cell types as a mode to control the pools of intracellular choline and betaine [36]. Since betaine can only be synthesized in mitochondria, the reduced amount of betaine in patient's blood could only be attributed to the reduced choline transport by POTS cells and mitochondria. CTL1/SLC44A1 is a choline/H⁺ antiport, driven by the membrane electrochemical gradient [43] and the mitochondrial choline transport is dependent on the membrane potential [209]. Choline deficiency in muscle cells reduces the amount of mitochondrial CTL1/SLC44A1 [39] and in the animal models reduced choline decreases the mitochondrial membrane potential [241, 242].

The electron transfer rates through the electron transport chain measured by the (OCR) was almost 3 times lower in POTS fibroblasts than in control fibroblasts, which could be the result of decreased expression and/or activity of TCA cycle or oxidative phosphorylation proteins, or another anomaly impairing electron transport chain function upstream of Complex III. Treatments with Oligomycin decreased OCR 65% in control fibroblasts, but only 50% in POTS, indicating more proton leakage across the inner mitochondrial membrane in the POTS cells. Following FCCP treatment, OCR increased 7-fold in control, while in POTS OCR only increased 4-fold. These results clearly indicated that the POTS cells have a decreased respiration capacity compared to controls and the mitochondria in those cells may be unable to provide adequate amounts of ATP during times of increased energy demand.

The measurement of glycolytic activity by ECAR assay showed that baseline ECAR was 40% lower in POTS fibroblasts compared to control cells, suggesting a block in the glycolytic pathway. Inhibition of ATP synthase caused glycolytic rates to increase in both cell cultures, as expected, but the ECAR response to Oligomycin was lower in the POTS fibroblasts. POTS cells also produce more reactive oxygen species (ROS) compared to controls. However, total mitochondrial dehydrogenase activity measured by MTT assay was increased in the POTS compared to control fibroblasts. Therefore, the respiratory capacity of the mitochondria was reduced in the POTS cells. The mitochondrial impairments in the POTS, a type of choline deficiency, are mostly caused by modified membrane phospholipid composition. Indeed, the composition of the mitochondrial membranes was previously found to be altered in choline deficiency affecting mitochondrial oxidative capacity and energy metabolism [243].

The cellular characteristics of the POTS fibroblasts described here represent a model of choline and CTL1/SLC44A1 deficiency, in which glycerolipid homeostasis and mitochondrial function

were impaired. Choline treatment of the POTS fibroblasts could restore glycerolipid pools and membrane function and offer a promising option for treating this patient. The choline deficiency in this patient may be a result of impaired supply of dietary choline by intestinal CTL1/SLC44A1 transporter [33, 244]. However, further experiments on intestinal cell choline metabolism are needed to confirm this hypothesis.

Table 4. 1: Metabolites measured from the POTS patient. Plasma or serum was isolated from blood collected in fasted state, and metabolites analysed by the chromatography assay described in Methods section.

Metabolite	Physiological range	POTS
Methionine	13.3-42.7 μ M	29.2
AdoMet	92.8 $\pm$ 16.2 nM	123
AdoHcy	27.8 $\pm$ 7.9 nM	21.9
AdoMet/AdoHcy	2.1-7.0	5.6
Homocysteine	5.1-13.9 μ M	<u>34.3</u>
Cystathionine	44-342 nM	<u>468</u>
Cysteine	203-369 μ M	<u>172</u>
Sarcosine	0.6-2.67 μ M	0.88
Phosphatidylcholine	1500-2559 μ M	2119
Choline	10.7-19.7 μ M	<u>5.69</u>
Betaine	26-67 μ M	<u>9.98</u>
Dimethylglycine	1.4-5.3 μ M	4.49
Methylmalonate	73-271 μ M	253
2-Methylcitrate	60-228 μ M	102
Glycine	152-413 μ M	163
Serine	97-267 μ M	<u>61</u>
α-Aminobutyrate	8-30 μ M	<u>6</u>

Underlined values represent altered metabolites in POTS

Table 4. 2: Relative amount of individual fatty acids identified by LC/MS/MS in POTS and control fibroblasts. Results are expressed as relative amount (%) of each species in the total lipids detected.

	POTS	Ctrl	p values
SFA			
PC			
16:0	47.26 ±1.97	43.9±0.27	0.07
18:0	3.531±0.13	3.627±0.40	0.39
PE			
16:0	27.16	27.45±0.33	
18:0	17.24±3.49	14.96±0.53	0.23
PS			
16:0	42.05±0.43	45.1±1.51	0.055*
CL			
16:0	47.62±0.86	50.18±0.94	0.052*
MUFA			
PC			
16:1	8.206	9.2±0.24	
18:1	31.06±0.34	31.15±0.88	0.45
PE			
18:1	21.34	19.05±0.44	
PS			
18:1	44.53±0.62	47.51±1.66	0.07
PUFA			
PC			
20:4	11.05±0.88	12.12±0.77	0.16
PE			
20:4	30.7±7.65	27.41±0.38	0.3
22:5	13.74±3.24	11.14±0.61	0.19
PS			
22:4	7.495±0.16	4.987±0.22	0.003*
22:5	5.918±0.35	4.802	
CL			
20:4/22:4	52.38±0.86	49.82±0.94	0.052*

*p≤0.05

5 Choline supplementation restores substrate balance and alleviates complications of Pcyt2 deficiency

Abstract

Choline plays a critical role in systemic lipid metabolism and hepatic function. Here we conducted a series of experiments to investigate the effects of choline supplementation on metabolic altered Pcyt2^{+/-} (HET) mice. In Pcyt2^{+/-} mice the flux through the CDP-ethanolamine pathway is reduced and the triacylglycerol (TAG) formation increased [245], resulting in adult-onset obesity and liver steatosis. We hypothesise that choline supplementation will eliminate the excess of diacylglycerol (DAG) by facilitating the flux through the CDP-choline pathway. We found that choline supplementation could reverse the HET phenotype by preventing weight gain and reducing liver steatosis. It facilitated the metabolic flux through the CDP-choline pathway and increased expression of genes involved in TAG degradation by lipolysis. In addition, lower levels of plasma C3 acylcarnitine and greater mitochondrial membrane potential in HET mice treated with choline indicates improvement in the flux through the TCA cycle and the respiratory chain activity. Metabolomics data also indicated a reduced activity of the methionine–homocysteine cycle in HET mice, which was restored by choline supplementation. Our data suggest that extra dietary choline could maintain lipid homeostasis and prevent the development or onset of metabolic syndrome.

5.1 Introduction

Choline is considered an essential nutrient for both humans and animals [4]. It is a precursor for the synthesis of the major phospholipid phosphatidylcholine (PC), of the neurotransmitter acetylcholine and of betaine, an important methyl-group donor. Choline also plays a critical role in systemic lipid metabolism and hepatic and muscle function [41, 220, 227, 246]. Choline homeostasis is regulated by metabolism and multiple protein-mediated transport systems in and out of the cells. The choline transporter-like protein 1 (CTL1/SLC44A1) transports choline in the plasma membrane and mitochondria [33, 35, 37] and is considered the most important choline transporter for *de novo* PC biosynthesis by the Kennedy pathway [31, 33, 37, 112]. There are two branches of this pathway, the CDP-choline pathway for the biosynthesis of PC, and the CDP-ethanolamine pathway for the biosynthesis of phosphatidylethanolamine (PE). The two pathways share the enzymes choline/ethanolamine kinase and choline/ethanolamine phosphotransferase, and the substrates ATP, CTP and diacylglycerol (DAG) [98, 99]. Functional distinction between the two pathways is mainly at the level of formation of CDP-choline and CDP-ethanolamine governed by CTP:cholinephosphate (CT/Pcyt1) and CTP:ethanolaminephosphate (ET/Pcyt2) cytidyltransferases. PC and PE are also metabolically connected through biosynthesis of phosphatidylserine (PS). They are substrates for the formation of PS in the base-exchange reactions catalysed by PS synthases, PSS-1 for PC and PSS-2 for PE. The newly formed PS contributes the membrane bilayers or could be transformed into PE (but not PC) by mitochondrial PS decarboxylase (PSD). Mostly in the liver, PE could be converted into PC in a three-step methylation reaction catalysed by phosphatidylethanolamine-N-methyltransferase (PEMT).

The CDP-choline pathway, as well as PSD, PSS and PEMT reactions have been extensively characterized [44, 65, 124, 132]. Strangely enough, there is no information available on how the impairments in the CDP-ethanolamine pathway impact the CDP-choline pathway or PC and PS catabolism. Our group have developed a heterozygous ($Pcyt2^{+/-}$) mouse model in which the flux through the CDP-ethanolamine pathway is reduced [245]. These mice also have reduced PE turnover and increased fatty acid and triacylglycerol (TAG) formation, and develop adult-onset obesity, dyslipidemia, fatty liver and insulin resistance, all well-known characteristics of the metabolic syndrome [245, 247]. We demonstrated that even before these symptoms appear, young $Pcyt2^{+/-}$ mice have elevated fatty acid synthesis, to esterify excess DAG unused in the CDP-ethanolamine pathway [117]. This process, however, elevates TAG and leads to obesity and insulin resistance in adults. Thus, $Pcyt2^{+/-}$ mice are a unique model for studying the contribution of defective membrane phospholipid biogenesis to the development of metabolic diseases.

Choline is directly consumed by the CDP-choline pathway [248] and we proposed that stimulation of this pathway by choline could be a good strategy to reverse the metabolic complications of $Pcyt2$ deficiency. In a previous study, our group found that choline supplementation in $Pcyt2^{+/-}$ mice could reduce liver steatosis, adipose tissue weight and modified the expression of lipid metabolic genes [179]. The expression of the lipogenic genes DGAT2, SREBP-1c, FAS, SCD1, which were higher in $Pcyt2^{+/-}$ mice, decreased after choline treatment. PPAR α and PGC1 α expression in turn were increased by choline treatments, indicating higher fatty acid oxidation. Here we conducted a series of experiments to further investigate the effects of choline supplementation on $Pcyt2^{+/-}$ pathologies. We hypothesise that increased choline availability will eliminate the excess of DAG (unused in the CDP-ethanolamine pathway or

formed by elevated lipogenesis) by facilitating the flux through the CDP-choline pathway and the mitochondrial fatty acid oxidation, and consequently reducing the obesity phenotype in the Pcyt2^{+/-} mice.

5.2 Methods

5.2.1 Animals

Generation of Pcyt2 deficient (Pcyt2 ^{+/-}) mice was described previously [245]. All procedures were approved by the University of Guelph's Animal Care Committee and were in accordance with guidelines of the Canadian Council on Animal Care. Mice were exposed to a 12-h light/12-h dark cycle beginning with light at 7:00 a.m. Male and female mice were fed a standardized diet (Harlan Teklad S-2335) ad libitum and had free access to water.

5.2.2 Genotyping

Pcyt2 genotypes (heterozygous Pcyt2 ^{+/-} and wild-type Pcyt2 ^{+/+}) were determined from tail genomic DNA. Tissues were digested with 200 µg of proteinase K at 55°C in a buffer (10 mM Tris-HCl pH 8.0, 0.1 M EDTA pH 8.0, 0.5% sodium dodecyl sulfate, 0.1 M NaCl). To eliminate RNA, 10 µg of RNase A was added. Genomic DNA was amplified by PCR using a common upstream primer, FP (CCTGGAACATCATGAGATCCTCCTG), in combination with either a downstream primer, RP (ATCGCACACACCCGCACGA), specific for the wild-type allele or primer N1 (TGCGAGGCCAGAGGCCACTTGTGTAGC), specific for the knockout allele. PCR was performed with 0.3 units of REDtaq DNA polymerase (Sigma). Initial denaturation was at 94°C for 5 min, followed by 32 cycles at 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The primer pairs FP/RP and FP/N1 yielded products of 450 and 305 bp, respectively [245].

Table 5. 1: Primer sequences, fragment size and melting temperature (T_m) for reverse transcription polymerase chain reaction (RT-PCR)

Gene	Primer Forward 5'→3'	Primer Reverse 5'→3'	T _m	Size (bp)
ATGL	CAACGCCACTCACATCTACGG	GGACACCTCAATAATGTTGGCAC	57°C	106
HSL	ACGCTACACAAAGGCTGCTT	TCGTTGCGTTTGTAGTGCTC	59°C	125
LPL	GCTCGCACGAGCGCTCCATT	CCTCGGGCAGGGTGAAGGGAA	59°C	351
CTL1	GAACGCTCTGCGAGTGGCTGC	CGGCTTTAGCTCTCGGGCGT	50°C	376
PCYT1	ATGCACAGAGTTCAGCTAAAG	GGGCTTACTAAAGTCAACTTCAA	50°C	170
BHMT	TGGATTGGAACCCCGAGTTG	GGTGTGCATGTCCAAACCAC	59°C	211
PSS1	CTGTTGTGCAATGGTGGTGG	GGCTGGCTTGGAACACAAAG	59°C	283
PSS2	GAGTGGCTGTCCCTGAAGAC	TCGTAGATCTCACGCATGGC	59°C	305
PSD	GTTTGCTGTCACGTGCCTGTG	CAGTGCAAGCCACATACGGG	59°C	205
PEMT	TGTTTGTGCTGTCCAGCTTC	TTCCAAAGATCCTTCATGGC	52°C	320
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	58°C	452

5.2.3 Choline treatments

Eight- to ten-month old mice were divided into three groups: control littermates (Pcyt2^{+/+}; WT), Pcyt2^{+/-} (HET) and Pcyt2^{+/-} treated with choline (HET+Cho). Het-Cho mice had access to water containing 2mg/ml choline (Sigma) for 4 weeks. For all groups, water intake was measured daily and body weight was measured every 7 days. Food intake was visually tracked. Male mice were used for the metabolic radiolabeling experiments whereas female mice were used for all other experiments. Mice were euthanized in fasted state after 4 weeks of treatment. Tissues were weighed, immediately used or frozen in liquid nitrogen and stored at -80°C.

5.2.4 In-vivo radiolabeling of the CDP-choline pathway

Experiments were conducted as previously described [245] with minor modifications. Male WT, HET and HET+Cho mice (n=6 each) were IP injected with 0.5 μCi (1 $\mu\text{Ci}/\mu\text{L}$) of [^{14}C]-oleic acid and 5 μCi (1 $\mu\text{Ci}/\mu\text{L}$) of [methyl- ^3H] choline chloride diluted in saline. After 1.5h mice were sacrificed and liver samples collected. The total liver lipids were extracted by the Bligh-Dyer method [193] and separated by TLC as before [41, 249]. The water-soluble metabolites choline, P-Cho and CDP-Cho were separated with ethanol/water/ammonia (48:95:7) and stained with phosphomolybdic acid in chloroform-ethanol (1:1) + 1% stannous chloride. ^3H -PC and ^{14}C -PC from the organic phase was isolated by chloroform-methanol-acetic acid-water (40:12:2:0.75) and stained with 15% sulfuric acid and 0.5% K_2CrO_7 . ^{14}C -labelled DAG and TAG were separated with heptane/isopropyl alcohol/acetic acid (60:40:3) and stained with iodine. The radioactivity of separated compounds was determined by LSC.

5.2.5 Immunoblotting

Frozen liver samples were homogenized in a cold lysis buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, and 10 mM NaF) containing protease (1/10) and phosphatase (1/100) inhibitor cocktails (Sigma). The lysate was centrifuged at 2,000 $\times g$ for 20 min at 4°C to remove cell debris, and 15 μg of lysate was mixed with a non-denaturing loading buffer (62mM Tris-HCl and 0.01% bromophenol blue in 10% glycerol) and separated on a native gel at 110V for 1.5h. Proteins were transferred to polyvinylidene difluoride membranes (Roche) and stained with Ponceau S for protein loading. Membranes were then blocked with 5% skim milk in PBS-T and incubated with the C-terminal CTL1/SLC44A1 antibody produced in our laboratory (ENS-627; 1:100 dilution with 5% skim milk in PBS-T) overnight at 4 °C. Membranes were washed 4 times with PBS-T and incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody

(1:10,000 in 5% skim milk in PBS-T) for 1 h. After washing with PBS-T, CTL1/SLC44A1 was visualized using a chemiluminescent substrate (Amersham). Reprobing the membrane with β -tubulin was used as an internal protein control. Experiments were performed twice in triplicates from WT, HET and HET+Cho mice.

5.2.6 Gene regulation by choline treatment

Liver mRNA was isolated from HET, WT and HET+Cho mice (5-8 each group) using Trizol reagent (Invitrogen). cDNA was synthesized from 2 μ g of total mRNA using a poly(dT) primer and Superscript II reverse transcriptase (Invitrogen). We analysed the expression of genes involved in the CDP-choline pathway (CTL1/SLC44A1 and CCT/Pcyt1), in the betaine pathway (betaine-homocysteine methyl-transferase, BHMT) and in the PC-PS-PE cycle (PSS1, PSS2, PSD, PEMT). Also the genes involved in lipolysis were analysed: adipose-tissue triglyceride lipase (ATGL), lipoprotein lipase (LPL), hormone-sensitive lipase (HSL). The amplification of G3PDH was used as a control. The primers and PCR conditions are described in Table 5.1. PCR products were resolved on 1.5% agarose gel and quantified using Image J densitometry software (NIH).

5.2.7 Measurement of mitochondrial membrane potential

Mitochondria from liver were prepared as described previously [33]. In brief, the liver sample (50mg) was incubated (10 min) and homogenised in 1 ml of RSB buffer (10mMNaCl, 1.5mM MgCl₂, 10mM Tris-HCl). Then, 1mL of MS buffer (210nM mannitol, 70mM sucrose, 5nM Tris-HCl and 1mM EDTA) was added and the homogenates centrifuged at 2500 rpm for 10 min. The obtained supernatants were centrifuged at 12,500 rpm for 20 min to yield the mitochondria fractions. The protein content was determined by the bicinchoninic acid assay kit (Pierce). The

membrane potential was determined by a fluorescence assay in a Flex station 3 microplate reader using 30µg of the mitochondrial protein and carbocyanine dye (JC-1) according to the Isolated Mitochondria Staining Kit (Sigma-Aldrich). When it concentrates in mitochondria, JC-1 emits a red fluorescence with excitation at 490nm and emission at 590nm. A reduction in the membrane potential releases JCI from mitochondria as a green fluorescence. The membrane potential dissipating agent valinomycin (1µg/mL) served as a negative control. Results were expressed as a mean red fluorescence $\pm$ SEM from 3 separate measurements.

5.2.8 Plasma metabolomic analysis

5.2.1.1 Blood acylcarnitines and amino acids were measured by MS/MS at the Ghent University as previously described [250]. Briefly, a drop of blood samples from WT, HET and HET+Cho female mice (n=3 per group) were collected on 903 Whatman cards (GE Health Care). The blood was extracted with a methanolic solution (100µL) containing labeled internal standard and then dried by a hot air blower. Butanolic-HCl (70 µL) was added to each sample, heated at 65°C for 15 min and evaporated. The butanol-derivatized samples were reconstituted with 100µL of acetonitrile and water (80:20). Samples were then ready for MS/MS analysis. An ACQUITY UPLC Quattro Premier from Waters instrument was used for the analysis.

5.2.7.2 Metabolomics analysis was performed by ^1H NMR spectroscopy at the NRC Institute for Nutrisciences and Health. Frozen plasma (30 µL) of HET, WT, HET+Cho mice were centrifuged and dissolved in standard NMR solutions. Spectra were acquired on a Bruker Avance III 600 MHz ^1H -NMR spectrometer (Bruker Biospin Ltd., Milton, ON, Canada) using standard protocols. 1D NOESY spectra with pre-saturation of the water signal during both the relaxation delay and mixing time were acquired with 128 scans. The ^1H -NMR chemical shifts were

referenced with respect to the internal DSS or DSS-d6 standards. The data were acquired and processed using TopSpin NMR data acquisition and processing Software (Bruker Biospin Ltd., East Milton, ON). The raw data were processed using Chenomx NMR Suite (Chenomx Inc, Edmonton, AB). For global profiling, the FID file of each sample was imported, with phase and baseline corrected, and chemical shift calibrated by referencing to DSS or DSS-d6 standards. The spectra were then binned into 0.02 ppm buckets from 0-10 ppm, with selected regions excluded for water (4.73-4.82 ppm) and DSS (-0.60-0.66, 1.72-1.80, 2.88-2.94, 4.73-4.82 ppm). For targeted profiling, the processed spectra were loaded into the Profiler module, and fit against 304 standard compounds built in the software. Using DSS or DSS-d6 concentration as reference, the concentrations for metabolites profiled with confidence were obtained through the fitting process. All the spectral binning and targeted profiling data, after being adjusted in response to different diluting factors for the two experiment batches, was exported as an MS Excel dataset for further analysis. For the multivariate data analysis, the datasets from spectral binning and targeted profiling were imported to SIMCA-P+ (Umetrics, Umea, Sweden) for conducting the principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA).

5.2.9 Statistical analysis

All experiments were performed with 6 to 10 mice per group, except for acylcarnitine analysis, which was performed in triplicates. One way-ANOVA was performed to compare means between WT, HET and HET+Cho groups. A $p \leq 0.05$ at 95% confidence interval was considered as significant. Graphics are represented as mean $\pm$ SEM. All statistical tests were performed with Graphpad Prism 4 software.

5.3 Results

5.3.1 Choline supplementation prevents weight gain and alleviates liver steatosis

Pcyt2^{+/-} (HET) mice are characterized by weight gain, adult-onset obesity and lipid accumulation in the liver [117, 245]. Supplementation with choline progressively decreased the weight gain in both male and female HET mice. Untreated male and female HET mice both continued to gain weight during the same four-week period (Figure 5.1A,B). At the end of the treatment, the weight of HET mice treated with choline (HET+Cho) was similar to WT mice. Treatment with choline stabilized the weight without affecting the food or water intake. Our previous study showed that adipose tissue weight was higher in HET mice compared to WT [117]. Choline treated groups (HET+Cho) had reduced adipose weight and adipocyte cell sizes [179]. HET mice also develop liver steatosis [117]. Choline treatment could reduce lipid droplet accumulation in the HET mice liver.

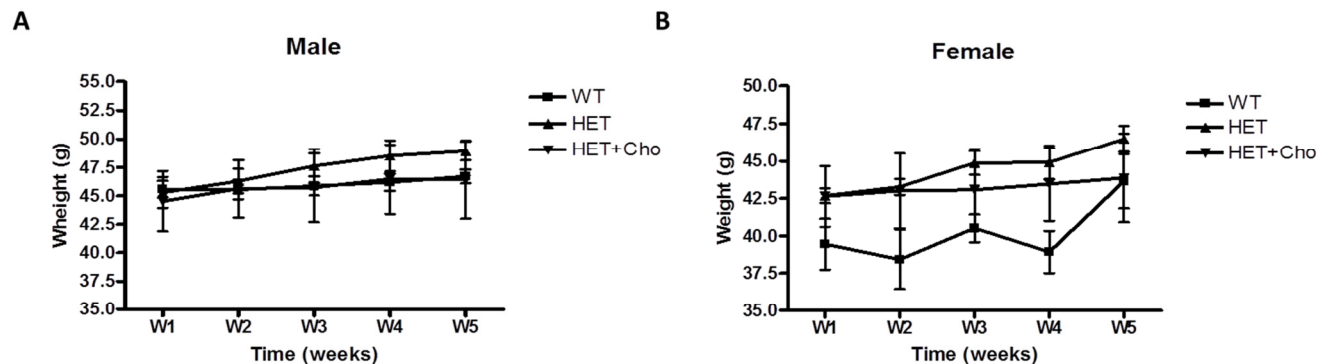


Figure 5. 1: Choline reduces weight gain in Pcyt2 HET mice. The weight change (in grams) during 4 weeks of treatment in male (A) and female (B) mice showed that choline treatment reduced the rate of weight gain in HET mice. Results are expressed as mean $\pm$ SEM.

5.3.2 Choline upregulates CDP-choline pathway for PC synthesis

The supplementation with choline was expected to stimulate PC synthesis by the CDP-choline pathway because HET mice also have abundant DAG levels [117]. Indeed, the choline radiolabeling (Figure 5.2) showed that the CDP-choline pathway was upregulated in the liver of HET+Cho mice. There was a decrease in [^3H]P-choline with simultaneous increase in [^3H]CDP-choline, showing that in the HET+Cho mice, PC synthesis was upregulated at the step catalysed by CCT/Pcyt1. HET-Cho mice also had significantly higher ^{14}C -oleate labelling of PC. PC synthesis in untreated HET mice and WT mice were similar, showing that Pcyt2 deficiency did not impact the CDP-choline pathway. Relative to WT, HET mice however had reduced [^3H]P-choline and unchanged [^3H]CDP-choline activities, implicating reduced PC catabolism in Pcyt2 deficient liver of HET mice.

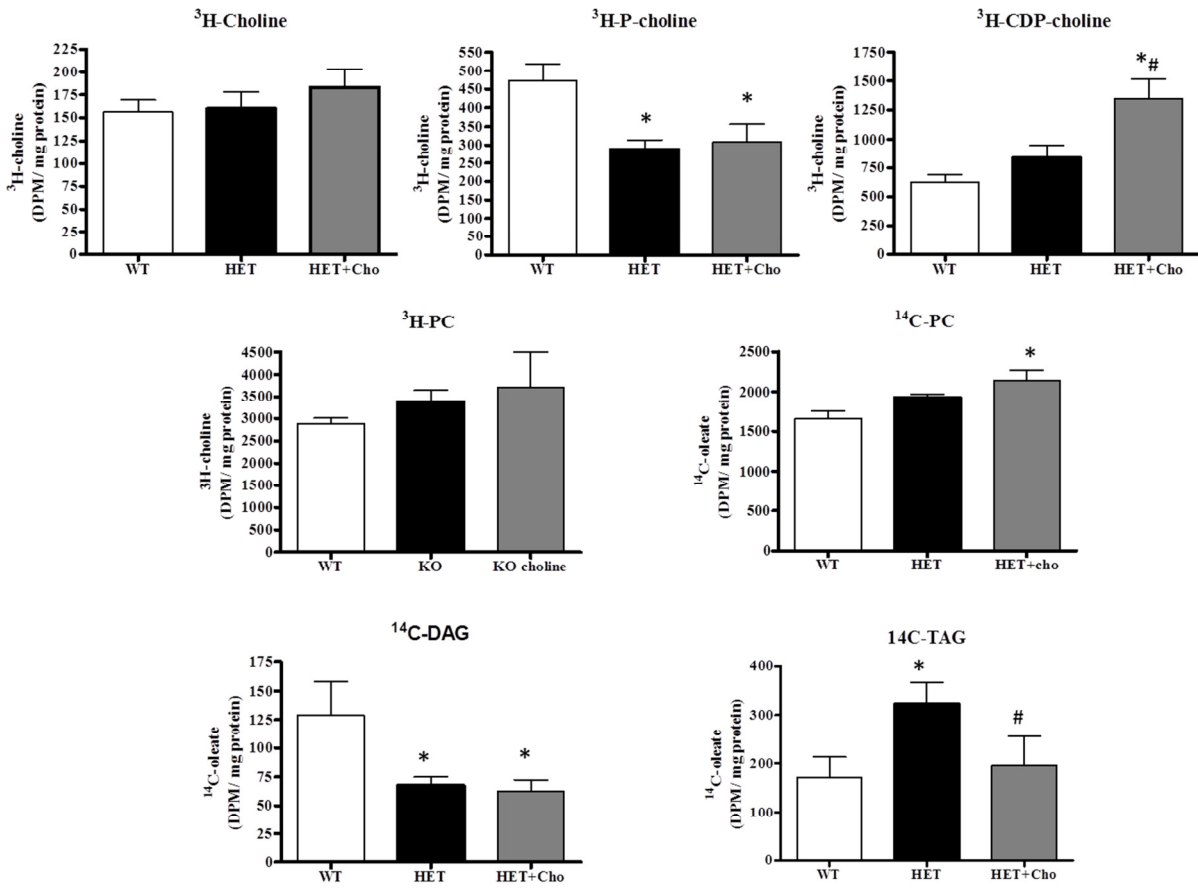


Figure 5. 2: In-vivo labeling of male mice with ^{14}C -Oleic acid and Methyl- ^3H - choline chloride. After recovered from intraperitoneal radionucleotide injections, mice were euthanized and liver tissue were isolated. Thin-layer chromatography was performed to separate CDP-choline intermediates, PC and TAG and DAG fractions and radioactivity of each fraction was measured by LSC. Liver analysis showed that ^3H -Choline (A) was not different between groups but its incorporation into P-Choline (B) was lower in HET and HET+choline groups. (C) CDP- ^3H -Choline was significantly higher in the HET mice treated with choline. (D,E) We did not observe a significant change in the ^3H - phosphatidylcholine (PC), but ^{14}C -PC was significantly higher in the HET+choline mice. (F,G) The ^{14}C -oleic acid incorporation into DAG was significantly lower in HET and HET+choline groups, but ^{14}C -oleic acid incorporation into TAG was strongly increased in HET mice only. Results are expressed as DPM/150mg tissue (mean $\pm$ SEM). * $p < 0.05$ compared to WT; # $p < 0.05$ compared to HET.

5.3.3 Choline does not modify expression of most phospholipid genes

We aimed to investigate how the expression of genes involved in choline metabolism and the PC-PE-PS cycle were regulated and if choline supplementation can affect these pathways in HET mice. The mRNA levels of CCT/Pcytl did not differ among WT, HET and HET+Cho groups (Figure 5.3B). Also BHMT which is central for the S-adenosyl methionine (AdoMet) production in the methionine-homocysteine cycle was not affected in HET and HET+Cho groups (Figure 5.3G). The expression of the PE producing gene PSD and the PE degrading genes PSS2 and PEMT were similar between all groups (Figure 5.3D,E,F). Only the PC degrading (choline releasing) PSS1 was upregulated in HET+Cho compared to HET groups (Figure 5.3C). The mRNA expression of the choline transporter CTL1/SLC441A was significantly increased in HET+Cho mice (Figure 5A) but the CTL1/SLC44A1 protein was significantly decreased in both HET and HET+Cho mice (2.4 and 2.1 folds, respectively; Figure 5.4). It is most likely that CTL1/SLC44A1 was down-regulated post-translationally by increased protein degradation as we demonstrated before [249].

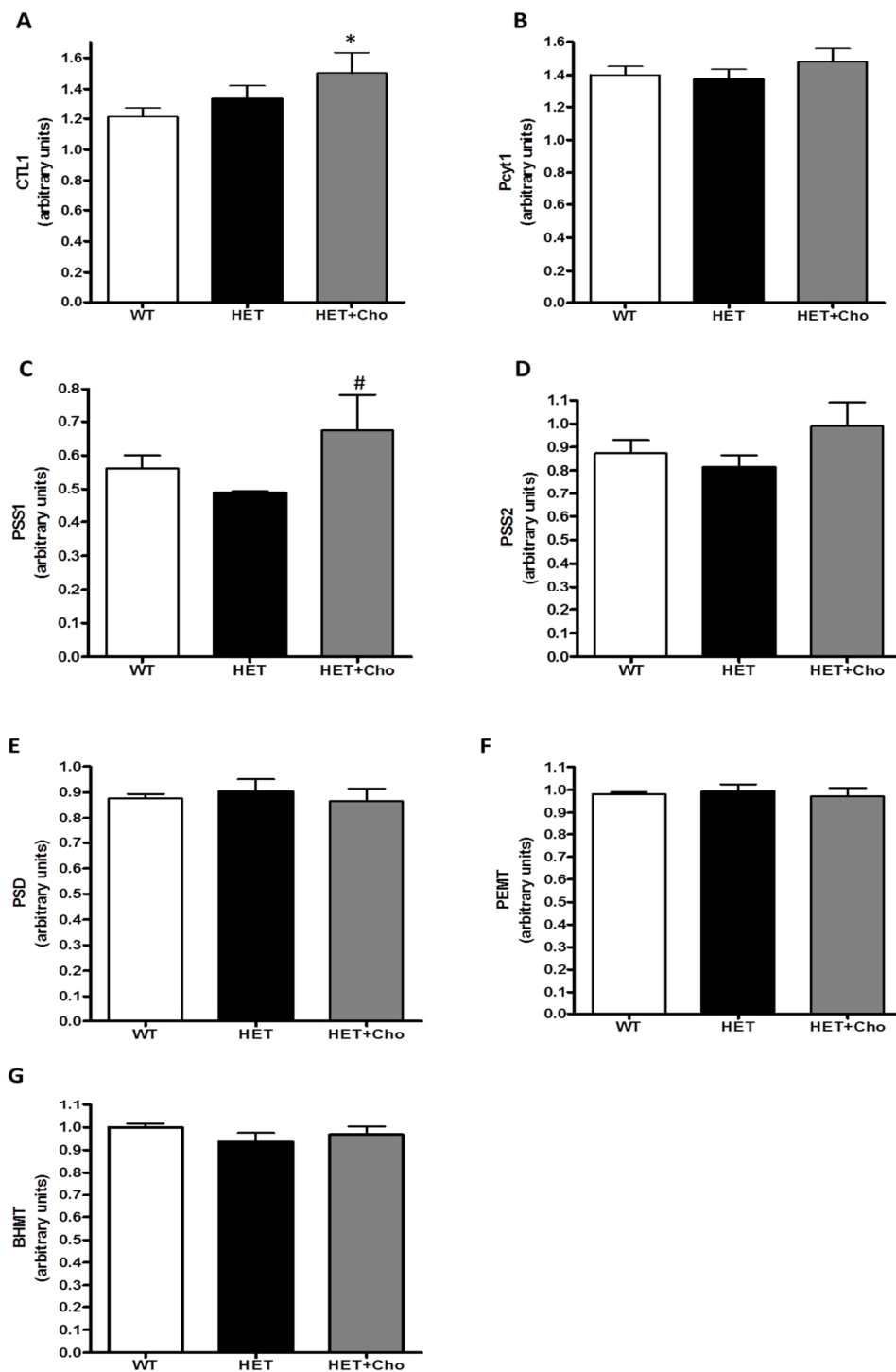


Figure 5. 3 mRNA expression of phospholipid metabolism genes analyzed by RT-PCR. CTL1 (A) and PSS1 (C) expression were significantly increased in HET+choline group compared to those in WT mice. Pcyt1 (B), PSS2 (D), PSD (E), PEMT (F) and BHMT (G) expression were not different between groups. Experiment was performed

with 6 mice per group. Results are expressed as arbitrary units (mean $\pm$ SEM) corrected by control G3PDH. * p <0.05 compared to WT

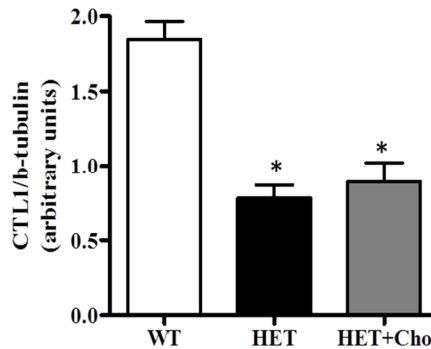


Figure 5. 4: HET mice had lower CTL1 protein content. The CTL1 protein was analysed in liver total protein lysate by immunoblotting. CTL1 protein was lower in both HET and HET treated with choline compared to that in WT mice. Intensity of CTL1 and β -tubulin bands was quantified using ImageJ software and expressed as arbitrary units (mean $\pm$ SEM). * p <0.05 compared to WT.

5.3.4 Choline increases expression of triglyceride and fatty acid metabolic genes

Because choline supplementation alleviated liver steatosis [179] we also investigated the expression of genes involved in fatty acid and TAG metabolism. The adipose triglyceride lipase (ATGL), which hydrolyses TAG to DAG, and the hormone-sensitive lipase (HSL), which hydrolyses DAG to monoacylglycerol (MAG), were both upregulated in the HET+Cho liver (Figure 5.5B,C) suggesting that the choline treatment stimulated lipolysis. The expression of the liver lipoprotein lipase (LPL), which is mainly active in the circulation, was not affected by choline treatments (Figure 5.5A).

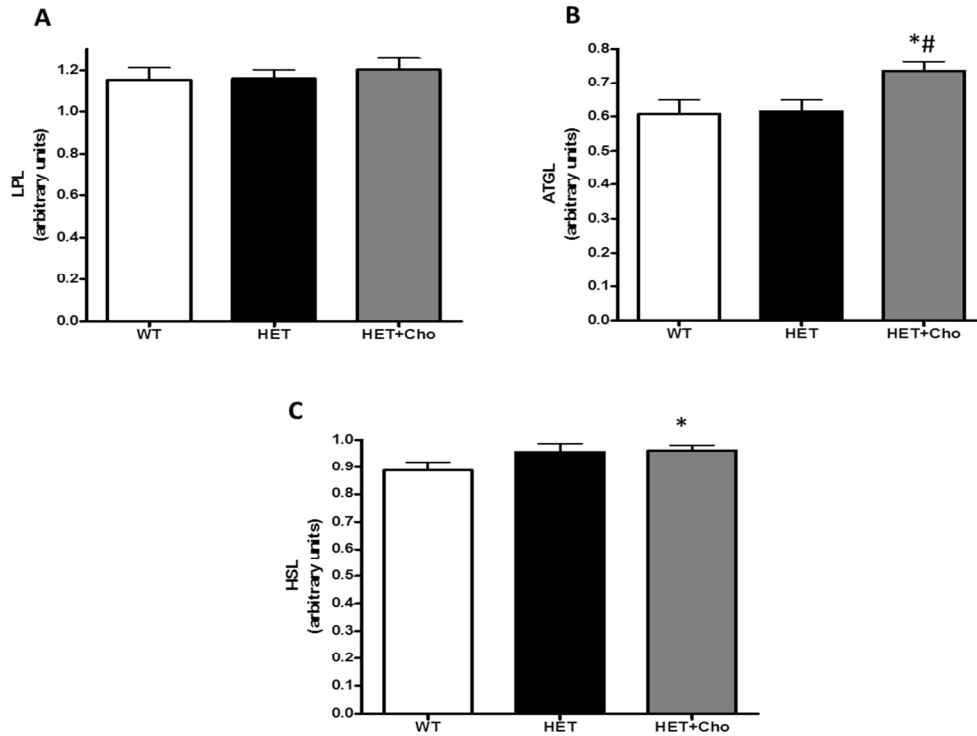


Figure 5. 5: mRNA expression of lipid metabolism genes analyzed by RT-PCR. (A,B,C) The expression of lipases ATGL and HSL, but not LPL, were increased in choline treated HET mice. Experiment was performed with 6 mice per group. Results are expressed as arbitrary units (mean $\pm$ SEM) corrected by control G3PDH. * $p < 0.05$ compared to WT; # $p < 0.05$ compared to HET.

Previous analyses by our group [179] showed that diacylglycerol acyltransferase 2 (DGAT2), which catalyze the synthesis of TAG from DAG and fatty acids, was downregulated by choline treatments. In addition, SREBP-1c (sterol regulatory element-binding protein) was upregulated in HET mice, corresponding to the increased expression of fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1). The expression of SREBP-1c, FAS and SCD were significantly reduced by choline treatment. Altogether, choline treatments reduced the expression of genes involved in FA and TAG synthesis by lipogenesis, which was in accordance with the reduced lipid accumulation observed in HET+Cho liver.

We initially showed that HET mice have an impaired ability to oxidize fatty acids [117]. Proliferator-activated receptor alpha (PPAR α) and peroxisome proliferator activated receptor co-activator 1 α (PGC1 α) influence the expression of important genes that affect fatty acid oxidation and mitochondrial biogenesis. The expression of PPAR α and PGC1 α was increased by choline treatment, suggesting improvements in fatty acid oxidation [179]. Interestingly, PPAR- γ , which is a well-known initiator of liver steatosis [251], was down-regulated by choline supplementation [179]. Taken together, choline treatment reduced TAG accumulation in HET+Cho liver by decreasing expression of the fatty acid and TAG biosynthetic genes (lipogenesis) and increasing the expression of TAG (lipolysis) and fatty acid (oxidation) catabolic genes. We also probed the effect of choline on mitochondrial function. We found that the mitochondrial membrane potential was modestly but significantly increased in HET+Cho liver mitochondria, suggesting improvements by choline treatments (Figure 5.6).

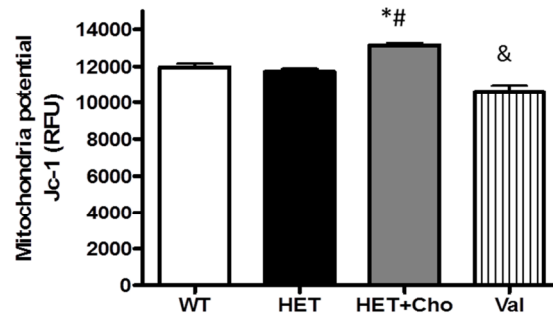
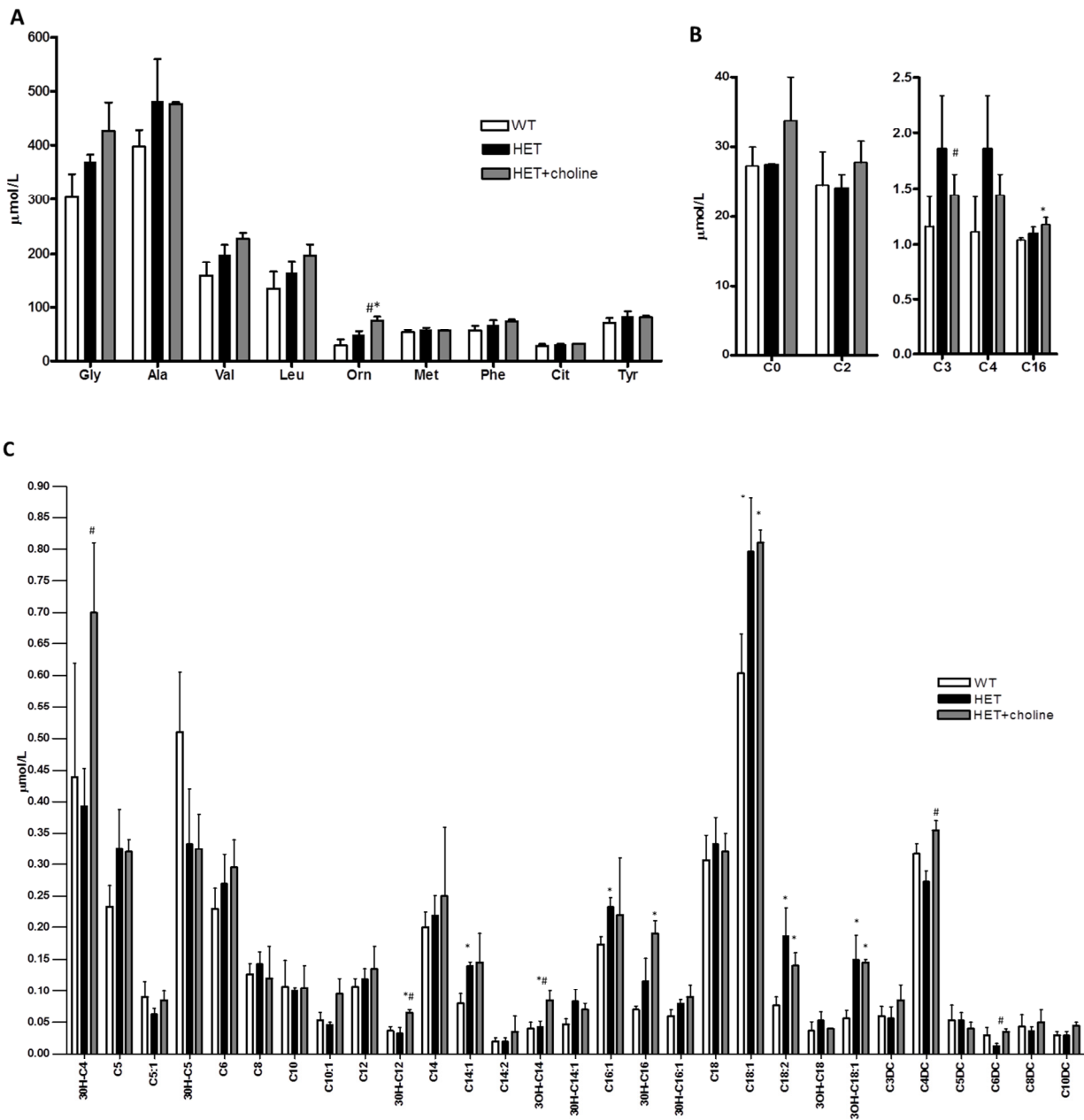


Figure 5. 6: Mitochondrial membrane potential was increased in HET+choline mice. Potential was measured using JC-1 staining and fluorescence was read at 590nm. Valinomycin (Val; 1 μ g/mL) was used as an uncoupler agent and negative control. Result is expressed as relative fluorescence units (RFU). *p<0.05 compared to WT; #p<0.05 compared to HET.

5.3.5 Amino acid and acylcarnitine profiling

MS analysis indicated that most of the circulating amino acids were elevated in female HET mice compared to WT littermate controls (Figure 5.7A). Choline treatments did not change the levels of amino acids in HET mice. Only plasma ornithine was significantly elevated in HET+Cho suggesting that choline stimulated arginine metabolism and the urea cycle. L-ornithine is known for promoting efficiency of energy consumption and excretion of ammonia, and it has been used in treatments of chronic hepatic encephalopathy, cirrhosis [252] and physical fatigue [253].

Among 34 acylcarnitines, multiple long-chain acylcarnitines (LCAC) were significantly elevated in HET plasma (C14:1, C:16:1, C18:1, C18:2 and 3OH-C18:1) (Figure 5.7B and C). The increased total LCACs (C14-C18 species) is a biomarker of incomplete β -oxidation in HET mice (Figure 5.7D). The LCAC profile in choline-treated HET (HET+Cho) was similar to untreated HET. However, the acetyl-CoA oxidative products were increased in HET+Cho group including methylmalonyl and methylglutaryl acylcarnitines and the hydroxi products 3OH-C4, 3OH-C12, 3OH-C14 showing the significant effect of choline on fatty acid oxidation. In addition, the level of C3 acylcarnitine, which is specifically derived from BCAA, was reduced in HET+Cho plasma (Figure 5.7B) suggesting a reduction in catabolism (preservation of essential BCAA) by choline treatments.



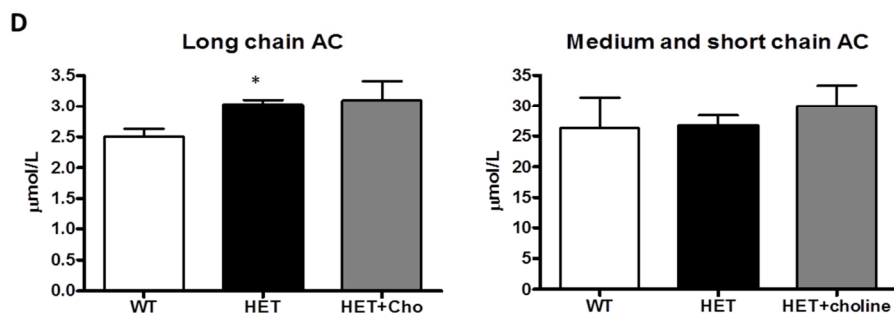
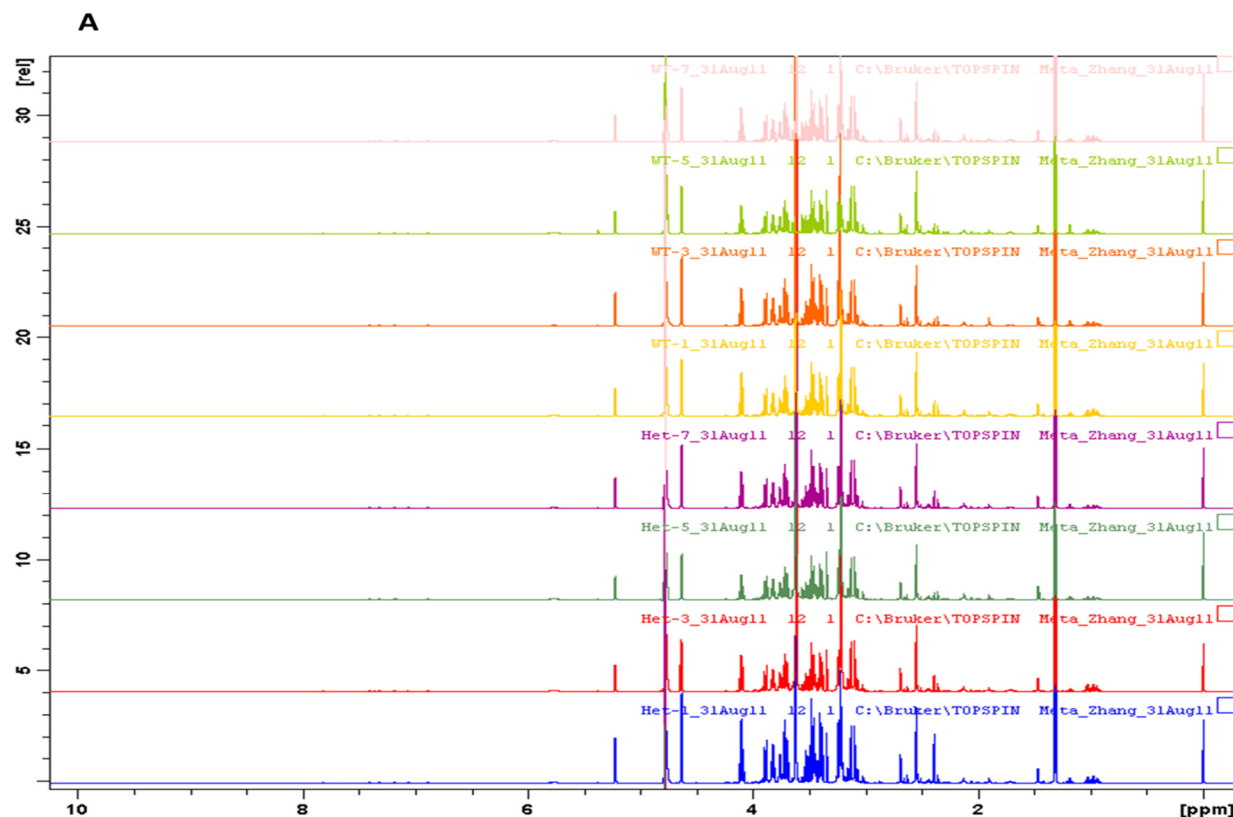


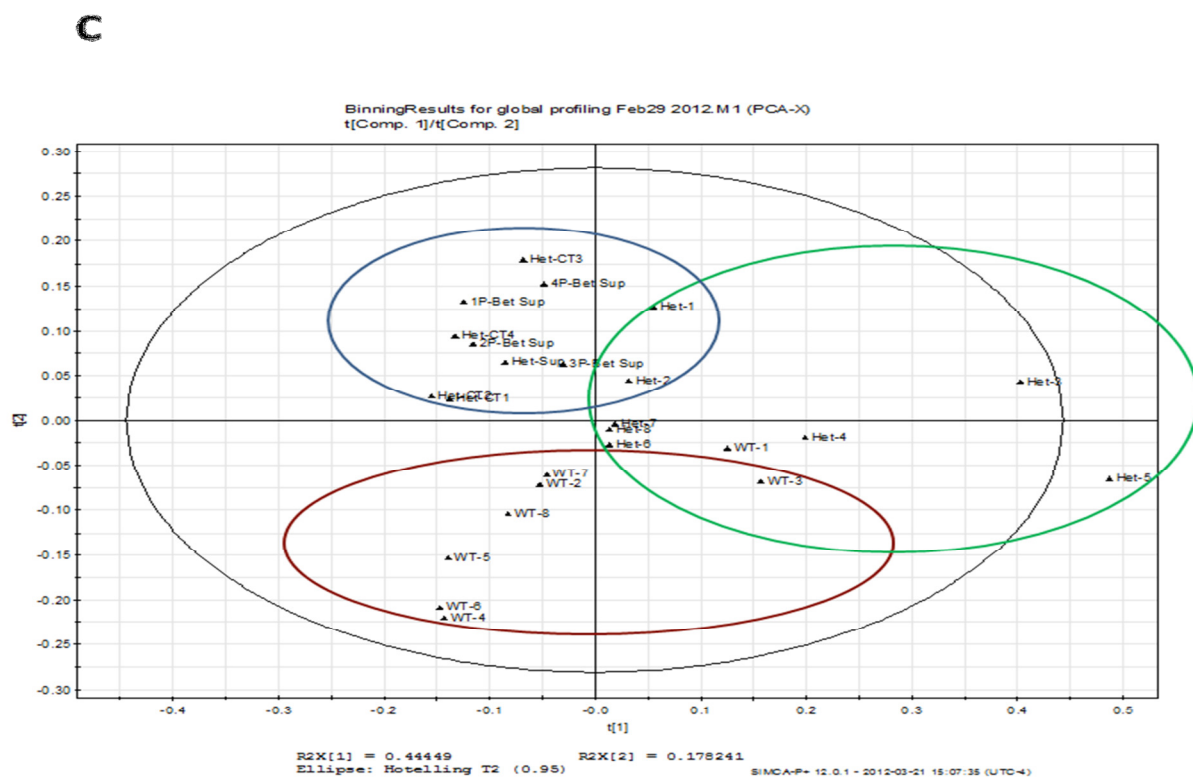
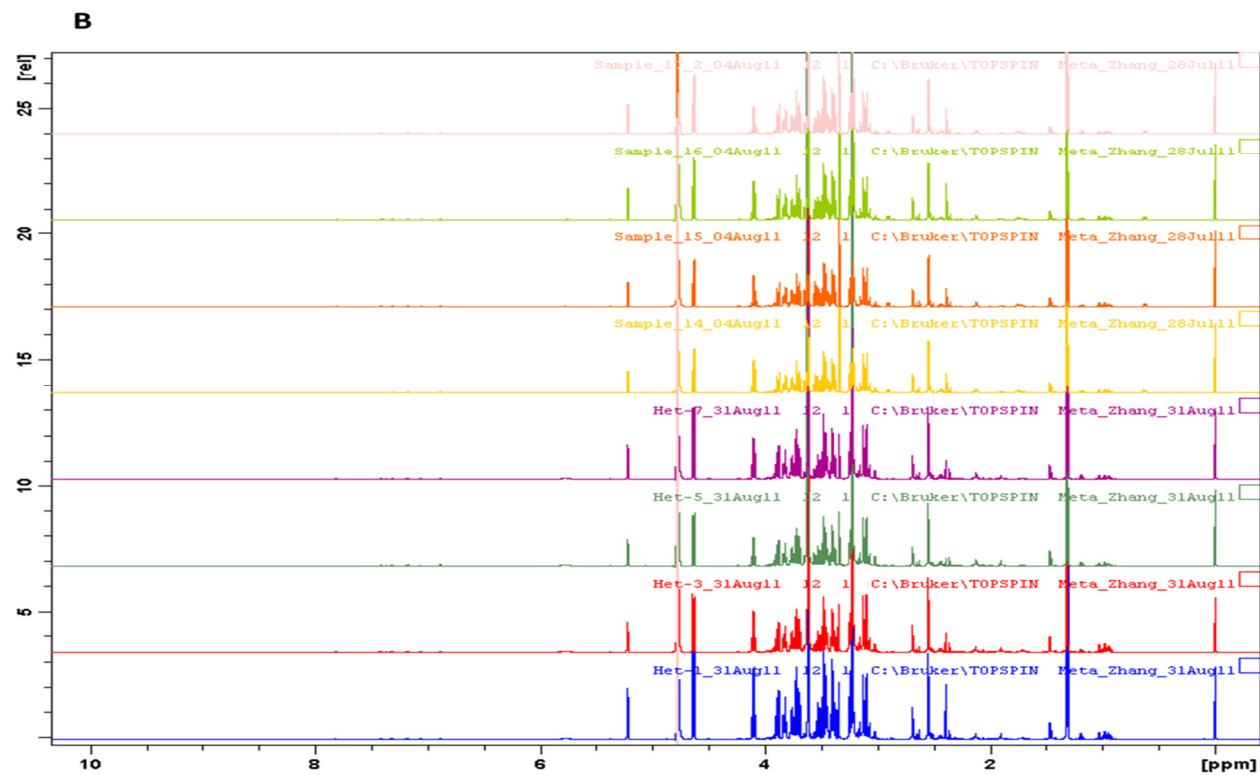
Figure 5. 7: Amino acid and acylcarnitine analysis in blood. (A) Amino acids detected in blood from WT, HET and HET+choline mice showed similar profile in these groups, but Ornithine (Orn) was increased in HET+choline group. Val=valine, Leu=leucine, Met=methionine, Phe=Phenylalanine, Tyr=tyrosine, Orn=ornithine, Cit=citrulline, Gly=glycine, Ala=alanine. (B,C) Individual acylcarnitine identified in blood are expressed as $\mu\text{mol/L}$. (D) Analysis of total long-chain (C14-C18) and medium to short-chain (C2-C12) acylcarnitines showed that HET mice have higher amount of long-chain acylcarnitines compared to WT (HET; $\bar{x}$ =3.02, SEM=0.08; WT; $\bar{x}$ =2.5, SEM=0.13). Results are expressed as mean $\pm$ SEM. * p <0.05 compared to WT; # p <0.05 compared to HET.

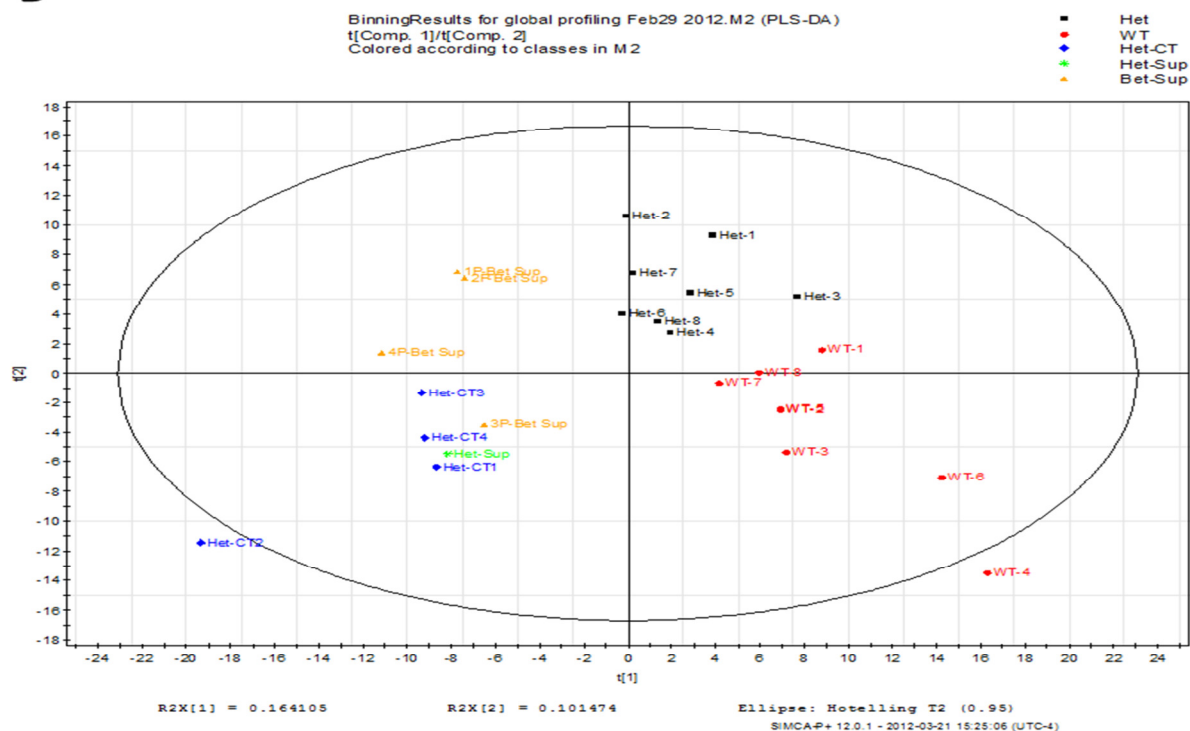
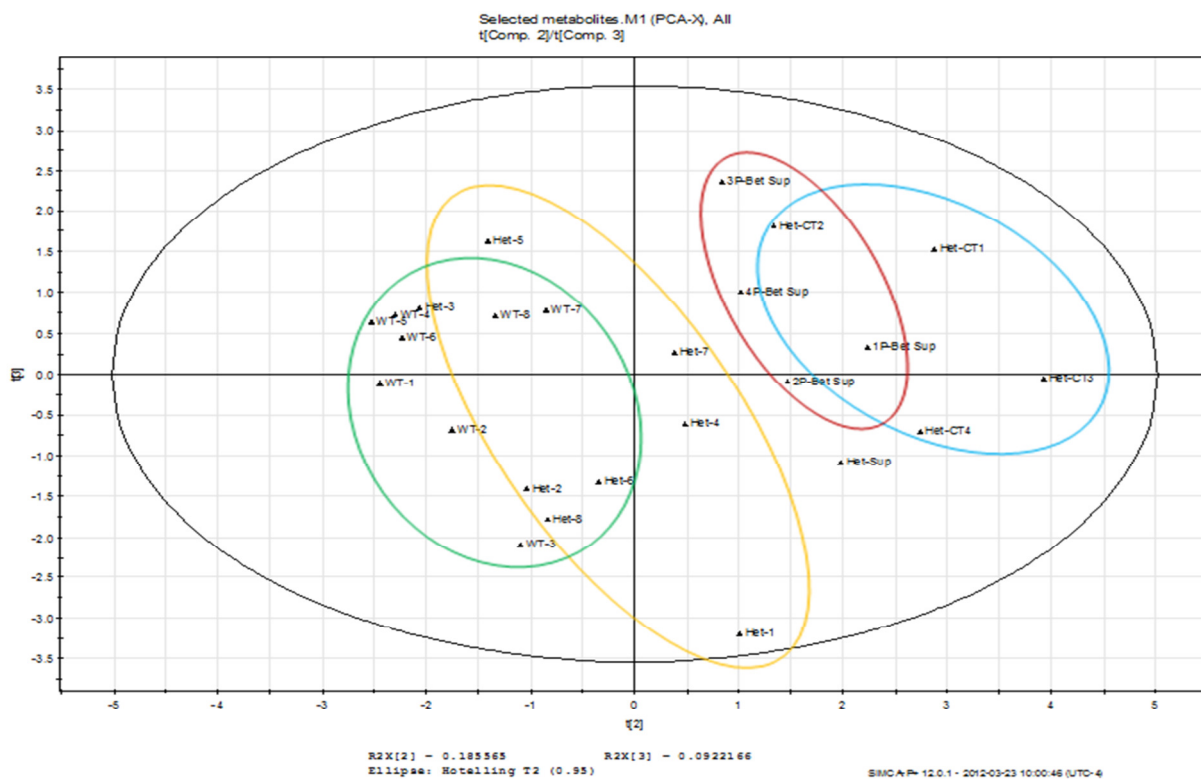
5.3.6 Metabolomic analysis by NMR

A visual comparison of ^1H -NMR spectra showed that the presence of detectable metabolites in WT, HET and HET+Cho groups was very similar (Figure 5.8A). The scores plots of PCA and PLS-DA analyses with all the samples included showed that there was a clear pattern of metabolite separation for WT, Het, and Het-Cho, also including an independent betaine study for comparison (HET-Bet) (Figure 5.8C,D). This analysis provided a general understanding of the separation patterns for the group of samples. It is generally challenging to assign metabolites without ambiguity just based on the NMR peak bins, due to complicated overlapping nature of ^1H -NMR spectra. Therefore, we used a targeted profiling approach to quantify metabolites that could be fitted with confidence using the Chenomx NMR Suite. The plasma concentrations of 27 metabolites were obtained and they are shown in Table 2. Among them, 19 metabolites with relatively higher abundance were selected for the multivariate data analysis. (Figure 5.8E,F).

Among those metabolites, acetate (acetyl-CoA), 3-hydroxybutyrate, creatine, sarcosine (intermediate in the synthesis of glycine), the amino acids threonine, taurine and lysine were reduced in HET+Cho plasma compared to WT and HET (Table 5.1). Glucose and glutamine (anaplerosis) remained increased and unmodified in HET and HET+choline groups, whereas glycine and glycerol (product of adipocyte lipolysis) were significantly increased with choline treatments. Dimethylglycine (DMG) was lower in HET mice and significantly increased in HET+Cho group. DMG is a metabolite of betaine in the methylation of homocysteine to form methionine by the betaine homocysteine methyltransferase (BHMT). Methionine then produces the main methylation agent S-adenosyl methionine (AdoMet). The increase in plasma DMG and glycine, the final metabolite of DMG, in HET+cho mice suggests higher activity of BHMT pathway.





D**E**

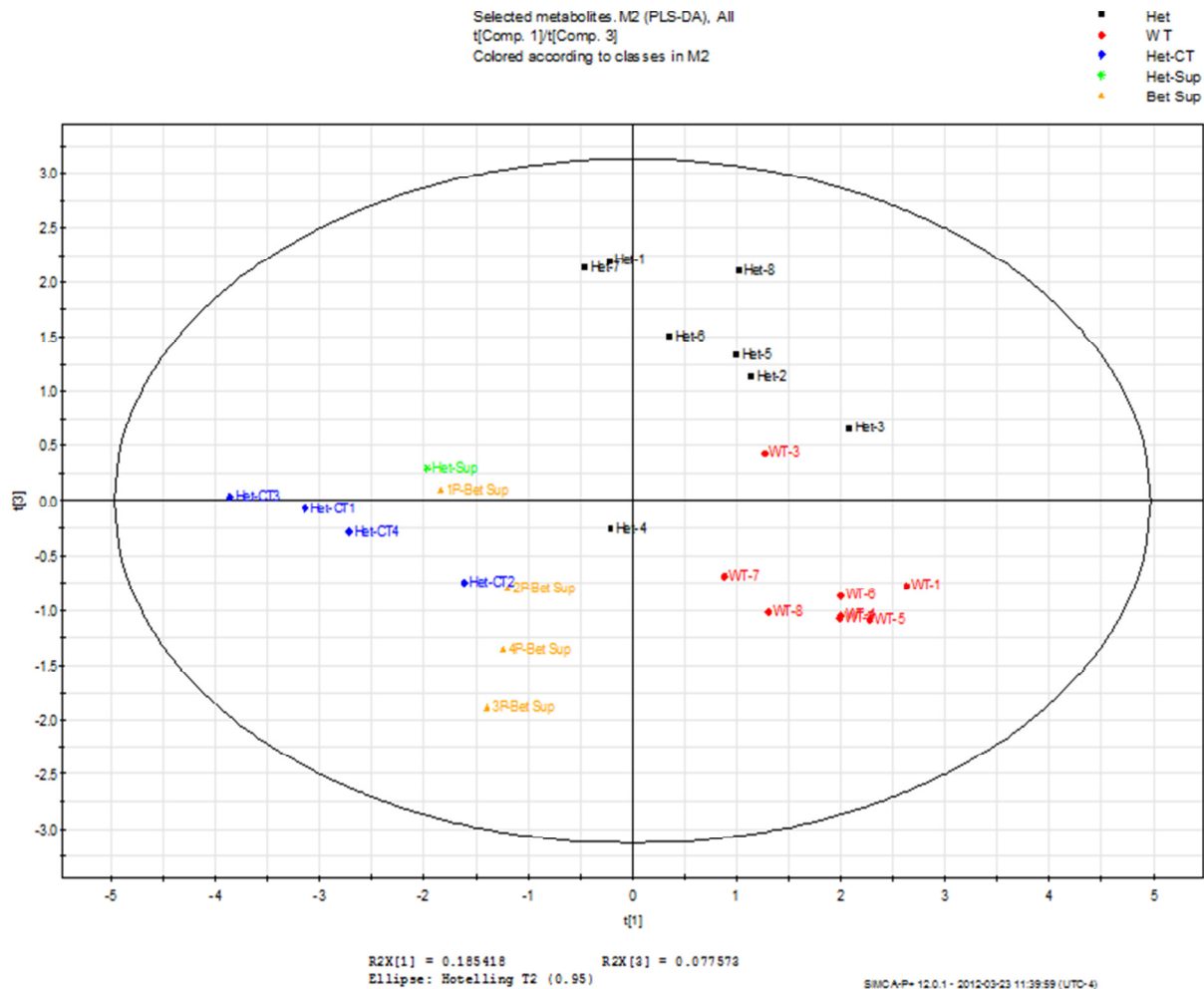
F

Figure 5. 8 Metabolomic analysis by ^1H -NMR spectra. Samples analysed: WT, HET (Het), HET+choline (Het-CT; Het-Sup), HET+betaine (Bet-Sup). (A) ^1H -NMR spectral comparison of 4 selected HET and 4 WT samples. (B) ^1H -NMR spectral comparison of 4 selected HET and 4 HET-choline (Sample 14-17) samples. (C) Scores plot of PCA on data from NMR spectral binning for all samples. (D) Scores plot of PLS-DA on data from NMR spectral binning for all samples. (E) Scores plot of PCA on data from NMR target profiling of metabolites for all samples (F) Scores plot of PLS-DA on data from NMR targeted profiling of metabolites for all samples.

Table 5. 2: Plasma concentration (mM) of metabolites identified by $^1\text{H-NMR}$. * $p < 0.05$ compared to WT; # $p < 0.05$ compared to HET.

	WT	HET	HET+Cho
2-OG	0.041	0.046	0.039
3-HB	0.229	0.210	0.106* [#]
HOAc	0.082	0.089	0.024* [#]
Ala	0.456	0.521	0.444
Bet	0.048	0.053	0.036 [#]
Cho	0.018	0.017	0.015*
Cre	0.143	0.141	0.102* [#]
Glu	10.08	12.96*	12.24*
Glm	0.474	0.614*	0.575*
GlyOH	1.19	0.72	2.26* [#]
Gly	0.254	0.276	0.282*
His	0.062	0.063	0.061
Isoleu	0.108	0.101	0.075
La	6.40	7.30	6.99
Leu	0.142	0.147	0.110
Lys	0.289	0.241*	0.190* [#]
MetOH	1.097	1.176	4.188* [#]
MMA	0.031	0.037	0.031
DMG	0.009	0.006*	0.010* [#]
Phe	0.073	0.083	0.063
Sar	0.010	0.009	0.003* [#]
Ser	0.178	0.165	0.180
Suc	0.161	0.257	0.403*
Tau	0.517	0.614	0.219* [#]
Thr	0.173	0.185	0.124*
Tyr	0.101	0.100	0.080
Val	0.242	0.232	0.258

2-OG 2-Oxoglutarate; 3-HB 3-Hydroxybutyrate; HOAc Acetate; Bet betaine; Cho choline; Cre Creatine; Glu glucose; GlyOH Glycerol; La lactate; MeOH methanol; MMA Methylmalonate; DMG N,N-Dimethylglycine;

5.4 Discussion

The current study determined the effects of choline supplementation in the Pcyt2 heterozygous mice (HET), having specifically reduced the activity of the CDP-ethanolamine Kennedy pathway. The main characteristics of HET mice are adult-onset obesity, liver steatosis, insulin resistance and hypertriglyceridemia. Choline supplementation reversed the HET phenotype by preventing weight gain and reducing liver steatosis as previously demonstrated by a reduction in the lipid droplets formation by histological analysis [179]. In addition circulating TAG levels

were reduced [179]. Altogether, we showed that choline facilitated the metabolic flux through the CDP-choline pathway, increased expression of multiple genes involved in TAG degradation by lipolysis, and reduced expression of lipid-generating-lipogenic genes, improving the liver and general health of HET mice.

5.4.1 Metabolic changes by choline supplementation

We previously demonstrated that the reduced rate of PE synthesis by the CDP-ethanolamine pathway in HET mice increases DAG availability and esterification to TAG [117]. TAG lipolysis was unmodified in HET and it was not responsible for TAG accumulation and development of liver steatosis [117]. We further established that HET mice have elevated fasting levels of very low density lipoproteins (VLDL) [117]. Then we showed that TAG accumulates in HET plasma as a result of both elevated liver VLDL (fasted) and intestinal chylomicron (fed) secretion, as well reduced plasma TAG clearance [254].

We hypothesized that choline supplementation would reduce TAG by redirecting unused DAG towards PC synthesis. Using in-vivo radiolabeling experiments, we indeed showed that choline increased the rate of PC synthesis by the CDP-choline pathway, while at the same time reducing glycerol incorporation into DAG and TAG. Interestingly, among the phospholipid genes CDP-choline pathway regulatory gene *Pcyt1* was not affected and only *PSS1* was mildly upregulated by choline treatments. This suggests that PC catabolism to produce PS (and release choline) could be elevated when PC synthesis was increased by choline treatments. It was then expected that the higher availability of choline would also upregulate CTL1 mediated choline transport in HET+Cho liver. We previously showed that choline treatments increase CTL1 protein content in cell culture (unpublished data, FASEB J.). Surprisingly, CTL1 protein content was dramatically reduced in HET liver and choline did not improve its levels. As the CTL1 mRNA was mildly

elevated, CTL1 protein was being degraded in HET liver. We previously demonstrated that an excess of palmitic acid induces autophagy and causes lysosomal degradation of CTL1 [249]. HET liver has shown an increased autophagy [255], which could be directing degradation of CTL1 protein. Further studies are needed to determine if longer treatments with choline could increase the amount of CTL1 protein in HET mice.

Choline and betaine supplementation have been found to alter DNA methylation and expression of genes involved in lipid metabolism. For example, betaine supplementation upregulated the expression of PPAR α and its target genes, thus alleviating hepatic TAG accumulation [52]. Choline supplementation was also found to regulate the expression of genes of cholesterol metabolism and prevent liver damage [256]. Here and in the previous study by Sivanesan, 2012, we found that HET mice supplemented with choline had higher expression of lipolysis genes (ATGL, HSL) and lower expression of lipogenic genes (FAS, SCD1, SREBP-1c) and TAG synthetic gene (DGAT1/2) than untreated HET mice [179]. In addition, PPAR α and PGC1 α expression was increased in the liver of choline supplemented mice. PPAR α and PGC1 α plays a vital role in prevention from liver steatosis and metabolic diseases. PPAR α stimulates the expression of genes critical for fatty acid oxidation, whereas PGC1 α , a transcriptional coactivator, regulates mitochondria biogenesis and energy metabolism. Taken together, choline was able to normalise the expression of several deregulated lipid and mitochondrial genes and restore energy homeostasis in HET mice by increasing lipolysis and fatty acid oxidation, and decreasing fatty acid and TAG synthesis.

5.4.2 Metabolomic analysis

Obesity and insulin resistance are linked with impaired fatty acid β -oxidation and a mismatch between β -oxidation and tricarboxylic acid (TCA) cycle [257]. Excessive supply of fatty acids

overloads β -oxidation, resulting in the efflux of acylcarnitines into the circulation. Even-chain species of medium and long chain acylcarnitines could arise from incomplete β -oxidation, whereas odd-chain species, C3 and C5, are derived from amino acid catabolism [258]. C4 acylcarnitine can be derived from both amino acid and fatty acids catabolism; C2 acetylcarnitine is the final product of β -oxidation and glycolysis and the universal degradation product of energy metabolism. Thus, acylcarnitine profile in blood is a useful indicator of mitochondrial energy metabolism and function. Indeed, the analysis of the acylcarnitine profile is used for the diagnosis of genetic defects in mitochondrial fatty acid oxidation and defects in the catabolism of branched chain amino acid (BCAA) [259].

Long chain acylcarnitines (LCAC) are increased in rat obesity [257, 260] and in obese and insulin resistant humans [261]. We demonstrated that HET mice, which are obese and insulin resistant, also have higher levels of LCAC compared with WT non-obese mice. Choline treatment did not reduce the levels of LCAC in HET mice. Interestingly, choline treated HET mice had increased hydroxi-derived acylcarnitines (3OH-C4, 3OH-C12, 3OH-C14), methylmalonylcarnitine (C4DC) and methylglutarylcarnitine (C6DC). 3OH-C4 was found to be increased in fasted states, and together with other short-chain acylcarnitines reflects a higher catabolism [262]. C4DC is generated through amino acid catabolism as a metabolite of valine, methionine and isoleucine and through acetyl-CoA metabolism. C6DC is derived from ketone/cholesterol synthesis intermediate and leucine catabolism. In addition, HET+Cho mice showed lower C3 acylcarnitine when compared to HET untreated. Alterations in odd-chain acylcarnitines, C3 and C5, reflect changes in BCAA catabolism. Increased catabolism of BCAA is correlated with obesity-associated insulin resistance and plasma C3 and C5 were found to be elevated in insulin resistant obese humans [263]. Similarly, C3 acylcarnitine is elevated in the

skeletal muscle of obese rats, consistent with the concept that BCAA also contribute to mitochondrial overload [258]. Choline treatment decreased the levels of C3 acylcarnitine in obese HET mice, suggesting that choline supplementation may reduce the essential amino acid catabolism and increase utilization of glucose for the TCA cycle activity.

Taken together HET mice showed an increase in long-chain acylcarnitines (LCAC), in agreement with the impaired fatty acid oxidation established earlier [117]. Choline treatments of HET mice showed higher fatty acid oxidation flux, as demonstrated by increased acylcarnitines in plasma. In addition, the flux through the TCA cycle and the respiratory chain also increased, as demonstrated by lower levels of plasma C3 acylcarnitine and greater mitochondrial membrane potential in HET-Cho liver. The improved fatty acid oxidation in HET-Cho mice is also manifested in the elevated liver lipolysis and reduced liver and plasma TAG levels [179].

In the present study we used a ^1H -NMR-based metabolomics approach [264-266] to observe general metabolic differences between WT, HET and HET-Cho mice. The concentration of plasma glucose was high in both HET and HET-Cho mice. The high glucose level in HET mice agrees with hyperglycemia and insulin resistance observed in these mice [117]. Choline supplementation did not seem to reduce the hyperglycemia in these mice. On the other hand, HET treated with choline had higher levels of glycerol, which indicates higher TAG/DAG lipolysis and that was in agreement with the gene expression data. Also, acetate (acetyl-CoA) and some amino acids (threonine, taurine and lysine) were reduced in HET-Cho plasma compared to the other groups, suggesting a higher activity of the TCA cycle.

Interesting changes were observed in metabolites of the methionine–homocysteine cycle after choline supplementation. The choline oxidation product betaine donates one methyl group to homocysteine to form methionine and dimethylglycine (DMG). DMG is further metabolized to

sarcosine and then glycine. DMG which was reduced in HET plasma increased after choline supplementation. This indicated that a reduced activity of the methionine–homocysteine cycle in HET mice was restored by choline supplementation. Together with the increase in DMG, HET-Cho plasma contained more glycine and less sarcosine, choline and betaine than HET plasma. Lower plasma choline and betaine after supplementation seems intriguing. This, however, shows that choline supplementation upregulated the major cellular metabolic pathways for choline utilization, the production of AdoMet and CDP-choline pathway, thereby both serving to prevent the increase of choline in the circulation. The increased AdoMet could potentially change DNA methylation status and induce epigenetic changes in gene expression; however, no increase in homocysteine or AdoMet levels in HET or HET-Cho plasma were observed by ^1H -NMR.

5.5 Conclusion

Choline supplementation could ameliorate symptoms of metabolic disease, such as obesity and liver steatosis, in mice deficient in CDP-ethanolamine pathway. The mechanism linking choline and lipid metabolism seems to involve several factors: changes in gene expression towards increasing lipolysis and FA oxidation and decreasing lipogenesis; increase in the rate of PC synthesis by the CDP-choline pathway and PC turnover; increased homocysteine-methionine cycle activity and improvement of mitochondrial function. Our data suggest that extra dietary choline could maintain lipid homeostasis and prevent the development or onset of metabolic syndrome.

6 Characterization of the CDP-choline pathway in Pcyt2 deficient cells

Abstract

The synthesis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by the Kennedy pathway needs to be simultaneously regulated to maintain the ratio between those membrane phospholipids. However, little is known about the mechanism for this regulation. Here we investigated the regulation of the CDP-choline pathway in Pcyt2 knocked-down human fibroblast (KD5). Consequently, the KD5 cells exhibited a significant reduced PE synthesis through the CDP-ethanolamine pathway. We demonstrated that the choline uptake and PC synthesis were upregulated in KD5 cells. Here was observed an increase in the expression of choline kinase ($CK\alpha$ and $CK\beta$), and an increase in P-choline synthesis and P-choline content in KD5 cells. These results indicate that CDP-choline pathway is upregulated at the level of choline uptake and phosphorylation, not at the rate limiting step of CDP-cho formation (Pcyt1). At the same time, the expression of phosphatidylserine decarboxylase (PSD) was upregulated, indicating a higher phosphatidylserine (PS) degradation to compensate for the impairment on the CDP-ethanolamine pathway.

6.1 *Introduction*

The maintenance of PC and PE homeostasis requires the coordination of the biosynthesis and degradation [65]. The exact mechanisms that are critical for this regulation, however, are not well established. It is known that Pcyt1, the rate limiting enzyme of PC synthesis, can be regulated by the membrane content [267], but there is no evidence regarding similar regulation of other enzymes involved in phospholipid homeostasis.

The role of the Kennedy pathway is to synthesize PC and PE from choline and ethanolamine and the common lipid intermediate diacylglycerol (DAG). Choline and ethanolamine are first transported into the cells. Choline is transported across the plasma membrane by choline transporter-like 1 (CTL1/SLC44A1). The transport system for ethanolamine remains poorly understood. After entering the cell, choline and ethanolamine are phosphorylated by choline and ethanolamine kinases (CK α and $-\beta$ and EK α and $-\beta$) [100]. The products of these kinases, phosphocholine and phosphoethanolamine, are then coupled with CTP by the specific and the regulatory pathway enzymes CDP:phosphocholine- and CDP:phosphoethanolamine cytidyltransferases (CCT/Pcyt1 and ECT/Pcyt2) to yield CDP-choline and CDP-ethanolamine, respectively. In the final steps, the CDP-choline and the CDP-ethanolamine derivatives are condensed with DAG, catalysed by multiple DAG:choline- and DAG:ethanolamine phosphotransferases (CPT, EPT, CEPT), to release CMP and produce PC and PE at the endoplasmic reticulum (ER). Phosphatidylserine (PS) can be synthesized from PC via a α -base-exchange reaction catalysed by PS synthase (PSS1). The newly formed PS then can be transformed into mitochondrial PE by PS decarboxylase (PSD).

It has been postulated that the synthesis of PC and PE by the Kennedy pathway is internally regulated. The enzyme CEPT could convert PC to DAG by reverse reaction and further to PE

when the PC:PE ratio is too high, maintaining the proper PC:PE ratio [65]. The bifunctional CEPT enzyme catalyses both PC and PE synthesis from DAG and these reactions are reversible, making this enzyme an important entity that serves to connect the PC and PE synthetic pathways [268, 269].

Because the synthesis of PE in mammalian cells occurs *via* both the CDP-ethanolamine Kennedy pathway and by the decarboxylation of PS, it is necessary for these pathways to be tightly regulated in order to maintain cellular PE homeostasis. In PSD Knock-out mice the hepatic CDP-ethanolamine pathway was stimulated, but the exact mechanism for this upregulation is not known [83]. Conversely, in *Pcyt2* liver Knock-out mice, where the activity of the CDP-ethanolamine pathway was blocked, PS content and PSD mRNA levels were increased [270], indicating that the cells produce PE by the PS decarboxylation pathway.

Here we investigated the regulation of the CDP-choline pathway in human fibroblast cells (KD5) with ~90% of *Pcyt2* knocked-down [255]. The KD5 cells have exhibited ~50% reduced rate of PE synthesis [255, 271]. We hypothesized that the rate of PC synthesis and PC conversion to PE via PS decarboxylation would be increased to maintain phospholipid membrane homeostasis.

6.2 Methods

6.2.1 Pcyt2 Knock-down cells

Pcyt2 shRNA of the human fibroblasts MCH58 have been previously described [271]. MCH58 fibroblasts infected with lentiviral particles of shRNA were selected for puromycin (2µg/mL) resistance and expanded to make stable cell lines [271]. The *Pcyt2* knock down-cell line is named here as KD5. MCH58 cells infected with an empty lentiviral vector were used as control.

Both KD5 and MCH58 controls cells were grown in Dulbecco's Modified Eagle Media (DMEM, high glucose, Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 2 μ g/mL puromycin at 37°C in 5% CO₂. The Pcyt2 deficiency in KD5 cells was confirmed through Western blot using anti-Pcyt2 α antibody and qRT-PCR. The KD5 cells showed approximately 90% reduction in Pcyt2 mRNA expression and 3.9 times less Pcyt2 protein expression compared to control cells [255, 271].

6.2.2 Radiolabeling of the CDP-choline pathway with [³H-methyl]choline

For pulse experiments, KD5 and controls were incubated for 0.5, 1.5 and 3h with 5 μ Ci [³H-methyl]choline (American Radiolabeled Chemicals-ARC) in 2ml of the DMEM 10% FBS. Cells were washed with PBS, and total lipids extracted by the Bligh-Dyer method [193]. An aliquot of cell suspension was used to measure the total protein concentration. The water-soluble metabolites choline, PCho and CDP-Cho were separated using the solvent system of ethanol/water/ammonia (48:95:7) and visualized with 1% phosphomolybdic acid in chloroform/ethanol (1:1) + 1% SnCl₂. The radioactivity was determined by liquid scintillation counting (LSC). The radiolabelled PC was determined by LSC after TLC separation with chloroform/methanol/acetic acid/water (40:12:2:0.75) and staining with 15% sulfuric acid and 0.5% K₂CrO₇.

For the equilibrium radiolabeling of the CDP-choline intermediates, KD5 and MCH58 cells were incubated for 24h with [³H-methyl]choline in 2ml of the DMEM 10% FBS. Cells were washed two times with PBS, scraped and total lipids extracted by Bligh-Dyer method. The TLC for water-soluble metabolites and PC were performed as described for pulse experiments. Results were repeated at least 2 times in triplicates.

6.2.3 Pool sizes of TAG, PC and PE

KD5 and control fibroblasts were radiolabeled with [^3H]glycerol (ARC) for 24h to determine the glycerolipid pool of TAG, PC and PE. The radiolabeled cells were washed twice with PBS and total lipids were extracted by the Bligh-Dyer method [193]. TAG lipids were separated in heptane/isopropyl alcohol/acetic acid (60:40:3) and stained in iodine vapour. PC and PE were separated with chloroform/methanol/acetic acid/water (40:12:2:0.75) and visualized with 15% sulfuric acid and 0.5% K_2CrO_7 . The lipid bands were collected from the plates and the radioactivity determined by LSC. The experiments were performed at least 3 times.

6.2.4 Choline uptake

KD5 and control fibroblasts were grown in 60mm plates until 90% confluent. Cells were washed with Krebs Ringer HEPES (KRH) buffer (130mM NaCl, 1.3mM KCl, 2.2mM CaCl_2 , 1.2mM MgSO_4 , 1.2mM KH_2PO_4 , 10mM HEPES pH 7.4 and 10mM glucose) and incubated 5 min with 5nM [^3H -methyl]choline (1 $\mu\text{Ci}/\text{mL}$; ARC). The labelled cells were washed with ice-cold KRH buffer containing 1mM of excess “cold” choline to stop the uptake and remove the extracellular radiolabeled choline. Cells were then lysed in 500 μL of the ice-cold lysis buffer (10mM Tris-HCl, 1mM EDTA, 10mM NaF). The [^3H -methyl]choline radioactivity was determined with LSC and the choline uptake calculated as DPM/mg/min. Protein amount was determined using a BCA protein assay (Thermo Scientific).

6.2.5 Expression of genes in the PC and PS metabolic pathway

Reverse-Transcription PCR was performed for the genes involved in the PC Kennedy pathway and the PC-PS-PE cycle. Total RNA from KD5 and control cells was isolated with TRIzol (Invitrogen). DNase I was used to eliminate genomic DNA and cDNA was then synthesized

from 2ug of mRNA using Superscript II reverse transcriptase (Invitrogen). Expression of multiple genes involved in the CDP-choline pathway, choline transport and the PC-PS-PE cycle were determined using gene specific primers (Table 6.1). Reactions were standardized by amplifying the GAPDH cDNA and relative band density quantified using ImageJ (NIH) software.

Table 6. 1: Genes and respective primers used for RT-PCR in KD5 and MCH58 cells

Gene	Primer Forward	Primer Reverse
CTL1/ SLC44A1	ACGTAGCTGGCAAGGTCTTC	GATCCAAATCAGGCCACCA
PTDSS1	CATGGTCGTTGCCGTTTT	TGTGTGTCGGTGAGGTAAGC
PTDSS2	TCTGGGACAAGTTGGATGGC	CCCTGCCACTTGTACGTCTT
CHKA	CCCAAGCAAGGTTTGATGCC	AGTAGTGAGCCACCCAAAGC
CHKB	AATGGTGCCGGGAGTACTTG	CCCTCTGGGAAGACTCCGTA
PCYT1A	AGAATGAGCGCTATGACGCA	TTTGCTGTGTAGCCCTCTG
PEMT	GGCCTGCTACTCTCTAAGCG	CCCAGTACATGGGGTTGTCC
PISD	CATCTGGACGTTTGGGGTGA	CGACTCCAGGGAGTAGGTGA
GPAM	GTGTTTTGGGAGAGGGAGGG	GAACACCCAAGACTCCCTGG

6.3 Results

6.3.1 Choline uptake and the CDP-choline pathway are up-regulated in Pcyt2 knock-down cells

We showed that choline uptake in KD5 cells was increased 1.45 fold compared to control MCH58 cells (Figure 6.1). The synthesis of PC and choline intermediates of the Kennedy

pathway were analyzed by pulse experiment with [^3H -methyl]choline. The rate of Choline and P-choline, but not CDP-choline, synthesis was also increased in KD5 cells (Figure 6.2 A,B,C). The rate of PC synthesis was 1.2 fold higher in KD5 cells compared to MCH58 cells based on the area under the curve (Figure 6.2D). Faster PC synthesis may compensate for the lower PE synthesis in the Pcyt2 deficient cells. This indicates higher choline uptake and P-choline synthesis to produce more choline, P-choline and PC.

The pool of choline intermediates analyzed after 24h labeling with [^3H -methyl]choline showed significantly higher levels of P-choline in KD5 cells than in MCH58 cells (Figure 6.3B). Choline, CDP-choline and PC pools were not different between KD5 and control fibroblasts (Figure 6.3A,C,D).

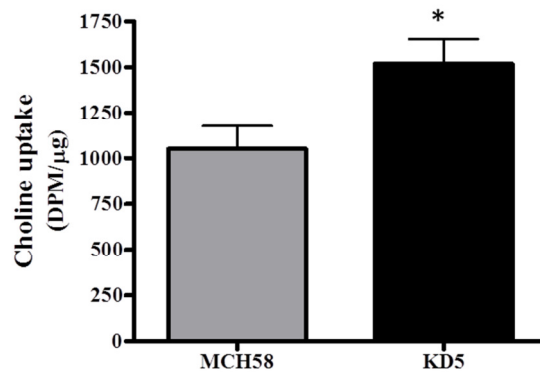


Figure 6. 1: Choline uptake is increased in KD5 cells. Choline uptake was measured using ^3H -choline in intact cells. Choline uptake into the cell was increased in KD5 cells ($\bar{x}$ =1519, SEM=135) compared to control MCH58 ($\bar{x}$ =1050, SEM=126.6). (Results are expressed as DPM/μg protein.) * p <0.05.

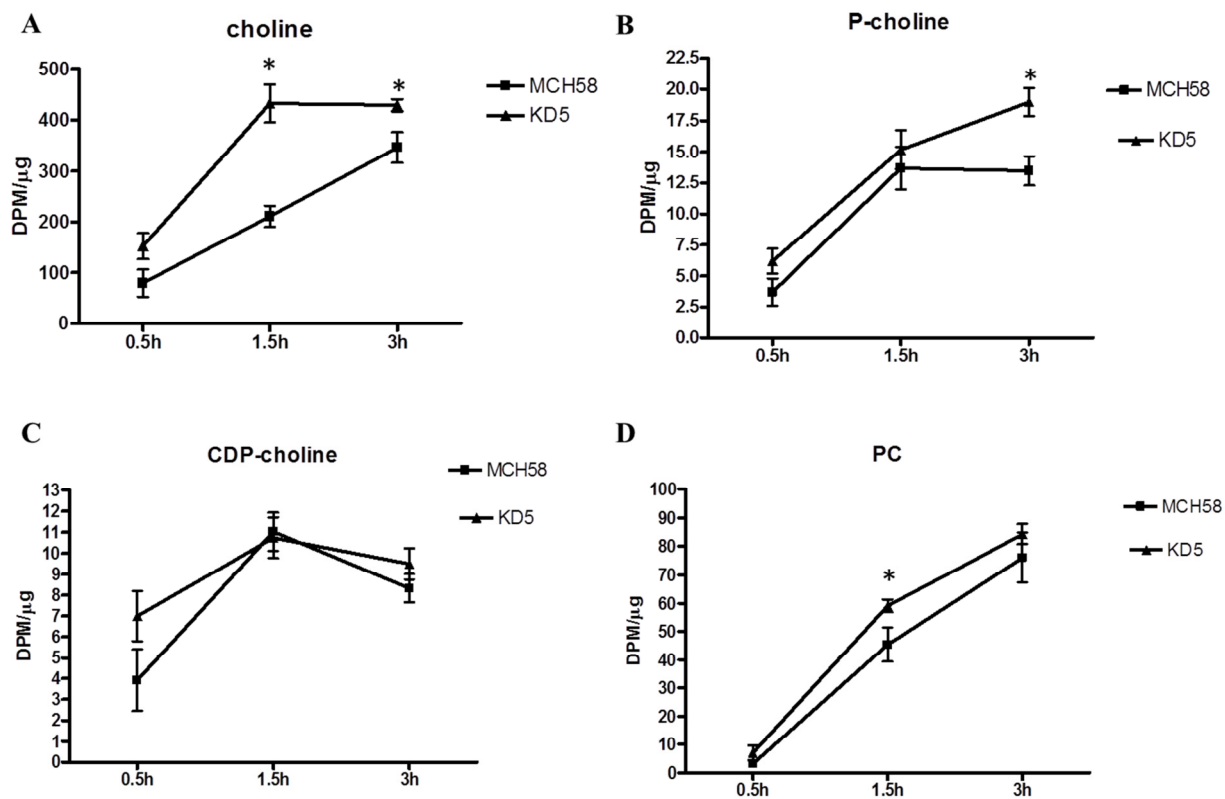


Figure 6. 2: PC synthesis is upregulated in KD5 cells. Intermediates of the Kennedy pathway and PC synthesis were analysed after 0.5h, 1.5h and 3h pulse with ^3H -choline. KD5 fibroblasts showed significantly increased synthesis of choline (A), P-Choline (B) and PC (D), but not CDP-choline (C), compared with control MCH58. Results are expressed as mean $\pm$ SEM; * $p < 0.05$.

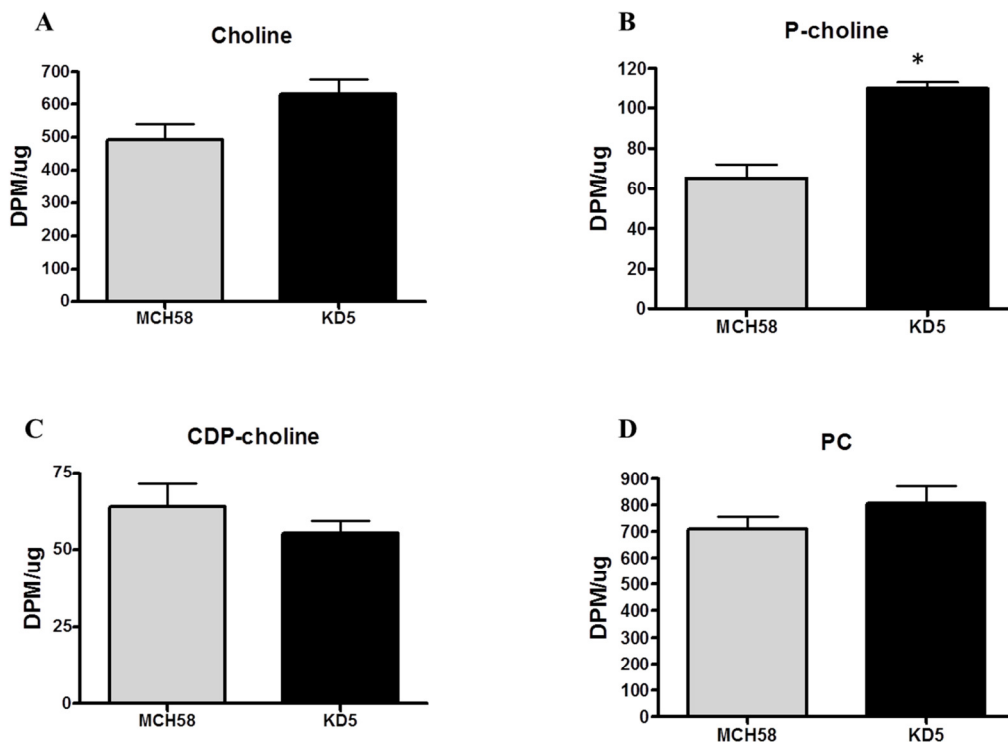


Figure 6. 3: CDP-choline intermediates content in Pcyt2 Knock-down cells. CDP-choline intermediates and PC pool size were measured after 24h of equilibrium radiolabeling with [^3H -methyl]choline in KD5 and MCH58 cells. P-choline content is increased in KD5 cells (* $p < 0.05$). Results are expressed as mean $\pm$ SEM.

6.3.2 Pool of glycerolipids is maintained in KD5 cells

PC, PE and TAG pool sizes were analyzed after 24h labeling with [^3H]glycerol. The incorporation of glycerol into PC, PE and TAG did not differ between KD5 and MCH58 cells (Figure 6.4 A, B, C).

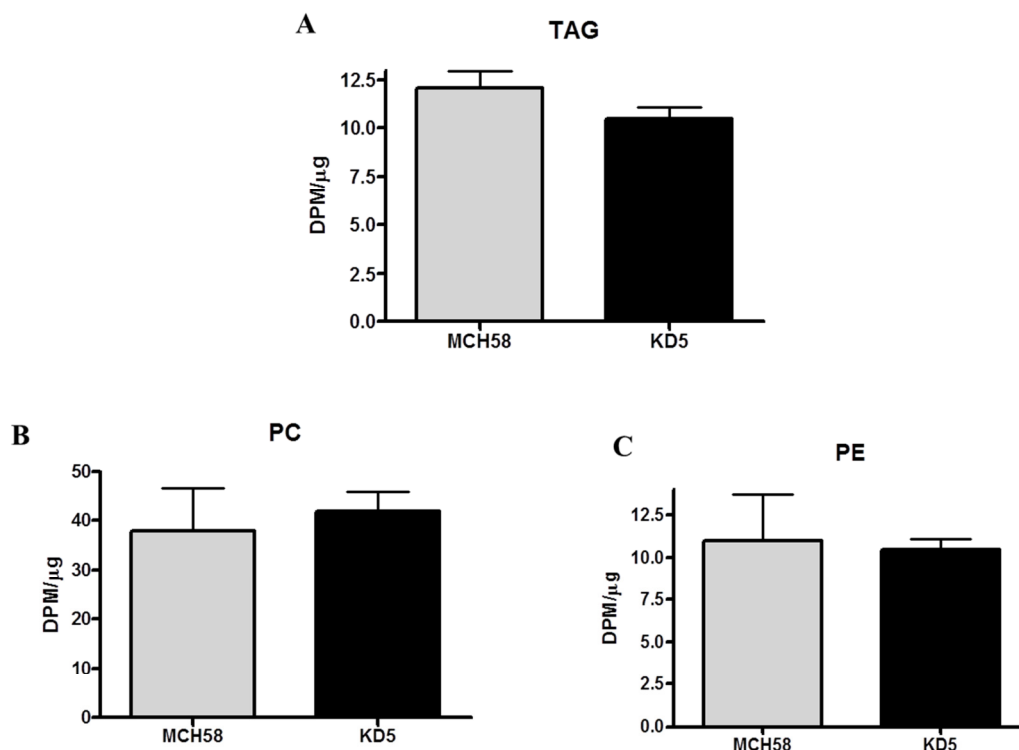


Figure 6. 4 : Pool of glycerolipids in Pcyt2 Knock-down cells. TAG (A), PC (B) and PE (C) pool size was measured after 24h of equilibrium radiolabeling with [^3H]glycerol in KD5 and MCH58 cells. TAG, PC and PE pool sizes are similar between KD5 and MCH58 fibroblasts. Results are expressed as mean $\pm$ SEM.

6.3.3 Expression of genes in the choline pathways

To better understand the regulation of the CDP-choline pathway in Pcyt2 deficient cells, the expression of genes in this pathway was analyzed. The expression of the major choline transporter, CTL1, and the rate limiting enzyme of the CDP-choline pathway, Pcyt1, did not differ between KD5 and MCH58 cells (Figure 6.5 A,B). Interestingly, choline kinase (CKA and CKB) expression was increased in KD5 cells by 1.16 and 1.26 fold, respectively (Figure 6.5 C,D). CK α and β (CKA and CKB) phosphorylate choline to produce P-choline. We observed an increase in CK expression as well as an increase in P-choline and PC synthesis.

We concluded that the increase in *de novo* PC synthesis would upregulate the PC-PS-PE reactions in order to compensate for the lower PE synthesis in KD5 cells. The expression of PSS1, which catalyzes the conversion of PC to PS, did not change in KD5 cells (Figure 6.5 E). However, the expression of PSD, which catalyzes the synthesis of PE from PS, was significantly increased (1.2 fold) in KD5 cells (Figure 6.5F). The upregulation of the PC-PS-PE cycle may be responsible for maintaining the equilibrium of these phospholipids.

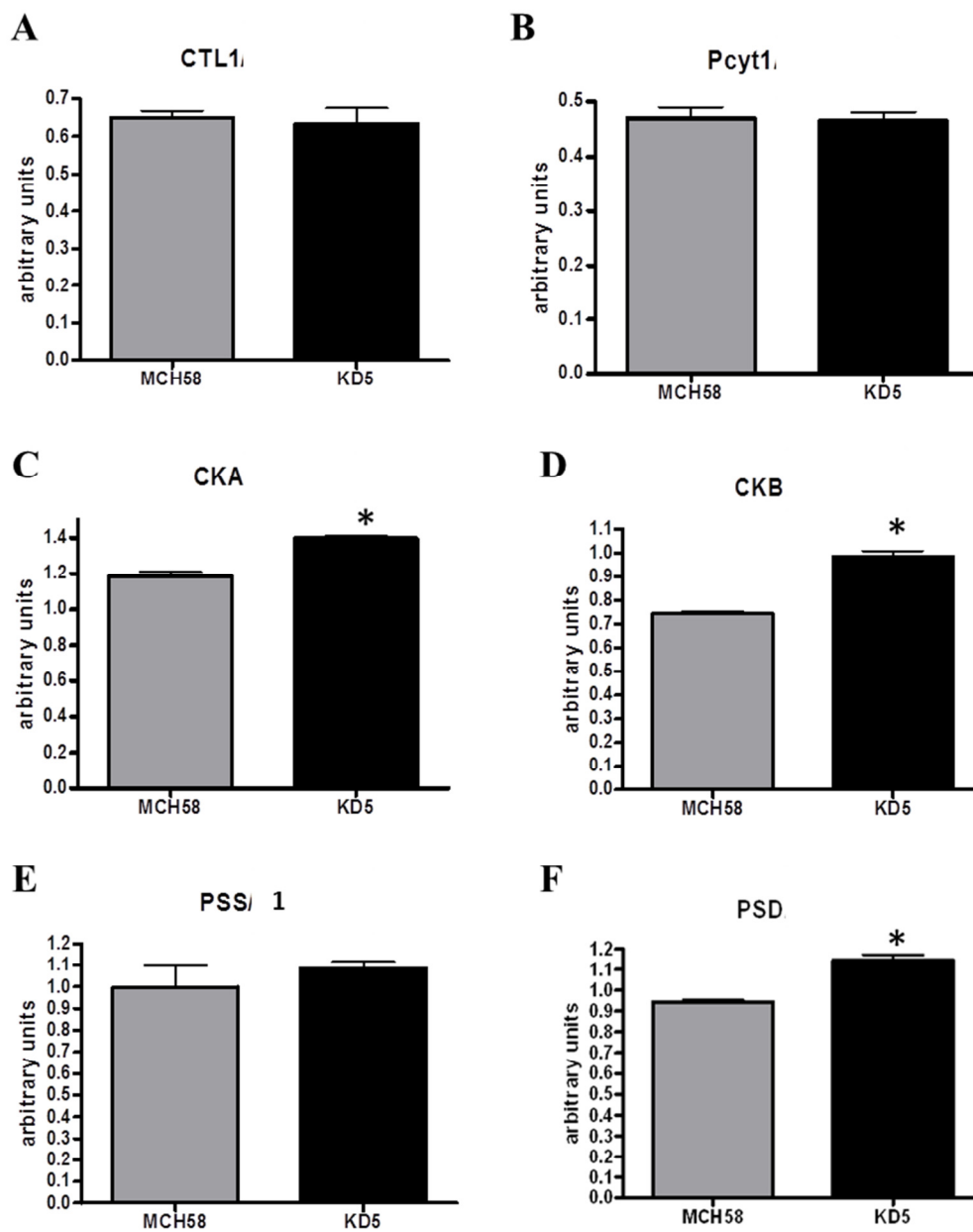


Figure 6. 5: mRNA expression of phospholipid metabolic genes analyzed by RT-PCR. Intensity of PCR bands was quantified using ImageJ software and expressed as arbitrary units (mean $\pm$ SEM). Results were normalized by the loading control GAPDH cDNA.

6.4 *Discussion*

The compositional diversity of mammalian membranes is related to chemical and physical properties of membranes, which is required to facilitate various membrane-associated functions. The phospholipid composition of membranes must therefore be constantly tightly regulated. The regulation of phospholipid composition is a complex task because of the vast array of phospholipid classes that exist. Little is known about the coordination of processes that are involved in maintaining the homeostasis of the major phospholipids (ie. PC and PE) in the bilayer membranes. PC and PE are made by the Kennedy pathway; however the regulatory mechanisms for the coordination of the CDP-choline and the CDP-ethanolamine branches of this pathway are still unclear.

Here we analysed the regulation of the Kennedy pathway in human fibroblasts that were knocked-down for the *Pcyt2* gene. Knock-down cells (KD5) have shown decreased PE synthesis through CDP-ethanolamine pathway, with the synthesis of CDP-ethanolamine from P-ethanolamine being strongly reduced compared to control cells [255]. We demonstrated that the rate of choline uptake and PC synthesis was higher in KD5 cells, indicating that the CDP-choline pathway was up-regulated when the CDP-ethanolamine pathway was impaired. We also observed an increase in the expression of CK α and CK β , and an increase in P-choline synthesis and content in KD5 cells. These results suggest that the choline uptake and phosphorylation are the major regulatory steps that control the stimulation of the CDP-choline pathway under conditions of reduced CDP-ethanolamine pathway.

CK α and CK β have the ability to function as both choline kinase and ethanolamine kinase, and the activity of CK could be regulated at the transcriptional level [272]. In addition, CK α and CK β expression could be regulated by the concentration of intracellular choline, which is increased in

KD5 cells. In these cells, the increase in CK expression could stimulate the phosphorylation of choline and PC synthesis in KD5 cells.

The mRNA expression of PSD was also increased in KD5 cells. PSD catalyzes the synthesis of PE from PS in the mitochondria, which is the second most important pathway for PE synthesis in mammalian cells [132]. Similar to our findings, hepatocytes with a downregulation of the CDP-ethanolamine pathway exhibited higher PSD mRNA expression [270]. The increase in PC synthesis along with an increase in PSD expression suggests that PC-PS-PE reactions are up-regulated when CDP-ethanolamine pathway was inhibited in an attempt to balance PE homeostasis. As observed in this study, the incorporation of ^3H -glycerol into PC and PE after 24h labeling is similar in KD5 and MCH58 cells. However, the incorporation of ^{14}C -ethanolamine into PE (a measurement of the CDP-ethanolamine pathway) was reduced in Knock-down cells [271]. This shows that PE levels are maintained by mechanisms aside from the CDP-ethanolamine pathway, and the PSD pathway was the only available pathway to produce PE. In this scenario, the levels of PS must be controlled by increasing PS synthesis from PC, which result in increased *de novo* PC synthesis by the CDP-choline pathway.

7 Integrative discussion

Until recently, the importance of choline and choline metabolites in the regulation of energy metabolism and lipid homeostasis has not been recognized. This thesis explored the interaction between choline, CTL1/SLC44A1 and lipid metabolism in different states of choline deficiency and metabolic diseases. First, the choline transport and metabolism were analysed in muscle cells treated with excess of fatty acids, as in obesity and insulin resistance states (Chapter 3). Second, choline metabolism and lipid homeostasis were characterized in the skin fibroblast cells isolated from a patient diagnosed with Postural Tachycardia Syndrome-POTS (Chapter 4). Third, choline transport and the CDP-choline pathway were studied in Pcyt2-deficient mice and Pcyt2-deficient human fibroblasts (Chapter 5 and 6). This work demonstrated the benefits of choline supplementation on balancing lipid metabolism and alleviating metabolic disease in POTS (Chapter 4) and Pcyt2^{+/-} obesity/metabolic syndrome (Chapter 5).

7.1 Regulation of choline transport and metabolism with fatty acids

The first work of this thesis (Chapter 3) provided new mechanisms for the regulation of choline transport by SLC44A1/CTL1 and CDP-choline pathway in the states of fatty acid overload. Our group initially established that choline deficiency down-regulates CTL1/SLC44A1 and PC synthesis, causing accumulation of TAG in the skeletal muscle cells C2C12 [41]. As described in Chapter 3, when C2C12 cells were treated with palmitic acid, CTL1/SLC44A1 protein became degraded resulting in reduced choline uptake. In contrast, oleic acid did not alter the CTL1/

SLC44A1 protein in the plasma membrane nor had an effect on choline uptake. Similarly to palmitic acid treated cells, Pcyt2^{+/-} mice, which have fatty liver and are obese, had reduced CTL1/SLC44A1 protein in the liver (Chapter 5). As in the case of C2C12 cells, the amount of CTL1/SLC44A1 mRNA was also not affected in the Pcyt2^{+/-} mice, suggesting that the transporter was similarly regulated at the posttranscriptional level. The excessive fatty acids availability in the liver of Pcyt2^{+/-} mice likely stimulated CTL1/SLC44A1 degradation by the autophagosomal/lysosomal pathway as shown in C2C12 cells. However, other mechanisms might have contributed to the reduced amount of CTL1/SLC44A1 in the liver of Pcyt2^{+/-} mice as well as C2C12 cells. It is well known that microRNAs negatively regulate translational processes. MicroRNAs are altered in the fatty liver induced by methionine/choline deficient diet, as well as in other fatty liver models [273-275]. In Pcyt2^{+/-} fatty liver, the expression of specific miRNAs may similarly changed, but if they contribute to reduced amounts of CTL1/ SLC44A1 needs to be established.

In C2C12 cells, fatty acids stimulated the synthesis of PC through the CDP-choline (Kennedy) pathway. The stimulation of PC synthesis by palmitic acid was less potent than the stimulation by oleic acid. This was also reinforced by lower choline transport after palmitic acid treatment. The up-regulation of the Kennedy pathway may be a mechanism for protection against accumulation of toxic lipid intermediates, as this pathway also utilizes DAG as a second substrate. However, in Chapter 3 we showed that choline transport and metabolism do not completely follow the fatty acid availability. In fact, there is a distinct utilization of palmitic acid and oleic acid in PC synthesis and in the regulation of choline transport in the C2C12 muscle cells. The distinct roles of the saturated palmitic acid, and the monounsaturated oleic acid, in the regulation of energy metabolism are well established. Palmitic acid causes insulin resistance

associated with mitochondrial oxidative stress and mitochondrial dysfunction [187, 189]. On the other hand, oleic acid is efficiently oxidised in the skeletal muscle mitochondria [276]. Our results showed that palmitic acid reduced choline uptake and only slightly increased PC and DAG synthesis, but not TAG synthesis in muscle cells. On the other hand, oleic acid did not modify choline uptake but effectively stimulate PC and TAG formation. Thus, we established that more efficient incorporation of oleic acid than palmitic acid into PC and TAG offers protection against the accumulation of the toxic intermediates DAG and ceramide, well-known causes of insulin resistance.

7.2 *Choline transporter-like 1 protein in the mitochondria*

Our group previously established that CTL1/SLC441A is a choline transporter in the outer mitochondrial membrane [36]. The mitochondrial transport provides choline for betaine synthesis (liver and kidney) and regulates the intracellular pools of choline [36]. Mitochondrial membranes are abundant in PC, which is synthesised in the endoplasmic reticulum and imported into the mitochondria through membrane contact sites. Mitochondrial PC can be metabolized by phospholipase D in the mitochondria or by phosphatidylserine synthase 1 (PSS1) in the mitochondria associated membranes (MAM), which both release free choline [177, 208]. The newly generated choline may be transported by the CTL1/SLC44A1 in and out of mitochondria to provide free choline for PC synthesis in the ER. Thus, CTL1/SLC44A1 may transport choline in and out of the mitochondria to regulate choline availability in different cellular compartments. In Chapter 3 was shown that the mitochondrial choline transport is differently regulated in relation to the plasma membrane choline transport. The transport of choline into the

mitochondria was inhibited by both, palmitic acid and oleic acid treatments, which offers a mechanism to preserve the cytosolic pools of choline when demands for PC synthesis increase. However, mitochondrial CTL1/SLC44A1 content was not drastically changed by palmitic acid, but was reduced by oleic acid. This suggests that the transport of choline into the mitochondria depends on other mechanisms besides CTL1/SLC44A1 levels. Choline uptake into the mitochondria strongly depends on the membrane potential [209]. Choline transport mediated by CTL1/SLC44A1 is inhibited by the acidification (high H^+) of extracellular medium and by a collapse of the plasma membrane electrochemical gradient, showing that CTL1/SLC44A1 operates as a choline/ proton anti-porter driven by the membrane potential [43]. Fatty acids are uncouplers of the electrochemical gradient in the mitochondria, mediated by the fatty acid-regulation of UCP1-3 [214, 277]. We found that the mitochondrial choline uptake and the mitochondrial membrane potential were both reduced by fatty acid treatments. Therefore, the mitochondrial uptake of choline does not solely depend on CTL1/SLC44A1 content and the proton [H^+] gradient could regulate choline uptake in mitochondria. While mitochondrial CTL1/SLC44A1 is the outer membrane transporter [36], the inner membrane transport system also exists and contributes to mitochondrial choline uptake [210]. Therefore, the regulation of choline uptake into mitochondria is complex and multiple factors are involved. These include changes in the electrochemical gradient, the content and the functionality of the choline transporter CTL1/SLC44A1, and a poorly characterized inner membrane transport system. Finally, the regulation of CTL1/SLC44A1 expression in the mitochondria depends on the trafficking of this transporter from intracellular stores to the mitochondria membrane. As observed with oleic acid treatments, the total cellular CTL1/SLC44A1 level was not affected, but the mitochondrial CTL1/SLC44A1 content was dramatically reduced. This indicates that oleic

acid limited the CTL1/SLC44A1 trafficking from the intracellular stores to the mitochondrial membranes, which could be an interesting topic for future investigations.

7.3 *Consequences of choline transporter deficiency*

Choline deficiency has a significant impact on liver function and lipid metabolism [46, 278]. However, the consequences of choline deficiency in extra-hepatic tissues are not well understood. Chapter 3 demonstrated that the lack of intracellular choline caused by impaired choline transport (CTL1/SLC44A1 activity) at both plasma and mitochondrial membranes has consequences on phospholipid and TAG metabolism. Chapter 4 showed that fibroblasts isolated from a POTS patient, who has normal plasma PC but very low plasma choline and betaine, are deficient in the CTL1/SLC44A1 protein and cellular choline transport. Therefore, POTS is the first known human case of choline/betaine deficiency, we identified to be caused by reduced expression and function of the choline transporter CTL1/SLC44A1. POTS fibroblasts consequently have reduced PC synthesis when compared to control fibroblasts. The POTS pool of phospholipids (PC, PE and PS) was reduced while the TAG pool was increased. Likewise, Chapter 3 and the previous studies in the liver and muscle cells have also shown that during choline deficiency, the reduced PC synthesis is linked with an accumulation of TAG [39, 232, 279].

The mitochondrial choline uptake by CTL1/SLC44A1 was also reduced in the POTS fibroblasts showing that the lack of mitochondrial choline transport may also triggers betaine deficiency in this patient. This might have severe consequences in the regulation of DNA methylation and osmolyte homeostasis. It is well known that the lack of dietary choline reduces betaine and

AdoMet synthesis and cause DNA hypomethylation [47, 280]. The membrane phospholipid composition of POTS fibroblasts was also modified. The composition of the mitochondrial membranes was previously found to be altered in choline deficient rats, affecting mitochondrial oxidative capacity and energy metabolism [243, 281].

Taken together, the patient fibroblasts represent the first case of CTL1/SLC44A1 deficiency. We conclude that the lack of choline transport has consequences on intracellular choline and glycerolipid homeostasis by reducing PC synthesis and increasing TAG, on betaine production and on mitochondrial function. What is causing reduction in CTL1/SLC44A1 expression is not known at the present. However, no mutations on CTL1/SLC44A1 gene promoter area were found.

7.4 Choline treatment and regulation of lipid metabolism

A conclusion from the second and third studies was that the supplementation of choline could balance glycerolipid and fatty acid metabolism and energy homeostasis. We showed the effect of choline treatment on two genetically altered states- the CTL1 deficient POTS fibroblasts which had impaired choline transport/CDP-choline pathway, and the Pcyt2^{+/-} mice which had impaired CDP-ethanolamine pathway.

Chronic treatments of POTS fibroblasts with excess choline stimulated the uptake of choline by increasing CTL1/SLC44A1 expression and the CDP-choline Kennedy pathway. One-month of excess choline supplementation also reversed the lipid phenotype of the CDP-ethanolamine pathway deficient mice Pcyt2^{+/-}. Choline supplementation reduced the expression of genes involved in fatty acid synthesis and increased the expression of genes involved in TAG

degradation. In addition, the methionine/homocysteine cycle which was reduced in $Pcyt2^{+/-}$ mice, was improved by choline supplementation. Similarly to our findings, choline and betaine supplementation improved metabolic disease by modifying the expression of fatty acid and lipid metabolic genes [52, 282]. The mechanism by which choline supplementation was able to restore TAG and fatty acid homeostasis in $Pcyt2^{+/-}$ mice includes several interrelated possibilities. Choline modified the expression of the key transcription factors such as SREBP1 and PPARalpha and therefore restored the expression of important lipogenic and lipolytic genes. Additional epigenetic changes (microRNA and gene promoter methylation) caused by choline supplementation could be involved. PC synthesis is essential for the secretion of VLDL-TAG and prevention of hepatic steatosis in the PEMT knockout mice [235]. In $Pcyt2^{+/-}$ mice, TAG accumulates in the liver and other organs and in the circulation. Choline treatments reduced the liver and plasma TAG, most likely by stimulating plasma and organ lipolysis, and by increasing VLDL secretion, but this could be affected by additional factors such as increased VLDL assembly and ApoB levels. Extra choline directly facilitated the utilization of DAG in the Kennedy pathway and increased PC turnover. Thus, under conditions of excessive fatty acids intake or production, as in obesity/metabolic syndrome or in choline deficiency/ POTS cells, extra choline is beneficial and protects the cells (muscle, fibroblasts and liver) from toxic lipid intermediates (fatty acids, DAG) and excessive TAG load.

7.5 Integration of CDP-choline and CDP-ethanolamine pathways

Since we showed that the CDP-choline and the CDP-ethanolamine pathways could both prevent lipid accumulation and regulate glycerolipid homeostasis, we also elucidated how the CDP-

choline pathway functions in conditions of reduced CDP-ethanolamine pathway, using *Pcyt2*^{+/-} mice (Chapter 5) and *Pcyt2* deficient human cells (Chapter 6). *Pcyt2*^{+/-} mice have reduced PE synthesis and degradation and maintain unchanged level of phospholipids (PE, PC and PS) [117]. However, *Pcyt2*^{+/-} mice accumulate TAG in the liver and other organs because excess DAG unused for PE synthesis is esterified to TAG, which also increases the fatty acid formation by lipogenesis [117]. When on normal choline (chow) diet, PC synthesis by the CDP-choline pathway is not affected in the *Pcyt2*^{+/-} mice. However, when these mice are supplemented with excess choline, the CDP-choline pathway became up-regulated, also reducing the fatty acid synthesis and TAG accumulation by increasing DAG utilization in the CDP-choline pathway. Thus, the balance between the CDP-choline and the CDP-ethanolamine pathways is important to prevent the fatty acid and TAG accumulation. The stimulation of the choline branch of the Kennedy pathway, when the ethanolamine branch of the pathway is impaired, also efficiently prevents TAG accumulation. The opposite conditions still need to be demonstrated in some CDP-choline pathway deficient models. It is however well-known that the supplementation of PC or PE could prevent/reduce obesity.

Lipid homeostasis is maintained by the coordination of synthesis and degradation. As shown in the *Pcyt2*^{+/-} mice, when the rate of PE synthesis is decreased the PE content was unchanged due to decreased PE degradation. In addition, choline supplementation increased PC synthesis and perhaps PC degradation to PS, as could be implied from the increased expression of PSS1. In contrast to *Pcyt2*^{+/-} mice, the *Pcyt2* deficient cells (KD5) showed reduced PE synthesis [271] but increased PC synthesis (PC content not changed) and do not accumulate TAG. The differences between the Kennedy pathway regulation in *Pcyt2*^{+/-} mice and KD5 cells arise from distinct metabolic and regulatory requirements in the whole-body organism and in the single cell type.

The cells in culture depend only on the substrates available in the media and those produced in the same cell type, whereas mice experience complex metabolic and regulatory interactions between various cell types and tissues.

There was an increase in choline uptake and PC synthesis in KD5 cells. In addition, the expression of PSD which converts PS to PE was increased, suggesting a stimulation of PE formation from PS in those cells. Similarly, the liver-specific knockout mice for the CDP-ethanolamine Kennedy pathway exhibited a higher PSD expression [270]. Therefore, PC synthesis and PS decarboxylation could compensate for the reduced CDP-ethanolamine Kennedy pathway. Interestingly, a higher synthesis of phosphocholine in KD5 cells suggests that choline uptake and/or choline phosphorylation are regulating CDP-choline pathway in these cells. Therefore, this needs to be further investigated by probing if the CDP-choline pathways could fully compensate for impairments in the CDP-ethanolamine pathway when extra choline is supplemented. The coordination between the two branches of the Kennedy pathway maintains the level and the ratio of the two main bilayer phospholipids, PE and PC, and therefore both pathways are critical for proper membrane function. Further studies are needed to better understand the contribution of the third membrane phospholipid-PS, expanding into the area of PS synthesis from PE and PC and mitochondrial PS degradation when some PE but not PC could be regenerated.

7.6 Conclusion and future directions

This thesis presented new approaches to better understand the effect of choline transport and metabolism in different metabolic states. The current study demonstrated that choline transporter

CTL1/SLC44A1, PC synthesis, and glycerolipid homeostasis are affected during fatty acid overload and in choline deficient states. Choline supplementation, however, upregulated PC synthesis and modified fatty acid and TAG homeostasis, showing that an increased supply of choline could have protective role against obesity and development of metabolic diseases. Longitudinal studies are warranted to elucidate if choline supplementation can protect the onset of metabolic diseases. Furthermore, this thesis demonstrated novel research in the interaction between CDP-choline and CDP-ethanolamine pathway to maintain PE and PC homeostasis.

The regulation of different type of fatty acids, such as n-3 and n-6 PUFA, on choline transport and phospholipid metabolism would be interesting to be examined. PUFAs are mainly incorporated into PE and PC phospholipids and their composition influences membrane fluidity and function [283]. The consumption of n-3 PUFA is associated with improvement of dyslipidemia and insulin resistance [284]. Treatment of choline deficient POTS cells and insulin resistant C2C12 cells with PUFA alone or together with choline may increase phospholipid synthesis and prevents TAG accumulation. In addition, different treatments may change the fatty acid composition of phospholipids and membrane fluidity.

The interaction of choline and one-carbon metabolism (BHMT cycle) was also established. The BHMT cycle was impaired in *Pcyt2*^{+/-} mice and increased after choline supplementation. Similarly, in the POTS patient, who showed impaired choline transporter CTL1/SLC44A1, BHMT cycle was down-regulated resulting in elevated plasma homocysteine. Hyperhomocysteinemia (high plasma Hcy) is an important risk factor for cardiovascular and neurodegenerative diseases and choline/betaine supplementation was found to reduce plasma Hcy [48]. However, the effect of choline supplementation on the betaine synthesis, BHMT pathway and gene regulation in POTS cells was not analysed. It would be important to

investigate those pathways further, in order to understand better the relationship between choline and one-carbon metabolism and to help with the patient prognosis and treatment.

In addition, the role of choline supplementation on mitochondrial function needs to be further studied. CTL1-deficient cells (POTS cells) have altered mitochondrial function together with impaired choline uptake. We demonstrated that choline supplementation improved choline uptake and restored glycerolipids in these cells, but the transporter role in the mitochondrial formation of betaine or cellular choline efflux still needs to be demonstrated. Mitochondrial potential, ATP production and fatty acid oxidation depends on the composition of mitochondrial membranes. Choline supplementation of the POTS cells, which had altered phospholipid (PC, PE) composition, may also restore the mitochondrial membranes and improve the mitochondrial function.

The mice study showed that choline supplementation could increase the expression of genes involved in fatty acid oxidation and mitochondrial biogenesis and increase mitochondrial potential. Further studies on the oxygen consumption rate and the extracellular acidification rate, are warranted in the *Pcyt2*^{+/-} mice supplemented with choline. Finally, the mechanisms of choline regulation of gene expression also need to be more firmly established. Choline has been evidenced to modify epigenetic marks on specific genes. Whether choline supplementation could alter the gene expression by altering gene methylation still needs to be demonstrated.

REFERENCES

- [1] Newberne PM. The methyl-deficiency model: History characteristics and research directions. *J. Nutr. Biochem.* 1993;4:618,-624.
- [2] Hershey JM, Soskin JS. Substitution of lecithin for raw pancreas in the diet of the depancreatized dog. *Am. J. Physiol.* 1931;98:74,-85.
- [3] Best CH, Huntsman ME. The effect of the components of lecithin upon deposition of fat in the liver. *J. Physiol.* 1932;75:405,-412.
- [4] Zeisel SH, Blusztajn JK. Choline and human nutrition. *Annu Rev Nutr* 1994;14:269-96.
- [5] Walkey CJ, Yu L, Agellon LB, Vance DE. Biochemical and evolutionary significance of phospholipid methylation. *J Biol Chem* 1998;273(42):27043-6.
- [6] Zeisel SH. Choline: An essential nutrient for humans. *Nutrition* 2000;16(7-8):669-71.
- [7] Fischer LM, daCosta K, Kwock L, Stewart P, Lu TS, Stabler S, et al. Sex and menopausal status influence human dietary requirements for the nutrient choline. *Am J Clin Nutr* 2007;85:1275,-1285.
- [8] Institute of medicine and national academy of sciences of the USA. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. National Academies Press (US), editor. Washington (DC): ; 1998.
- [9] Fischer LM, Searce JA, Mar MH, Patel JR, Blanchard RT, Macintosh BA, et al. Ad libitum choline intake in healthy individuals meets or exceeds the proposed adequate intake level. *J Nutr* 2005;135(4):826-9.
- [10] Lewis ED, Subhan FB, Bell RC, McCargar LJ, Curtis JM, Jacobs RL, et al. Estimation of choline intake from 24 h dietary intake recalls and contribution of egg and milk consumption to intake among pregnant and lactating women in alberta. *Br J Nutr* 2014;112(1):112-21.
- [11] Mygind VL, Evans SE, Peddie MC, Miller JC, Houghton LA. Estimation of usual intake and food sources of choline and betaine in new zealand reproductive age women. *Asia Pac J Clin Nutr* 2013;22(2):319-24.
- [12] Zeisel SH. Choline deficiency. *J Nutr Biochem* 1990;1(7):332-49.
- [13] Canty DJ, Zeisel SH. Lecithin and choline in human health and disease. *Nutr Rev* 1994;52(10):327-39.

- [14] Liapi C, Feskou I, Zarros A, Galanopoulou P, Tsakiris S. Effects of gestational and lactational choline deprivation on brain antioxidant status, acetylcholinesterase, (na(+),K(+))- and mg(2+)-ATPase activities in offspring rats. *Clin Chem Lab Med* 2007;45(5):651-6.
- [15] Yao ZM, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem* 1988;263(6):2998-3004.
- [16] Yao ZM, Vance DE. Reduction in VLDL, but not HDL, in plasma of rats deficient in choline. *Biochem Cell Biol* 1990;68(2):552-8.
- [17] Buchman AL, Dubin MD, Moukarzel AA, Jenden DJ, Roch M, Rice KM, et al. Choline deficiency: A cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. *Hepatology* 1995;22(5):1399-403.
- [18] Sheard NF, Tayek JA, Bistran BR, Blackburn GL, Zeisel SH. Plasma choline concentration in humans fed parenterally. *Am J Clin Nutr* 1986;43(2):219-24.
- [19] Burt ME, Hanin I, Brennan MF. Choline deficiency associated with total parenteral nutrition. *Lancet* 1980;2(8195 pt 1):638-9.
- [20] Carroll C, Williams L. Choline deficiency in rats as influenced by dietary energy somics. . *Nutr. Rep. Internat.* 1982;25:773.
- [21] Subbaiah PV, Ganguly J. Transesterification of lysolecithin in the intestinal mucosa of rats. *Indian J Biochem* 1971;8(4):197-203.
- [22] Le Kim D, Betzing H. Intestinal absorption of polyunsaturated phosphatidylcholine in the rat. *Hoppe Seylers Z Physiol Chem* 1976;357(9):1321-31.
- [23] Zeisel SH, Growdon JH, Wurtman RJ, Magil SG, Logue M. Normal plasma choline responses to ingested lecithin. *Neurology* 1980;30(11):1226-9.
- [24] Katz-Brull R, Degani H. Kinetics of choline transport and phosphorylation in human breast cancer cells; NMR application of the zero trans method. *Anticancer Res* 1996;16(3B):1375-80.
- [25] Riley SP, Talbot NJ, Ahmed MJ, Jouhal K, Hendry BM. Characterization of human erythrocyte choline transport in chronic renal failure. *Nephrol Dial Transplant* 1997;12(9):1921-7.
- [26] Iorio E, Mezzanzanica D, Alberti P, Spadaro F, Ramoni C, D'Ascenzo S, et al. Alterations of choline phospholipid metabolism in ovarian tumor progression. *Cancer Res* 2005;65(20):9369-76.
- [27] Villa AM, Caporizzo E, Papagni A, Miozzo L, Del Buttero P, Grilli MD, et al. Choline and phosphatidylcholine fluorescent derivatives localization in carcinoma cells studied by laser scanning confocal fluorescence microscopy. *Eur J Cancer* 2005;41(10):1453-9.

- [28] Deves R, Krupka RM. The binding and translocation steps in transport as related to substrate structure. A study of the choline carrier of erythrocytes. *Biochim Biophys Acta* 1979;557(2):469-85.
- [29] Sawada N, Takanaga H, Matsuo H, Naito M, Tsuruo T, Sawada Y. Choline uptake by mouse brain capillary endothelial cells in culture. *J Pharm Pharmacol* 1999;51(7):847-52.
- [30] van Rossum GD, Boyd CA. Sodium-dependent and -independent choline uptake by type II epithelial cells from rat lung. *J Membr Biol* 1998;162(2):147-56.
- [31] Michel V, Yuan Z, Ramsbair S, Bakovic M. Choline transport for phospholipid synthesis. *Exp Biol Med* (Maywood) 2006;231(5):490-504.
- [32] O'Regan S, Traiffort E, Ruat M, Cha N, Compaore D, Meunier FM. An electric lobe suppressor for a yeast choline transport mutation belongs to a new family of transporter-like proteins. *Proc Natl Acad Sci U S A* 2000;97(4):1835-40.
- [33] Yuan Z, Tie A, Tarnopolsky M, Bakovic M. Genomic organization, promoter activity, and expression of the human choline transporter-like protein 1. *Physiol Genomics* 2006;26(1):76-90.
- [34] Wille S, Szekeres A, Majdic O, Prager E, Staffler G, Stockl J, et al. Characterization of CDw92 as a member of the choline transporter-like protein family regulated specifically on dendritic cells. *J Immunol* 2001;167(10):5795-804.
- [35] Yuan Z, Wagner L, Poloumienko A, Bakovic M. Identification and expression of a mouse muscle-specific CTL1 gene. *Gene* 2004;341:305-12.
- [36] Michel V, Bakovic M. The solute carrier 44A1 is a mitochondrial protein and mediates choline transport. *FASEB J* 2009;23(8):2749-58.
- [37] Fullerton MD, Wagner L, Yuan Z, Bakovic M. Impaired trafficking of choline transporter-like protein-1 at plasma membrane and inhibition of choline transport in THP-1 monocyte-derived macrophages. *Am J Physiol Cell Physiol* 2006;290(4):C1230-8.
- [38] Inazu M, Takeda H, Matsumiya T. Molecular and functional characterization of an Na^{+} -independent choline transporter in rat astrocytes. *J Neurochem* 2005;94(5):1427-37.
- [39] Michel V, Singh RK, Bakovic M. The impact of choline availability on muscle lipid metabolism. *Food Funct* 2011;2(1):53-62.
- [40] Brandes AH, Ward CS, Ronen SM. 17-allylamino-17-demethoxygeldanamycin treatment results in a magnetic resonance spectroscopy-detectable elevation in choline-containing metabolites associated with increased expression of choline transporter SLC44A1 and phospholipase A2. *Breast Cancer Res* 2010;12(5):R84.

- [41] Michel V, Singh RK, Bakovic M. The impact of choline availability on muscle lipid metabolism. *Food Funct* 2011;2(1):53-62.
- [42] Kaplan CP, Porter RK, Brand MD. The choline transporter is the major site of control of choline oxidation in isolated rat liver mitochondria. *FEBS Lett* 1993;321(1):24-6.
- [43] Yabuki M, Inazu M, Yamada T, Tajima H, Matsumiya T. Molecular and functional characterization of choline transporter in rat renal tubule epithelial NRK-52E cells. *Arch Biochem Biophys* 2009;485(1):88-96.
- [44] Vance DE, Ridgway ND. The methylation of phosphatidylethanolamine. *Prog Lipid Res* 1988;27(1):61-79.
- [45] Holm PI, Hustad S, Ueland PM, Vollset SE, Grotmol T, Schneede J. Modulation of the homocysteine-betaine relationship by methylenetetrahydrofolate reductase 677 C->t genotypes and B-vitamin status in a large-scale epidemiological study. *J Clin Endocrinol Metab* 2007;92(4):1535-41.
- [46] da Costa KA, Gaffney CE, Fischer LM, Zeisel SH. Choline deficiency in mice and humans is associated with increased plasma homocysteine concentration after a methionine load. *Am J Clin Nutr* 2005;81(2):440-4.
- [47] Zeisel SH, Zola T, daCosta KA, Pomfret EA. Effect of choline deficiency on S-adenosylmethionine and methionine concentrations in rat liver. *Biochem J* 1989;259(3):725-9.
- [48] Obeid R. The metabolic burden of methyl donor deficiency with focus on the betaine homocysteine methyltransferase pathway. *Nutrients* 2013;5(9):3481-95.
- [49] Niculescu MD, Craciunescu CN, Zeisel SH. Dietary choline deficiency alters global and gene-specific DNA methylation in the developing hippocampus of mouse fetal brains. *FASEB J* 2006;20(1):43-9.
- [50] Mehedint MG, Craciunescu CN, Zeisel SH. Maternal dietary choline deficiency alters angiogenesis in fetal mouse hippocampus. *Proc Natl Acad Sci U S A* 2010;107(29):12834-9.
- [51] Cordero P, Campion J, Milagro FI, Martinez JA. Transcriptomic and epigenetic changes in early liver steatosis associated to obesity: Effect of dietary methyl donor supplementation. *Mol Genet Metab* 2013;110(3):388-95.
- [52] Wang L, Chen L, Tan Y, Wei J, Chang Y, Jin T, et al. Betaine supplement alleviates hepatic triglyceride accumulation of apolipoprotein E deficient mice via reducing methylation of peroxisomal proliferator-activated receptor alpha promoter. *Lipids Health Dis* 2013;12:34,511X-12-34.
- [53] Zabaras-Krick B. Betaine improves energy utilisation. *Intern. Pig Top.* 1997;12:12,-14.

- [54] Loest CA, Titgemeyer EC, Drouillard JS, Coetzer CM, Hunter RD, Bindel DJ, et al. Supplemental betaine and peroxide-treated feather meal for finishing cattle. *J Anim Sci* 2002;80(9):2234-40.
- [55] Yan J, Winter LB, Burns-Whitmore B, Vermeulen F, Caudill MA. Plasma choline metabolites associate with metabolic stress among young overweight men in a genotype-specific manner. *Nutr Diabetes* 2012;2:e49.
- [56] Courtenay ES, Capp MW, Anderson CF, Record MT, Jr. Vapor pressure osmometry studies of osmolyte-protein interactions: Implications for the action of osmoprotectants in vivo and for the interpretation of "osmotic stress" experiments in vitro. *Biochemistry* 2000;39(15):4455-71.
- [57] Horio M, Ito A, Matsuoka Y, Moriyama T, Orita Y, Takenaka M, et al. Apoptosis induced by hypertonicity in madin darley canine kidney cells: Protective effect of betaine. *Nephrol Dial Transplant* 2001;16(3):483-90.
- [58] Delgado-Reyes CV, Garrow TA. High sodium chloride intake decreases betaine-homocysteine S-methyltransferase expression in guinea pig liver and kidney. *Am J Physiol Regul Integr Comp Physiol* 2005;288(1):R182-7.
- [59] Ueland PM. Choline and betaine in health and disease. *J Inherit Metab Dis* 2011;34(1):3-15.
- [60] Burg MB, Ferraris JD. Intracellular organic osmolytes: Function and regulation. *J Biol Chem* 2008;283(12):7309-13.
- [61] O'Donoghue N, Sweeney T, Donagh R, Clarke KJ, Porter RK. Control of choline oxidation in rat kidney mitochondria. *Biochim Biophys Acta* 2009;1787(9):1135-9.
- [62] van Meer G, Voelker DR, Feigenson GW. Membrane lipids: Where they are and how they behave. *Nat Rev Mol Cell Biol* 2008;9(2):112-24.
- [63] Vance JE, Vance DE. Phospholipid biosynthesis in mammalian cells. *Biochem Cell Biol* 2004;82(1):113-28.
- [64] Fadeel B, Xue D. The ins and outs of phospholipid asymmetry in the plasma membrane: Roles in health and disease. *Crit Rev Biochem Mol Biol* 2009;44(5):264-77.
- [65] Hermansson M, Hokynar K, Somerharju P. Mechanisms of glycerophospholipid homeostasis in mammalian cells. *Prog Lipid Res* 2011;50(3):240-57.
- [66] Vance DE, Vance JE. Physiological consequences of disruption of mammalian phospholipid biosynthetic genes. *J Lipid Res* 2009;50 Suppl:S132-7.
- [67] Owen JS, Bruckdorfer KR, Day RC, McIntyre N. Decreased erythrocyte membrane fluidity and altered lipid composition in human liver disease. *J Lipid Res* 1982;23(1):124-32.

- [68] Colbeau A, Nachbaur J, Vignais PM. Enzymic characterization and lipid composition of rat liver subcellular membranes. *Biochim Biophys Acta* 1971;249(2):462-92.
- [69] Zinser E, Daum G. Isolation and biochemical characterization of organelles from the yeast, *saccharomyces cerevisiae*. *Yeast* 1995;11(6):493-536.
- [70] Osman C, Voelker DR, Langer T. Making heads or tails of phospholipids in mitochondria. *J Cell Biol* 2011;192(1):7-16.
- [71] Houtkooper RH, Vaz FM. Cardiolipin, the heart of mitochondrial metabolism. *Cell Mol Life Sci* 2008;65(16):2493-506.
- [72] Yin H, Zhu M. Free radical oxidation of cardiolipin: Chemical mechanisms, detection and implication in apoptosis, mitochondrial dysfunction and human diseases. *Free Radic Res* 2012;46(8):959-74.
- [73] Kagan VE, Tyurin VA, Jiang J, Tyurina YY, Ritov VB, Amoscato AA, et al. Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors. *Nat Chem Biol* 2005;1(4):223-32.
- [74] Berliner JA, Leitinger N, Tsimikas S. The role of oxidized phospholipids in atherosclerosis. *J Lipid Res* 2009;50 Suppl:S207-12.
- [75] Hammad LA, Wu G, Saleh MM, Klouckova I, Dobrolecki LE, Hickey RJ, et al. Elevated levels of hydroxylated phosphocholine lipids in the blood serum of breast cancer patients. *Rapid Commun Mass Spectrom* 2009;23(6):863-76.
- [76] Barth PG, Scholte HR, Berden JA, Van der Klei-Van Moorsel JM, Luyt-Houwen IE, Van 't Veer-Korthof ET, et al. An X-linked mitochondrial disease affecting cardiac muscle, skeletal muscle and neutrophil leucocytes. *J Neurol Sci* 1983;62(1-3):327-55.
- [77] Montine TJ, Montine KS, McMahan W, Markesbery WR, Quinn JF, Morrow JD. F2-isoprostanes in alzheimer and other neurodegenerative diseases. *Antioxid Redox Signal* 2005;7(1-2):269-75.
- [78] Porter FD, Scherrer DE, Lanier MH, Langmade SJ, Molugu V, Gale SE, et al. Cholesterol oxidation products are sensitive and specific blood-based biomarkers for niemann-pick C1 disease. *Sci Transl Med* 2010;2(56):56ra81.
- [79] Raza Shaikh S, Brown DA. Models of plasma membrane organization can be applied to mitochondrial membranes to target human health and disease with polyunsaturated fatty acids. *Prostaglandins Leukot Essent Fatty Acids* 2013;88(1):21-5.
- [80] Pfeiffer K, Gohil V, Stuart RA, Hunte C, Brandt U, Greenberg ML, et al. Cardiolipin stabilizes respiratory chain supercomplexes. *J Biol Chem* 2003;278(52):52873-80.

- [81] Claypool SM, Oktay Y, Boontheung P, Loo JA, Koehler CM. Cardiolipin defines the interactome of the major ADP/ATP carrier protein of the mitochondrial inner membrane. *J Cell Biol* 2008;182(5):937-50.
- [82] Kawasaki K, Kuge O, Chang SC, Heacock PN, Rho M, Suzuki K, et al. Isolation of a chinese hamster ovary (CHO) cDNA encoding phosphatidylglycerophosphate (PGP) synthase, expression of which corrects the mitochondrial abnormalities of a PGP synthase-defective mutant of CHO-K1 cells. *J Biol Chem* 1999;274(3):1828-34.
- [83] Steenbergen R, Nanowski TS, Beigneux A, Kulinski A, Young SG, Vance JE. Disruption of the phosphatidylserine decarboxylase gene in mice causes embryonic lethality and mitochondrial defects. *J Biol Chem* 2005;280(48):40032-40.
- [84] Choi SY, Huang P, Jenkins GM, Chan DC, Schiller J, Frohman MA. A common lipid links mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. *Nat Cell Biol* 2006;8(11):1255-62.
- [85] Joshi AS, Thompson MN, Fei N, Huttemann M, Greenberg ML. Cardiolipin and mitochondrial phosphatidylethanolamine have overlapping functions in mitochondrial fusion in *saccharomyces cerevisiae*. *J Biol Chem* 2012;287(21):17589-97.
- [86] Claypool SM, Boontheung P, McCaffery JM, Loo JA, Koehler CM. The cardiolipin transacylase, tafazzin, associates with two distinct respiratory components providing insight into barth syndrome. *Mol Biol Cell* 2008;19(12):5143-55.
- [87] Yang JS, Gad H, Lee SY, Mironov A, Zhang L, Beznoussenko GV, et al. A role for phosphatidic acid in COPI vesicle fission yields insights into golgi maintenance. *Nat Cell Biol* 2008;10(10):1146-53.
- [88] Huang H, Gao Q, Peng X, Choi SY, Sarma K, Ren H, et al. piRNA-associated germline nuage formation and spermatogenesis require MitoPLD profusogenic mitochondrial-surface lipid signaling. *Dev Cell* 2011;20(3):376-87.
- [89] Nakanishi H, de los Santos P, Neiman AM. Positive and negative regulation of a SNARE protein by control of intracellular localization. *Mol Biol Cell* 2004;15(4):1802-15.
- [90] Chen H, Chomyn A, Chan DC. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *J Biol Chem* 2005;280(28):26185-92.
- [91] Han GS, Carman GM. Characterization of the human LPIN1-encoded phosphatidate phosphatase isoforms. *J Biol Chem* 2010;285(19):14628-38.
- [92] Monteiro JP, Oliveira PJ, Jurado AS. Mitochondrial membrane lipid remodeling in pathophysiology: A new target for diet and therapeutic interventions. *Prog Lipid Res* 2013;52(4):513-28.

- [93] Kiebish MA, Han X, Cheng H, Chuang JH, Seyfried TN. Cardiolipin and electron transport chain abnormalities in mouse brain tumor mitochondria: Lipidomic evidence supporting the warburg theory of cancer. *J Lipid Res* 2008;49(12):2545-56.
- [94] Chen Q, Lesnefsky EJ. Depletion of cardiolipin and cytochrome c during ischemia increases hydrogen peroxide production from the electron transport chain. *Free Radic Biol Med* 2006;40(6):976-82.
- [95] Reibel DK, O'Rourke B, Foster KA, Hutchinson H, Uboh CE, Kent RL. Altered phospholipid metabolism in pressure-overload hypertrophied hearts. *Am J Physiol* 1986;250(1 Pt 2):H1-6.
- [96] Han X, Yang J, Cheng H, Yang K, Abendschein DR, Gross RW. Shotgun lipidomics identifies cardiolipin depletion in diabetic myocardium linking altered substrate utilization with mitochondrial dysfunction. *Biochemistry* 2005;44(50):16684-94.
- [97] Petrosillo G, Portincasa P, Grattagliano I, Casanova G, Matera M, Ruggiero FM, et al. Mitochondrial dysfunction in rat with nonalcoholic fatty liver involvement of complex I, reactive oxygen species and cardiolipin. *Biochim Biophys Acta* 2007;1767(10):1260-7.
- [98] KENNEDY EP. Biosynthesis of phospholipides. *Fed Proc* 1957;16(3):847-53.
- [99] KENNEDY EP, WEISS SB. The function of cytidine coenzymes in the biosynthesis of phospholipides. *J Biol Chem* 1956;222(1):193-214.
- [100] Aoyama C, Yamazaki N, Terada H, Ishidate K. Structure and characterization of the genes for murine choline/ethanolamine kinase isozymes alpha and beta. *J Lipid Res* 2000;41(3):452-64.
- [101] Vermeulen PS, Tijburg LB, Geelen MJ, van Golde LM. Immunological characterization, lipid dependence, and subcellular localization of CTP:Phosphoethanolamine cytidyltransferase purified from rat liver. comparison with CTP:Phosphocholine cytidyltransferase. *J Biol Chem* 1993;268(10):7458-64.
- [102] Nakashima A, Hosaka K, Nikawa J. Cloning of a human cDNA for CTP-phosphoethanolamine cytidyltransferase by complementation in vivo of a yeast mutant. *J Biol Chem* 1997;272(14):9567-72.
- [103] Tijburg LB, Geelen MJ, Van Golde LM. Biosynthesis of phosphatidylethanolamine via the CDP-ethanolamine route is an important pathway in isolated rat hepatocytes. *Biochem Biophys Res Commun* 1989;160(3):1275-80.
- [104] Tijburg LB, Geelen MJ, van Golde LM. Regulation of the biosynthesis of triacylglycerol, phosphatidylcholine and phosphatidylethanolamine in the liver. *Biochim Biophys Acta* 1989;1004(1):1-19.

[105] Bakovic M, Fullerton MD, Michel V. Metabolic and molecular aspects of ethanolamine phospholipid biosynthesis: The role of CTP:Phosphoethanolamine cytidylyltransferase (Pcyt2). *Biochem Cell Biol* 2007;85(3):283-300.

[106] Sugimoto H, Banchio C, Vance DE. Transcriptional regulation of phosphatidylcholine biosynthesis. *Prog Lipid Res* 2008;47(3):204-20.

[107] Pelech SL, Vance DE. Regulation of rat liver cytosolic CTP: Phosphocholine cytidylyltransferase by phosphorylation and dephosphorylation. *J Biol Chem* 1982;257(23):14198-202.

[108] Weinhold PA, Charles LG, Feldman DA. Microsomal CTP:Choline phosphate cytidylyltransferase: Kinetic mechanism of fatty acid stimulation. *Biochim Biophys Acta* 1991;1086(1):57-62.

[109] Weinhold PA, Charles L, Rounsifer ME, Feldman DA. Control of phosphatidylcholine synthesis in hep G2 cells. effect of fatty acids on the activity and immunoreactive content of choline phosphate cytidylyltransferase. *J Biol Chem* 1991;266(10):6093-100.

[110] Wang Y, MacDonald JI, Kent C. Regulation of CTP:Phosphocholine cytidylyltransferase in HeLa cells. effect of oleate on phosphorylation and intracellular localization. *J Biol Chem* 1993;268(8):5512-8.

[111] Cornell R, Vance DE. Translocation of CTP: Phosphocholine cytidylyltransferase from cytosol to membranes in HeLa cells: Stimulation by fatty acid, fatty alcohol, mono- and diacylglycerol. *Biochim Biophys Acta* 1987;919(1):26-36.

[112] Michel V, Bakovic M. The ubiquitous choline transporter SLC44A1. *Cent Nerv Syst Agents Med Chem* 2012;12(2):70-81.

[113] Ross BM, Moszczynska A, Blusztajn JK, Sherwin A, Lozano A, Kish SJ. Phospholipid biosynthetic enzymes in human brain. *Lipids* 1997;32(4):351-8.

[114] Houweling M, Tjburg LB, Vaartjes WJ, van Golde LM. Phosphatidylethanolamine metabolism in rat liver after partial hepatectomy. control of biosynthesis of phosphatidylethanolamine by the availability of ethanolamine. *Biochem J* 1992;283 (Pt 1)(Pt 1):55-61.

[115] Bleijerveld OB, Klein W, Vaandrager AB, Helms JB, Houweling M. Control of the CDPethanolamine pathway in mammalian cells: Effect of CTP:Phosphoethanolamine cytidylyltransferase overexpression and the amount of intracellular diacylglycerol. *Biochem J* 2004;379(Pt 3):711-9.

[116] Lykidis A, Wang J, Karim MA, Jackowski S. Overexpression of a mammalian ethanolamine-specific kinase accelerates the CDP-ethanolamine pathway. *J Biol Chem* 2001;276(3):2174-9.

- [117] Fullerton MD, Hakimuddin F, Bonen A, Bakovic M. The development of a metabolic disease phenotype in CTP:Phosphoethanolamine cytidyltransferase-deficient mice. *J Biol Chem* 2009;284(38):25704-13.
- [118] McMaster CR, Tardi PG, Choy PC. Modulation of phosphatidylethanolamine biosynthesis by exogenous ethanolamine and analogues in the hamster heart. *Mol Cell Biochem* 1992;116(1-2):69-73.
- [119] Vance JE, Shiao YJ. Intracellular trafficking of phospholipids: Import of phosphatidylserine into mitochondria. *Anticancer Res* 1996;16(3B):1333-9.
- [120] DeLong CJ, Shen YJ, Thomas MJ, Cui Z. Molecular distinction of phosphatidylcholine synthesis between the CDP-choline pathway and phosphatidylethanolamine methylation pathway. *J Biol Chem* 1999;274(42):29683-8.
- [121] Cui Z, Houweling M, Vance DE. Expression of phosphatidylethanolamine N-methyltransferase-2 in McArdle-RH7777 hepatoma cells inhibits the CDP-choline pathway for phosphatidylcholine biosynthesis via decreased gene expression of CTP:Phosphocholine cytidyltransferase. *Biochem J* 1995;312 (Pt 3)(Pt 3):939-45.
- [122] Walkey CJ, Donohue LR, Bronson R, Agellon LB, Vance DE. Disruption of the murine gene encoding phosphatidylethanolamine N-methyltransferase. *Proc Natl Acad Sci U S A* 1997;94(24):12880-5.
- [123] Noga AA, Zhao Y, Vance DE. An unexpected requirement for phosphatidylethanolamine N-methyltransferase in the secretion of very low density lipoproteins. *J Biol Chem* 2002;277(44):42358-65.
- [124] Vance JE, Steenbergen R. Metabolism and functions of phosphatidylserine. *Prog Lipid Res* 2005;44(4):207-34.
- [125] Kodaki T, Nikawa J, Hosaka K, Yamashita S. Functional analysis of the regulatory region of the yeast phosphatidylserine synthase gene, PSS. *J Bacteriol* 1991;173(24):7992-5.
- [126] Kanfer JN, McCartney D, Hattori H. Regulation of the choline, ethanolamine and serine base exchange enzyme activities of rat brain microsomes by phosphorylation and dephosphorylation. *FEBS Lett* 1988;240(1-2):101-4.
- [127] Nishijima M, Kuge O, Akamatsu Y. Phosphatidylserine biosynthesis in cultured chinese hamster ovary cells. I. inhibition of de novo phosphatidylserine biosynthesis by exogenous phosphatidylserine and its efficient incorporation. *J Biol Chem* 1986;261(13):5784-9.
- [128] Kuge O, Saito K, Nishijima M. Control of phosphatidylserine synthase II activity in chinese hamster ovary cells. *J Biol Chem* 1999;274(34):23844-9.

- [129] Stone SJ, Cui Z, Vance JE. Cloning and expression of mouse liver phosphatidylserine synthase-1 cDNA. overexpression in rat hepatoma cells inhibits the CDP-ethanolamine pathway for phosphatidylethanolamine biosynthesis. *J Biol Chem* 1998;273(13):7293-302.
- [130] Stone SJ, Vance JE. Cloning and expression of murine liver phosphatidylserine synthase (PSS)-2: Differential regulation of phospholipid metabolism by PSS1 and PSS2. *Biochem J* 1999;342 (Pt 1)(Pt 1):57-64.
- [131] Shiao YJ, Vance JE. Evidence for an ethanolamine cycle: Differential recycling of the ethanolamine moiety of phosphatidylethanolamine derived from phosphatidylserine and ethanolamine. *Biochem J* 1995;310 (Pt 2)(Pt 2):673-9.
- [132] Vance JE, Tasseva G. Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. *Biochim Biophys Acta* 2013;1831(3):543-54.
- [133] Jiang F, Rizavi HS, Greenberg ML. Cardiolipin is not essential for the growth of *saccharomyces cerevisiae* on fermentable or non-fermentable carbon sources. *Mol Microbiol* 1997;26(3):481-91.
- [134] Jiang F, Ryan MT, Schlame M, Zhao M, Gu Z, Klingenberg M, et al. Absence of cardiolipin in the *crd1* null mutant results in decreased mitochondrial membrane potential and reduced mitochondrial function. *J Biol Chem* 2000;275(29):22387-94.
- [135] Gohil VM, Greenberg ML. Mitochondrial membrane biogenesis: Phospholipids and proteins go hand in hand. *J Cell Biol* 2009;184(4):469-72.
- [136] Kutik S, Rissler M, Guan XL, Guiard B, Shui G, Gebert N, et al. The translocator maintenance protein Tam41 is required for mitochondrial cardiolipin biosynthesis. *J Cell Biol* 2008;183(7):1213-21.
- [137] Hatch GM. Cell biology of cardiac mitochondrial phospholipids. *Biochem Cell Biol* 2004;82(1):99-112.
- [138] Kiyasu Jy, Pieringer Ra, Paulus H, Kennedy Ep. The biosynthesis of phosphatidylglycerol. *J Biol Chem* 1963;238:2293-8.
- [139] Brindley DN, Waggoner DW. Mammalian lipid phosphate phosphohydrolases. *J Biol Chem* 1998;273(38):24281-4.
- [140] Choi HS, Su WM, Morgan JM, Han GS, Xu Z, Karanasios E, et al. Phosphorylation of phosphatidate phosphatase regulates its membrane association and physiological functions in *saccharomyces cerevisiae*: Identification of SER(602), THR(723), AND SER(744) as the sites phosphorylated by CDC28 (CDK1)-encoded cyclin-dependent kinase. *J Biol Chem* 2011;286(2):1486-98.

- [141] Wu WI, Carman GM. Regulation of phosphatidate phosphatase activity from the yeast *saccharomyces cerevisiae* by phospholipids. *Biochemistry* 1996;35(12):3790-6.
- [142] Kent C. Eukaryotic phospholipid biosynthesis. *Annu Rev Biochem* 1995;64:315-43.
- [143] Chakraborty TR, Vancura A, Baliya VS, Haldar D. Phosphatidic acid synthesis in mitochondria. topography of formation and transmembrane migration. *J Biol Chem* 1999;274(42):29786-90.
- [144] MacDonald JI, Sprecher H. Phospholipid fatty acid remodeling in mammalian cells. *Biochim Biophys Acta* 1991;1084(2):105-21.
- [145] Schlame M, Ren M. Barth syndrome, a human disorder of cardiolipin metabolism. *FEBS Lett* 2006;580(23):5450-5.
- [146] Shindou H, Hishikawa D, Harayama T, Yuki K, Shimizu T. Recent progress on acyl CoA: Lysophospholipid acyltransferase research. *J Lipid Res* 2009;50 Suppl:S46-51.
- [147] Shimizu T. Lipid mediators in health and disease: Enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation. *Annu Rev Pharmacol Toxicol* 2009;49:123-50.
- [148] Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: Dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol* 2008;8(5):349-61.
- [149] Vangaveti V, Baune BT, Kennedy RL. Hydroxyoctadecadienoic acids: Novel regulators of macrophage differentiation and atherogenesis. *Ther Adv Endocrinol Metab* 2010;1(2):51-60.
- [150] Piomelli D, Sasso O. Peripheral gating of pain signals by endogenous lipid mediators. *Nat Neurosci* 2014;17(2):164-74.
- [151] Bridges JP, Ikegami M, Brilli LL, Chen X, Mason RJ, Shannon JM. LPCAT1 regulates surfactant phospholipid synthesis and is required for transitioning to air breathing in mice. *J Clin Invest* 2010;120(5):1736-48.
- [152] Stace CL, Ktistakis NT. Phosphatidic acid- and phosphatidylserine-binding proteins. *Biochim Biophys Acta* 2006;1761(8):913-26.
- [153] Vicinanza M, D'Angelo G, Di Campli A, De Matteis MA. Function and dysfunction of the PI system in membrane trafficking. *EMBO J* 2008;27(19):2457-70.
- [154] Hishikawa D, Hashidate T, Shimizu T, Shindou H. Diversity and function of membrane glycerophospholipids generated by the remodeling pathway in mammalian cells. *J Lipid Res* 2014;55(5):799-807.

- [155] Monteiro JP, Pereira CV, Silva AM, Maciel E, Baldeiras I, Peixoto F, et al. Rapeseed oil-rich diet alters hepatic mitochondrial membrane lipid composition and disrupts bioenergetics. *Arch Toxicol* 2013.
- [156] Guderley H, Kraffe E, Bureau W, Bureau DP. Dietary fatty acid composition changes mitochondrial phospholipids and oxidative capacities in rainbow trout red muscle. *J Comp Physiol B* 2008;178(3):385-99.
- [157] Herbst EA, Paglialunga S, Gerling C, Whitfield J, Mukai K, Chabowski A, et al. Omega-3 supplementation alters mitochondrial membrane composition and respiration kinetics in human skeletal muscle. *J Physiol* 2014;592(Pt 6):1341-52.
- [158] Divakaran P, Venkataraman A. Effect of dietary fats on oxidative phosphorylation and fatty acid profile of rat liver mitochondria. *J Nutr* 1977;107(9):1621-31.
- [159] Yamaoka S, Urade R, Kito M. Mitochondrial function in rats is affected by modification of membrane phospholipids with dietary sardine oil. *J Nutr* 1988;118(3):290-6.
- [160] Clandinin MT, Field CJ, Hargreaves K, Morson L, Zsigmond E. Role of diet fat in subcellular structure and function. *Can J Physiol Pharmacol* 1985;63(5):546-56.
- [161] Yamaoka S, Urade R, Kito M. Cardiolipin molecular species in rat heart mitochondria are sensitive to essential fatty acid-deficient dietary lipids. *J Nutr* 1990;120(5):415-21.
- [162] Valianpour F, Wanders RJ, Overmars H, Vaz FM, Barth PG, van Gennip AH. Linoleic acid supplementation of Barth syndrome fibroblasts restores cardiolipin levels: Implications for treatment. *J Lipid Res* 2003;44(3):560-6.
- [163] Hulbert AJ, Turner N, Storlien LH, Else PL. Dietary fats and membrane function: Implications for metabolism and disease. *Biol Rev Camb Philos Soc* 2005;80(1):155-69.
- [164] Takeuchi H, Matsuo T, Tokuyama K, Shimomura Y, Suzuki M. Diet-induced thermogenesis is lower in rats fed a lard diet than in those fed a high oleic acid safflower oil diet, a safflower oil diet or a linseed oil diet. *J Nutr* 1995;125(4):920-5.
- [165] Shimomura Y, Tamura T, Suzuki M. Less body fat accumulation in rats fed a safflower oil diet than in rats fed a beef tallow diet. *J Nutr* 1990;120(11):1291-6.
- [166] Pan DA, Storlien LH. Dietary lipid profile is a determinant of tissue phospholipid fatty acid composition and rate of weight gain in rats. *J Nutr* 1993;123(3):512-9.
- [167] Pehowich DJ. Thyroid hormone status and membrane n-3 fatty acid content influence mitochondrial proton leak. *Biochim Biophys Acta* 1999;1411(1):192-200.

- [168] Tijburg LB, Nishimaki-Mogami T, Vance DE. Evidence that the rate of phosphatidylcholine catabolism is regulated in cultured rat hepatocytes. *Biochim Biophys Acta* 1991;1085(2):167-77.
- [169] Morash SC, Cook HW, Spence MW. Phosphatidylcholine metabolism in cultured cells: Catabolism via glycerophosphocholine. *Biochim Biophys Acta* 1988;961(2):194-202.
- [170] De Windt LJ, Reneman RS, Van der Vusse GJ, Van Bilsen M. Phospholipase A2-mediated hydrolysis of cardiac phospholipids: The use of molecular and transgenic techniques. *Mol Cell Biochem* 1998;180(1-2):65-73.
- [171] Zhang XH, Zhao C, Seleznev K, Song K, Manfredi JJ, Ma ZA. Disruption of G1-phase phospholipid turnover by inhibition of Ca²⁺-independent phospholipase A2 induces a p53-dependent cell-cycle arrest in G1 phase. *J Cell Sci* 2006;119(Pt 6):1005-15.
- [172] Mancuso DJ, Sims HF, Han X, Jenkins CM, Guan SP, Yang K, et al. Genetic ablation of calcium-independent phospholipase A2gamma leads to alterations in mitochondrial lipid metabolism and function resulting in a deficient mitochondrial bioenergetic phenotype. *J Biol Chem* 2007;282(48):34611-22.
- [173] Fukami K, Inanobe S, Kanemaru K, Nakamura Y. Phospholipase C is a key enzyme regulating intracellular calcium and modulating the phosphoinositide balance. *Prog Lipid Res* 2010;49(4):429-37.
- [174] Hii CS, Edwards YS, Murray AW. Phorbol ester-stimulated hydrolysis of phosphatidylcholine and phosphatidylethanolamine by phospholipase D in HeLa cells. evidence that the basal turnover of phosphoglycerides does not involve phospholipase D. *J Biol Chem* 1991;266(30):20238-43.
- [175] Minahk C, Kim KW, Nelson R, Trigatti B, Lehner R, Vance DE. Conversion of low density lipoprotein-associated phosphatidylcholine to triacylglycerol by primary hepatocytes. *J Biol Chem* 2008;283(10):6449-58.
- [176] Gao Q, Frohman MA. Roles for the lipid-signaling enzyme MitoPLD in mitochondrial dynamics, piRNA biogenesis, and spermatogenesis. *BMB Rep* 2012;45(1):7-13.
- [177] Jin JK, Kim NH, Lee YJ, Kim YS, Choi EK, Kozlowski PB, et al. Phospholipase D1 is up-regulated in the mitochondrial fraction from the brains of alzheimer's disease patients. *Neurosci Lett* 2006;407(3):263-7.
- [178] Tvrzicka E, Kremmyda LS, Stankova B, Zak A. Fatty acids as biocompounds: Their role in human metabolism, health and disease--a review. part 1: Classification, dietary sources and biological functions. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 2011;155(2):117-30.

- [179] Sivanesan S. Amelioration of metabolic syndrome with choline and betaine diets.[dissertation], University of Guelph; 2012.
- [180] Machova E, O'Regan S, Newcombe J, Meunier FM, Prentice J, Dove R, et al. Detection of choline transporter-like 1 protein CTL1 in neuroblastoma x glioma cells and in the CNS, and its role in choline uptake. *J Neurochem* 2009;110(4):1297-309.
- [181] Nakamura T, Fujiwara R, Ishiguro N, Oyabu M, Nakanishi T, Shirasaka Y, et al. Involvement of choline transporter-like proteins, CTL1 and CTL2, in glucocorticoid-induced acceleration of phosphatidylcholine synthesis via increased choline uptake. *Biol Pharm Bull* 2010;33(4):691-6.
- [182] Ishiguro N, Oyabu M, Sato T, Maeda T, Minami H, Tamai I. Decreased biosynthesis of lung surfactant constituent phosphatidylcholine due to inhibition of choline transporter by gefitinib in lung alveolar cells. *Pharm Res* 2008;25(2):417-27.
- [183] Uchida Y, Inazu M, Takeda H, Yamada T, Tajima H, Matsumiya T. Expression and functional characterization of choline transporter in human keratinocytes. *J Pharmacol Sci* 2009;109(1):102-9.
- [184] Hu W, Bielawski J, Samad F, Merrill AH,Jr, Cowart LA. Palmitate increases sphingosine-1-phosphate in C2C12 myotubes via upregulation of sphingosine kinase message and activity. *J Lipid Res* 2009;50(9):1852-62.
- [185] Taube A, Eckardt K, Eckel J. Role of lipid-derived mediators in skeletal muscle insulin resistance. *Am J Physiol Endocrinol Metab* 2009;297(5):E1004-12.
- [186] Yang C, Aye CC, Li X, Diaz Ramos A, Zorzano A, Mora S. Mitochondrial dysfunction in insulin resistance: Differential contributions of chronic insulin and saturated fatty acid exposure in muscle cells. *Biosci Rep* 2012;32(5):465-78.
- [187] Hirabara SM, Curi R, Maechler P. Saturated fatty acid-induced insulin resistance is associated with mitochondrial dysfunction in skeletal muscle cells. *J Cell Physiol* 2010;222(1):187-94.
- [188] Henique C, Mansouri A, Fumey G, Lenoir V, Girard J, Bouillaud F, et al. Increased mitochondrial fatty acid oxidation is sufficient to protect skeletal muscle cells from palmitate-induced apoptosis. *J Biol Chem* 2010;285(47):36818-27.
- [189] Yuzefovych L, Wilson G, Rachek L. Different effects of oleate vs. palmitate on mitochondrial function, apoptosis, and insulin signaling in L6 skeletal muscle cells: Role of oxidative stress. *Am J Physiol Endocrinol Metab* 2010;299(6):E1096-105.
- [190] Michel VH. Expression, subcellular localization and function of the solute carrier 44A1 [dissertation]. ; 2010.

- [191] Romero-Calvo I, Ocon B, Martinez-Moya P, Suarez MD, Zarzuelo A, Martinez-Augustin O, et al. Reversible ponceau staining as a loading control alternative to actin in western blots. *Anal Biochem* 2010;401(2):318-20.
- [192] Gilda JE, Gomes AV. Stain-free total protein staining is a superior loading control to beta-actin for western blots. *Anal Biochem* 2013;440(2):186-8.
- [193] BLIGH EG, DYER WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;37(8):911-7.
- [194] Graeve M, Janssen D. Improved separation and quantification of neutral and polar lipid classes by HPLC-ELSD using a monolithic silica phase: Application to exceptional marine lipids. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009;877(20-21):1815-9.
- [195] FOLCH J, LEES M, SLOANE STANLEY GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957;226(1):497-509.
- [196] Chavez JA, Summers SA. Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. *Arch Biochem Biophys* 2003;419(2):101-9.
- [197] Zucker LM. Hereditary obesity in the rat associated with hyperlipemia. *Ann N Y Acad Sci* 1965;131(1):447-58.
- [198] Jacobs RL, Zhao Y, Koonen DP, Sletten T, Su B, Lingrell S, et al. Impaired de novo choline synthesis explains why phosphatidylethanolamine N-methyltransferase-deficient mice are protected from diet-induced obesity. *J Biol Chem* 2010;285(29):22403-13.
- [199] Pelech SL, Cook HW, Paddon HB, Vance DE. Membrane-bound CTP:Phosphocholine cytidyltransferase regulates the rate of phosphatidylcholine synthesis in HeLa cells treated with unsaturated fatty acids. *Biochim Biophys Acta* 1984;795(3):433-40.
- [200] Coll T, Eyre E, Rodriguez-Calvo R, Palomer X, Sanchez RM, Merlos M, et al. Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells. *J Biol Chem* 2008;283(17):11107-16.
- [201] Jacobs RL, Zhao Y, Koonen DP, Sletten T, Su B, Lingrell S, et al. Impaired de novo choline synthesis explains why phosphatidylethanolamine N-methyltransferase-deficient mice are protected from diet-induced obesity. *J Biol Chem* 2010;285(29):22403-13.
- [202] Baburina I, Jackowski S. Cellular responses to excess phospholipid. *J Biol Chem* 1999;274(14):9400-8.
- [203] Komiya K, Uchida T, Ueno T, Koike M, Abe H, Hirose T, et al. Free fatty acids stimulate autophagy in pancreatic beta-cells via JNK pathway. *Biochem Biophys Res Commun* 2010;401(4):561-7.

- [204] Tan SH, Shui G, Zhou J, Li JJ, Bay BH, Wenk MR, et al. Induction of autophagy by palmitic acid via protein kinase C-mediated signaling pathway independent of mTOR (mammalian target of rapamycin). *J Biol Chem* 2012;287(18):14364-76.
- [205] Choi SE, Lee SM, Lee YJ, Li LJ, Lee SJ, Lee JH, et al. Protective role of autophagy in palmitate-induced INS-1 beta-cell death. *Endocrinology* 2009;150(1):126-34.
- [206] Singh R, Cuervo AM. Lipophagy: Connecting autophagy and lipid metabolism. *Int J Cell Biol* 2012;2012:282041.
- [207] Boslem E, MacIntosh G, Preston AM, Bartley C, Busch AK, Fuller M, et al. A lipidomic screen of palmitate-treated MIN6 beta-cells links sphingolipid metabolites with endoplasmic reticulum (ER) stress and impaired protein trafficking. *Biochem J* 2011;435(1):267-76.
- [208] Stone SJ, Vance JE. Phosphatidylserine synthase-1 and -2 are localized to mitochondria-associated membranes. *J Biol Chem* 2000;275(44):34534-40.
- [209] O'Donoghue N, Sweeney T, Donagh R, Clarke KJ, Porter RK. Control of choline oxidation in rat kidney mitochondria. *Biochim Biophys Acta* 2009;1787(9):1135-9.
- [210] Porter RK, Scott JM, Brand MD. Choline transport into rat liver mitochondria. *Biochem Soc Trans* 1992;20(3):248S.
- [211] van der Veen JN, Lingrell S, da Silva RP, Jacobs RL, Vance DE. The concentration of phosphatidylethanolamine in mitochondria can modulate ATP production and glucose metabolism in mice. *Diabetes* 2014.
- [212] Li Z, Agellon LB, Allen TM, Umeda M, Jewell L, Mason A, et al. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab* 2006;3(5):321-31.
- [213] Al-Makdissy N, Younsi M, Pierre S, Ziegler O, Donner M. Sphingomyelin/cholesterol ratio: An important determinant of glucose transport mediated by GLUT-1 in 3T3-L1 preadipocytes. *Cell Signal* 2003;15(11):1019-30.
- [214] Hirabara SM, Silveira LR, Alberici LC, Leandro CV, Lambertucci RH, Polimeno GC, et al. Acute effect of fatty acids on metabolism and mitochondrial coupling in skeletal muscle. *Biochim Biophys Acta* 2006;1757(1):57-66.
- [215] Benarroch EE. Postural tachycardia syndrome: A heterogeneous and multifactorial disorder. *Mayo Clin Proc* 2012;87(12):1214-25.
- [216] Gallazzini M, Burg MB. What's new about osmotic regulation of glycerophosphocholine. *Physiology (Bethesda)* 2009;24:245-9.

- [217] Gibellini F, Smith TK. The Kennedy pathway--de novo synthesis of phosphatidylethanolamine and phosphatidylcholine. *IUBMB Life* 2010;62(6):414-28.
- [218] Zeisel SH. Choline: An important nutrient in brain development, liver function and carcinogenesis. *J Am Coll Nutr* 1992;11(5):473-81.
- [219] Filipowicz CM, McCauley RB. The effect of choline deficiency on the outer membranes of rat liver mitochondria. *Biochim Biophys Acta* 1983;734(2):373-7.
- [220] Zeisel SH, Da Costa KA, Franklin PD, Alexander EA, Lamont JT, Sheard NF, et al. Choline, an essential nutrient for humans. *FASEB J* 1991;5(7):2093-8.
- [221] Lin C, Wu R. **Choline oxidation and choline dehydrogenase**. *Journal of Protein Chemistry* 1986;5(3):193-200.
- [222] Koc H, Mar MH, Ranasinghe A, Swenberg JA, Zeisel SH. Quantitation of choline and its metabolites in tissues and foods by liquid chromatography/electrospray ionization-isotope dilution mass spectrometry. *Anal Chem* 2002;74(18):4734-40.
- [223] Davis SR, Quinlivan EP, Shelnut KP, Maneval DR, Ghandour H, Capdevila A, et al. The methylenetetrahydrofolate reductase 677C->T polymorphism and dietary folate restriction affect plasma one-carbon metabolites and red blood cell folate concentrations and distribution in women. *J Nutr* 2005;135(5):1040-4.
- [224] Stabler SP, Allen RH. Quantification of serum and urinary S-adenosylmethionine and S-adenosylhomocysteine by stable-isotope-dilution liquid chromatography-mass spectrometry. *Clin Chem* 2004;50(2):365-72.
- [225] Han X, Gross RW. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: A bridge to lipidomics. *J Lipid Res* 2003;44(6):1071-9.
- [226] Ejsing CS, Sampaio JL, Surendranath V, Duchoslav E, Ekroos K, Klemm RW, et al. Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proc Natl Acad Sci U S A* 2009;106(7):2136-41.
- [227] Fischer LM, daCosta KA, Kwock L, Stewart PW, Lu TS, Stabler SP, et al. Sex and menopausal status influence human dietary requirements for the nutrient choline. *Am J Clin Nutr* 2007;85(5):1275-85.
- [228] Vance DE, Walkey CJ, Cui Z. Phosphatidylethanolamine N-methyltransferase from liver. *Biochim Biophys Acta* 1997;1348(1-2):142-50.
- [229] Fernandez-Roig S, Cavalle-Busquets P, Fernandez-Ballart JD, Ballesteros M, Berrocal-Zaragoza MI, Salat-Batlle J, et al. Low folate status enhances pregnancy changes in plasma

betaine and dimethylglycine concentrations and the association between betaine and homocysteine. *Am J Clin Nutr* 2013;97(6):1252-9.

[230] Dominguez-Salas P, Moore SE, Cole D, da Costa KA, Cox SE, Dyer RA, et al. DNA methylation potential: Dietary intake and blood concentrations of one-carbon metabolites and cofactors in rural african women. *Am J Clin Nutr* 2013;97(6):1217-27.

[231] Zeisel SH, Zola T, daCosta KA, Pomfret EA. Effect of choline deficiency on S-adenosylmethionine and methionine concentrations in rat liver. *Biochem J* 1989;259(3):725-9.

[232] da Costa KA, Badea M, Fischer LM, Zeisel SH. Elevated serum creatine phosphokinase in choline-deficient humans: Mechanistic studies in C2C12 mouse myoblasts. *Am J Clin Nutr* 2004;80(1):163-70.

[233] Lombardi B, Ugazio G, Raick AN. Choline-deficiency fatty liver: Relation of plasma phospholipids to liver triglycerides. *Am J Physiol* 1966;210(1):31-6.

[234] Li Z, Agellon LB, Vance DE. Phosphatidylcholine homeostasis and liver failure. *J Biol Chem* 2005;280(45):37798-802.

[235] Vance DE. Role of phosphatidylcholine biosynthesis in the regulation of lipoprotein homeostasis. *Curr Opin Lipidol* 2008;19(3):229-34.

[236] Bakewell L, Burdge GC, Calder PC. Polyunsaturated fatty acid concentrations in young men and women consuming their habitual diets. *Br J Nutr* 2006;96(1):93-9.

[237] De Smet CH, Cox R, Brouwers JF, de Kroon AI. Yeast cells accumulate excess endogenous palmitate in phosphatidylcholine by acyl chain remodeling involving the phospholipase B Plb1p. *Biochim Biophys Acta* 2013;1831(6):1167-76.

[238] Tanaka K, Fukuda R, Ono Y, Eguchi H, Nagasawa S, Nakatani Y, et al. Incorporation and remodeling of extracellular phosphatidylcholine with short acyl residues in *saccharomyces cerevisiae*. *Biochim Biophys Acta* 2008;1781(8):391-9.

[239] Boumann HA, Gubbens J, Koorengel MC, Oh CS, Martin CE, Heck AJ, et al. Depletion of phosphatidylcholine in yeast induces shortening and increased saturation of the lipid acyl chains: Evidence for regulation of intrinsic membrane curvature in a eukaryote. *Mol Biol Cell* 2006;17(2):1006-17.

[240] Puri P, Baillie RA, Wiest MM, Mirshahi F, Choudhury J, Cheung O, et al. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology* 2007;46(4):1081-90.

[241] Guo WX, Pye QN, Williamson KS, Stewart CA, Hensley KL, Kotake Y, et al. Mitochondrial dysfunction in choline deficiency-induced apoptosis in cultured rat hepatocytes. *Free Radic Biol Med* 2005;39(5):641-50.

- [242] Teodoro JS, Rolo AP, Duarte FV, Simoes AM, Palmeira CM. Differential alterations in mitochondrial function induced by a choline-deficient diet: Understanding fatty liver disease progression. *Mitochondrion* 2008;8(5-6):367-76.
- [243] Filipowicz CM, McCauley RB. The effect of choline deficiency on the outer membranes of rat liver mitochondria. *Biochim Biophys Acta* 1983;734(2):373-7.
- [244] Kamath AV, Darling IM, Morris ME. Choline uptake in human intestinal caco-2 cells is carrier-mediated. *J Nutr* 2003;133(8):2607-11.
- [245] Fullerton MD, Hakimuddin F, Bakovic M. Developmental and metabolic effects of disruption of the mouse CTP:Phosphoethanolamine cytidylyltransferase gene (Pcvt2). *Mol Cell Biol* 2007;27(9):3327-36.
- [246] da Costa KA, Niculescu MD, Craciunescu CN, Fischer LM, Zeisel SH. Choline deficiency increases lymphocyte apoptosis and DNA damage in humans. *Am J Clin Nutr* 2006;84(1):88-94.
- [247] Fullerton MD, Bakovic M. Complementation of the metabolic defect in CTP:Phosphoethanolamine cytidylyltransferase (Pcvt2)-deficient primary hepatocytes. *Metabolism* 2010;59(12):1691-700.
- [248] Zeisel SH. Dietary choline deficiency causes DNA strand breaks and alters epigenetic marks on DNA and histones. *Mutat Res* 2012;733(1-2):34-8.
- [249] Schenkel LC, Bakovic M. Palmitic acid and oleic acid differentially regulate choline transporter-like 1 levels and glycerolipid metabolism in skeletal muscle cells. *Lipids* 2014.
- [250] Zytkevich TH, Fitzgerald EF, Marsden D, Larson CA, Shih VE, Johnson DM, et al. Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: A two-year summary from the new england newborn screening program. *Clin Chem* 2001;47(11):1945-55.
- [251] Moran-Salvador E, Lopez-Parra M, Garcia-Alonso V, Titos E, Martinez-Clemente M, Gonzalez-Periz A, et al. Role for PPARgamma in obesity-induced hepatic steatosis as determined by hepatocyte- and macrophage-specific conditional knockouts. *FASEB J* 2011;25(8):2538-50.
- [252] Stauch S, Kircheis G, Adler G, Beckh K, Ditschuneit H, Gortelmeyer R, et al. Oral L-ornithine-L-aspartate therapy of chronic hepatic encephalopathy: Results of a placebo-controlled double-blind study. *J Hepatol* 1998;28(5):856-64.
- [253] Sugino T, Shirai T, Kajimoto Y, Kajimoto O. L-ornithine supplementation attenuates physical fatigue in healthy volunteers by modulating lipid and amino acid metabolism. *Nutr Res* 2008;28(11):738-43.

- [254] Singh RK, Fullerton MD, Vine D, Bakovic M. Mechanism of hypertriglyceridemia in CTP:Phosphoethanolamine cytidyltransferase-deficient mice. *J Lipid Res* 2012;53(9):1811-22.
- [255] Pereira L. The role of a defect in the CDP-ethanolamine pathway in autophagy [dissertation]. University of Guelph; 2012.
- [256] Al Rajabi A, Castro GS, da Silva RP, Nelson RC, Thiesen A, Vannucchi H, et al. Choline supplementation protects against liver damage by normalizing cholesterol metabolism in *pemt/ldlr* knockout mice fed a high-fat diet. *J Nutr* 2014;144(3):252-7.
- [257] Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* 2008;7(1):45-56.
- [258] Sampey BP, Freemerman AJ, Zhang J, Kuan PF, Galanko JA, O'Connell TM, et al. Metabolomic profiling reveals mitochondrial-derived lipid biomarkers that drive obesity-associated inflammation. *PLoS One* 2012;7(6):e38812.
- [259] Vreken P, van Lint AE, Bootsma AH, Overmars H, Wanders RJ, van Gennip AH. Rapid diagnosis of organic acidemias and fatty-acid oxidation defects by quantitative electrospray tandem-MS acyl-carnitine analysis in plasma. *Adv Exp Med Biol* 1999;466:327-37.
- [260] Koves TR, Li P, An J, Akimoto T, Slentz D, Ilkayeva O, et al. Peroxisome proliferator-activated receptor-gamma co-activator 1alpha-mediated metabolic remodeling of skeletal myocytes mimics exercise training and reverses lipid-induced mitochondrial inefficiency. *J Biol Chem* 2005;280(39):33588-98.
- [261] Mihalik SJ, Goodpaster BH, Kelley DE, Chace DH, Vockley J, Toledo FG, et al. Increased levels of plasma acylcarnitines in obesity and type 2 diabetes and identification of a marker of glucolipotoxicity. *Obesity (Silver Spring)* 2010;18(9):1695-700.
- [262] Soeters MR, Serlie MJ, Sauerwein HP, Duran M, Ruiter JP, Kulik W, et al. Characterization of D-3-hydroxybutyrylcarnitine (ketocarnitine): An identified ketosis-induced metabolite. *Metabolism* 2012;61(7):966-73.
- [263] Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* 2009;9(4):311-26.
- [264] Dumas ME, Barton RH, Toye A, Cloarec O, Blancher C, Rothwell A, et al. Metabolic profiling reveals a contribution of gut microbiota to fatty liver phenotype in insulin-resistant mice. *Proc Natl Acad Sci U S A* 2006;103(33):12511-6.
- [265] Dong F, Zhang L, Hao F, Tang H, Wang Y. Systemic responses of mice to dextran sulfate sodium-induced acute ulcerative colitis using ¹H NMR spectroscopy. *J Proteome Res* 2013;12(6):2958-66.

- [266] Dawiskiba T, Deja S, Mulak A, Zabek A, Jawien E, Pawelka D, et al. Serum and urine metabolomic fingerprinting in diagnostics of inflammatory bowel diseases. *World J Gastroenterol* 2014;20(1):163-74.
- [267] Attard GS, Templer RH, Smith WS, Hunt AN, Jackowski S. Modulation of CTP:Phosphocholine cytidylyltransferase by membrane curvature elastic stress. *Proc Natl Acad Sci U S A* 2000;97(16):9032-6.
- [268] Bjornstad P, Bremer J. In vivo studies on pathways for the biosynthesis of lecithin in the rat. *J Lipid Res* 1966;7(1):38-45.
- [269] Roberti R, Mancini A, Freysz L, Binaglia L. Reversibility of the reactions catalyzed by cholinephosphotransferase and ethanolaminephosphotransferase solubilized from rat-brain microsomes. *Biochim Biophys Acta* 1992;1165(2):183-8.
- [270] Leonardi R, Frank MW, Jackson PD, Rock CO, Jackowski S. Elimination of the CDP-ethanolamine pathway disrupts hepatic lipid homeostasis. *J Biol Chem* 2009;284(40):27077-89.
- [271] Gohil VM, Zhu L, Baker CD, Cracan V, Yaseen A, Jain M, et al. Meclizine inhibits mitochondrial respiration through direct targeting of cytosolic phosphoethanolamine metabolism. *J Biol Chem* 2013;288(49):35387-95.
- [272] Gallego-Ortega D, Gomez del Pulgar T, Valdes-Mora F, Cebrian A, Lacal JC. Involvement of human choline kinase alpha and beta in carcinogenesis: A different role in lipid metabolism and biological functions. *Adv Enzyme Regul* 2011;51(1):183-94.
- [273] Dolganiuc A, Petrasek J, Kodys K, Catalano D, Mandrekar P, Velayudham A, et al. MicroRNA expression profile in lieber-DeCarli diet-induced alcoholic and methionine choline deficient diet-induced nonalcoholic steatohepatitis models in mice. *Alcohol Clin Exp Res* 2009;33(10):1704-10.
- [274] Gori M, Arciello M, Balsano C. MicroRNAs in nonalcoholic fatty liver disease: Novel biomarkers and prognostic tools during the transition from steatosis to hepatocarcinoma. *Biomed Res Int* 2014;2014:741465.
- [275] Cermelli S, Ruggieri A, Marrero JA, Ioannou GN, Beretta L. Circulating microRNAs in patients with chronic hepatitis C and non-alcoholic fatty liver disease. *PLoS One* 2011;6(8):e23937.
- [276] Lim JH, Gerhart-Hines Z, Dominy JE, Lee Y, Kim S, Tabata M, et al. Oleic acid stimulates complete oxidation of fatty acids through protein kinase A-dependent activation of SIRT1-PGC1alpha complex. *J Biol Chem* 2013;288(10):7117-26.
- [277] Tonkonogi M, Krook A, Walsh B, Sahlin K. Endurance training increases stimulation of uncoupling of skeletal muscle mitochondria in humans by non-esterified fatty acids: An uncoupling-protein-mediated effect? *Biochem J* 2000;351 Pt 3:805-10.

- [278] Kohlmeier M, da Costa KA, Fischer LM, Zeisel SH. Genetic variation of folate-mediated one-carbon transfer pathway predicts susceptibility to choline deficiency in humans. *Proc Natl Acad Sci U S A* 2005;102(44):16025-30.
- [279] Yao ZM, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem* 1988;263(6):2998-3004.
- [280] Tsujiuchi T, Tsutsumi M, Sasaki Y, Takahama M, Konishi Y. Hypomethylation of CpG sites and c-myc gene overexpression in hepatocellular carcinomas, but not hyperplastic nodules, induced by a choline-deficient L-amino acid-defined diet in rats. *Jpn J Cancer Res* 1999;90(9):909-13.
- [281] James SJ, Cross DR, Miller BJ. Alterations in nucleotide pools in rats fed diets deficient in choline, methionine and/or folic acid. *Carcinogenesis* 1992;13(12):2471-4.
- [282] Goselink RM, van Baal J, Widjaja HC, Dekker RA, Zom RL, de Veth MJ, et al. Effect of rumen-protected choline supplementation on liver and adipose gene expression during the transition period in dairy cattle. *J Dairy Sci* 2013;96(2):1102-16.
- [283] Colussi G, Catena C, Baroselli S, Nadalini E, Lapenna R, Chiuch A, et al. Omega-3 fatty acids: From biochemistry to their clinical use in the prevention of cardiovascular disease. *Recent Pat Cardiovasc Drug Discov* 2007;2(1):13-21.
- [284] Barre DE. The role of consumption of alpha-linolenic, eicosapentaenoic and docosahexaenoic acids in human metabolic syndrome and type 2 diabetes--a mini-review. *J Oleo Sci* 2007;56(7):319-25.