

Quantifying mitochondria substrate utilisation in a heterogenous cancer metabolism landscape

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Faculty of Medicine and Health

The University of Sydney

June 2023

Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all assistance received in preparing this thesis and sources have been acknowledged.

Ms Nancy Tonya Santiappillai

Acknowledgements

Completing my doctoral thesis would not have been possible without the guidance and support from many people. I would like to express my deepest appreciation to those that helped me along this journey.

Thank you to my supervisor Dr Andrew Hoy for providing me the greatest opportunity to pursue my career in research. Your perseverance, confidence, and encouragement were constant inspirations to me. In all aspects I am truly thankful for your support during the pandemic and throughout my PhD to continually challenge me to do my best. I also extend my thanks to my co-supervisor Dr Lake-Ee Quek. I am so grateful for your unlimited support throughout my PhD, willingness to share your depth of mass-spec knowledge with me, and for always pushing me to exceed my potential. Your kind nature and enthusiasm towards research always motivated me throughout my candidature. Thank you also to my co-supervisor Prof. Lisa Butler for sharing your expertise on prostate cancer metabolism and valuable feedback from project design to reviewing my thesis.

Thank you to all the past members of the Hoy lab including Masih and Anabel who helped me in the lab. A special thanks to Mariam for your continual support, technical expertise, and positive attitude during our very long incubations and endless sample preps into the late nights. I couldn't have finished my PhD without you! I would like to thank Yue for being my go-to bioinformatics expert and always one slack message away. To Julia and Zeyad, thank you for all your help and support with my experimental designs, and the entire Butler lab for being so welcoming during my time in Adelaide.

I would like to thank Prof. Jean Yang for your guidance and expertise. Thank you to Dr Atul Bhatnagar and Dr David Fuentes from Sydney Mass Spectrometry for your technical support. I would like to thank Dr Helen McGuire, Sydney Cytometry, and Sydney Microscopy & Microanalysis for helping me to access their equipment and complete my final experiments this year. Thank you to The Australian Rotary Health and The University of Sydney for co-funding my scholarship. Thanks also to every collaborating lab that helped me source all the cell lines, without which my projects would not be possible.

To Thy, Nunki, Susannah, and all my friends in Sydney, Melbourne, and Auckland. Thank you all so much for your continuous support and generosity! Completing my PhD during the pandemic made the experience even more challenging, and I will never forget all your kind words of encouragement that got me to the finish line. To Paul, thank you for the countless times you were always there for me, for listening to all my practice presentations, trying to understand my mass-spec difficulties, and your unwavering belief in me.

Finally, to my parents Amma and Dada. It is hard to put into words how much I appreciate your unconditional love, support, guidance, and belief in me. Although we were separated for most of the time during the pandemic, you were both there for me every day and supported me throughout my lonely lockdowns and quarantines. I wouldn't be where I am today without you both and completing my PhD is a true testament of your love and continual blessings.

To my parents

Authorship Attribution Statement (Chapter 2)

Chapter 2 of this thesis is prepared as a manuscript for publication as Santiappillai N.T.S, Cao Y., Hakeem-Sanni M., Yang J., Quek L.E., Hoy A.J. A ratio-based metabolome approach reveals common metabolic targets across cancer types.

I designed experiments and provided intellectual input; extracted, analysed, and visualised the data; interpreted the batch-corrected heatmap analyses by Cao Y; wrote and edited the draft manuscripts. In the case where I am not the corresponding author of an item, permission to include the material has been granted by the corresponding author.

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Authorship Attribution Statement (Chapter 3)

Chapter 3 of this thesis is prepared as a manuscript for publication as Santiappillai N.T.S, Hakeem-Sanni M., Withy A., Butler L.M., Quek L.E., Hoy A.J. Mitochondrial long chain fatty acid oxidation is a minor carbon source in prostate cancer.

I designed experiments and provided intellectual input; extracted, analysed, and visualised the data; wrote and edited the draft manuscripts. In the case where I am not the corresponding author of an item, permission to include the material has been granted by the corresponding author.

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Dr Andrew James Hoy

Date

Authorship Attribution Statement (Chapter 4)

Chapter 4 of this thesis is prepared as a manuscript for publication as Santiappillai N.T.S, Hakeem-Sanni M., Ghasemi M., Withy A., Quek L.E., Hoy A.J. Fatty acids are not a significant contributor to the TCA cycle in cancer cell lines.

I designed experiments and provided intellectual input; extracted, analysed, and visualised the data; wrote and edited the draft manuscripts. In the case where I am not the corresponding author of an item, permission to include the material has been granted by the corresponding author.

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Presentations that have arisen as a direct result of this thesis

Santiappillai N.T.S., Cao Y., Hakeem-Sanni M., Yang J., Quek L.E., Hoy A.J. A comprehensive assessment of cancer cell metabolism. Presented at: The Australia & New Zealand Metabolomics Network webinar series 2020: SCIEX Early Career Researchers' Competition; 2020 December 16; virtual presentation.

Santiappillai N.T.S., Cao Y., Hakeem-Sanni M., Yang J., Quek L.E., Hoy A.J. Targeted metabolomic profiling of cancer cell lines of varying origins. Presented at: The Charles Perkins Centre Chronic Disease Themes Workshop; 2021 June 8; Sydney, NSW, Australia.

Santiappillai N.T.S., Cao Y., Hakeem-Sanni M., Yang J., Quek L.E., Hoy A.J. A comprehensive assessment of cancer cell metabolism. Presented virtually at: The Charles Perkins Centre Biology Domain Series; 2021 August 19; virtual presentation.

Santiappillai N.T.S., Cao Y., Hakeem-Sanni M., Yang J., Quek L.E., Hoy A.J. Targeted metabolomic profiling of cell lines of varying origins. Presented at: The Sydney Cancer Conference 2021; 2021 September 9-10; virtual presentation.

Santiappillai N.T.S., Hakeem-Sanni M., Butler L.M., Quek L.E., Hoy A.J. Fatty acid oxidation is a minor substrate source to the TCA cycle in prostate cancer cells. Presented at: The Cancer Research Network 2021 Postgraduate & Early Career Researcher Cancer Research Symposium; 2021 December 9; virtual presentation.

Santiappillai N.T.S., Hakeem-Sanni M., Butler L.M., Quek L.E., Hoy A.J. Fatty acid oxidation is a minor substrate source to the TCA cycle in a panel of prostate cancer cells. Presented at: The Cancer Metabolism Showcase and Workshop 2022; 2022 April 4-6; virtual presentation.

Santiappillai N.T.S., Hakeem-Sanni M., Butler L.M., Quek L.E., Hoy A.J. Fatty acid oxidation inhibition by etomoxir did not adversely impact TCA cycle fluxes in prostate cancer cells. Presented at: The Cancer and Metabolism Conference 2022; 2022 July 17-19; London, UK.

Santiappillai N.T.S., Hakeem-Sanni M., Butler L.M., Quek L.E., Hoy A.J. Quantifying the mitochondrial fates of extracellular fatty acids in prostate cancer. Presented at: The Keystone Tumor Metabolism Symposia 2022; 2022 September 5-8; Keystone, CO, USA.

Santiappillai N.T.S., Hakeem-Sanni M., Butler L.M., Quek L.E., Hoy A.J. Quantifying the mitochondrial fates of extracellular fatty acids in prostate cancer cells. Presented at: The 4th Australian Cancer and Metabolism Meeting; 2022 December 7-9; Melbourne, VIC, Australia.

Awards:

The Australian Rotary Health/Rotary Club of Blacktown City ‘Mel Grey’ PhD Scholarship (2020-2023)

Cancer Research Network 2020 Postgraduate and Early Career Researcher Conference and Education Award (2020)

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Abstract

Cancer metabolism is characterised by dynamic substrate usage to meet bioenergetic and synthetic demands required to support tumour progression. The tricarboxylic acid (TCA) cycle is a central hub of many nutrients to fuel the electron transport chain and energy production, and generate signalling molecules and precursors for biomass synthesis. Projects within this thesis set out to comprehensively define the relationships of metabolic substrates within the mitochondria and identify common metabolic phenotypes amongst different cancer types.

In study 1, a ratio-metric metabolome analysis was performed on a panel of cancer cells from different lineages. This approach identified subsets of cancer cells that utilised glucose and glutamine differently by pathways converging at the TCA cycle. The insights from this project provide evidence to support that subsets of cancer cells are more sensitive to central carbon metabolism targeting therapeutics, irrespective of tissue lineage. In study 2, a parallel tracing strategy was performed in prostate cancer cells and discovered that extracellular long chain fatty acids (LCFAs) were minor carbon sources to the TCA cycle, compared to glucose and glutamine. This study also showed that exogenous LCFAs are incorporated into cardiolipins and that this was, in part, regulated at the mitochondrial entry of fatty acids (FAs). Study 3 expanded the observations of study 2 and found LCFAs were again minor carbon sources to the TCA cycle in a panel of cancer cells, irrespective of FA oxidation activity.

This thesis provides new insights into how cancer cells from different tissue lineages metabolise exogenous substrates within the mitochondria. Overall, the studies in this thesis identified common metabolic signatures within subsets of cancer cells that could provide new approaches to drug repurposing strategies beyond the scope of a particular cancer type.

List of Abbreviations

3PG	3-phosphoglycerate
A-kg	oxoglutarate
ACC	acetyl-CoA carboxylase
ACLY	ATP-citrate lyase
ACSL	acyl-CoA synthetase
ADSL	adenylosuccinate lyase
ALCAT1	acyl-CoA:lysoCL acyltransferase 1
ATP	adenosine triphosphate
BCA	Bicinchoninic assay
BRAF	V-Raf Murine Sarcoma Viral Oncogene Homolog B
BSA	bovine serum albumin
CACT	carnitine acyl-carnitine translocase
CD36	cluster of differentiation 36
CDP-DAG	CDP-diacylglycerol
CL	cardiolipin
CLS1	cardiolipin synthase
CO ₂	carbon dioxide
CPT	carnitine palmitoyl transferase
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELOVL	elongation of very long-chain fatty acid
ER	endoplasmic reticulum
ETC	electron transport chain
F6P	fructose-1,6-bisphosphate
FA	fatty acid
FABP	fatty acid binding protein
FACS	fluorescence-activated cell sorting
FADH ₂	flavin adenine dinucleotide
FADS	fatty acid desaturase
FAO	fatty acid oxidation
FASN	fatty acid synthase
FATP	fatty acid transport protein
FBS	fetal bovine serum
FCCP	carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone)

G6P	glucose-6-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gln	glutamine
GLS	glutaminase
Glu	glutamate
GLUT	glucose transporter
GPL	glycerophospholipid
HER2	Human epidermal growth factor receptor 2
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IMM	inner mitochondrial membrane
KEGG	Kyoto encyclopedia of genes and genomes
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LCFA	long chain fatty acid
LCFA-CoA	long chain fatty acyl-CoA
LDH	lactate dehydrogenase
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
MCFA	medium chain fatty acid
MCT	monocarboxylate transporter
MLCL	monolyso-cardiolipin
MLCLAT1	monolysoCL acyltransferase 1
MPC	mitochondrial pyruvate carrier
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFA	mono-unsaturated fatty acid
NAD ⁺	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide plus hydrogen
NADPH	nicotinamide adenine dinucleotide phosphate plus hydrogen
OCR	oxygen consumption rate
OMM	outer mitochondrial membrane
OXPHOS	oxidative phosphorylation
PA	phosphatidic acid
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCa	prostate cancer
PDE5	phosphodiesterase 5
PE	phosphatidylethanolamine

PEP	phosphoenolpyruvate
PG	phosphatidylglycerol
PGP	phosphatidylglycerol phosphate
PGS1	phosphatidylglycerol phosphate synthase
PLA2	phospholipase A2
PPP	pentose phosphate pathway
PTPMP1	protein-tyrosine phosphatase mitochondrial 1
PUFA	poly-unsaturated fatty acid
PVDF	polyvinylidene fluoride
RIPA	radioimmunoprecipitation assay
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SCD	delta-9 stearoyl-CoA desaturase
SCFA	short chain fatty acid
SDH	succinate dehydrogenase
SFA	saturated fatty acid
TAMM41	TAM41 translocator assembly and maintenance homolog
TCA	tricarboxylic acid

1. Introduction

Cancer remains a major health burden and leading cause of death in Australia and worldwide. Although cancer incidence and mortality rates are declining due to early screening methods, therapeutic advancements, and improved control of risk factors (Luo et al., 2022, Siegel et al., 2023), projections into the next 25-years indicate that there will be a 51% increase in new cancer cases and 36% increase in cancer-related mortality (Luo et al., 2022). These changes may, in part, be explained by the growing obesity epidemic and related lifestyle factors, including poor diet and physical inactivity, which strongly correlate with cancer aggressiveness (Avgerinos et al., 2019, Lauby-Secretan et al., 2016, Saha et al., 2023). Preventative cancer strategies, including an active lifestyle and balanced diet, provide a new opportunity to tackle the onset of cancers. Therefore, building a clearer understanding of how nutrients support cancer metabolism will lead to more effective and durable therapeutics and, ultimately better patient outcomes.

Cancer cells exhibit altered nutrient uptake and utilisation compared to normal tissues (Pavlova et al., 2022, Rohatgi et al., 2022). Often, metabolic dysregulation manifests as an over-reliance on specific nutrients to supply copious cellular building blocks and sustain high bioenergetic demands. Metabolically targeting precision therapies in development inhibit these so-called metabolic vulnerabilities that differentiate malignant cells from normal cells, to selectively eliminate the former (Stine et al., 2022). Significant therapeutic opportunities have emerged to target metabolic reprogramming in cancers driven by oncogenic mutations and altered gene expression but have had limited clinical success due to the adaptive resistance in tumours (Nagarajan et al., 2016, Seth Nanda et al., 2020). Exploiting the tight co-ordinations of metabolic pathway activities and oncogene expressions have provided effective strategies to overcome resistance and reduce tumour growth (Altea-Manzano et al., 2020, Garcia-Bermudez et al., 2020, Hopkins et al., 2018, Pisarsky et al., 2016). This is because unforeseen escape mechanisms emerging from deregulated signalling cascades could be prevented by targeting key pathways generating biomass or energy.

In broadest terms, all cancer cells have the same metabolic processes and rely on the same fundamental needs for nutrients. In this respect, there is an emerging theme of shared metabolic vulnerabilities from pan-cancer studies (i.e., across a diverse and heterogeneous spectrum of cancer types) (Barretina et al., 2012, Garnett et al., 2012), as well as from cancer-type specific studies (Hensley et al., 2016, Kamphorst et al., 2015, Maher et al., 2012). For example, the BRAF (V-Raf Murine Sarcoma Viral Oncogene Homolog B) V600E mutation upregulates glycolytic activity in melanoma to drive tumour progression and has been exploited for the clinical use of BRAF-targeting inhibitors (Parmenter et al., 2014). Importantly, BRAF mutations are frequently observed in other cancer types, including papillary thyroid, colon, and head and neck cancers, and thus BRAF inhibition could be an applicable clinical therapy in multiple cancers beyond melanoma (Barretina et al., 2012). This means we can begin to predict the efficacy of a metabolic drug developed for a specific cancer-type to multiple cancers. These shared vulnerabilities represent untapped opportunities to repurpose approved therapeutics for rare cancers. However, to rationalise these vulnerabilities, the first step is to have a clear mechanistic understanding of how heterogeneous cancer cell-types utilise nutrients.

This thesis aims to take a pan-cancer approach to build a clearer understanding on how significant nutrients, such as glucose, glutamine, and fatty acids, support cancer metabolism. The projects within this thesis provide new insights into the prevalent relationships of cellular metabolism and substrate dependencies of cancer cells that could help propel the advancements of targeting common metabolic vulnerabilities among diverse cancer types.

1.1 Cancer metabolism

The reprogramming of cancer cell metabolism and the reliance on nutrients is extremely dynamic (Fendt et al., 2020, Lehuédé et al., 2016). Specifically, cancer cells can adapt to nutrient depleted conditions by increasing the utilisation of other substrates to fuel metabolic pathways and maintain tumour progression, termed metabolic flexibility (Bergers and Fendt, 2021, Li et al., 2022). Significant efforts have been made to study the interplay between major carbon substrates, glucose and glutamine, and their contributions to metabolic pathways in cancers including osteosarcoma (Mullen et al., 2011),

colon cancer (Reckzeh et al., 2019), and lung cancer (Momcilovic et al., 2018). Substrate sources such as fatty acids (Dueregger et al., 2015, Mashimo et al., 2014, Vriens et al., 2019) and other amino acids (Hosios et al., 2016, Zhang et al., 2014) are gaining attention as alternative metabolic resources that support tumourigenesis. However, their contributions are still largely underappreciated compared to glucose and glutamine. The co-operation of metabolic pathways means studying substrates or pathways in isolation is likely ineffective. The following sections of this chapter will highlight common metabolic phenotypes shared across cancer types and our current understanding of glucose, glutamine, and fatty acid metabolism (Figure 1.1).

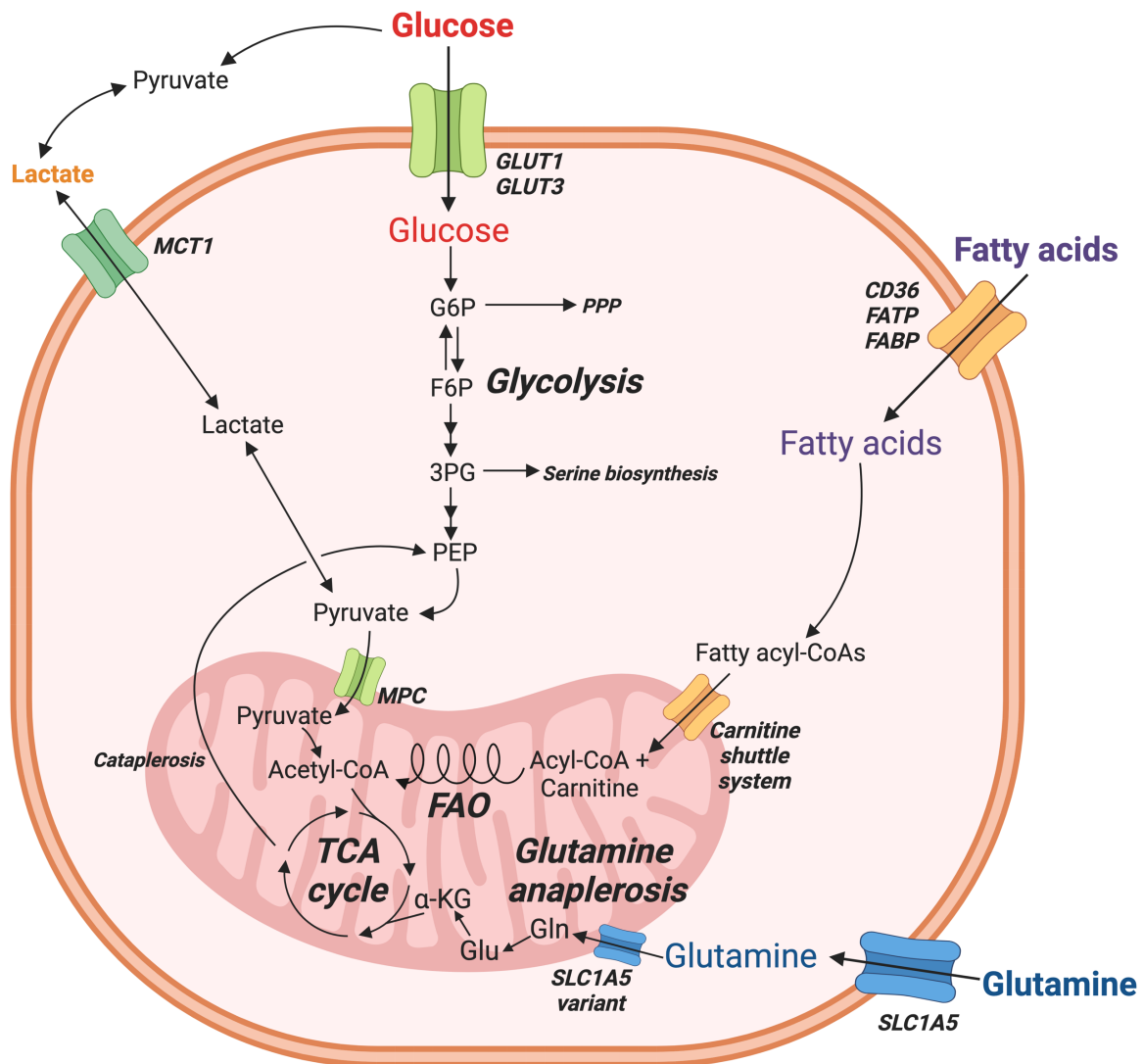


Figure 1.1: A simplified illustration of the converging glucose, glutamine, and fatty acid metabolism pathways.

Glucose enters the cell via GLUT transporters and is converted by a series of reactions to pyruvate (glycolysis). The PPP branches off from glycolysis at G6P, and serine synthesis from 3PG. Glucose can be converted exogenously to pyruvate and lactate, which is then transported into the cell via MCT1. Pyruvate is transported into the mitochondria via MPC and converted to acetyl-CoA, which is oxidised by the TCA cycle. Oxaloacetate can be exported from the TCA cycle (cataplerosis) and utilised for PEP formation. Glutamine enters the cell via the SLC1A5 transporter. Glutamine can undergo glutamine anaplerosis within the mitochondria following entry through the SLC1A5 variant. Glutamine-derived oxoglutarate fuels the TCA cycle. FAs enter the cell via FA transporters and are then activated to fatty acyl-CoAs before being transported into the mitochondria via the carnitine shuttle system. Mitochondrial fatty acids undergo oxidation to acetyl-CoA that enters the TCA cycle. 3PG, 3-phosphoglycerate; FA, fatty acid; FAO, fatty acid oxidation; FGP, fructose-1,6-bisphosphate; G6P, glucose-6-phosphate; GLUT, glucose transporter; MCT, monocarboxylate transporter; MPC, mitochondrial pyruvate carrier; PEP, phosphoenolpyruvate; PPP, pentose phosphate pathway; TCA, tricarboxylic acid. Figure was created with Biorender.com.

1.1.1 Glucose metabolism

Starting in the 1920s, both Cori and Warburg identified that cancers exhibit high levels of glucose uptake and lactate secretion even in the presence of abundant oxygen (Cori and Cori, 1925, Warburg et al., 1927). This phenotype was named The Warburg Effect and at the time was proposed to arise from impaired mitochondrial respiration and occurred in all cancers (Warburg, 1956). Now close to a century later from those seminal observations, there is significant evidence that The Warburg effect is not caused by impaired mitochondrial respiration (DeBerardinis and Chandel, 2020, Maher et al., 2012), nor occurs in all cancer types (Jose et al., 2011). Many assumptions proposed by Warburg have been challenged over time. Pyruvate kinase splice form M2 catalyses the conversion of phosphoenolpyruvate (PEP) to pyruvate at the end of glycolysis and is an essential process that helps fuel the TCA cycle (Zhang et al., 2019) and is upregulated in many cancer types (Bluemlein et al., 2011). Additionally, the high rate of lactate production from glucose functions as a compensatory mechanism when the mitochondrial nicotinamide adenine dinucleotide plus hydrogen (NADH) shuttle becomes saturated (Luengo et al., 2021, Wang et al., 2022). Lactate that is produced by lung and pancreatic cancer cells is a fundamental carbon source for the TCA cycle to support tumour growth (Figure 1.1) (Faubert et al., 2017, Hui et al., 2017) and not just a by-product of glycolysis. Early-stage prostate cancer (PCa), which has a substrate preference for lipids over glucose (Dueregger et al., 2015, Mah et al., 2020), is a prime example of how the Warburg effect does not occur in all cancer types.

There is now a much greater understanding of cancer cell glucose metabolism beyond lactate production to fuel many branching pathways from glycolysis to generate nucleotides, amino acids, and lipids for tumour growth and survival. Specifically, the pentose phosphate pathway (PPP) branches off from glucose-6-phosphate (G6P) to produce ribonucleotides for RNA and DNA synthesis, and nicotinamide adenine dinucleotide phosphate plus hydrogen (NADPH) for lipid biosynthesis and redox homeostasis (Figure 1.1) (Jiang et al., 2014). Additionally, 3-phosphoglycerate (3PG) is a crucial substrate for serine synthesis and the conversion to glycine that facilitates redox homeostasis and folate metabolism (Hay, 2016, Locasale et al., 2011). Glucose can also be synthesised by a series of reactions known as

gluconeogenesis from the cataplerosis of the TCA cycle metabolite oxalacetate and conversion to PEP by phosphoenolpyruvate carboxykinase (Montal et al., 2015).

Inhibiting glucose metabolism is an effective strategy to prevent tumour growth, but cancer cells can respond with adaptive mechanisms to utilise other nutrients and maintain tumour growth. For example, under pharmacological glucose transporter (GLUT) inhibition, colon cancer cells shift to a reliance on glutamine and require additional glutamine inhibition to effectively reduce cancer cell growth (Reckzeh et al., 2019). At the same time, pancreatic cancer cells can upregulate glutamine and glutamate utilisation by the TCA cycle to maintain oxidative phosphorylation (OXPHOS) and cell survival when glycolysis is suppressed (Shiratori et al., 2019). Thus, inhibiting glucose metabolism alone is insufficient for tumour suppression and highlights the need to understand how metabolic compensation occurs.

1.1.2 Glutamine metabolism

Glutamine is the most abundant amino acid in plasma (Cruzat et al., 2018, Mc et al., 1957), and is extremely versatile as it serves as both a carbon source for the TCA cycle (Kodama et al., 2020), lipogenesis and redox homeostasis via reductive carboxylation (Jiang et al., 2016, Metallo et al., 2012), and a nitrogen donor for nucleotide biosynthesis (Kodama et al., 2020, Liu et al., 2012). Many cancers exhibit increased dependency or addiction to glutamine (Jin et al., 2020, Nilsson et al., 2020, Quek et al., 2022, Wise et al., 2008). Glutamine metabolism is a prime target for anticancer exploitation (Altman et al., 2016). There are many classes of compounds targeting glutamine metabolism in pre-clinical stages (Jin et al., 2023). One of the most well-characterised and developed therapies in clinical trials is CB-839, a potent glutaminase 1 (GLS1) inhibitor that acts to inhibit glutamine conversion to glutamate and subsequent feeding into the TCA cycle (Gross et al., 2014, Ren et al., 2020). Some cancer cells exhibit excessive glutamine uptake, to the point that glutamine becomes a conditionally essential nutrient (Wise and Thompson, 2010). As a result, inhibiting glutamine uptake by targeting transporters such as SLC1A5 (ASCR2) (Figure 1.1) is effective at suppressing tumour growth in several cancer types, including melanoma (Wang et al., 2014), non-small cell lung cancer (Hassanein et al., 2015),

acute myeloid leukemia (Willems et al., 2013), endometrial (Marshall et al., 2017), and breast cancer (van Geldermalsen et al., 2016). More recently, a variant of SLC1A5 (SLC1A5_var) was discovered as the mitochondrial glutamine transporter (Figure 1.1) that has essential functions for glutamine anaplerosis, TCA cycle activity and cancer growth (Yoo et al., 2020). This study found that when SLC1A5_var is downregulated, a significant reduction of glutamine-derived TCA cycle metabolite concentration is observed, which is not seen when the cytoplasmic glutamine transporter SLC1A5 is inhibited (Yoo et al., 2020), and thus paves the way for new glutamine targeting drugs in cancer.

Despite promising results for many glutamine metabolism targeting compounds (Halama and Suhre, 2022), monotherapy success in clinical trials is often limited as cancer cells become refractory to inhibitors by switching to other substrates, such as to asparagine (Krall et al., 2016) and fatty acid pathways (Biancur et al., 2017). Clearly, it is still difficult to anticipate how glutamine is used by cancer cells that are metabolically flexible and can utilise other nutrients when glutamine pathways are impaired. For example, during GLS1 inhibition or knockdown, fatty acids or pyruvate carboxylation are upregulated as a compensatory measure to replenish TCA cycle metabolites and maintain tumour cell growth in pancreatic cancer and glioblastoma (Biancur et al., 2017, Cheng et al., 2011). Remarkably, these studies showed that despite pancreatic and glioblastoma cancers being avid glutamine consumers, they can still dynamically adapt to glutamine-deprived settings to maintain metabolic activity required for cell survival. Conversely, glutamine can also be a rescue mechanism to maintain proliferation when other pathways like glycolysis are impaired, such as upon mitochondria pyruvate carrier inhibition (Yang et al., 2014) and glucose deprivation (Yang et al., 2009). Therefore, targeting glutamine dependency and glucose flexibility in combination is leading to promising synergistic results to improve cancer survival (Méndez-Lucas et al., 2020, Momcilovic et al., 2018, Reckzeh et al., 2019). These studies highlight the interplay between glucose and glutamine metabolism in cancers and the potential for co-targeting adaptive metabolic activities.

1.1.3 Fatty acid metabolism

Extending beyond the dynamic interplay of glucose and glutamine metabolism in cancer, other substrates such as exogenous fatty acids (FAs), can also support tumour growth (Figure 1.1) (Hoy et al., 2021). FAs are now considered essential substrates for cancer cells as they participate in many cellular processes, including acting as building blocks for membranes, e.g., glycerophospholipids (GPLs), substrates for mitochondrial ATP and NADH generation that buffer against oxidative stress, and as mediators of intracellular signalling for tumour progression (Butler et al., 2021, Butler et al., 2020, Nagarajan et al., 2021). What distinguishes FAs from glucose and glutamine is the molecular diversity of FAs that give rise to very complex fates and functions. FAs differ in the number of carbons (chain length): short chain (SCFA, < 6C), medium chain (MCFA, 6-12C), long chain (LCFA, 12-20C), or very long chain (VLCFA, ≥ 20 C) FAs. Additionally, they can differ by the number of double bonds: (SFA, 0 double bonds), mono-unsaturated (MUFA, 1 double bond), and poly-unsaturated (PUFA, ≥ 2 or more double bonds) FAs (Harayama and Riezman, 2018). Further complexity of FA metabolism compared to glucose and glutamine also arises from diverse sources of FAs (Figure 1.2). Recent insights from lung, breast, and prostate cancer cells have shown that the majority of total cellular lipid carbons are derived from exogenous FA sources (60-80%), while the remainder is from endogenous sources synthesised from glucose and glutamine-derived carbons (Figure 1.2) (Balaban et al., 2019, Balaban et al., 2017, Hosios et al., 2016). Moreover, cancer cell FA metabolism is also highly flexible, like glucose and glutamine, as they can shift to a reliance on FA synthesis to maintain cancer survival when exogenous FA sources are limited (Daniëls et al., 2014, Peck et al., 2016). The next sections highlight the reprogramming of FA synthesis, uptake, and modifications within cancers.

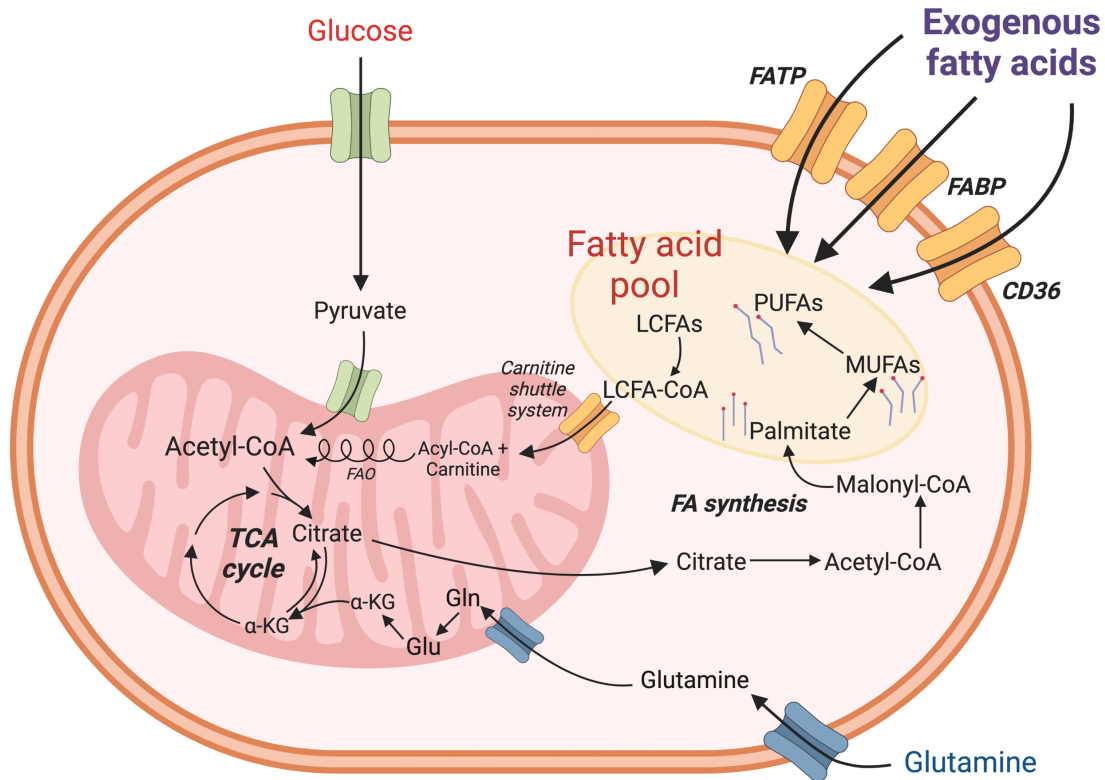


Figure 1.2: Fatty acid uptake and synthesis pathways.

Fatty acids from exogenous sources are transported into cells by CD36, FABP, and FATP transporters, contributing to most of the FA pool in cancer cells. Carbon sources going into FA synthesis can be derived from glucose and glutamine to form palmitate. Palmitate is modified to MUFAs and PUFAs. LCFAs enter the mitochondria via CPT1. α -kg, oxoglutarate; CD36, cluster of differentiation 36; FA, fatty acid; FABP, fatty acid binding protein; FAO, fatty acid oxidation; FATP, fatty acid transport protein; Gln, glutamine; Glu, glutamate; LCFA, long chain fatty acid; LCFA-CoA, long chain fatty acyl-CoA; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; TCA, tricarboxylic acid. Figure was created with Biorender.com.

1.1.3.1 *De novo* FA synthesis

The *de novo* synthesis of FAs is increased in cancer cells and is essential for cancer progression (Röhrig and Schulze, 2016). Briefly, glucose or glutamine can fuel the TCA cycle in the mitochondria to generate citrate, which is cleaved to acetyl-CoA in the cytoplasm by ATP-citrate lyase (ACLY). Additionally, glutamate-derived oxoglutarate can also undergo reductive metabolism to cytoplasmic citrate. Acetyl-CoA carboxylases (ACC) then catalyses the conversion of acetyl-CoA to malonyl-CoA, which is then condensed by a series of reactions to the FA palmitate by FA synthase (FASN) (Figure 1.2) (Röhrig and Schulze, 2016). These sequential reactions to produce FAs have been therapeutically

exploited to reduce cancer growth, specifically, FASN (Zadra et al., 2019, Zaytseva et al., 2018), ACC (Hunt et al., 2017, Li et al., 2013), and ACLY (Gu et al., 2021, Shah et al., 2016).

Targeting *de novo* FA synthesis is an effective pre-clinical monotherapy, however, following prolonged treatment, cancers have been shown to upregulate exogenous FA uptake as a compensatory mechanism to maintain proliferation and growth (Cao et al., 2017, Drury et al., 2020). Therefore, the ability of cancer cells to metabolically switch between FA synthesis and uptake and maintain cancer growth has provided a new therapeutic avenue for co-targeting strategies and aversion to therapy resistance (Daniëls et al., 2014, Watt et al., 2019). The ability for FA uptake to adaptively maintain cancer growth questions the essential nature of *de novo* FA synthesis in cancer cells. Therefore, the focus of the remaining sections will elucidate the role of exogenous FAs in cancers and their respective substrate dependencies.

1.1.3.2 Exogenous fatty acid uptake

Membrane associated transporters facilitate the uptake of LCFAs across the plasma membrane from the tumour microenvironment. The most widely characterised FA transporters include cluster of differentiation 36 (CD36), FA transport proteins (FATPs), and FA binding proteins (FABPs) (Figure 1.2) (Nagarajan et al., 2021). These FA transporters are generally upregulated and support cancer progression, with examples including CD36 in ovarian (Ladanyi et al., 2018), breast (Feng et al., 2019), and prostate cancer (Watt et al., 2019), as well as FABP in colorectal cancer (Kawaguchi et al., 2016), and FATP in melanoma (Zhang et al., 2018). FA transporters, such as CD36, are susceptible to inhibition or knockdown in many cancer types including prostate (Watt et al., 2019), liver (Nath et al., 2015), oral (Pascual et al., 2017), and breast (Feng et al., 2019) cancers.

Like the adaptive interplay between glucose and glutamine, the depletion of exogenous FAs forces cancer cells to shift towards a greater utilisation of endogenous FAs to maintain cancer growth (Cao et al., 2017, Drury et al., 2020). The role of the adaptive utilisations of FA sources remains to be fully understood for therapeutic advantages, as shown by a recent study in breast cancer cells that evaded inhibition of human epidermal growth factor receptor 2 (HER2) regulated FA synthesis by upregulating

CD36-mediated FA uptake that induces HER2 drug resistance (Feng et al., 2019). Therefore, FA metabolism is adaptive and can sustain cancer growth when FA sources or synthesis are impaired.

1.1.3.3 FA modifications

Following either FA uptake or *de novo* synthesis, FAs can be modified via several mechanisms such as activation, elongation, and desaturation prior to downstream roles such as GPLs that provide membrane integrity. Activation of FAs is essential for their use in metabolic pathways and occurs by their conversion into fatty acyl-CoAs catalysed by Acyl-CoA synthetases (ACSLs) (Nagarajan et al., 2021). Once activated, these fatty acyl-CoAs can then undergo either elongation or desaturation, in part to maintain and regulate their chain length. Desaturation, or the introduction of a double bond, is catalysed by the function of desaturase enzymes including Delta-9 stearoyl-CoA desaturase (SCD), which is upregulated in bladder (Presler et al., 2018), lung (Noto et al., 2013), prostate (Fritz et al., 2010), breast (Vriens et al., 2019) cancers, or FA-desaturases (FADS1-3), which are also upregulated in many cancer types (Heravi et al., 2022). The dependence of SCD activity in breast and prostate cancers has been associated with the availability of exogenous FA sources, with cancer cells shifting to endogenous FA sources under lipid deprivation to enhance the levels of MUFAs in the cellular lipid pool (Peck et al., 2016). Fatty acyl-CoAs can also be elongated by having two-carbon units added by elongation of very long-chain fatty acid enzymes (ELOVL1-7). The elongation of fatty acyl chains in GPLs, by ELOVL activity is upregulated and linked to poor prognosis in several cancer types including prostate (Centenera et al., 2021), liver (Zhang et al., 2022), renal cell carcinoma (Nitta et al., 2022) and lung cancer (Marien et al., 2016). Furthermore, ELOVL knockdown reduces cancer progression (Centenera et al., 2021, Marien et al., 2016, Nitta et al., 2022, Su et al., 2018).

The upregulation of FA remodelling in cancer is linked to the essential role that FAs play to form membrane lipids (see review Harayama and Riezman (2018)). The diversity brought to FAs and lipids by desaturase and elongase enzymes can govern cellular membrane curvature, lipid-lipid interactions, and contribute to overall lipid homeostasis, essential for cancer growth and survival (Harayama and Riezman, 2018, Lisec et al., 2019, Sen et al., 2023). Specifically, the ratio of MUFAs to SFAs and

PUFAs is important to maintain cellular homeostasis and survival, as excess SFAs induce lipotoxicity while increased levels of PUFAs leads to elevated susceptibility to peroxidation (Scott et al., 2022). Moreover, cancer cell membranes enriched with *de novo* synthesised SFAs are less permeable to chemotherapeutics and renders cancers less sensitive to cytotoxicity (Rysman et al., 2010). On the other hand, PUFAs are essential components to increase membrane fluidity and flexibility compared to MUFAs (Harayama and Shimizu, 2020). Taken together, modified FAs and the ratios to SFAs serve vital functions to maintain FA homeostasis in cancer cells and warrants further investigation to understand how diverse FAs are used for membrane lipids in cancers.

1.2 Mitochondrial metabolism is a convergent point for multiple metabolic pathways

Mitochondria are the bioenergetic hubs within cancer cells where multiple substrates such as glucose, glutamine, and FAs are catabolised (Figure 1.3). The interplay between glycolysis and glutamine anaplerosis that converges at the TCA cycle has been widely studied, however, the role of FAs in the context of these pathways remains underappreciated. One approach to evaluate this knowledge gap is by quantifying the relative amount of metabolites entering the TCA cycle and specifically LCFAs that undergo regulated uptake to the mitochondria (Fritz and Yue, 1963). As a key contributor to drug resistance is substrate switching as described above, understanding the capacity and constraints of how TCA cycle substrates are utilised will be important in the design of novel metabolic therapeutic and co-targeting strategies.

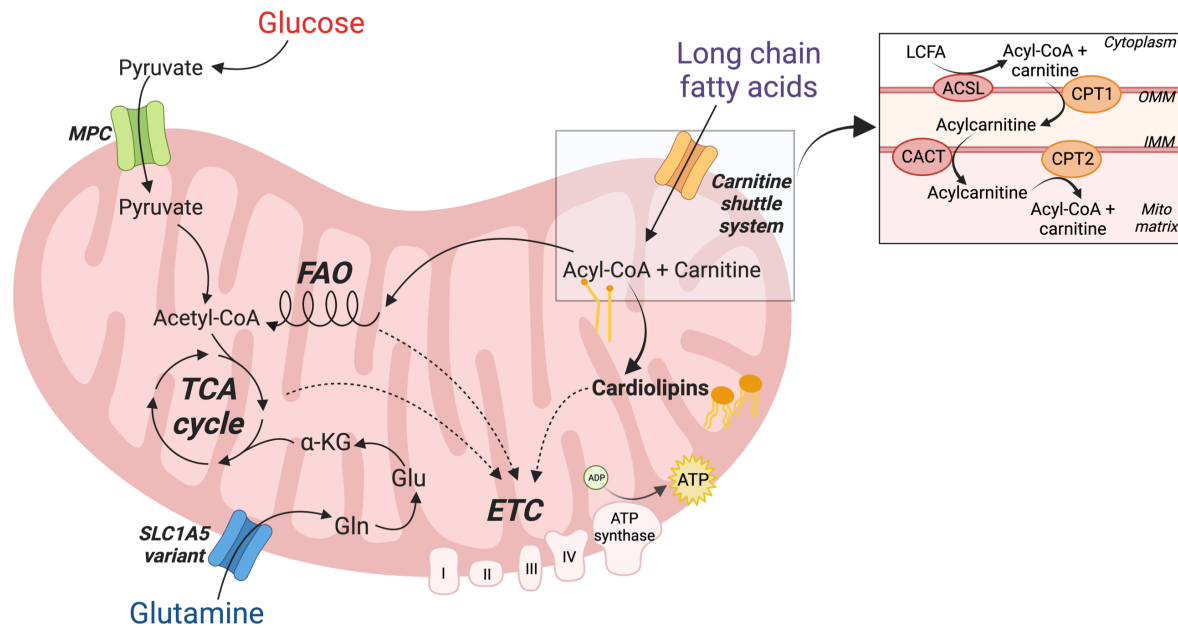


Figure 1.3: Mitochondrial metabolism in the context of fatty acids.

Glucose-derived pyruvate is transported into the mitochondria via MPC and converted to acetyl-CoA which is oxidised by the TCA cycle. Glutamine enters the mitochondria through the SLC1A5 variant and undergoes glutamine anaplerosis to provide oxoglutarate to the TCA cycle. LCFAs are impermeable to the mitochondrial membrane and are transported into the mitochondria via the carnitine shuttle system. LCFA is transformed into acyl-CoA and carnitine by ACSL. CPT1 located at the OMM converts acyl-CoA and carnitine into acyl-carnitine. CACT transports acyl-carnitine across the IMM. CPT2 converts acyl-carnitines back to acyl-CoA and carnitine inside the mitochondrial matrix. Acyl-CoAs enter the shortening cyclical series of FAO that produces acetyl-CoA for the TCA cycle. FAO and the TCA cycle provide reducing equivalents for the ETC. Acyl-CoAs are donated to the acyl-chains of cardiolipins that structurally support the ETC complexes I-IV. ACSL, acyl-CoA synthetase; α -kg, oxoglutarate; CACT, carnitine acyl-carnitine translocase; CPT, carnitine palmitoyl transferase; ETC, electron transport chain; FAO, fatty acid oxidation; Gln, glutamine; Glu, glutamate; IMM, inner mitochondrial membrane; LCFA, long chain fatty acid; MPC, mitochondrial pyruvate carrier; OMM, outer mitochondrial membrane; TCA, tricarboxylic acid. Figure was created with Biorender.com.

1.2.1 Tricarboxylic acid cycle

The TCA cycle (also known as the citric acid cycle or Krebs cycle) is a major metabolic hub within the mitochondria that generates energy and metabolic intermediates. Briefly, the TCA cycle comprises of a series of reactions that produce reducing equivalents NADH and flavin adenine dinucleotide (FADH_2) that are used by the electron transport chain (ETC) and OXPHOS to generate ATP (Spinelli and Haigis, 2018) (Figure 1.3). The TCA cycle functions also extend beyond bioenergetics through the actions of

metabolites that act as signalling mediators. For example, oxoglutarate regulates cell death under hypoxic conditions (Tennant et al., 2009), and the oncometabolite succinate accumulates when succinate dehydrogenase is mutated and results in increased OXPHOS, ROS, and oxidative damage (Eijkelenkamp et al., 2020).

Both glucose-derived pyruvate and glutamine are major substrates for TCA cycle replenishment or anaplerosis that is essential for tumour growth and survival (Yang et al., 2014). Pyruvate sits at the endpoint of glycolysis in the cytosol and is transported across the mitochondrial membrane via mitochondrial pyruvate carriers (MPC) (Figure 1.3) (Schell and Rutter, 2013). Once in the mitochondria, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase complex (Park et al., 2018) or to oxaloacetate by pyruvate carboxylase (Fan et al., 2009, Kiesel et al., 2021). Glutamine is another anaplerotic fuel for the TCA cycle. It is first converted to glutamate in the mitochondria by GLS and subsequently to oxoglutarate by either glutamate dehydrogenase or aminotransferases (Figure 1.3) (Moreadith and Lehninger, 1984). Importantly, both glucose and glutamine can compensate for each other to maintain TCA cycle activity. For example, glucose-derived pyruvate carboxylation is increased in response to GLS inhibition in glioblastoma (Cheng et al., 2011), whereas glutamine anaplerosis increases in glioma and glioblastoma cells treated with an MPC inhibitor (Yang et al., 2014).

The contributions of glucose and glutamine to the TCA cycle vary dependent on cancer type. For example, glucose-derived pyruvate is a predominant TCA cycle carbon source for non-small cell lung cancer (Chen et al., 2016, Sellers et al., 2015), while glutamine is a major substrate source in melanoma and multiple myeloma (Gonsalves et al., 2020, Ratnikov et al., 2015). In addition to cancer-type specific preferences, glutamine utilisation can also vary within cancers such as in breast cancer where triple negative breast cancer subtypes have greater glutamine anaplerosis flux through the TCA cycle compared to luminal A subtypes (Quek et al., 2022).

The roles of TCA cycle substrate selections have primarily focused on glucose and glutamine, however, these are not the only TCA cycle fuelling nutrients. The seminal fluxomics study by Hui et al. (2020) infused 15 labelled circulating metabolites in both fed and fasted mice and quantified the contributions

of these metabolites to the TCA cycle in 11 tissues. Fundamentally, they observed that the contribution of fuel sources to the TCA cycle occurred in a tissue-specific manner, such as the brain that predominantly used glucose, compared to cardiac tissues that mainly utilised fatty acids. Importantly, across all tissues Hui et al. (2020) concluded that of all 15 circulating nutrients, lactate and fatty acids were the major TCA cycle contributors in nearly all tissues. This study highlights the missed opportunity to assess the contribution of exogenous LCFAs in the context of other substrates in a cancer setting. Some studies have proposed LCFAs to be the predominant carbon sources for the TCA cycle in cancers via fatty acid oxidation (FAO) that produces acetyl-CoA as its end-product (Carracedo et al., 2013, Ma et al., 2018), yet fail to quantify this contribution to other major substrates such as glucose and glutamine. Therefore, there is a need to determine whether FAO substantially contribute carbons to the TCA cycle compared to other substrates in cancers.

1.2.2 Long chain fatty acid entry to the mitochondria

LCFAs are constricted to regulated uptake to the mitochondria by the carnitine shuttle system, to which the gatekeeper carnitine palmitoyltransferase 1 (CPT1) is located in the outer mitochondrial membrane (Fritz and Yue, 1963, Kamp and Hamilton, 2006). CPT1 produces acyl-carnitine from acyl-CoA and carnitine (Fritz and Yue, 1963), and the commonly studied CPT1a isoform is genetically overexpressed and linked to the progression of many cancer types including acute myeloid leukemia (Shi et al., 2016), glioblastoma (Shim et al., 2022), and breast cancer (Jariwala et al., 2021). Acyl-carnitines are shuttled across the mitochondrial matrix by carnitine/acyl-carnitine translocase (CACT). CPT2 is located on the matrix side of the inner mitochondrial membrane (IMM) and catalyses the conversion of acyl-carnitines back to fatty acyl-CoAs (Figure 1.3) (McGarry and Brown, 1997).

1.2.3 CPT1a and mitochondrial fatty acid oxidation

Fatty acyl-CoAs within the mitochondria are substrates for the cyclic process known as FAO, which sequentially shortens acyl-CoAs by two carbons each cycle and generates NADH and FADH₂ as by-products and the 2-carbon acetyl-CoA as the end-product. The overexpression of FAO enzymes has been linked to the progression of tumour growth and poor survival in cancers such as prostate (Nassar

et al., 2020), lung (Padanad et al., 2016), and glioma (Lin et al., 2017). Additionally, FAO activity is increased in many cancer types including breast cancer (Linher-Melville et al., 2011), glioma (Lin et al., 2017), prostate cancer (Balaban et al., 2019), and acute myeloid leukemia (Skrede and Iversen, 1995). The importance of FAO to cancer growth is commonly explained by the sole partitioning of the CPT1a-mediated entry of LCFAs to FAO and the use of the end-product acetyl-CoA for the TCA cycle and subsequent ATP production (Camarda et al., 2016, Ma et al., 2018, Pike et al., 2011) (Figure 1.3). However, the importance of FAO to the TCA cycle has yet to be studied in the context of major carbon donors such as glucose or glutamine in multiple cancer types, and so it remains unclear if FAO is in fact an important fuel for the TCA cycle. Further, the inhibition of CPT1a decreases FAO and ATP production and leads to reduced cell viability in cancers (Lin et al., 2017, Pike et al., 2011, Schlaepfer et al., 2014, Tirado-Vélez et al., 2012). These studies have led to the dogmatic view that CPT1a is the sole rate-limiting enzyme for FAO. However, this view does not consider other essential fates of mitochondrial LCFAs downstream of CPT1a that may also explain decreased ATP production and cancer cell viability. Additionally, the significant off-target effects exhibited by the commonly used CPT1a inhibitor etomoxir (Yao et al., 2018) question the interpretation of many studies that have implicated a role for CPT1 in regulating FAO and the TCA cycle. Further investigations could provide insights into the role of mitochondrial destined LCFAs downstream of CPT1a to FAO and the TCA cycle compared to other mitochondrial fates.

1.2.4 CPT1 and mitochondrial lipids

CPT1 inhibition reduces FAO, ATP levels, and mitochondrial membrane potential (Zhang et al., 2021) and also leads to disrupted mitochondria membrane structure (van Meer et al., 2008, Yao et al., 2018), and mitochondrial morphology (O'Connor et al., 2018, Yao et al., 2018). Importantly, the decreased FAO activity and ATP production have contributed to classifying CPT1a as the “sole rate limiting enzyme for FAO”, however, it remains unclear how CPT1 regulates mitochondrial homeostasis. Importantly, this creates a knowledge gap to determine if CPT1a is restricted to solely regulating FAO and provides the basis for determining whether CPT1a-mediated mitochondrial entry of LCFAs impacts

other mitochondrial fates that underpin the changes in mitochondrial homeostasis following CPT1 inhibition.

Two critical studies have demonstrated that CPT1 influences mitochondrial lipid composition (Yao et al., 2018, Zhang et al., 2021). Specifically, they observed that CPT1 loss-of-function decreased complex lipid levels within isolated mitochondria from BT549 triple-negative breast cancer cells (Yao et al., 2018), and within whole cell lysates from pancreatic, breast, colorectal, and lung cancer cells (Zhang et al., 2021). Across both studies, the mitochondrial exclusive GPL, cardiolipins (CLs) pose an interesting fate for LCFAs downstream of CPT1, that could explain the energy crisis and disrupted mitochondrial morphology observed upon CPT1 inhibition. This is because CLs function to maintain mitochondrial morphology and ETC complex structures (Paradies et al., 2014) (Figure 1.3). Therefore, investigating if CPT1-mediated mitochondrial entry of LCFAs impacts CL homeostasis may provide a new window into the diverse roles of CPT1a in cancers, beyond its characterisation as a FAO rate limiting enzyme.

1.2.5 Cardiolipins

CLs are an essential membrane lipid exclusively located within the IMM and account for ~20% of mitochondrial phospholipid content in mammalian cells (Horvath and Daum, 2013). CLs are inherently more diverse than other GPL classes due to their four fatty acyl-chain structure (Falabella et al., 2021). The conical structure of CLs supports their functions to maintain mitochondrial membrane and cristae structures (Huang et al., 2006, Ikon and Ryan, 2017, Kondadi et al., 2020), mitophagy (Shen et al., 2017), apoptosis (Gonzalvez and Gottlieb, 2007, McMillin and Dowhan, 2002), and stabilising the ETC complexes I-IV to form respiratory supercomplexes and support OXPHOS (Fiedorczuk et al., 2016, Paradies et al., 2014, Schwall et al., 2012).

1.2.5.1 Cardiolipin synthesis and remodelling pathways

CLs are synthesised from ER-derived constituents and further remodelled before translocating to the IMM (Figure 1.4). Briefly, *de novo* CL synthesis involves a series of reactions that utilise phosphatidic acid (PA) that is sourced from the endoplasmic reticulum (ER) and transported to the mitochondria via

ER-mitochondrial contact sites (Osman et al., 2011). The first reaction of the CL synthesis pathway is the conversion of PA to CDP-diacylglycerol (CDP-DAG) which leads to phosphatidylglycerol (PG) formation (Zhang et al., 2011). The final step of biosynthesis uses two PGs to form a nascent CL catalysed by CL synthase (Chen et al., 2006) (Figure 1.4). The enzymes involved in synthesising nascent CLs do not exhibit acyl-chain specificity (Hostetler et al., 1975), and so further acyl-chain remodelling must take place to form mature CLs that facilitate the mitochondrial structure and membrane functions of CLs (Schlame et al., 2000). The remodelling pathways are essential to form the predominant 18:2 acyl-chain composition of CLs found in mammalian cells (Oemer et al., 2021, Taylor and Hatch, 2009). The remodelling of CLs is initiated by a de-acylation step (removal of one acyl-chain) to form monolyso-CLs (MLCL), which then undergo a re-acylation step to incorporate acyl-chains of specific saturation and chain lengths (Figure 1.4) (Lu 2015). The re-acylation step utilises acyl-CoAs as acyl-chain donors facilitated by acyl-CoA:lysoCL acyltransferase 1 (ALCAT1) located at the ER (Cao et al., 2004), or monolyso-CL acyltransferase 1 (MLCLAT1) at the IMM (Taylor and Hatch, 2009). Another re-acylation process involves the enzyme Tafazzin that uses acyl-chains donated from phosphatidylcholine or phosphatidylethanolamine that have no specific acyl chain specificity (Houtkooper et al., 2009) (Figure 1.4).

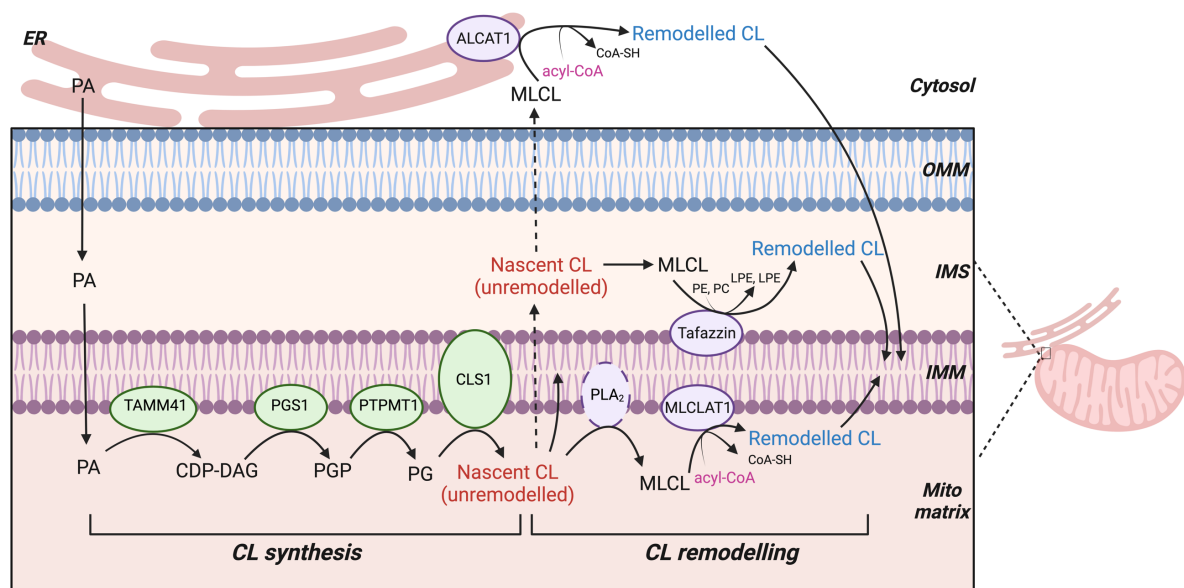


Figure 1.4: Cardiolipin synthesis and remodelling pathways.

CL is synthesised in the IMM and undergoes further remodelling. PA is transported from the ER to the IMM where it is converted to the nascent CL (unremodelled) following a series of systematic reactions. Nascent CL is trafficked to the cytosol where de-acylation (removal of one acyl-chain) occurs to form MLCL, which is re-acylated by the ER-located ALCLAT1 enzyme. MLCL in the mitochondrial matrix can be re-acylated by IMM-located MLCLAT1, or in the IMS by Tafazzin. MLCLAT1 and ALCAT1 utilise acyl-CoAs, while Tafazzin utilises PE or PC for the re-acylation steps. Once assembled, CLs are located at the IMM. ALCAT1, acyl-CoA:lysocardiolipin acyltransferase 1; CDP-DAG, cytidine diphosphate-diacylglycerol; CL, cardiolipin; CLS1, cardiolipin synthase 1; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; IMS, intermembrane space; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MLCL, monolysocardiolipin; MLCLAT1; MLCL acyltransferase 1; OMM, outer mitochondrial membrane; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PGP, phosphatidylglycerol phosphate; PGS1, phosphatidylglycerol phosphate synthase; PLA₂, phospholipase A₂; PTPMP1, protein-tyrosine phosphatase mitochondrial 1; TAMM41, TAM41 translocator assembly and maintenance homolog. Figure was created with Biorender.com.

Critically, recent studies have shown that CLs regulate apoptosis by peroxidation, which causes the subsequent release of proapoptotic cytochrome c (Schug and Gottlieb, 2009, Zhong et al., 2017). Further, remodelling FAs are essential for cancer survival as SCD inhibition sensitises cancer cells to CL-induced apoptosis (Peck et al., 2016). CLs are also essential for stabilising ETC complex I-IV structures for ATP production (Musatov and Sedláč, 2017, Paradies et al., 2014). Namely, as CPT1 knockdowns in cancer cells caused both impaired ATP production and decreased CL levels (Yao et al., 2018, Zhang et al., 2021), it is possible that the contribution of CPT1 to cellular bioenergetics is, in part, mediated through CL biology.

1.3 Identifying pan-cancer metabolic phenotypes

Metabolic pathways supporting cellular growth, such as glycolysis, glutamine anaplerosis, and FA metabolism, are reprogrammed in cancers to meet their high bioenergetic demands as described in previous sections. More commonly, investigations to identify these metabolic reprogramming events are typically carried out in cancer-type specific studies that focus on one tissue lineage, such as the utilisation of lactate to fuel the TCA cycle in non-small cell lung cancers (Faubert et al., 2017). Yet, observations of the same metabolic alterations and associated targetable vulnerabilities are not confined within that single cancer type, possibly due to metabolic convergence. For example, targeting the

uptake of glutamine can suppress tumour growth in several cancer types including melanoma, non-small cell lung cancer, acute myeloid leukemia, and breast cancer (Hassanein et al., 2015, van Geldermalsen et al., 2016, Wang et al., 2014, Willems et al., 2013). Cancers from different tissue lineages are susceptible to the same targetable metabolic vulnerabilities, highlighting the potential advantage of pan-cancer studies to identify shared metabolic signatures beyond specific cancer types. Li *et al* (2019) demonstrated in their seminal pan-cancer study that asparaginase therapy can be utilised in solid liver and stomach tumours, beyond its characterised therapeutic usage in acute lymphoblastic leukemia. Further studies have systematically profiled cancer metabolomes, and provided evidence that metabolic phenotypes are commonly observed amongst diverse cancer types (Cherkaoui et al., 2022, Ortmayr et al., 2019, Shorthouse et al., 2022). Together, pan-cancer studies can be utilised to identify common perturbations of altered cancer metabolism that lead to decreased cancer cell viability and increased survival rates (Shorthouse et al., 2022). Systematic profiling of diverse cancer metabolomes can indicate sensitivities to anti-cancer strategies and advance the development and translation of therapies from one cancer type to another (Pushpakom et al., 2019).

1.4 Summary and Thesis Aims

The TCA cycle is a bioenergetic hub within the mitochondria and provides an excellent opportunity to study relative substrate utilisations in cancer cells. The mitochondrial metabolism field has already identified the clear interplay between glucose and glutamine metabolism that can adaptively sustain cancer growth when either substrate pathway is impaired. However, the relationship of glucose and glutamine to exogenous FA metabolism remains to be determined. LCFAs that enter the mitochondria are assumed to be solely partitioned to FAO, with the majority, if not all, of FA carbons going into the TCA cycle. Yet, the contribution of LCFA oxidation to the TCA cycle remains to be assessed in the context of glucose and glutamine in cancers spanning diverse FAO capacities. A current flaw in the dogmatic view of CPT1a is that it solely regulates the entry of LCFAs to FAO. Further investigation is required to identify the mitochondrial fates of LCFAs after CPT1a entry, beyond what is known about FAO, the TCA cycle, and the associated ETC. It is known that CLs utilise intra-mitochondrial acyl-

CoAs for remodelling pathways and serve roles in mitochondrial structure and bioenergetics in non-cancer tissues. CLs pose a new area to investigate mitochondrial FA metabolism in cancers. Therefore, quantifying the incorporation of intra-mitochondrial LCFAs downstream of CPT1a into the TCA cycle in the context of glucose and glutamine, or to CLs, can lead to a greater understanding of FA utilisations in cancer.

This thesis aimed to understand how cancers utilise exogenous nutrients within the mitochondria, and to identify novel metabolic phenotypes and dependencies that may be targetable in the future. This thesis contains three research projects that address this overarching aim of substrate utilisation. The first project (Chapter 2) used a pan-cancer approach and aimed to characterise metabolic phenotypes of 57 cell lines to determine whether metabolic signatures can be identified within subsets of diverse cancer types. This project provided an objective that insights into common metabolic signatures would uncover potential metabolic vulnerabilities across cancers. The second research project (Chapter 3) aimed to quantify the contribution of exogenous LCFAs to the TCA cycle relative to glucose and glutamine in prostate cancer cells. This project hypothesised that LCFAs are a major carbon source for the TCA cycle and CLs following CPT1a-mediated entry. The third project (Chapter 4) aimed to expand our findings of the second project (Chapter 3) using a pan-cancer approach to test if cancer cells with various FAO capacities differentially utilise LCFAs to feed carbons to the TCA cycle. This project hypothesised that diverse cancer types would hold a spectrum of FAO activity that could determine the magnitude of carbon contribution from LCFAs to the TCA cycle. Together, this thesis aimed to identify metabolic phenotypes within glucose, glutamine, and FA metabolism, that could reveal opportunities to repurpose metabolic-targeting therapies beyond specific cancer types.

Chapter 2

A ratio-based metabolome approach reveals common metabolic targets within a subset of diverse cancer types.

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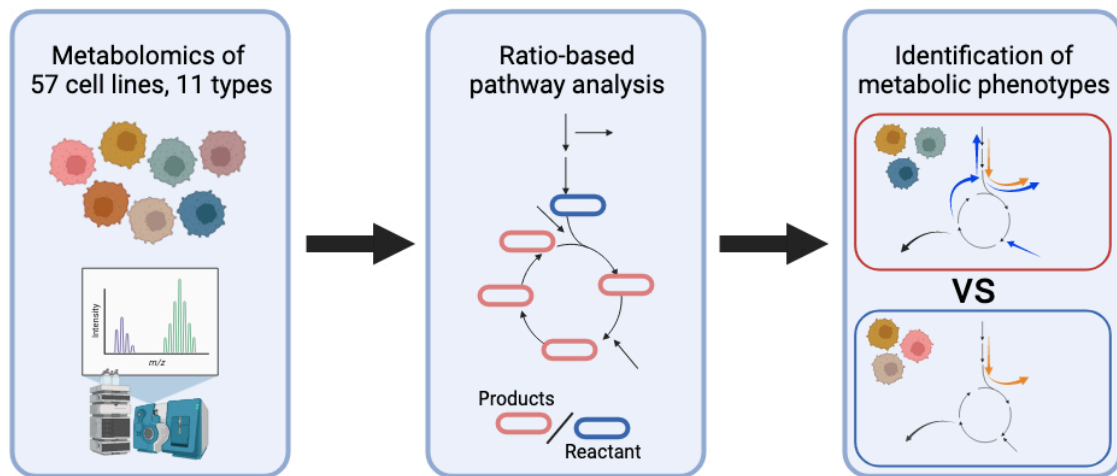
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Abstract

Intrinsic metabolic drivers and genetic alterations are essential for cancer growth and survival and have influenced the development of precision targeting therapies. Large-scale metabolomic analysis of cell lines from different tissue origins have identified common metabolic phenotypes in multiple cancers. However, the signatures unveiled from these untargeted experiments are often obscured by secondary metabolic pathways that are not exploitable for anti-cancer therapies. Here, we chose a targeted metabolomics approach to explore specific metabolic pathways that are reprogrammed to support cancer proliferation. To identify metabolic phenotypes centred on central carbon and amino acid pathways we profiled the intracellular metabolome of 57 cell lines from 11 origin sites, of which 49 were tumour cells. We undertook a ratio-based approach to assess relationships between reactant and product metabolites and from this, we identified a subset of cells from different tissue origins that exhibited an increased conversion of glucose and glutamine into central carbon metabolism. We validated this metabolic signature using stable isotope tracing and *in silico* analyses of drug sensitivity and loss-of-function databases. Together, our study showcases the potential to identify common metabolic signatures that may lead to new clinical treatment strategies, independent of the underlying genetic heterogeneity and tissue lineage differences that exist among cancer types.

Synopsis



Ratio-based metabolome pathway analysis of 49 tumour and 8 normal epithelial cell lines reveals common metabolic signatures within subsets of diverse cancer types that can be used to identify targetable vulnerabilities.

- Metabolite abundances drive cancer cells to cluster independent of origin site, oncogenic drivers, and culturing conditions.
- Ratio-based metabolome analysis identifies patterns of pathway activity not observed from metabolite abundances alone.
- Metabolic activity can be associated with the differential usage of glucose and glutamine between subsets of diverse cancer types by substrate uptake, stable isotope tracing, oxygen consumption rates, drug sensitivity and loss-of-function screens.

Introduction

Cancer cells exhibit a heterogeneous metabolic landscape that supports the dynamic plasticity of nutrient utilisation for tumour growth and survival (Altea-Manzano *et al*, 2020; Fendt *et al*, 2020; Pavlova *et al*, 2022). Many novel insights into the metabolic signatures of cancers have arisen from studies taking a cancer-type specific or metabolic pathway focused approach (for example, Hensley *et al*, 2016; Kamphorst *et al*, 2015; Wang *et al*, 2023). More recently, several studies have reported the outcomes of high-throughput metabolomics using diverse cancer cell line panels such as the NCI-60 (60 cell lines, Jain *et al*, 2012; Ortmayr *et al*, 2019), CCLE (928 cell lines, Li *et al*, 2019), and other pan-cancer panels containing 180 cell lines (Cherkaoui *et al*, 2022) or 173 cell lines (Shorthouse *et al*, 2022). These studies aimed to elucidate the links between cancer metabolism phenotypes to transcriptional regulation (Ortmayr *et al*, 2019), or genetic alterations and dependencies (Li *et al*, 2019), that correlate to drug-sensitivities (Shorthouse *et al*, 2022) or metabolite dependencies (Jain *et al*, 2012). Notably, Cherkaoui and colleagues (2022) took a top-down approach by clustering the metabolome of 180 cancer cells acquired by untargeted metabolomics across 49 KEGG metabolic pathways. Inferring pathway activity for each cell line, their results identified two clusters broadly characterised by higher carbohydrate metabolic activity or aerobic mitochondrial activity that correlated with epithelial or mesenchymal status, respectively. Here, we employed a targeted approach to characterise substrate utilisation of high-flux central metabolism through glucose and glutamine tracing in a panel of 57 proliferating cells, from tumour and benign epithelial tissue types spanning 11 cancer origin sites. We aimed to identify common but nontrivial metabolic signatures that contribute to proliferation and are targetable metabolic vulnerabilities within subsets of diverse cancer types.

Results

Metabolic phenotyping of cells from 11 tissue origins.

We first set out to identify common metabolic phenotypes associated with central carbon (glycolysis, TCA cycle, pentose phosphate pathway) and amino acid metabolism pathways. To achieve this, 57 adherent cell lines (49 tumour- and 8 normal epithelial-derived) from 11 cancer types were cultured in basal media conditions (Appendix Table 1). Intracellular lysates were extracted and analysed using a targeted metabolomics approach. Samples were generated in triplicate across 6 batches including control cell lines to perform batch correction, ensuring consistency throughout the complete data set (Fig 1A). K-means algorithms with Pearson correlations of the batch-corrected dataset identified 4 distinct clusters of cells (Fig 1B). All clusters contained both tumour and normal cell lines from different tissue origins (Fig 1B). There were some instances where cell lines from the same tissue origin clustered together (Fig 1B), similar to previous pan-cancer metabolome studies (Shorthouse *et al*, 2022), such as Cluster 3 that was enriched with prostate cancer cells and Cluster 4 with endometrial cancer cells (Fig 1B). Importantly, the overall pattern observed in the 4 clusters of proliferating cells were not due to culturing media conditions, tissue type (normal epithelial vs. tumour), cancer type, tissue origin, or the mutation status of common oncogenic drivers that have been reported to influence cell metabolism (Cairns *et al*, 2011; Jia *et al*, 2008; Jones & Thompson, 2009; Oermann *et al*, 2012; Vousden & Ryan, 2009) (Fig 1C). There was no clear organisation of metabolites into pathways when clustered (Fig 1B), and so we were unable to infer how pathway activity differs between clusters. Nonetheless, these findings show that common metabolite signatures could be identified from cancers cells of diverse origins.

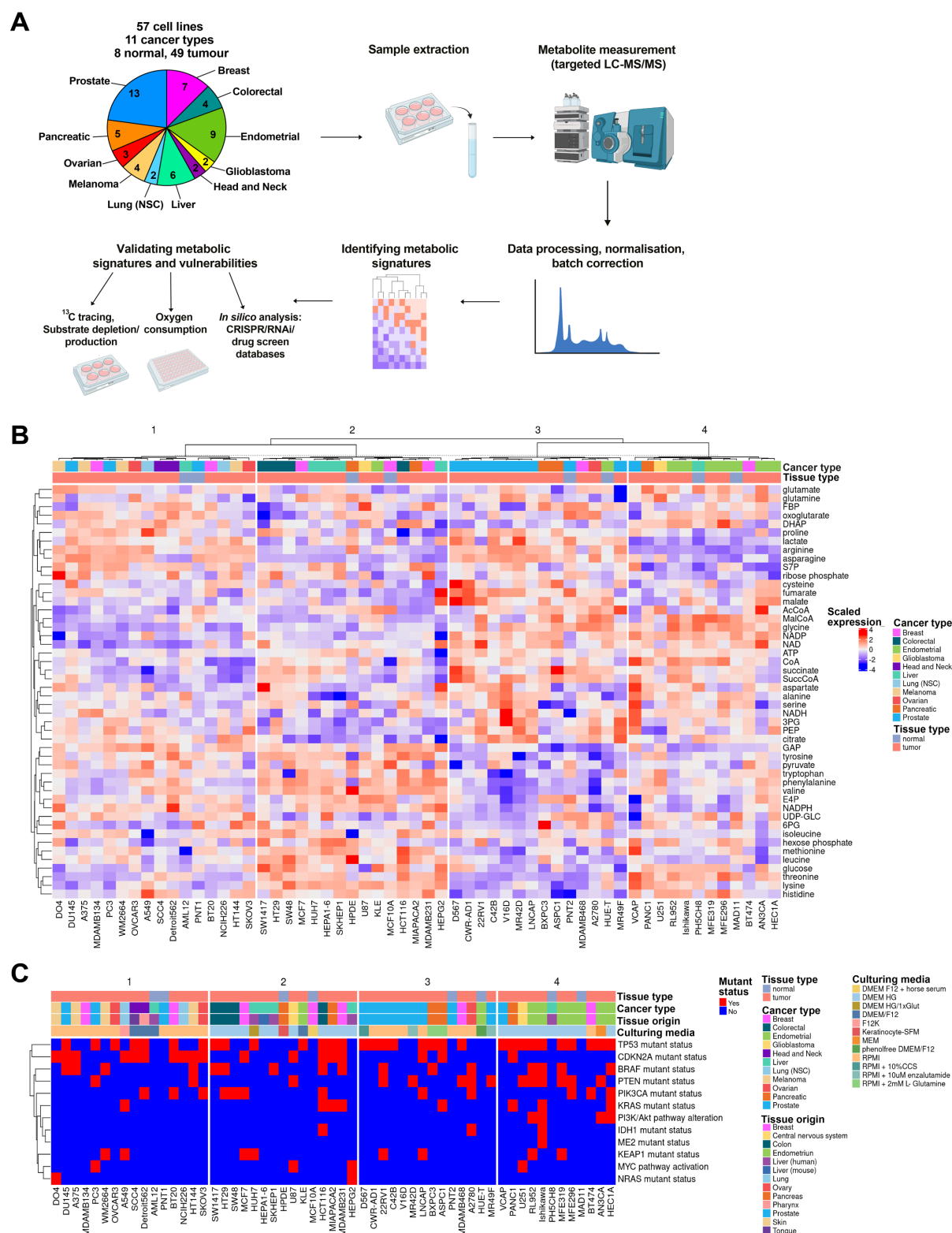


Figure 1: The metabolome landscape of cells from 11 tissue origin sites.

- A. Schematic of the workflow for targeted metabolomics profiling of 57 cell lines to identify and validate metabolic signatures.
- B. Heatmap of scaled metabolite expression across central carbon and amino acid metabolism within the cell line panel ($n=57$). Cell lines colour coded by cancer type and tissue type.

- C. Clusters of cell lines from (B) appended with colour coded legends for mutant status of common oncogenic drivers, culturing media conditions, tissue origins, cancer type, and tissue types.

Metabolite ratio-based approach uncovers metabolic signatures.

Since our aim was to determine whether clusters of cancer cell lines exhibited common patterns of metabolic activity, we transformed our metabolite abundances into ratios. We calculated the ratio between an upstream precursor or reactant metabolite (applied as the denominator) and downstream pathway product metabolites for each central carbon and major amino acid metabolism pathway. This transformation was based on the idea that metabolite conversion forms a cascade and therefore intrinsic correlations may exist between reactant and product that provide biologically meaningful insights into pathway activity. For example, glucose is the precursor of glycolysis and so ratios were calculated for the abundances of glycolytic metabolites relative to glucose (Fig 2B).

K-means algorithms with Pearson correlations of the entire metabolite ratio dataset spanning metabolic pathways of interest identified 5 distinct clusters of cells (Appendix Fig S1A). These clusters indicated high (red) or low (blue) pathway patterns (Appendix Fig S1A) that were not clearly observed from metabolite abundances alone (Fig 1). Each cluster was composed of cells from different tissue origins, tissue type, cancer type, mutation status of common oncogenic drivers, and culturing conditions (Appendix Fig S1B). To interpret differences in metabolic pathway patterns, the primary heatmap (Appendix Fig S1A) was separated into individual panels that conserved the cell clusters (Fig 2A-G). For example, the cells in Cluster 3 had greater pentose phosphate pathway (PPP) metabolite ratios compared to Cluster 2, suggesting the PPP of cells within Cluster 3 may be utilised or regulated in a different manner (Fig 2A). The ratio pattern for PPP Cluster 3 (red) was replicated for glycolysis (Fig 2B), but inverse patterns (blue) were seen in other pathways, including the TCA cycle (Fig 2C). For example, Cluster 3 had noticeably lower TCA cycle metabolite ratios compared to Cluster 2 and 4 (Fig 2C), which may indicate an inversion of glycolytic and TCA cycle metabolism. Structuring metabolite ratios into pathway formats enabled the discrimination of pathway activities and precursor usage among subsets of cells from different tissue origins. Furthermore, our ratio approach identified similar patterns between pathways not commonly connected, such as methionine metabolism and the TCA cycle (Fig

2C, 2E). Together, our analysis reveals novel ways in which cancer cells from different lineages may be related and poses an interesting area of future investigation.

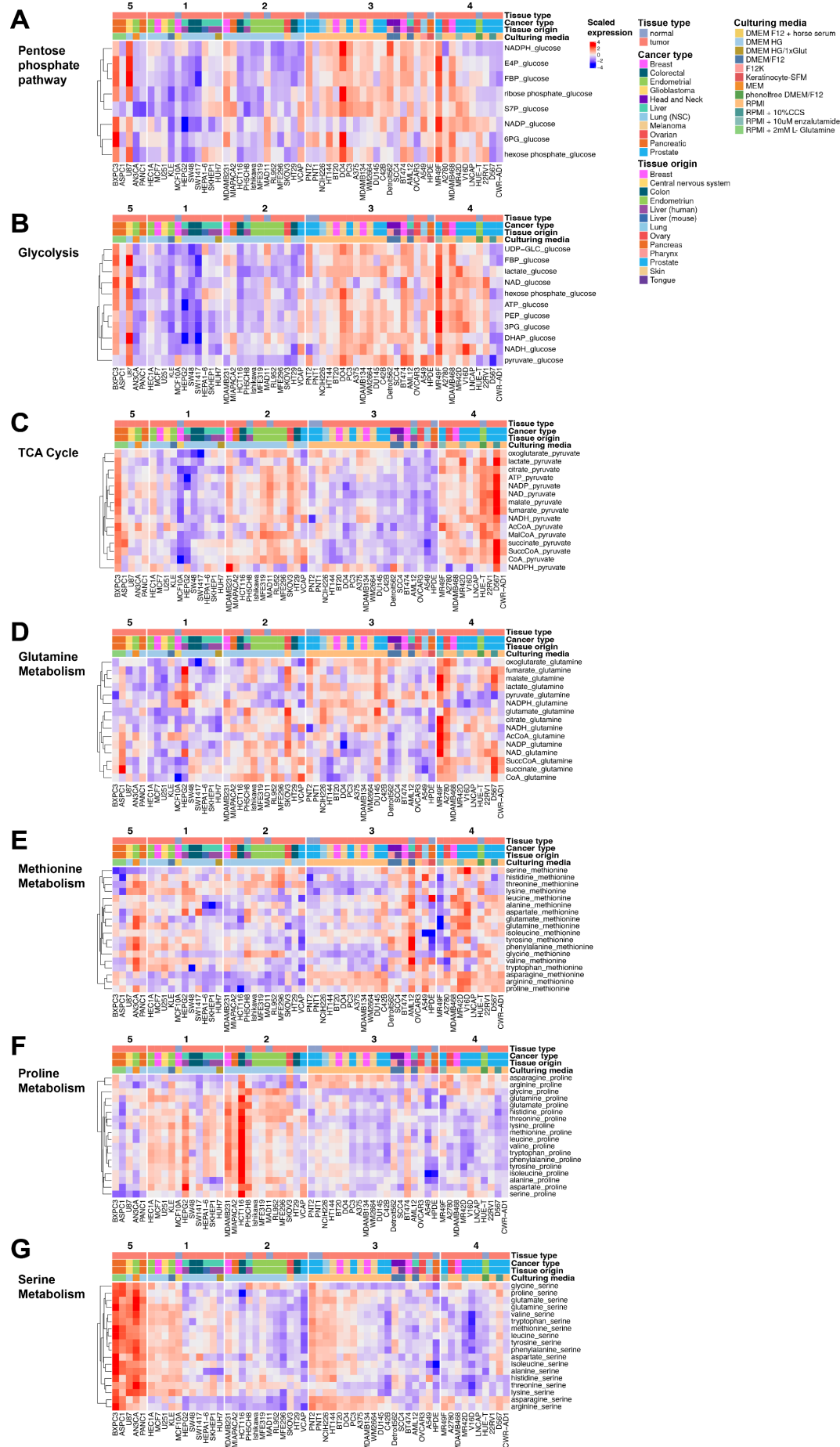


Figure 2: Identification of metabolic signatures from ratio-based clustering.

A-G. Metabolite ratio heatmaps formed by ratios of (A) pentose phosphate pathway and (B) glycolysis pathway metabolites to glucose, (C) TCA cycle pathway metabolites to pyruvate, (D) glutamine metabolism metabolites to glutamine, (E) methionine metabolism to methionine, (F) proline to proline, and (G) serine metabolism metabolites to serine.

Substrate utilisation profiles explain observed metabolite ratio patterns.

Next, we closely examined the differences in TCA cycle metabolism since it is an essential hub where various substrates and pathways converge and is critical for energy production and cell viability (Spinelli & Haigis, 2018) (Fig 3A). From the identified 5 clusters formed in Figure 2, we chose to contrast Cluster 3 (C3) and Cluster 4 (C4) as they have distinctive TCA cycle metabolite levels relative to pyruvate (Fig 3B). Firstly, we confirmed that Cluster 4 cells had greater TCA cycle metabolite levels relative to pyruvate (Multiple unpaired t-tests, P -value <0.001), except for oxoglutarate and NADPH (Fig 3C). Likewise, Cluster 4 cells in general had greater succinate and malate and low pyruvate levels compared to cells in Cluster 3 (Fig 3D). As pyruvate was used to assess the ratio-metric activity of the TCA cycle, we extended our analysis to lactate, which is synonymous with Warburg metabolism in cancer (Smith *et al*, 2016). To this effect, we calculated the ratio of lactate to pyruvate, which is associated with the equilibrium constant of lactate dehydrogenase (LDH). Cells in Cluster 4 had a greater lactate to pyruvate ratio than cells in Cluster 3 (Student t-test, $P=0.001$) (Fig 3E), but there was no difference in the NADH/NAD⁺ ratios (Appendix Fig S2A), which are co-factors of LDH and heavily influence LDH activity (Luengo *et al*, 2021). Thus, the greater production of lactate in Cluster 4 cells compared to Cluster 3 cells was unlikely driven by excess NADH, but possibly due to carbon surplus in the TCA cycle.

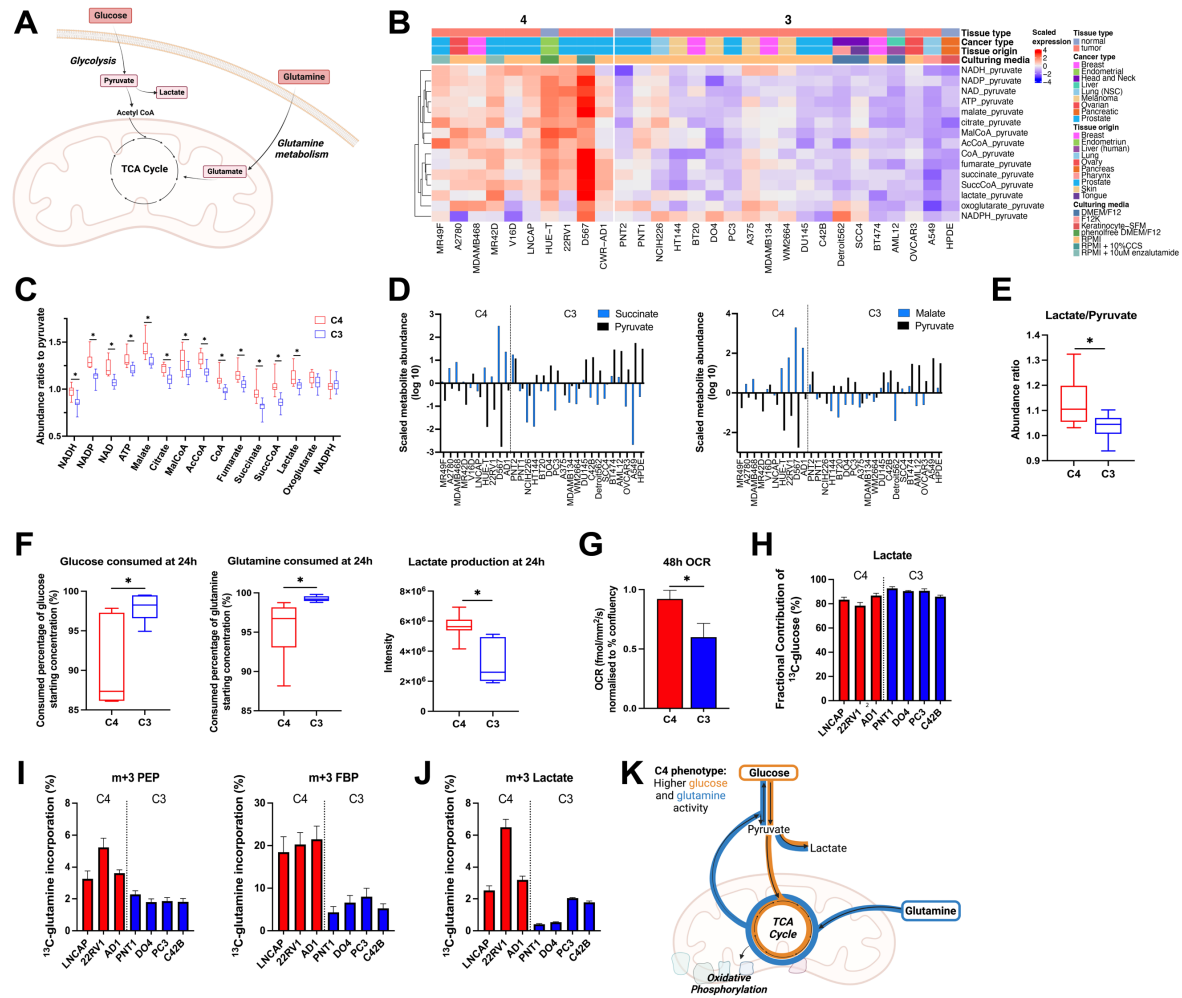


Figure 3: Validation of ratio patterns uncovers substrate dependencies for cell clusters.

- Schematic of glucose and glutamine sources to the TCA cycle.
- Scaled metabolite ratio heatmap of Clusters 4 and 3, derived from TCA cycle pathway metabolites calculated against the precursor pyruvate.
- Abundance ratios of TCA cycle metabolites to pyruvate (C4 n=10, C3 n=19, multiple unpaired t-tests, *p<0.05, error min to max values).
- Waterfall plots comparing the scaled metabolite abundance (log₁₀) of Clusters 3 and 4 of succinate and pyruvate (left), and malate and pyruvate (right).
- Abundance ratios of lactate to pyruvate (error min to max values) (C4 n=10, C3 n=19).
- Glucose and glutamine consumption (consumed % of starting substrate concentrations) and lactate production intensity at 24 hours (C4 n=8, C3 n=6, error min to max values).
- Oxygen consumption rates (OCR) at 48 hours normalised to % confluency (C4 n=6, C3 n=5, 3 biological replicates and 4 technical replicates per cell line, mean ± standard error of the mean).
- Fractional contribution of [U-¹³C]-glucose to intracellular lactate (C4 n=3, C3 n=4, 3 biological replicates and 3 technical replicates per cell line, mean ± standard error of the mean).
- Fractional contribution of [U-¹³C]-glutamine to m+3 FBP (fructose1,6-bisphosphate), and m+3 PEP (phosphoenolpyruvate) (C4 n=3, C3 n=4, 3 biological replicates and 3 technical replicates per cell line, mean ± standard error of the mean).
- Fractional contribution of [U-¹³C]-glutamine to intracellular lactate (C4 n=3, C3 n=4, 3 biological replicates and 3 technical replicates per cell line, mean ± standard error of the mean).
- Schematic of the C4 phenotype of greater utilisation from glucose to the TCA cycle and lactate metabolism, and glutamine to the TCA cycle, gluconeogenesis, and lactate.

Data information: (E-G) *P<0.05 vs Cluster 4 by Unpaired student's t-test.

Glycolysis and glutamine anaplerosis both feed into the TCA cycle and can produce lactate as a by-product (Smith *et al*, 2016). We next sought to determine whether cells in Cluster 4 produce more lactate due to excessive consumption of glucose and/or glutamine. We measured glucose and glutamine consumption and lactate production over 24 hours in a subset of Cluster 3 and 4 cells and observed cell line-dependent differences in substrate consumption and production (Appendix Fig S2B). Nonetheless, cells in Cluster 4 produced more lactate compared to those in Cluster 3 (Student t-test, $P=0.006$) (Fig 3F), which was consistent with greater TCA cycle metabolite levels and lactate to pyruvate ratios (Fig 3E). However, the net consumption of glucose (Student t-test, $P\text{-value}=0.02$) and glutamine (Student t-test, $P=0.04$) were lower in Cluster 4 cells (Fig 3F). It was plausible that Cluster 4 cells are more carbon-efficient at utilising glucose and glutamine despite the lower rate of consumption. To test this, we quantified oxygen consumption rate as a readout of oxidative phosphorylation (OXPHOS) activity that is coupled to the TCA cycle. In line with our prediction, cells in Cluster 4 possessed greater OXPHOS activity than Cluster 3 cells (Student t-test, $P=0.04$) (Fig 3G, Appendix Fig S3C). These results suggest that Cluster 4 cells more efficiently use glucose and glutamine via respiration, and that the spill over from pyruvate into lactate cannot be explained by higher substrate uptake fluxes.

To further resolve the intersection of glucose and glutamine metabolism at pyruvate, we performed [U- ^{13}C]-glucose and [U- ^{13}C]-glutamine tracing experiments in a subset of cells from Clusters 3 and 4. Lactate was largely produced from glucose (78-92% enrichment), although no differences in lactate or pyruvate enrichment were observed between Clusters 3 and 4 (Fig 3H, Appendix Fig S2D). Further, there was no difference in the enrichment of TCA cycle metabolites from glucose between cell clusters (Appendix Fig S2E), which suggested utilisation may differ at the point of branching pathways from the TCA cycle. Overall, tracing glucose did not reveal how cells from Clusters 3 and 4 differed by pathways converging at glucose to pyruvate. Therefore, we turned our attention to investigating the fate of exogenous glutamine, as it is a major carbon source to the TCA cycle (Spinelli & Haigis, 2018). From our ^{13}C -glutamine experiments, we detected a greater cataplerotic efflux of glutamine in Cluster 4 cells compared to Cluster 3 cells (Fig 3I, J). Specifically, there were strikingly greater levels of m+3 fructose1,6-bisphosphate, and to a lesser extent phosphoenolpyruvate (PEP) (Fig 3I). This pattern of

enrichment indicates an increased use of glutamine carbon for gluconeogenesis in Cluster 4 cells. We also detected more glutamine carbons incorporated in lactate (Fig 3J), via either PEP carboxykinase or malic enzymes (Mansouri *et al*, 2017; Montal *et al*, 2015). As such, the increased lactate production and more abundant TCA cycle metabolites in cells of Cluster 4 is likely explained by increased glutamine cataplerotic efflux (Fig 3C, F). Taken together, our metabolite ratio-based approach identified clusters of diverse cells from various tissue origins that use glucose and glutamine in the TCA cycle and fuel lactate production, gluconeogenesis and OXPHOS to a greater extent than other cells (Fig 3K).

Ratio-based phenotypes provide new opportunities for targeting metabolic vulnerabilities.

We identified cells in Cluster 4 to have more efficient glucose and glutamine respiration, and a greater glutamine cataplerotic efflux that feeds gluconeogenesis and lactate production compared to cells in Cluster 3 (Fig 3). We next set out to verify whether these metabolic differences translated to actionable vulnerabilities (Fig 4A, B). To do so, we performed *in silico* analyses using the pan-cancer DEMETER (Tsherniak *et al*, 2017) and Project Score (Behan *et al*, 2019) genetic loss-of-function screens, and the drug sensitivity PRISM (Corsello *et al*, 2020) and GDSC2 (Yang *et al*, 2012) datasets. We cross-referenced these large-scale screens with cell lines from our identified Clusters 3 and 4 and narrowed the target selection to those that overlap with KEGG metabolic pathways (Kanehisa & Goto, 2000). Unless indicated otherwise, all comparative analyses of Cluster 4 are expressed relative to Cluster 3.

We first tested the hypothesis that cells in Cluster 4 were more susceptible to depletion of genes associated with glycolysis, glutamine metabolism and OXPHOS (Fig 4B). In line with our hypothesis, cells in Cluster 4 had greater sensitivity to genetic impairment of OXPHOS complexes I-V and glutaminase (GLS) isoforms (Multiple unpaired t-tests, $P < 0.05$) (Fig 4C). Moreover, we observed a trend for greater sensitivity to genetic deletion of succinate dehydrogenase (SDHD) (Student t-test, $P = 0.06$), and lactate dehydrogenase (LDHD) (Student t-test, $P = 0.08$) in cells from Cluster 4 (Appendix Fig S3A). The confidence of our ratio-metric approach to identify metabolic phenotypes and associated vulnerabilities was supported by the fact that we observed several genes involved in lactate/pyruvate metabolism, glycolysis/gluconeogenesis, OXPHOS, and TCA cycle pathways in the top 20 gene knockouts with the greatest fitness score differences between Cluster 4 and 3 cells, including SDH (Appendix Fig S3B, DEMETER) and LDHD (Appendix Fig S3B, Project Score). Aligned with the ratio-based analyses of our targeted metabolomics, we verified that Cluster 4 cells have a greater sensitivity to drug and gene impairments of OXPHOS, glutamine metabolism, TCA cycle, and pyruvate/lactate pathway (Fig 4C). The success of our ratio-metric approach to identify targetable vulnerabilities in cancer cells of diverse origins provides an opportunity to improve anti-cancer targeting therapies and patient outcomes.

Finally, we assessed whether differential sensitivity between Clusters 3 and 4 may extend beyond glucose and glutamine metabolism. By datamining high-throughput loss-of-function and drug sensitivity screens that span the spectrum of KEGG annotated metabolic pathways, we identified the top 20 drugs or gene knockouts that had the greatest differences in area under the curve (AUC) z-scores or fitness scores between cells in Clusters 4 and 3 (Fig 4A, D, E). We next developed a scoring system to consolidate the top 20 sensitive pathways in Cluster 4 cells from all drug and loss-of-function databases. Interestingly, purine metabolism was the most vulnerable metabolic pathway in Cluster 4 cells (Appendix Fig S3C, Fig 4D, E). Specifically, Cluster 4 cells were more sensitive to inhibition of phosphodiesterase (PDE5) by avanafil, that supports purine associated cGMP (Lv *et al*, 2020; Pantziarka *et al*, 2018), and genetic knockout of adenylosuccinate lyase (ADSL) that catalyses multiple reactions within the purine pathway (De Vitto *et al*, 2021). Proliferating cancer cells are vulnerable to

purine metabolism impairment (Lane & Fan, 2015). Purine synthesis consumes glucose- and glutamine-derived aspartate produced via the TCA cycle (Kodama *et al*, 2020; Mullen & Singh, 2023). The increased sensitivity to inhibition of purine metabolism may therefore correlate with a greater cataplerotic conversion of glucose and glutamine in Cluster 4. Although our ratio-metric approach was limited to central carbon metabolism, we identified metabolic vulnerabilities among peripheral pathways that reflected how glucose and glutamine are utilised (Fig 4D, E).

Overall, the results highlight the potential of a ratio-based approach to differentiate substrate utilisation profiles. This new approach can be used to gain a greater insight into the commonalities of pathway mechanisms within subsets of diverse cancer types and identify targetable vulnerabilities.

Discussion

In this study, we identified targetable vulnerabilities in central carbon metabolism pathways using metabolic signatures generated from a ratio-based approach. We systematically analysed a panel of 57 proliferating cells of both tumour and normal epithelial tissue types from a targeted metabolite panel of central carbon and amino acid pathways. Focusing on the relationship between pyruvate and the TCA cycle, we revealed metabolic relationships of converging pathways to and from the TCA cycle that are overlooked when analysing metabolite abundances alone. In contrast to many pan-cancer studies, we found that metabolic signatures occurred independent of genetic oncogene drivers, culturing conditions, and tissue origins (Cherkaoui *et al*, 2022; Lagziel *et al*, 2020; Shorthouse *et al*, 2022). We verified our ratio-approach by demonstrating that drug sensitivity and gene knockout screens matched our model of Cluster 4 metabolic vulnerabilities. Taken together, our results suggest that metabolic phenotypes identified in subsets of diverse cancer types could lead to novel therapeutic approaches to target metabolism.

The ratio-metric approach we utilised explored the relationships of reactant and product metabolites of central carbon and amino acid pathways. This approach identified a subset of cancer cell lines from different tissue origins that had greater TCA cycle metabolite ratios relative to pyruvate and more efficiently utilised glucose and glutamine (Cluster 4 cells). By investigating pathways outside the direct uptake of glucose and glutamine, we observed a greater conversion of glutamine into precursors that fed into the auxiliary pathways of the TCA cycle, namely gluconeogenesis and lactate metabolism. The role of glutamine for gluconeogenesis has been shown in previous studies that described the function of phosphoenol carboxykinase that catalyses the reaction from oxaloacetate to PEP in colon, brain, and lung cancer cells for biomass generation and tumour growth (Grasmann *et al*, 2019; Liu *et al*, 2018; Vincent *et al*, 2015). Additionally, glutamine-derived production of lactate via glutaminolysis occurs in glioblastoma cells to produce NADPH and support fatty acid synthesis (DeBerardinis *et al*, 2007). OXPHOS activity was also increased reflecting higher glutamine anaplerosis and could be corroborated

by the knowledge of the tight coupling of TCA cycle and OXPHOS pathways, critical for energy production, cancer cell proliferation and viability (Ashton *et al*, 2018; Sica *et al*, 2020).

Complementing our ratio-metric based metabolic phenotypes, drug sensitivity and loss-of-function screens further supported the glucose and glutamine dependent relationships observed in Cluster 4 cells. For instance, among the top 20 drug sensitivity and gene knockouts we observed Cluster 4 cells to have a significant sensitivity to glutaminase (GLS) knockout. This association was complemented by other studies that have identified targeting GLS as an effective therapy in several cancer types such as triple-negative breast cancer, non-small cell lung cancer, and head and neck cancer (Gross *et al*, 2014; van den Heuvel *et al*, 2012; Wicker *et al*, 2021). Moreover, our analysis reached beyond the scope of our targeted metabolomics panel to identify that Cluster 4 cells are more sensitive to purine metabolism, which can also adaptively utilise glucose across cancers and shift to a reliance on glutamine to advance cancer progression (Kodama *et al*, 2020; Mullen & Singh, 2023). Consequently, our ratio-based method may provide a fast-tracked strategy to identify metabolic phenotypes and substrate dependencies for therapeutic implications across subsets of diverse cancer types.

While our ratio-based approach provided promising results to identify metabolic phenotypes in diverse cancer types, there were limitations to this method. Notably, we focused on the TCA cycle as a convergent point for glucose and glutamine utilisation, however whether these relationships are consistent for other TCA cycle substrates such as branched chain amino acids and fatty acids (Neinast *et al*, 2019; Schoors *et al*, 2015) remains to be seen. Additionally, we observed differential metabolite-ratios were less evident in other pathways such as glutamine metabolism which uses glutamine as a precursor metabolite for the ratio analysis. Therefore, our ratio-approach may be most effective at identifying metabolic phenotypes when choosing a precursor metabolite that is a convergent point for multiple substrates. For example, nucleotide metabolism is an essential process for metastasis in multiple cancer types and utilises both glucose and glutamine (Mullen & Singh, 2023). Future investigation into other metabolic hubs not covered in our targeted approach could identify common metabolic signatures and targetable vulnerabilities within subsets of diverse cancer types.

Our ratio-metric approach identified common metabolic signatures within subsets of diverse cancer types reflecting differential substrate utilisation. We anticipate that studying metabolic pathway relationships that span different tissue lineages will substantially contribute to the ongoing efforts to profile cancer metabolism for future therapeutic advances and the repurposing of metabolic targeting-based therapeutics among diverse cancer types.

Materials and Methods

Reagents and Tools Table

Reagent/Resource	Reference or Source	Identifier or Catalog Number
Experimental Models		
MCF10A	ATCC	ATCC CRL-10317 RRID: CVCL_0598
MDA-MB-231	ATCC	ATCC HTB-26 RRID: CVCL_0062
BT474	ATCC	ATCC HTB-20 RRID: CVCL_0179
MDA-MB-468	ATCC	ATCC HTB-132
MCF7	ATCC	ATCC HTB-22
BT20	ATCC	ATCC HTB-19
MDA-MB-134	ATCC	ATCC HTB-23
PNT1	ECACC	ECACC 95012614 RRID: CVCL_2163
PNT2	Prof. Lisa Butler, SAHMRI	Nassar <i>et al</i> , 2020
C4-2B	ATCC	ATCC CRL-3315
PC-3	ATCC	ATCC CRL-1435
22Rv1	ATCC	ATCC CRL-2505 RRID: CVCL_1045
LNCaP	ATCC	ATCC CRL-1740 RRID: CVCL_1379
DU145	A/Prof. Luke Selth, Flinders University	ATCC HTB-81
CWR-AD1	A/Prof. Luke Selth, Flinders University	Li <i>et al</i> , 2013; Nyquist <i>et al</i> , 2013
CWR-D567	A/Prof. Luke Selth, Flinders University	Nyquist <i>et al</i> , 2013
VCAP	Prof. Lisa Butler, SAHMRI	Nassar <i>et al</i> , 2020
V16D	Prof. Lisa Butler, SAHMRI	Nassar <i>et al</i> , 2020
MR49F	Prof. Lisa Butler, SAHMRI	Bishop <i>et al</i> , 2017
MR42D	Prof. Lisa Butler, SAHMRI	Bishop <i>et al</i> , 2017
AML12	Prof. Rob Parton, University of Queensland	Nagarajan <i>et al</i> , 2019
PH5CH8	A/Prof. Susan McLennan, University of Sydney	Nagarajan <i>et al</i> , 2019
HEPG2	A/Prof. Susan McLennan, University of Sydney	Nagarajan <i>et al</i> , 2019
HUH7	Prof. Mark Gorrell, University of Sydney	RRID: CVCL_0336
SKHEP1	Prof. Mark Gorrell, University of Sydney	RRID: CVCL_0525
HEPA1-6	Prof. Mark Gorrell, University of Sydney	RRID: CVCL_0327
HUE-T	Dr Frances Byrne, UNSW	Byrne <i>et al</i> , 2014

MAD11	Dr Frances Byrne, UNSW	Byrne <i>et al</i> , 2014
Ishikawa	Prof. Kyle Hoehn, UNSW	RRID: CVCL_2529
MFE296	Prof. Kyle Hoehn, UNSW	RRID: CVCL_1406
MFE319	Prof. Kyle Hoehn, UNSW	RRID: CVCL_2112
AN3CA	Prof. Kyle Hoehn, UNSW	RRID: CVCL_0028
RL952	Prof. Kyle Hoehn, UNSW	RRID: CVCL_0505
KLE	Prof. Kyle Hoehn, UNSW	RRID: CVCL_1329
HEC1A	Prof. Kyle Hoehn, UNSW	RRID: CVCL_0293
U251	Prof. Lenka Munoz, University of Sydney	RRID: CVCL_0021
U87	Prof. Lenka Munoz, University of Sydney	RRID: CVCL_3429
HPDE	A/Prof Thomas Grewal, University of Sydney	RRID: CVCL_4376
PANC1	A/Prof Thomas Grewal, University of Sydney	RRID: CVCL_0480
MIAPACA-2	A/Prof Thomas Grewal, University of Sydney	RRID: CVCL_0428
BXPC3	A/Prof Thomas Grewal, University of Sydney	RRID: CVCL_0186
ASPC1	A/Prof Thomas Grewal, University of Sydney	RRID: CVCL_0152
A2780	A/Prof. David Croucher, Garvan Institute	RRID: CVCL_0134
OVCAR3	A/Prof. David Croucher, Garvan Institute	RRID: CVCL_0465
SKOV3	A/Prof. David Croucher, Garvan Institute	RRID: CVCL_0532
A375	Dr Lorey Smith, Peter MacCallum Cancer Centre	ATCC CRL-1619 RRID: CVCL_0132
HT144	Dr Lorey Smith, Peter MacCallum Cancer Centre	ATCC HTB-63 RRID: CVCL_0318
WM266.4	Dr Lorey Smith, Peter MacCallum Cancer Centre	ATCC CRL-1676 RRID: CVCL_2765
DO4-M1	Dr Lorey Smith, Peter MacCallum Cancer Centre	Parmenter <i>et al</i> , 2014
A549	Dr Lorey Smith, Peter MacCallum Cancer Centre	Chen <i>et al</i> , 2019
NCI-H226	Dr Lorey Smith, Peter MacCallum Cancer Centre	RRID: CVCL_1544
Detroit562	A/Prof Thomas Grewal, University of Sydney	RRID: CVCL_1171
SCC4	A/Prof Thomas Grewal, University of Sydney	RRID: CVCL_1684
HT29	A/Prof. Kellie Charles, University of Sydney	RRID: CVCL_A8EZ
HCT116	A/Prof. Kellie Charles, University of Sydney	RRID: CVCL_0291

SW-48	A/Prof. Kellie Charles, University of Sydney	RRID: CVCL_1724
SW-1417	A/Prof. Kellie Charles, University of Sydney	RRID: CVCL_1717
Chemicals, Enzymes and other reagents		
Enzalutamide MDV3100	Selleckchem	Cat#S1250
D-Glucose	Sigma-Aldrich	Cat#273053
L-Glutamine	Gibco	Cat#25030081
Fatty acid free BSA	Bovogen	Cat#BSAS0.10
D-Glucose (U-13C6, 99%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-1396
L-Glutamine (U-13C5, 99%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-1822-H
Software		
GraphPad Prism V9	GraphPad Software	https://www.graphpad.com/
MATLAB	Mathworks	https://au.mathworks.com/products/matlab.html
MSConvert	N/A	Chambers <i>et al</i> , 2012
MSDial	Riken	http://prime.psc.riken.jp/compms/msdial/main.html
Biorender	Biorender	https://biorender.com/
Other		
Resipher	Lucid Scientific	https://lucidsci.com/
Incucyte-SX5	Sartorius	https://www.sartorius.com/

Cell lines and culture conditions

A total of 57 adherent cell lines (8 normal and 49 tumour) spanning 11 cancer types were used in this study. Cell lines obtained from vendors or that were kindly provided by academic labs, overlapped with major cell line resources such as the Cancer Cell Line Encyclopedia (CCLE) and National Cancer Institute 60 panel (NCI60) (Appendix table 1). Mutational status of common oncogenes was characterised by cross-referencing the Cellosaurus database, COSMIC database, and Depmap portal. Culturing media were supplemented with 10% fetal bovine serum (Cytiva Hyclone) and 1% penicillin/streptomycin (Gibco) unless stated otherwise (Appendix table 1). Cells were incubated at

37°C and 5% CO₂. The full panel of cells were generated across 6 batches over a period of 3 years due to COVID restrictions and included overlapping cell lines to correct for batch effects.

Metabolomics experiments

Cells were seeded in triplicate in 6-well plates at a density of 5×10^5 cells/well in 2mL of media. After 24 hours, the media was removed and cells were washed once with 2mL ice-cold 0.9% w/v NaCl. Cells were then scraped with 300µL of extraction buffer, EB, (1:1 LC/MS methanol:water (Optima) + 0.1x internal standards comprised of non-endogenous polar metabolites (2-morpholinoethanesulfonic acid, D-camphor-10-sulfonic acid and deuterated thymidine) and transferred to a 1.5mL microcentrifuge tube. A further 300µL of EB was added to the well and combined in the tube. 600uL Chloroform (Honeywell) was added before vortexing and incubating on ice for 10 minutes. Tubes were vortexed briefly and centrifuged at 15, 000xg for 10min at 4°C. The aqueous layer was collected and dried without heat, using a Savant SpeedVac (Thermo Fisher). Dried samples were resuspended in 40µL Amide buffer A (20mM ammonium acetate, 20mM ammonium hydroxide, 95:5 HPLC H₂O: Acetonitrile (v/v)) and vortexed and centrifuged at 15, 000xg for 5min at 4°C. 20µL of supernatant was transferred to HPLC vials containing 20µL acetonitrile for LC-MS analysis of amino acids and glutamine metabolites. The remaining 20µL of resuspended sample was transferred to HPLC vials containing 20µL LC-MS H₂O for LCMS analysis of glycolytic, pentose phosphate pathway (PPP), and TCA cycle metabolites. Amino acids and glutamine metabolites were measured using the Vanquish-TSQ Altis (Thermo) LC-MS/MS system. Analyte separation was achieved using a Poroshell 120 HILIC-Z Column (2.1x150mm, 2.7µm) (Agilent) at ambient temperature. The pair of buffers used were Amide buffer A and 100% acetonitrile (Buffer B), flowed at 200µL/min; injection volume of 5µL. Glycolytic, PPP and TCA cycle metabolites were measured using 1260 Infinity (Agilent)-QTRAP6500+ (AB Sciex) LC-MS/MS system. Analyte separation was achieved using a Synergi 2.5µm Hydro-RP 100A LC Column (100x2mm) at ambient temperature. The pair of buffers used were 95:5 (v/v) water:acetonitrile containing 10mM tributylamine and 15mM acetic acid (Buffer A) and 100%

acetonitrile (Buffer B), flowed at 200 μ L/min; injection volume of 5 μ L. Raw data from both LC-MS/MS systems were extracted using MSConvert (Chambers *et al*, 2012) and in-house MATLAB scripts. Concentrations of metabolites were calculated against a standard curve of polar and amino acid metabolite standards similarly extracted as above. Log10 normalisation was performed on the metabolite concentration data.

Metabolomics batch correction

Cell line samples were quantified over 6 batches during the experiment including overlapping cell lines across each batch. To eliminate potential batch effects, we applied the normalisation method: Removing Unwanted Variation-III (Kim *et al*, 2021; Molania *et al*, 2019). The cell lines that were measured across multiple runs were used as replicates for the batch correction algorithm and to assess the quality of the batch correction output.

Metabolomics clustering analysis

Clustering was performed on the batch-corrected matrix using K-means algorithms with Pearson correlation as the distance matrix (Gu *et al*, 2016). This was repeated 1000 times and the consensus taken to ensure a more robust clustering result. The clustering strategy was applied to both the original batch-corrected matrix and the ratio-transformed matrix. To confirm that the obtained clustering was not influenced by potential confounding factors, such as culturing conditions and common oncogenic gene mutations and tissue type, we added a colour-coded legend on culturing conditions and mutational information to the clustering heatmap.

The original batch-corrected matrix was subset to targeted metabolites from several key metabolic pathways including the TCA cycle, pentose phosphate pathway, glycolysis, amino acid metabolism, and glutamine metabolism, followed by clustering analysis. For the ratio-transformed matrix, batch-corrected abundance ratios were calculated between a precursor metabolite for each pathway (used as the denominator) and the remaining pathway metabolites. The corresponding precursor (denominator)

metabolites were pyruvate for the TCA cycle, glucose for PPP and glycolysis, and glutamine for the glutamine metabolism pathway. For amino acid pathways, serine, proline, and methionine were used as the precursor metabolites. The clustering structure determined from the processes above were also used to visualise the oncogene mutation status, tissue type, cancer type, tissue origin, and culturing media type of the cell lines using a heatmap and to inspect the relationship of these variables with the determined clusters.

U-¹³C stable isotope tracing

Cells were seeded in triplicate in 6-well plates at a density of 5×10^5 cells/well in 2mL of media. After 24 hours wells were washed with warm PBS and media replaced with 600 μ L of DMEM no glucose, no glutamine media supplemented with 2% (wt/vol) FA-free BSA, and 5mM glucose and 1mM glutamine, replaced with their respective U-¹³C forms. Cells were incubated in U-¹³C containing medium for 6 hours. Samples were extracted and measured using the Vanquish-TSQ Altis and Agilent-QTRAP6500+.

Extracellular substrate experiments

Cells were seeded in triplicate in 6-well plates at a density of 5×10^5 cells/well in 2mL of media. After 24 hours wells were washed with warm PBS and media replaced with 1mL of DMEM no glucose, no glutamine media supplemented with 5mM glucose, 1mM glutamine and 150 μ M palmitate. 100 μ L of extracellular media were collected from wells at 3, 6, 12, 24 hour timepoints. Media samples were centrifuged at 16, 000xg for 5min at 4°C and the supernatant collected for subsequent extraction and LC-MS analysis.

To extract media samples for LC-MS, 20 μ L of supernatant media was first diluted with 80 μ L water and vortexed, and then 10 μ L of the diluted media was transferred to 90 μ L of extraction buffer containing 1:1 (v/v) acetonitrile and methanol + 1x internal standards (non-endogenous standards) at -

30°C. The mixture was centrifuged at 12,000xg for 5min at 4°C and transferred into HPLC vials for LC-MS analysis measured using the Vanquish-TSQ Altis.

Oxygen consumption rate

Cells were seeded in 96-well plates (Nunc) in 100µL basal medium. Oxygen consumption rates (OCR) were continuously measured using Resipher (Lucid Scientific) at 37°C, 10% CO₂ as per manufacturer instructions over 48 hours, starting 24 hours post-seeding. Media volumes were replenished at 24 hours. Cell-free wells contained 200µL of PBS to avoid evaporation. To account for differences in cell growth over 48 hours, parallel plates were similarly cultured, and media replenished at 24 hours for cell confluency measured using IncuCyte-SX5 (Sartorius).

Analysis of drug and CRISPR databases

Drug sensitivity area under the curve (AUC) data were downloaded from the PRISM (Corsello *et al*, 2020) and GDSC2 (Yang *et al*, 2012) databases. Loss-of-function fitness score data were downloaded from the DEMETER (Tsherniak *et al*, 2017) and Project Score (Behan *et al*, 2019) databases. First, cell lines were filtered by overlapping cell lines within our panel, and then inhibitor or loss-of-function gene targets were filtered by KEGG metabolic pathway genes (Kanehisa & Goto, 2000). We then performed a differential expression analysis on the drug response of the cell lines belonging to clusters of interest, specifically the response of Cluster 3 cell lines versus Cluster 4 cell lines. The top 20 drugs or gene targets with the greatest differential response between Clusters 3 and 4 were identified.

Quantification and statistical analysis

For all quantifications, at least 3 technical replicates and 3 independent biological replicates were used for each sample group. Data are presented as mean ± standard error of the mean (SEM), mean ± standard deviation (SD), or mean ± min to max values, as indicated in figure legends. Data were analysed using unpaired Student's t-test or multiple unpaired t-tests. Statistical significance is indicated in all figures

by the following annotations * $p < 0.05$. P values > 0.05 were considered not statistically different. Prism (GraphPad V9) was used for statistical analysis. R-studio was used for clustering methods and heatmaps. Schematic diagrams were created with Biorender.com.

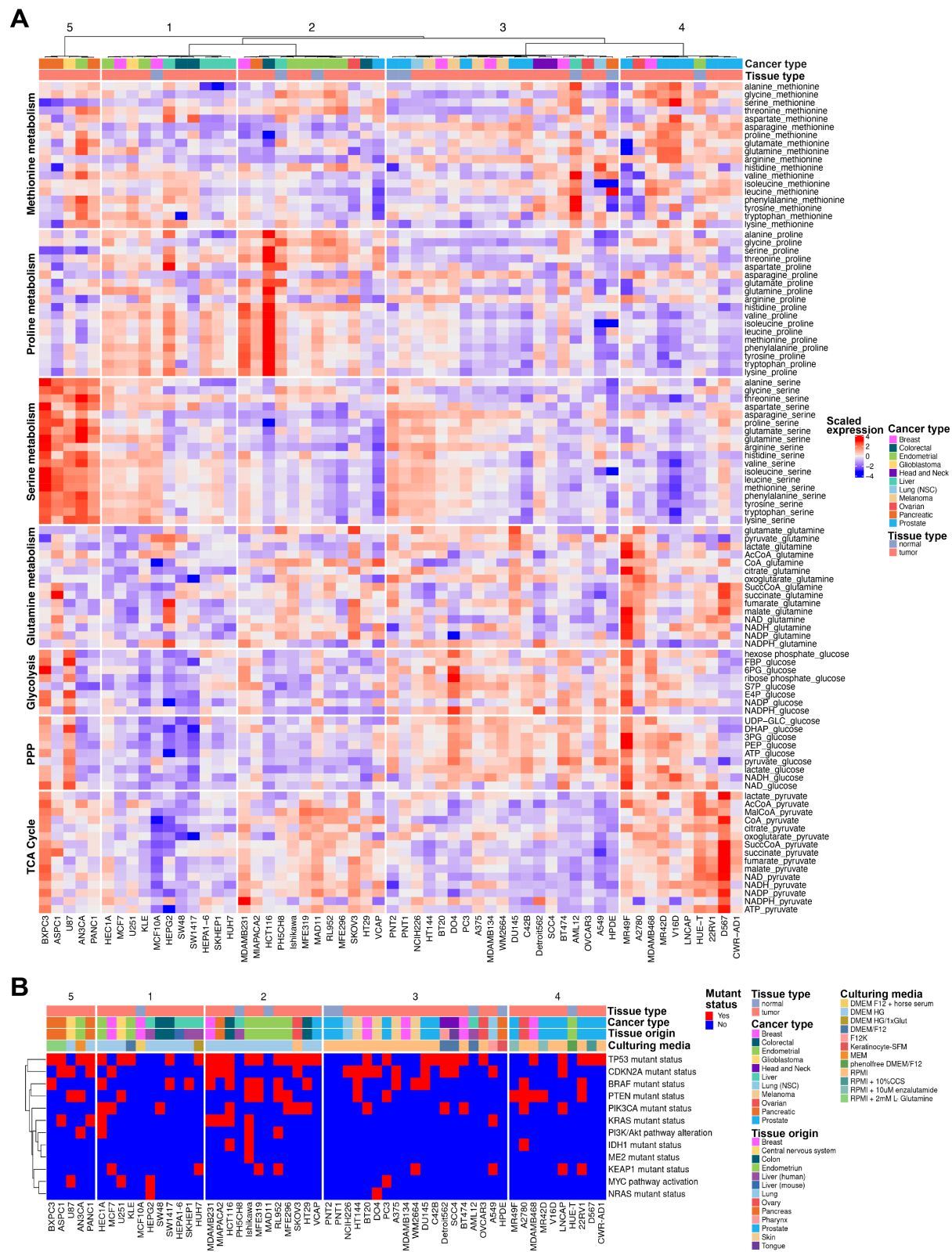
Appendix

Table 1: Cell line details

Cell line	Cancer type	Tissue type	Culture media	Overlap with CCLE (Yes/No)	Overlap with NCI60 (Yes/No)
MCF10A	Breast	normal	DMEM F12 + horse serum + EGF + hydrocortisone + insulin	N	N
MDA-MB-231		tumour	DMEM HG	Y	Y
BT474		tumour	RPMI	Y	N
MDA-MB-468		tumour	RPMI	Y	Y
MCF7		tumour	DMEM HG	Y	Y
BT20		tumour	RPMI	Y	N
MDA-MB-134		tumour	RPMI	Y	N
PNT1	Prostate	normal	RPMI	N	N
PNT2		tumour	RPMI	N	N
C4-2B		tumour	RPMI	Y	Y
PC-3		tumour	RPMI	Y	N
22Rv1		tumour	RPMI	Y	N
LNCaP		tumour	RPMI	Y	Y
DU145		tumour	RPMI	N	N
CWR-AD1		tumour	RPMI	N	N
CWR-D567		tumour	RPMI + 10%CCS	N	N
VCAP		tumour	DMEM HG	Y	N
V16D		tumour	RPMI	N	N
MR49F		tumour	RPMI + 10µM enzalutamide	N	N
MR42D		normal	RPMI + 10µM enzalutamide	N	N
AML12	Liver	normal	DMEM/F12	N	N
PH5CH8		normal	DMEM HG	N	N
HEPG2		tumour	DMEM HG	Y	N
HUH7		tumour	DMEM HG/1xGlut	Y	N
SKHEP1		tumour	DMEM HG	Y	N
HEPA1-6		tumour	DMEM HG	N	N

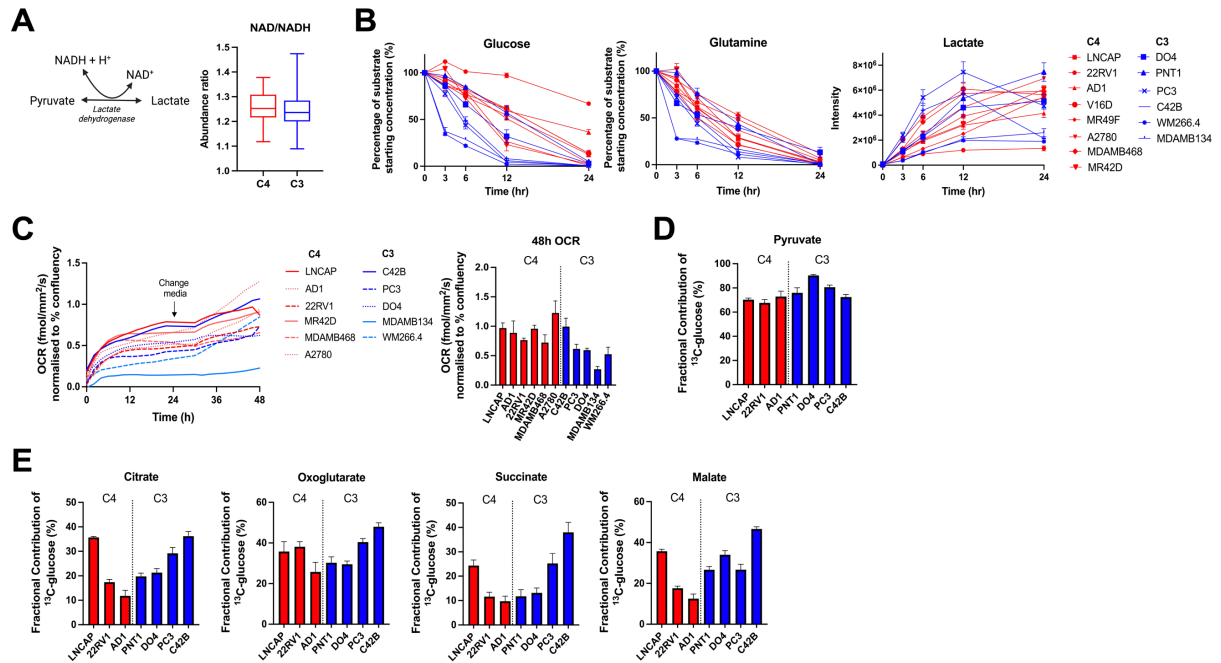
HUE-T	Endometrial	normal	Phenol-free DMEM/F12	N	N
MAD11		normal	DMEM HG	N	N
Ishikawa		tumour	DMEM HG	Y	N
MFE296		tumour	DMEM HG	Y	N
MFE319		tumour	DMEM HG	Y	N
AN3CA		tumour	MEM	Y	N
RL952		tumour	DMEM HG	Y	N
KLE		tumour	DMEM/F12	Y	N
HEC1A		tumour	DMEM HG	Y	N
U251	Glioblastoma	tumour	DMEM HG	Y	Y
U87		tumour	DMEM HG	Y	N
HPDE	Pancreatic	normal	Keratinocyte-SFM	N	N
PANC1		tumour	DMEM HG	Y	N
MIAPACA-2		tumour	DMEM HG	Y	N
BXPC3		tumour	RPMI + 2mM L-Glutamine	Y	N
ASPC1		tumour	RPMI + 2mM L-Glutamine	Y	N
A2780	Ovarian	tumour	RPMI	Y	N
OVCAR3		tumour	RPMI	Y	Y
SKOV3		tumour	RPMI	Y	Y
A375	Melanoma	tumour	RPMI	Y	N
HT144		tumour	RPMI	Y	N
WM266.4		tumour	RPMI	Y	N
DO4-M1		tumour	RPMI	N	N
A549	Lung (NSC)	tumour	F12K	Y	Y
NCI-H226		tumour	RPMI	Y	Y
Detroit562	Head and Neck	tumour	DMEM/F12	Y	N
SCC4		tumour	DMEM/F12	Y	N
HT29	Colorectal	tumour	DMEM HG	Y	Y
HCT116		tumour	DMEM HG	Y	Y
SW-48		tumour	DMEM HG	Y	N
SW-1417		tumour	DMEM HG	Y	N

Appendix Figures



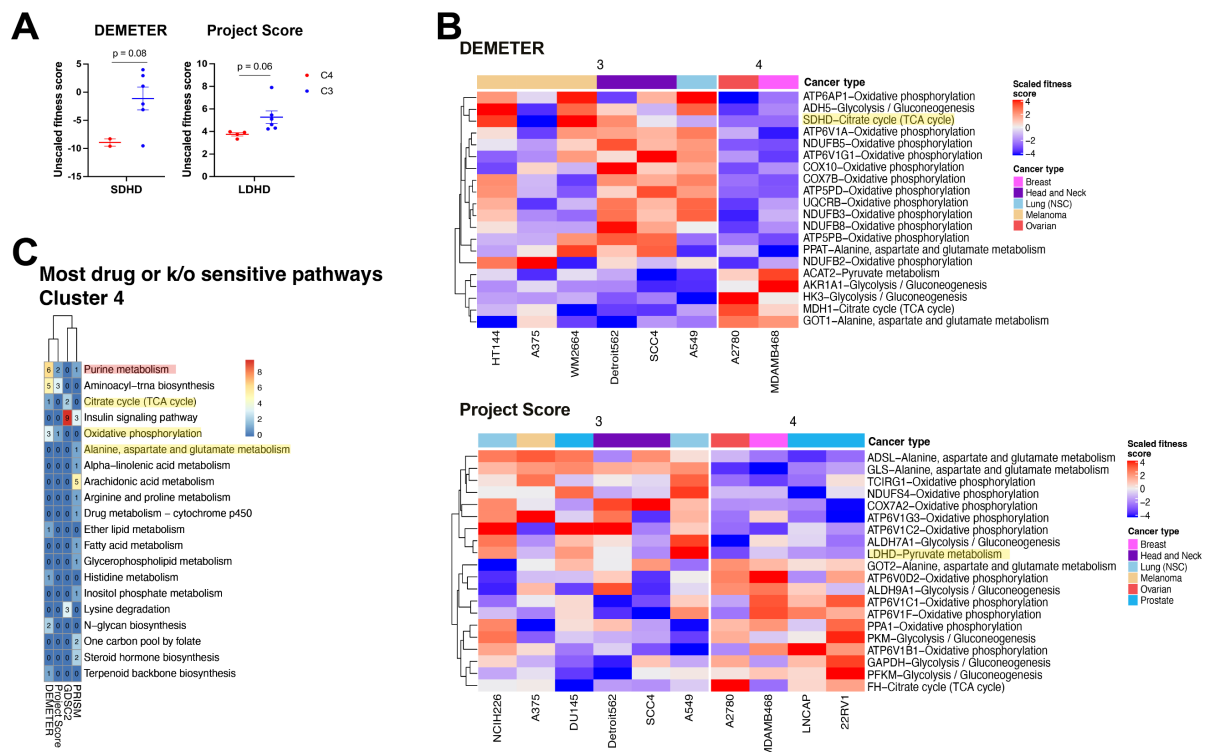
Appendix Figure S1: Ratio-based metabolic of all targeted metabolic pathways.

- Heatmap of scaled metabolite ratios by pathways covered in the targeted metabolomics approach. Ratios calculated for all metabolites of a specific pathway against a precursor metabolite.
- Clusters of cell lines from (A) appended with colour coded legends for mutant status of common oncogenic drivers, culturing media conditions and tissue origins.



Appendix Figure S2: Substrate preferences for cell line clusters identified by TCA cycle ratios.

- Schematic of the lactate dehydrogenase catalysed reaction. Comparison of Clusters 4 and 3 ratios of lactate to pyruvate (Unpaired student t-test, ns $p > 0.05$, error min to max values).
- Glucose and glutamine consumption and lactate production over a time course to 24 hours (C4 n=8, C3 n=6, 3 biological replicates and 3 technical replicates per cell line, mean $\pm$ standard error of the mean).
- Oxygen consumption rates (OCR) measured over a continuous time course to 48 hours, normalised to % confluency. Media changed at 24 hours (left). OCR measurement for cell lines at 48 hours (right) (C4 n=6, C3 n=5, 3 biological replicates and 4 technical replicates per cell line).
- Fractional contribution of [U- ^{13}C]-glucose to intracellular pyruvate (C4 n=3, C3 n=4, 3 biological replicates and 3 technical replicates per cell line, mean $\pm$ standard error of the mean).
- Fractional contribution of [U- ^{13}C]-glucose to TCA metabolites citrate, oxoglutarate, succinate and malate (C4 n=3, C3 n=4, 3 biological replicates and 3 technical replicates per cell line, mean $\pm$ standard error of the mean).



Appendix Figure S3: Loss of function and drug database screen validation of ratio-based metabolite clusters.

- Genes related to the glucose and glutamine utilisation phenotype of C4 with trends of greater sensitivity to gene knockouts. (Unpaired student t-test, ns $p > 0.05$, mean $\pm$ standard error of the mean, $n = 2-6$).
- Top 20 gene knockouts and associated KEGG pathways with the greatest fitness scores between C4 and C3 in DEMETER and Project Score databases from glycolysis, TCA cycle, pyruvate metabolism, glycolysis/gluconeogenesis, and alanine, aspartate and glutamate metabolism pathways. Drugs and pathways matching C4 vulnerability model are highlighted in yellow.
- Metabolic pathways associated with the top 20 drug and gene knockout sensitives for Cluster 4 for each database. Drugs and pathways matching C4 vulnerability model (yellow) and the top hit purine metabolism (red) are highlighted.

Acknowledgements

N.T.S. is supported by the Australian Rotary Health/Rotary Club of Blacktown City ‘Mel Grey’ PhD scholarship. A.J.H. is supported by a Robinson Fellowship. This work was supported by funding from The University of Sydney. We thank the Sydney Mass Spectrometry facility for access to LC-MS instruments; and all collaborators who kindly donated cell lines.

Author contributions

Nancy T Santiappillai: Project administration, formal analysis, investigation, validation, visualisation, writing – original draft, writing -review & editing. **Yue Cao:** Formal analysis, methodology, software, validation, visualisation. **Mariam Hakeem-Sanni:** Investigation. **Jean Yang:** Methodology, supervision. **Lake-Ee Quek:** Conceptualisation, methodology, project administration, software, supervision, writing – review & editing. **Andrew J Hoy:** Conceptualisation, funding acquisition, project administration, resources, supervision, writing – review & editing.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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Chapter 3

Mitochondrial long chain fatty acid oxidation is a minor TCA cycle carbon source in prostate cancer.

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Summary

Mitochondrial fatty acid oxidation (FAO) has been proposed to be a major bioenergetic pathway in prostate cancer. However, when evaluating the proportion and magnitude of long chain fatty acids (LCFAs) that are oxidised, it is important to consider this relative to other mitochondrial substrates. Here, we found extracellular LCFAs are minor sources of carbon entering the TCA cycle compared to glucose and glutamine, despite being assimilated in the mitochondria as acyl-carnitines. Instead, LCFAs were incorporated into cardiolipins in substantial amounts (>50%) within 6 hours, suggesting a key role in supporting cardiolipin turnover. Knockdown of the rate-limiting enzyme CPT1a did not impair TCA cycle activity but reduced the incorporation of LCFAs into cardiolipins. Surprisingly, CPT1a had a marginal role in regulating cellular respiration via FAO compared to cardiolipin remodelling. We propose that observations of a CPT1a-dependent reduction of FAO and mitochondrial function can be explained, at least in part, by perturbed cardiolipin maturation and reduced oxidative phosphorylation efficiency.

Keywords

Fatty acid oxidation, parallel stable isotope tracing, TCA cycle, cardiolipin, prostate, metabolomics

Introduction

Fatty acids (FAs) are important substrates for the progression and function of cancers including prostate cancer (PCa) cells that predominantly uptake and incorporate extracellular long chain FAs (LCFAs) into their total lipid pools compared to *de novo* synthesised FAs¹. Known lipid metabolic reprogramming in PCa include: the overexpression of lipid metabolism genes²⁻⁴, enhanced FAO compared to benign epithelial cells and tissues^{1,5,6}, substantial sensitivity to FA oxidation (FAO) and FA synthesis inhibition^{7,8}, lipid profile alterations associated with PCa progression⁸, and increased citrate oxidation for lipogenesis^{9,10}. Consequently, PCa is characterised as a lipid-dependent cancer type and FAO is claimed to be the predominant bioenergetic source^{11,12} because evidence for FAO metabolic reprogramming was not presented in the context of other mitochondria substrates. There are contradictions showing PCa cells consume non-trivial amounts of glucose and glutamine^{6,13} and can shift along a spectrum of glucose and lipid utilisation from benign to castration resistant PCa^{14,15}. This highlights a lingering ambiguity of the proportions in which these substrates fuel the tricarboxylic acid (TCA) cycle.

The contemporary view is that mitochondria LCFAs are almost entirely shunted into beta-oxidation, producing NADH, FADH₂ for the electron transport chain (ETC) and acetyl-CoA for the TCA cycle¹⁶. LCFAs enter the mitochondria through the carnitine shuttle system that includes the carnitine palmitoyltransferase isoform (CPT1a) located in the outer mitochondrial membrane¹⁷. CPT1a is viewed as the rate-limiting step of FAO because its inhibition or knockdown decreases ATP production and causes cell death in cancers^{1,18-20}. However, since overexpression of CPT1a is strongly associated with PCa progression²¹, we question whether there are other significant mitochondrial processes in PCa that could explain the observed cell death and decreased ATP production when CPT1a is inhibited. In breast cancer, Yao *et al*²² (2018) showed that CPT1a is needed for anabolic processes. Namely, they reported decreased mitochondrial lipid levels and altered mitochondrial morphology in CPT1a knockdowns. Together, these results raise the possibility that CPT1a is essential for maintaining intra-mitochondrial lipid homeostasis and questions the sole commitment of mitochondria LCFAs into beta-oxidation. In

this study, we set out to test whether FAO provides a significant carbon source to the TCA cycle in PCa and determine if LCFAs contribute to other significant mitochondrial fates.

Results

Glucose and glutamine are the major TCA cycle carbon sources in prostate cancer cells.

We set out to quantify, in parallel, the conversion of extracellular glucose, glutamine and LCFAs into TCA cycle metabolites in PCa cells. We first established the baseline FAO rates for a panel of PCa cells spanning the spectrum of disease from benign PNT1 cells to late-stage PC-3 cells. We know that PCa cells have greater FAO rates compared to benign cells^{1,5,6}, and using radiolabelled palmitate, here showed 2-4- fold greater FAO rates of PCa cells compared to PNT1 benign cells (Figure 1A). These differences in baseline FAO rates could not be explained by cellular active mitochondria amount (Figure 1B and 1C), nor by CPT1a protein levels (Figure 1D and 1E). We then performed parallel [U-¹³C] isotope tracing experiments to quantify the relative incorporation of uniformly labelled palmitate (FA 16:0), glucose, and glutamine carbons into TCA cycle metabolites. Palmitate was chosen because it is the most abundant FA circulating in humans²³. We determined that 6 hours was the optimal endpoint for our tracing experiments as isotopic steady-state was reached, and media substrates were not depleted (>50% of starting levels) (Figure S1A). Further, 3 to 6 hours of label exposure has been shown to reach isotopic steady state from ¹³C-glucose and -glutamine^{24,25}. We separately noted that doubling of media volume to 2mL was necessary for a 12 to 24 hour experiment, in order to maintain media substrates above 50% (Figure S1B).

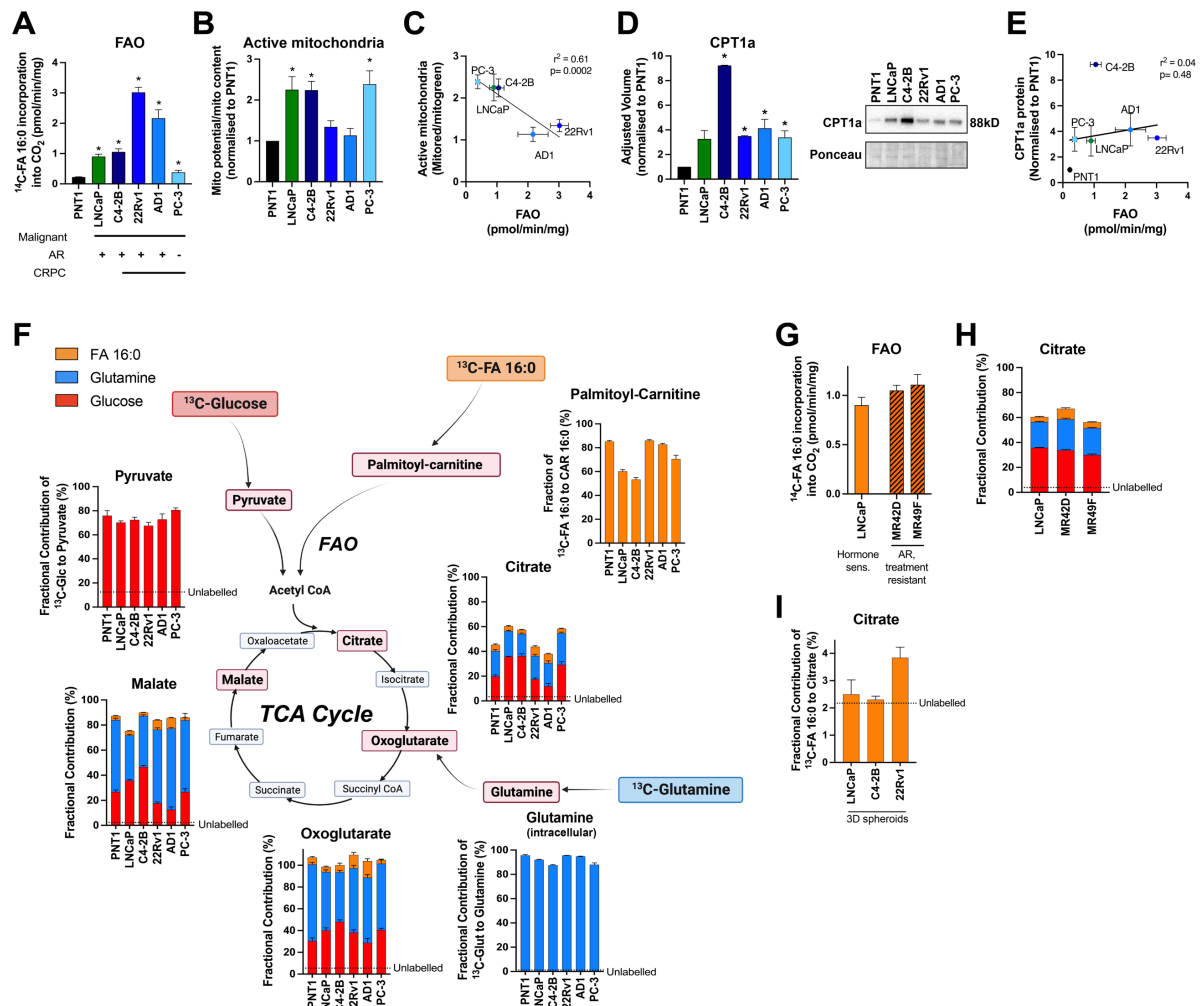


Figure 1 Palmitate is a minor TCA cycle substrate source compared to glucose and glutamine.

- (A) Fatty acid [$1\text{-}^{14}\text{C}$]-FA 16:0 oxidation rates. N=3-5 per cell line. FAO rates were compared to PNT1 cells.
- (B) Active mitochondria as a fraction of mitochondrial potential to mitochondrial content normalised to PNT1 cells. N=3-4 per cell line.
- (C) Correlation of active mitochondria content to FAO excluding PNT1 cells. Error bars represent $\pm$ SD. P and r^2 values by linear regression.
- (D) Quantification of CPT1a protein expression normalised to PNT1 cells. N=3 per cell line. A representative immunoblot is shown.
- (E) Correlation of CPT1a protein levels to FAO. Error bars represent $\pm$ SD. P and r^2 values by linear regression.
- (F) Fractional contribution of [$\text{U-}^{13}\text{C}$]-glucose to pyruvate (red), [$\text{U-}^{13}\text{C}$]-glutamine to intracellular glutamine (blue), [$\text{U-}^{13}\text{C}$]-FA 16:0 to palmitoyl-carnitine (orange) after 6 hours of labelling. [$\text{U-}^{13}\text{C}$]-glucose, glutamine, or 16:0 to citrate, oxoglutarate and malate. N=3 per cell line. Level of natural enrichment of metabolite standards indicated.
- (G) Fatty acid [$1\text{-}^{14}\text{C}$]-16:0 oxidation rates of treatment resistant MR42D and MR49F cells compared to treatment sensitive LNCaP cells. N=3 per cell line.
- (H) Fractional contribution of [$\text{U-}^{13}\text{C}$]-glucose, glutamine, and FA 16:0 to citrate in MR42D, MR49F and LNCaP cells. N=3 per cell line.
- (I) Fractional contribution of [$\text{U-}^{13}\text{C}$]-FA 16:0 to citrate in LNCaP, C4-2B and 22RV1 3D spheroid models. N=3 per cell line.

Graphs show mean $\pm$ SEM unless stated otherwise. * $p < 0.05$ by One-way ANOVA with Dunnett's multiple comparisons test, unless stated otherwise. See also Figure S1.

After 6 hours, we achieved sufficient labelling of the respective intracellular TCA cycle precursor pools: pyruvate >65%, glutamine >85%, and palmitoyl-carnitine >60% (Figure 1F). This also confirmed the uptake of labelled substrates. The fractional contribution of [U-¹³C]-glucose and [U-¹³C]-glutamine, was calculated as the weighted sum by the number of labelled carbons²⁶, and combined, reached between 60-80% for all measured TCA cycle metabolites (Figure 1F). In contrast, extracellular [U-¹³C]-palmitate contributed less than 15% of carbons to TCA cycle metabolites (Figure 1F and S1C-D). 22Rv1 and AD1 cells achieved the greatest ¹³C-palmitate enrichment, which was consistent with their high FAO rates (Figure S1C). In general, the fractional contribution of palmitate to TCA cycle metabolites weakly correlated with FAO and inversely correlated with active mitochondria content (Figure S1D and S1E). Overall, non-trivial amounts of carbon from extracellular palmitate oxidation did enter the TCA cycle, but this influx is dwarfed by glucose and glutamine.

Next, we explored two additional PCa models known to exhibit higher FAO, in a bid to detect increased incorporation of palmitate carbons among TCA cycle metabolites. Treatment resistant PCa cells MR42D and MR49F²⁷ have been reported to exhibit enhanced FA uptake and oxidation^{28,29}. However, in our hands, FAO rates for both cell lines were not different to treatment sensitive LNCaP cells (Figure 1G). Parallel tracing results for MR42D and MR49F cells reiterated that palmitate fractional contributions were not different from LNCaP cells (4-8.5%), as citrate was largely derived from glucose (35%) and glutamine (20%) (Figure 1H and S1G). The same parallel tracing was also performed on 3D PCa spheroid models as they have been shown to exhibit a more oxidative phenotype than 2D cell cultures³⁰⁻³². However, we still observed minimal contribution of palmitate into TCA cycle metabolites (<6%) (Figure 1I and S1J). In both our treatment resistant and 3D spheroid models, we verified that media glucose, glutamine, and palmitate were still sufficient prior to harvesting cells at 6 hours (Figure S1A and S1H), and that substantial ¹³C labelling of precursor pools were achieved (Figure S1F and S1I). Altogether, glucose and glutamine remained the major TCA cycle substrates compared to palmitate in a range of prostate cells regardless of disease stage pathophysiology, treatment responsiveness, or multicellular microenvironment.

Palmitate oxidation increases in the absence of glucose and glutamine but is insufficient to maintain TCA cycle function.

In basal conditions, we showed FAO is a marginal TCA cycle input (Figure 1). However, cancer metabolism is highly dynamic and adapts to glucose and glutamine deprived settings^{33,34}. We next examined whether FAO in PCa cells can be increased by withdrawing glucose, and hence we would see a greater palmitate incorporation into the TCA cycle. Compared to baseline FAO rates in the presence of 5mM glucose, FAO in glucose-free conditions were 1- to 5- fold greater across the panel of prostate cells (Figure 2A). Furthermore, FAO rates increased across a dose-dependent glucose titration (0mM to 5mM), most significantly in complete glucose deprivation (Figure S2A).

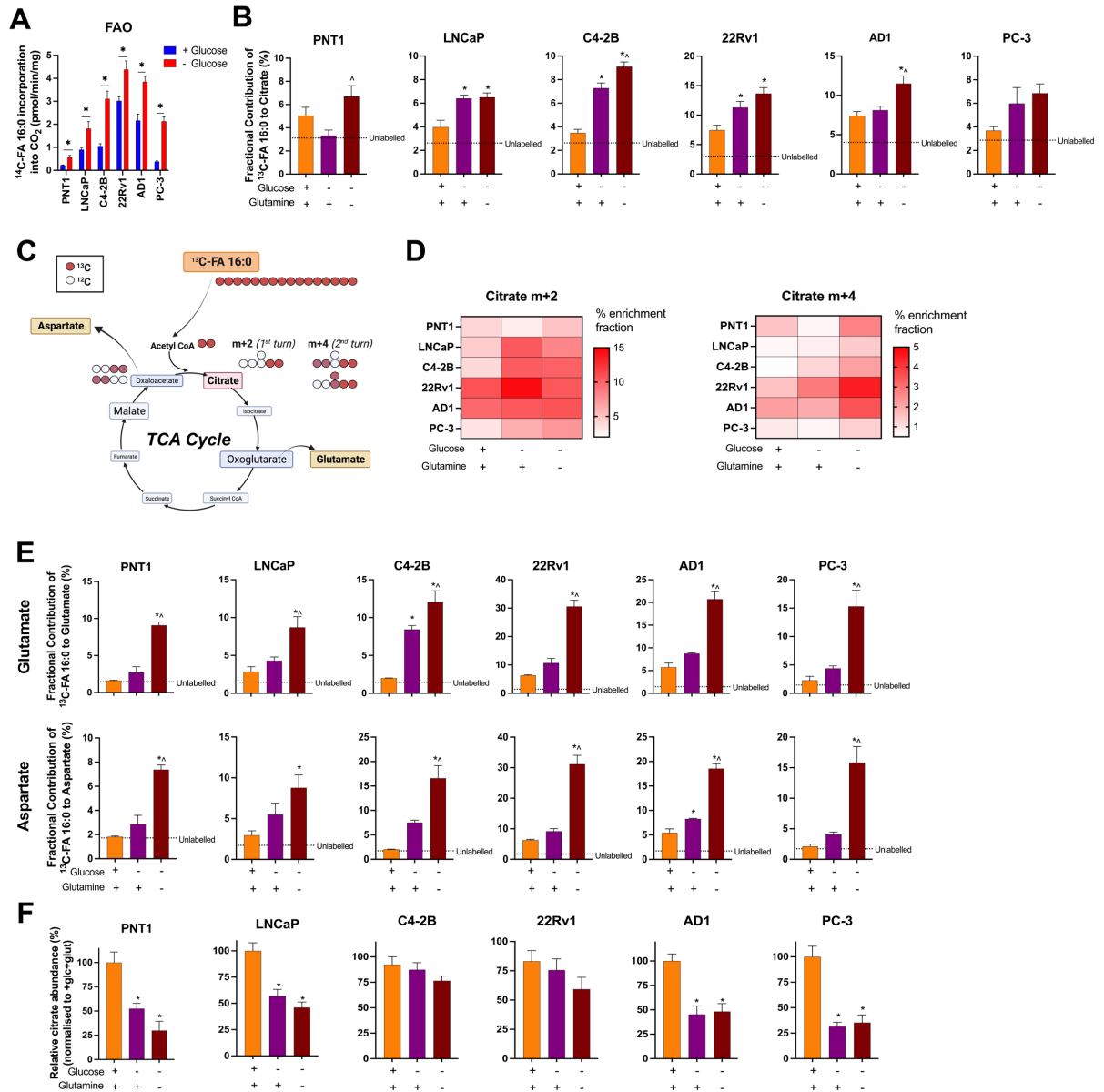


Figure 2 Palmitate alone is insufficient at maintaining TCA cycle activity.

- (A) [1-¹⁴C]-FA 16:0 oxidation rates in the presence (blue) or absence (red) of glucose. N=3-5 per cell line.
- (B) Fractional contribution of [U-¹³C]-FA 16:0 to citrate with or without glucose and/or glutamine. N=3 per cell line.
- (C) Schematic tracing conversion of [U-¹³C]-FA 16:0 into citrate m+2 and m+4 isotopologues.
- (D) Enrichment fraction (%) of [U-¹³C]-FA 16:0 to m+2 and m+4 citrate isotopologues in +glucose/+ glutamine, - glucose/+glutamine, and -glucose/-glutamine media conditions. N=3 per cell line.
- (E) Fractional contribution of [U-¹³C]-FA 16:0 to glutamate and aspartate +glucose/ glutamine, - glucose/+glutamine, or -glucose/-glutamine. N=3 per cell line.
- (F) Relative abundance (normalised to +glucose/+glutamine) of [U-¹³C]-FA 16:0 to citrate. N=3 per cell line.

Graphs show mean $\pm$ SEM. P<0.05 by One-way ANOVA with Dunnett's multiple comparisons test, *compared to +glucose/+glutamine, ^compared to -glucose/+glutamine. See also Figure S2.

We quantified the extent that extracellular palmitate could compensate for the absence of glucose and/or glutamine. We performed palmitate tracing in glucose-free and glucose-/ glutamine-free media conditions for 6 hours. We verified the short period of nutrient deprivation had no significant impact on metabolic activity (MTT) or cell viability (Figure S2B and S2C). The fractional contribution of palmitate to citrate was increased in glucose-free and glucose-/ glutamine-free conditions, but it remained minimal (~4% to ~6-8%) (Figure 2B). PNT1 benign and AD1 PCa cells showed increased palmitate labelling to citrate only in the absence of both glutamine and glucose, but not glucose alone (Figure 2B). These results suggest that PNT1 and AD1 cells mobilised glutamine first instead of palmitate to compensate for glucose deprivation. In the absence of glucose and glutamine, palmitate produced markedly more m+2 and m+4 citrate isotopologues, which represent palmitate utilisation in the first and second turns of the TCA cycle, respectively (Figure 2C and 2D). This suggests that palmitate carbons could sustain TCA cycle turnover under glucose and glutamine deprivation.

We next sought to identify other dynamic changes in the usage of palmitate-derived carbons during glucose and glutamine deprivation. We focused on cataplerosis-derived metabolites glutamate and aspartate, which are produced from oxoglutarate and oxaloacetate respectively (Figure 2C). Firstly, we observed essentially no incorporation of ^{13}C -palmitate into glutamate and aspartate in cells cultured in media containing physiological levels of glucose and glutamine (Figure 2E). In glucose-free, and most strikingly in glucose-/glutamine-free conditions, we saw significant ^{13}C -palmitate labelling into glutamate and aspartate (Figure 2C and 2E). As aspartate synthesis is essential for cell proliferation³⁵ and glutamate for glutathione synthesis and redox homeostasis³⁶, our results suggest that prostate cells, in the absence of glucose and/or glutamine, can upregulate cataplerosis reactions in an attempt to maintain cellular function and proliferation. Together, these data demonstrate the dynamic adaptability of palmitate to enhance the supply of carbons to the TCA cycle when prostate cells are deprived of glucose and/or glutamine.

FAO has been proposed to be the dominant bioenergetic source for PCa cells^{11,12}, and so we next investigated whether the increased palmitate utilisation upon glucose and/or glutamine deprivation could

maintain TCA cycle activity. In striking contrast to palmitate enrichment patterns, the abundances of TCA metabolites citrate, malate and oxoglutarate were reduced in glucose-free and glucose-/ glutamine-free settings compared to glucose and glutamine replete settings (Figure 2F and S2D). Importantly, these results preceded the cell death that was observed between 6 and 24 hours (Figure S2B and S2C). The inverted TCA cycle enrichment and abundance patterns demonstrate that in the absence of glucose and/or glutamine prostate cells increase FAO rates to incorporate more palmitate carbon into the TCA cycle but fail to maintain TCA cycle activity and support cataplerosis.

Extracellular LCFAs are a minor contributing fuel source to the TCA cycle.

In light of the minimal utilisation of palmitate to the TCA cycle (Figure 1 and 2), we next questioned if this was a palmitate-specific phenotype. To address this, we repeated our tests with a panel of ^{13}C -FAs that include non-essential (16:0, 18:0, 18:1) and essential (18:2, 18:3) LCFAs of varying saturation and carbon chain lengths. Surprisingly, a similar trend of minimal contribution (2-18%) to TCA cycle metabolites was observed across prostate cells, despite the diversity of LCFAs (Figure 3A). 22Rv1 and AD1 cells had the greatest palmitate labelling and FAO rates amongst prostate cells (Figure 1A and S1C), and consistently exhibited greater utilisation of LCFAs by the TCA cycle compared to an unlabelled control (Figure 3A). However, overall enrichment from LCFAs, irrespective of FA saturation status, chain length or cell line trends, remained minimal relative to extracellular glucose and glutamine (Figure 1C). Moreover, there was no clear substrate preference observed between palmitate and other LCFAs to feed carbons to the TCA cycle (Figure 1C). These results were all observed with substantial labelling of respective acyl-carnitine species (0.8-1, ratio m^n/m_0+m^n ; Figure 3B). Collectively, we show that in the presence of physiological glucose and glutamine, diverse extracellular LCFAs were not major carbon sources for the TCA cycle, despite substantial acyl-carnitine precursor labelling.

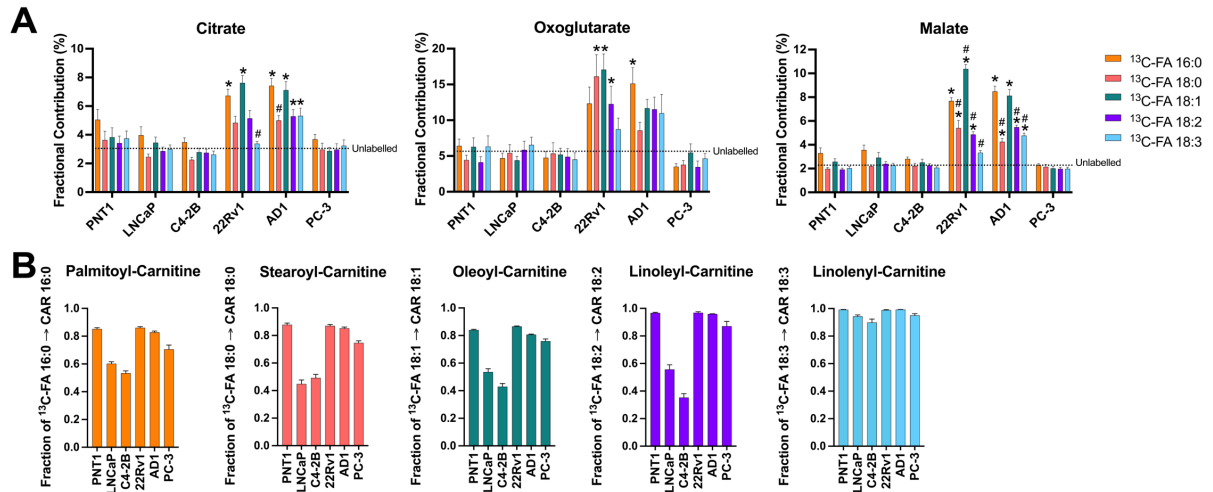


Figure 3 LCFAs of varying saturations are minor contributing substrate sources to the TCA cycles.

(A) Fractional contribution of [U-¹³C]-FA 16:0, 18:0, 18:1, 18:2, 18:3 to citrate, oxoglutarate, malate. N=3 per cell line.

(B) Fractional contribution of acyl-carnitines associated with the [U-¹³C]-FA substrates. N=3 per cell line.

Graphs show mean $\pm$ SEM unless stated otherwise. $P < 0.05$ by Two-way ANOVA with Tukey's multiple comparisons test, *compared to unlabelled control (dotted line), #compared to [U-¹³C]-FA 16:0. See also Figure S3.

The disconnect between the substantial conversion of LCFA enrichments to acyl-carnitines and the lack of conversion to TCA cycle intermediates could be explained by incomplete FAO, which generates medium chain acyl-carnitine species instead of fully oxidising LCFAs to acetyl-CoA^{37,38}. In agreement with this hypothesis, FA 16:0, 18:0, and 18:1 greatly enriched shortened acyl-carnitine species (Figure S3). Specifically, FA 16:0 significantly enriched shortened CAR 14:0 to CAR 10:0, FA 18:0 enriched CAR 16:0 to CAR 12:0, and FA 18:1 enriched CAR 16:1 to CAR 12:1. The greatest enrichments to shortened acyl-carnitines were observed in PNT1, 22Rv1, AD1 and PC-3 cells (Figure S3), that also had the greatest incorporation of FAs into precursor acyl-carnitines (Figure 3B). These data provide evidence that labelled LCFAs are not completely converted to the TCA-cycle precursor acetyl-CoA, which further supports our findings that LCFAs are minor carbon sources for the TCA cycle.

Extracellular LCFAs support the rapid turnover of cardiolipin pools.

LCFAs were not completely oxidised to acetyl-CoA and minimally contributed carbons to the TCA cycle, but were substantially present as acyl-carnitines (Figure 3 and S3). This led us to question which pathways could be consuming extracellular LCFAs entering the mitochondria via CPT1a. Yao and colleagues²² were the first to observe a reduction of mitochondrial complex lipids following CPT1a knockdown in BT549 breast cancer cells. Out of their observed lipidome results, cardiolipins (CLs) piqued our interest as they are exclusively located within the inner mitochondrial membrane (IMM) and account for 10-20% of mitochondria phospholipid content³⁹. CLs also provide essential structural functions to mitochondria membrane and cristae⁴⁰, and ETC complexes I-IV for ATP production⁴¹⁻⁴³. CL loss-of-function coincidentally matched some of the CPT1a impairments in cancers, namely disrupted mitochondrial morphology^{22,44}, reduced ATP production²⁰, and impaired cell viability and tumour growth^{1,18,19}. CLs are composed of four fatty acyl chains of different saturation and chain lengths⁴⁵. As CL remodelling functions to mature nascent CLs by the re-acylation from donated acyl-CoAs⁴⁶, it was conceivable that this process could be a significant consumer of mitochondria LCFAs. Therefore, we next investigated the contribution of extracellular LCFAs to CL synthesis and remodelling (Figure 4A). First, we examined the enrichments of CL pools from extracellular [U-¹³C]-16:0, 18:0, 18:1, and 18:2 LCFAs. Surprisingly, >50% enrichment was achieved within the 6 hours of ¹³C label exposure indicating substantial LCFA turnover of CL pools (Figure 4B), in stark contrast to the marginal arrival of LCFA label into the TCA cycle (Figure 1-3). Consistently across our panel of prostate cells, FA 18:2 was preferentially incorporated into CLs (1.3 to 3.7-fold greater) compared to FAs 16:0, 18:0, and 18:1 (Figure S4A). This was consistent with literature showing that the majority of CL acyl chains are composed of 18:2, and CLs containing 18:2 acyl-chains have the greatest turnover in cardiomyocytes and HeLa cells^{47,48}. Another striking observation was that FAs 16:0, 18:0, and 18:2 were directly incorporated into the acyl-chains of CLs (Figure S4B). For instance, CL 68:2 is composed of 16:0 and 18:1 acyl-chains and was predominantly labelled by 16:0, while CL 70:3 is also composed of 16:0 and 18:1 acyl-chains but was predominantly labelled by 18:1 (Figure 4C). Similarly, CL 72:5

is composed of 18:1 and 18:2 chains and was almost entirely labelled by 18:2 (Figure 4C). Cell line differences between PCa and benign PNT1 cells were also observed within these patterns of enrichment into CLs, however, no obvious pattern could be discerned (Figure 4C). Collectively, prostate cells display significant turnover of CL acyl-chains derived from extracellular LCFAs.

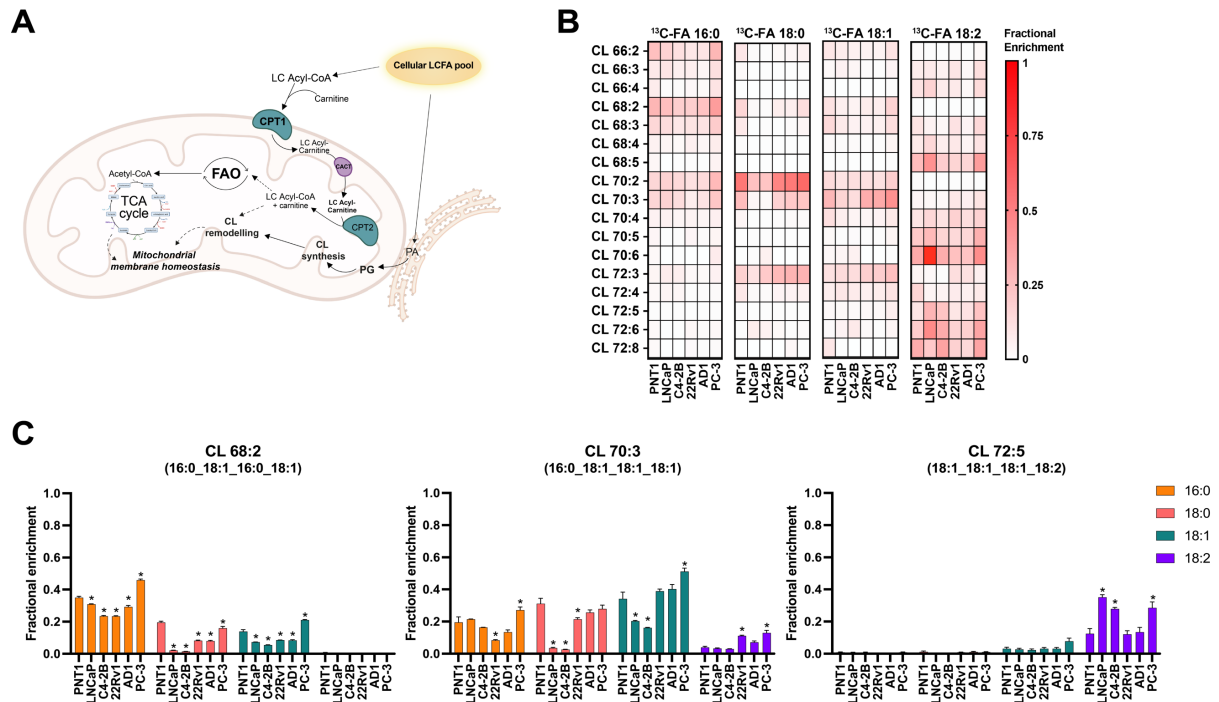


Figure 4 Fast incorporation of all LCFAs into cardiolipin pools.

- (A) Schematic depicting fates of FAs entering the mitochondria including FAO and CL pathways.
 (B) Fractional enrichment of $[U-^{13}C]$ -FA 16:0, 18:0, 18:1, 18:2 (150 μ M) tracing to CL species for 6 hours. N=3 per cell line.
 (C) Fractional enrichment of $[U-^{13}C]$ -FA 16:0, 18:0, 18:1, 18:2 to CL 68:2, CL 70:3, CL 72:5 species. N=3 per cell line.

Graphs show mean $\pm$ SEM. P<0.05 by Two-way ANOVA with Tukey's multiple comparisons test, *PCa cells vs PNT1 cells for each FA. See also Figure S4.

Knockdown of CPT1a decreases FAO and incorporation of LCFAs into CL.

The high incorporation of LCFAs into CLs across all prostate cells was in stark contrast to the magnitude of enrichment into TCA cycle metabolites (Figure 3 and 4). FAs are incorporated into CLs via multiple starting points, not exclusively competing with FAO for mitochondria FAs supplied by CPT1a. While these mitochondrial acyl-CoAs are substrates for CL remodelling acyltransferases

MLCLAT1⁴⁶ and ALCAT1⁴⁹, CLs are also assembled by the *de novo* pathway producing nascent CLs from phosphatidylglycerol (PG)⁵⁰, and the remodelling enzyme Tafazzin exchanging acyl-chains with phosphatidylcholine and phosphatidylethanolamine⁵¹. To tease out the association between CPT1a and CL remodelling, we quantified the incorporation of FA 18:2 in cells with a CPT1a knockdown because 18:2 has been reported to be predominantly incorporated into CLs via remodelling pathways^{46,47} (Figure 5A). We included FA 16:0 as it can be incorporated via both *de novo* synthesis and remodelling^{52,45}. Further, we chose C4-2B cells for this experiment as they had the greatest level of CPT1a protein in our panel of prostate cells (Figure 1D). In C4-2B cells with CPT1a knockdown (reduced by 89%) (Figure 5B), decreased palmitate oxidation (Figure 5C) and abundance of ¹³C-labelled 18:2 and 16:0 acyl-carnitines compared to scrambled non-targeting control cells (Figure 5D) were observed as expected. CPT1a^{KD} did not alter citrate enrichment from FA 18:2, but slightly decreased enrichment from FA 16:0 (Figure 5E).

Using MS ion counts, we determined the relative amount of ¹³C-labelled and total FA 18:2 among detected CL species. With this, we showed that CPT1a^{KD} significantly reduced the incorporation of FA 18:2 into the total pool of CL species by 19% to 15% (Figure 5F), and into individual CL species (Figure 5G, left). The same analysis in CPT1a^{KD} cells cultured with ¹³C-FA 16:0 showed no clear pattern of reduced enrichments (Figure 5F and 5G). The lack of an effect from CPT1a^{KD} on FA 16:0 enrichment patterns were as expected because FA 16:0 can be incorporated into CLs via the *de novo* pathway independent of CPT1a and mitochondrial acyl-CoAs⁵⁰. Therefore, we evaluated PG enrichment data from the perspective that PG and CL synthesis are not downstream of CPT1a. The PG enrichment differences (delta) between CPT1a^{KD} and scrambled control cells for ¹³C-18:2 and 16:0 were not altered (Figure 5H, S5B and S5C). Altogether, by contrasting reductions in CL FA 18:2 ¹³C enrichments against both 16:0 and PG, we showed that only FA 18:2 enrichments to CLs were specifically impacted by CPT1a loss-of function (Figure 5H). Thus, we inferred that CPT1a regulates the incorporation of 18:2 into CL, and that CL remodelling is a pathway downstream of CPT1a that utilises substantially greater amounts of mitochondrial FAs than FAO and the TCA cycle (Figure 5I).

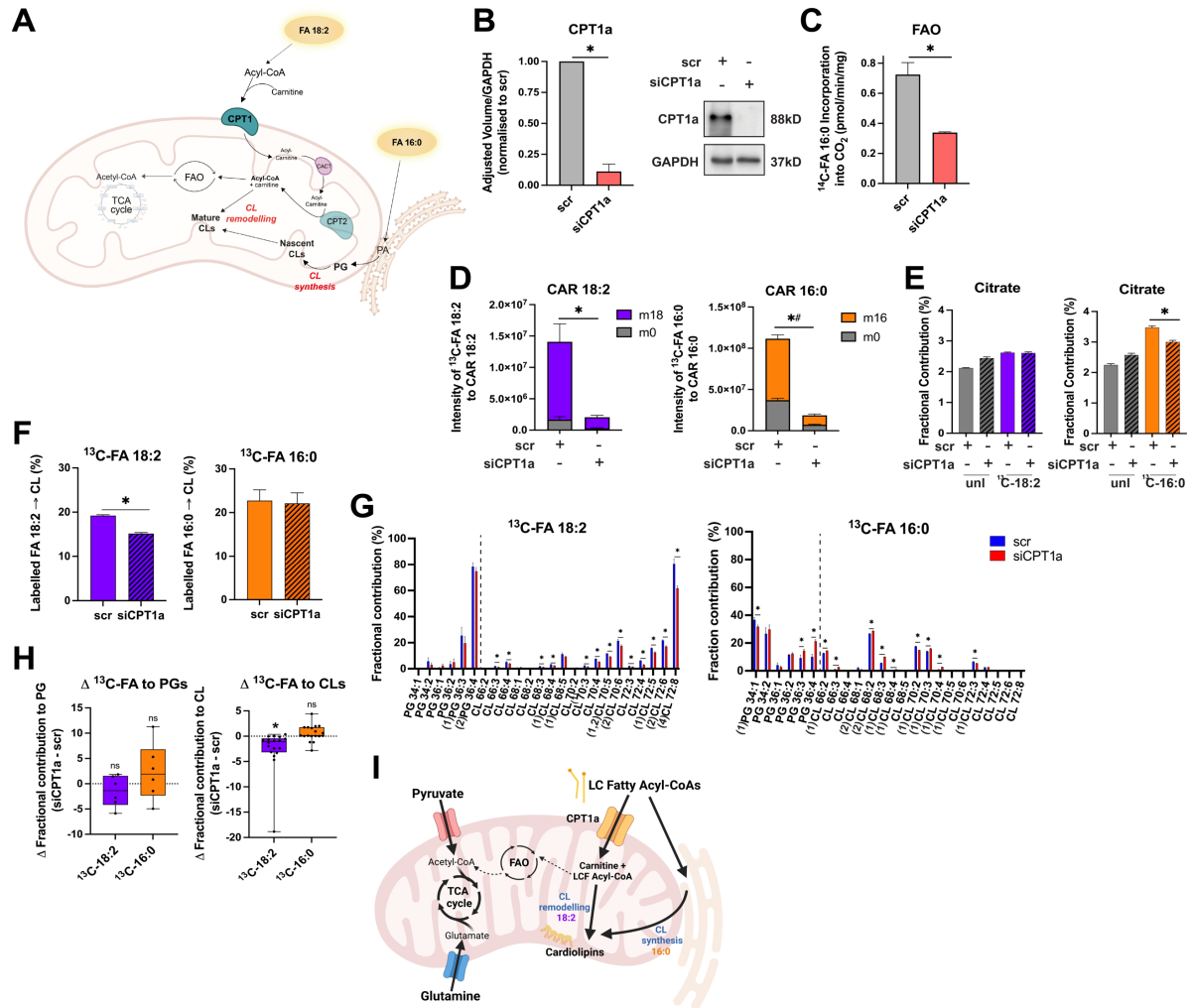


Figure 5 CPT1a knockdown reduced ¹³C-18:2 derived CL remodelling.

- Schematic depicting expected FA 18:2 to CL remodelling downstream of CPT1a and FA 16:0 to CL synthesis pathways derived from the endoplasmic reticulum.
- CPT1a knockdown by reverse siRNA transfection in C4-2B cells for 72 hours quantified by immunoblot. Representative immunoblots shown. N=3 per group.
- [1-¹⁴C]-FA 16:0 oxidation rates in non-targeting scrambled (scr) and siCPT1a C4-2B cells. N=3 per group.
- Intensity of [U-¹³C]-FA 18:2 or 16:0 to carnitine species following siCPT1a treatment. N=3 per group. Statistically significant differences between labelled (m16 orange or m18 purple) intensities (*) or unlabelled (m0 grey) intensities (#).
- Fractional contribution of unlabelled or [U-¹³C]-FA 18:2 or 16:0 to citrate in scr or siCPT1a C4-2B cells. N=3 per group.
- Percentage of labelled CL pools from [U-¹³C]-FA 18:2 or 16:0. N=3 per group.
- Fractional contribution of [U-¹³C]-FA 18:2 or 16:0 to PG and CL species. Annotated n values on the x-axis indicate the number of 18:2 or 16:0 containing acyl-chains. N=3 per group. Multiple unpaired t-tests.
- Delta differences between fractional contribution of [U-¹³C]-FA 18:2 or 16:0 to PG and CL species. N=3 per group. Statistically significant differences within [U-¹³C]-FA 18:2 or 16:0 groups to 0 difference shown. Error bars represent $\pm$ min to max values. Multiple unpaired t-tests.
- Schematic depicting major carbon contributions from glucose and glutamine into the TCA cycle in comparison to the substantial contributions of LCFA-derived acyl-CoAs for CLs either by CL remodelling downstream of CPT1a or CL synthesis.

Graphs show mean $\pm$ SEM unless stated otherwise. ns not significant, *p<0.05 between siCPT1a and scr control groups by student's t-test unless stated otherwise. See also Figure S5.

Discussion

The assumption that FAO is the predominant bioenergetic pathway in PCa is a culmination of the observed enhanced FAO rates compared to benign cells^{1,5,6}, low glycolytic activity at early disease stages^{14,15}, and sensitivity to targeting the FAO pathway⁷. However, this view first proposed by Liu¹¹ (2006) failed to consider the contribution of the major respiration substrates glucose and glutamine. CPT1a has been implicated as the rate-limiting step of FAO because its inhibition results in decreased ATP production and cancer cell death^{1,18-20}, and assumes that LCFAs are entirely diverted to FAO and the TCA cycle. Here, we generated evidence disputing this dogmatic view by showing extracellular LCFAs are minor carbon sources to the TCA cycle compared to glucose and glutamine in PCa, despite their increased FAO rates compared to benign cells. We also showed that LCFAs are not completely oxidised to acetyl-CoA and that rapid CL turnover consumes LCFAs. Further, that CPT1a regulates the incorporation of FA 18:2 into CLs. Together, our data shows a substantial role for CPT1a to regulate CL turnover in PCa and provides the foundation to investigate the functions of mitochondria lipid metabolism for PCa bioenergetics.

In a range of experiments, we show that FAO cannot meet the anaplerotic demand provided by glucose and glutamine to maintain TCA cycle activity, despite PCa cells having intrinsically higher rates of FAO compared to benign cells. Surprisingly, these results were also seen across the spectrum of PCa progression from benign to late-stage, which contradicted previous studies that indicated benign prostate cells and progressive castration resistant PCa exhibit high glycolysis rates compared to a lipid reliant phenotype in early-stage PCa^{14,15}. Previous evidence has pointed towards a greater utilisation of glucose in progressive PCa stages (Gleason score >7) for ¹⁸F-fluorodeoxyglucose PET/CT scans and the diagnosis of patients⁵³. Our results suggest that clinical strategies could exploit the substantial glucose and glutamine utilisation phenotype to the TCA cycle that spans PCa disease stages for earlier diagnosis. Furthermore, inhibiting reactions within FAO, like the use of trimetazidine, leads to decreased PCa viability⁷. At the same time, decreased PCa cell viability follows CPT1a inhibition^{19,21}. Interpreting these results in the context of ours provided us with the explanation that LCFAs following

CPT1a mitochondria entry are not fully oxidised to acetyl-CoA for respiration, but instead undergo incomplete oxidation to serve other cellular functions. We detected ^{13}C -labelled LCFAs as shortened acyl-carnitines. These intermediates have been associated with histone acetylation functions^{54,55} and could explain how incomplete FAO contributes to cancer survival. Our results that FAO is unable to maintain TCA cycle activity also suggested that acetyl-CoA formed from FAO downstream of CPT1a does not supply enough carbons into the TCA cycle compared to pyruvate-derived acetyl-CoA. Moreover, we showed that CPT1a^{KD} does not impact the levels of LCFAs fuelling TCA cycle metabolites but does decrease acyl-carnitine levels. We concluded that LCFAs entering the mitochondria must provide essential functions for PCa viability that are not exclusive to FAO.

LCFA entry into the mitochondria via CPT1 was found to be associated with complex lipid pools in cancers^{22,56}. From these studies, the mitochondria exclusive CL piqued our interest as it is essential for mitochondria function and bioenergetics⁵⁷. Our results showed the rapid turnover of CLs from LCFAs in PCa cells (>50%) and gave supporting evidence that LCFA oxidations, that only enriched <20% of TCA cycle metabolites, were not the exclusive mitochondria pathways for LCFAs. Similar to previous reports, our results showed FA 18:2 was predominantly incorporated into the acyl-chains of CLs compared to other LCFAs^{47,48}. These results were especially important as cancer cells that substantially incorporate exogenous 18:2 into CL acyl-chains have enhanced stimulation of ETC complex I activity⁴⁷ and a greater susceptibility to apoptosis⁵⁸. CPT1 may be supporting the high CL turnover based on results by Yao *et al*²²(2018) and Zhang *et al*⁵⁶(2021). Our results support this hypothesis in PCa, as depletion of CPT1a in C4-2B cells reduced CL turnover from ^{13}C -labelled 18:2, a major substrate consumed by the CL remodelling pathway⁴⁶. These results suggest that the regulation of FA 18:2 CL remodelling by CPT1a has functional effects on respiration and apoptosis^{47,58}, that could be adversely affected upon CPT1a impairment. Taken together, our data supported CLs as a prominent fate for LCFAs entering the mitochondria via CPT1a compared to the FAO derived TCA cycle.

Our study used ^{13}C -labelling experiments to characterise the roles of extracellular substrates to mitochondrial pathways, however there are limitations that warrant mentioning. We focused on

extracellular LCFAs as the major precursors for the PCa lipid pool¹. As the combined TCA cycle metabolite contributions from LCFAs, glucose, and glutamine did not reach 100%, further experiments could include other major TCA cycle carbon sources like lactate⁵⁹ or branched chain amino acids⁶⁰ that could help solidify our conclusion of FAO being a minor carbon source to the TCA cycle. We also gave evidence for incomplete FAO in PCa cells, however further investigation into the roles of shortened acyl-carnitines may provide insights into other fates of LCFAs, independent of CLs that we focused in on. Finally, we showed for the first time in PCa that CPT1a is associated to CL remodelling pathways, but further experiments could help explain if the energy crisis from CPT1a inhibition in cancers is linked to CLs and their role in ETC complex structure⁵⁷ instead of FAO.

Together, our findings provided evidence that LCFA oxidation following CPT1a mitochondria entry is not the predominant carbon source to the TCA cycle in prostate cells compared to glucose and glutamine. We showed for the first time that CLs are rapidly turned over from LCFAs in PCa and that CPT1a plays an important role for CL remodelling pathways instead of FAO and the TCA cycle. This study provides the foundation to explore the CPT1a-regulated intra-mitochondrial roles of LCFAs to CLs, instead of the TCA cycle, for PCa bioenergetics and cell viability.

Methods

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-CPT1A	Abcam	Cat#Ab128568; RRID: AB_11141632
Rabbit monoclonal anti-GAPDH	Cell Signalling Technology	Cat#2118; RRID: AB_561053
Chemicals, peptides, and recombinant proteins		
Enzalutamide MDV3100	Selleckchem	Cat#S1250
Lipofectamine RNAiMAX transfection reagent	Thermo Fisher Scientific	Cat#13778075
[1- ¹⁴ C] Palmitic acid	Perkin Elmer	Cat#NEC075H250UC
Palmitic acid	Sigma-Aldrich	Cat#P0500
D-Glucose	Sigma-Aldrich	Cat#273053
L-Glutamine	Gibco	Cat#25030081
Fatty acid free BSA	Bovogen	Cat#BSAS0.10
L-carnitine hydrochloride	Sigma-Aldrich	Cat#C0283
Mitotracker Green	Thermo Fisher Scientific	Cat#M7514
Mitotracker Red	Thermo Fisher Scientific	Cat#M7512
FCCP	Sigma-Aldrich	Cat#SML2959
DTT	Sigma-Aldrich	Cat#D0632
Protease and Phosphatase Inhibitor Cocktail, EDTA free	Astral Scientific	Cat#T-2496
Thiazolyl blue tetrazolium bromide MTT reagent	Sigma-Aldrich	Cat#M2128
Linoleic acid	Sigma Aldrich	Cat#L1012
D-Glucose (U-13C6, 99%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-1396
L-Glutamine (U-13C5, 99%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-1822-H

Palmitic acid (U-13C16, 98%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-409
Stearic acid (U-13C18, 98%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-6990
Linoleic acid (U-13C18, 98%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-10487
Linolenic acid (U-13C18, 98%)	Cambridge Isotope Laboratories, Inc.	Cat#8386
Critical commercial assays		
Pierce BCA Protein assay kit	Thermo Fisher Scientific	Cat#23225
Immobilon Crescendo Western HRP substrate	Merck Millipore	Cat#WBLUR0500
CellCarrier Spheroid ULA 96-well microplates	Perkin Elmer	Cat#6055330
Experimental models: Cell lines		
PNT1	ECACC	95012614
LNCaP	ATCC	CRL-1740
C4-2B	ATCC	CRL-3315
22Rv1	ATCC	CRL-2505
CWR-R1-AD1	A/Prof. Luke Selth, Flinders University	Nyquist 2013 ⁶¹ , Li 2013 ⁶²
PC-3	ATCC	CRL-1435
MR42D	Prof. Lisa Butler, SAHMRI	Bishop 2017 ²⁷
MR49F	Prof. Lisa Butler, SAHMRI	Bishop 2017 ²⁷
Oligonucleotides		
ON-TARGETplus Human CPT1A siRNA SMARTpool	Horizon Discovery	Cat#L-009749-00-0005
ON-TARGETplus Non-targeting Control pool	Horizon Discovery	Cat# D-001810-10-05
Software and algorithms		
GraphPad Prism V9	GraphPad Software	https://www.graphpad.com/
Image Lab 6.1	Bio-Rad	https://www.bio-rad.com/en-au/product/image-lab-software

FlowJo 2	FlowJo LLC	https://www.flowjo.com/
MATLAB	Mathworks	https://au.mathworks.com/products/matlab.html
MSConvert	N/A	Chambers 2012 ⁶³
MSDial	Riken	http://prime.psc.riken.jp/compms/msdial/main.html
Biorender	Biorender	https://biorender.com/

Method details

Cell lines and culture conditions

Human benign prostate epithelial cell line PNT1 and human prostate carcinoma cells lines LNCaP (androgen receptor (AR) +, hormone sensitive), C4-2B, 22Rv1, AD1 (AR+, androgen independent), and PC-3 (AR-, hormone resistant) cells were cultured in RPMI1640 medium (Life Technologies), supplemented with 10% FBS (Cytiva Hyclone), and 1% penicillin/streptomycin (Gibco). MR42D and MR49F (hormone resistant, treatment resistant) cells were cultured in RPMI 1640 medium (Life Technologies), supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 μ M enzalutamide. All cells were incubated at 37°C and 5% CO₂.

Spheroid culturing

Cells were seeded in 96-well Spheroid microplates, at a density of 5000 cells/well (LNCaP), 8000 cells/well (22Rv1), and 2000 cells/well (C4-2B), in RPMI medium in 100 μ L per well. An additional 100 μ L of medium was added to cells at 24h and left un-agitated for a further 24h until spheroids formed.

Cell Transfection

C4-2B cells were transfected for 72 hours using RNAiMAX transfection reagent and 25 pmol pooled CPT1a siRNA or non-targeting scrambled control, according to the manufacturer's instructions. After 72 hours, cells were used for subsequent radiolabelling, western immunoblotting, and ¹³C-labelling experiments.

¹⁴C-labelling

Cells were washed in warm PBS and incubated in 600µL media containing 0.1 mmol/L cold palmitate, [1-¹⁴C]-palmitate (0.1µCi/mL) conjugated to 2% (wt/vol) FA-free BSA and 1mmol/L -carnitine in RPMI glucose-free medium (Gibco) for 4 hours. Fatty acid oxidation rates were determined from ¹⁴CO₂ production as previously described¹ and supplemented with 0mM or 5mM glucose to measure metabolic flexibility. Medium for dose dependent glucose deprivation results were supplemented with 0mM, 1mM, 3mM, 5mM glucose. Protein content was measured by BCA protein assay and absorbances read at 540 nm using a TECAN infinite M1000 pro plate reader.

Mitotracker

Cells were seeded in 12-well plates at a density of 2x10⁵ cells/well in RPMI medium. After 24 hours, cells were washed in warm PBS and obtained by trypsinisation and collected in serum-free RPMI medium. The cell suspension was centrifuged at 1500 rpm for 5min in fluorescence-activated cell sorting (FACS) tubes. Cells were subsequently stained with 200nM Mitotracker Red and Mitotracker Green at 37°C for 30mins. Additionally, unstained, red only, green only, and 2.5µM FCCP treated cells were included as experimental controls. Cells were centrifuged at 1500 rpm for 5min and fixed with 4% paraformaldehyde at 37°C for 10min. Cells were washed with warm PBS and centrifuged at 1500 rpm for 5min and resuspended in 150µL PBS before the samples were read on a BD LSR Fortessa flow cytometer. Data analysis was performed using FlowJo software.

Immunoblotting

Cells were seeded in 6-well plates (5x10⁵). After 24 hours, cells were lysed in RIPA buffer containing 1% DTT and 0.1% protease and phosphatase inhibitor cocktail. Protein content was measured by BCA protein assay and absorbances read at 540 nm using a TECAN infinite M1000 pro plate reader. Cell lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore). Ponceau staining or GAPDH was used as a loading control, and then membranes were immunoblotted for CPT1a. Chemiluminescence was performed using Western HRP Substrate and

imaged using the Bio-Rad ChemiDoc MP Imaging System (Bio-Rad Laboratories) and Image Lab software.

Cell viability

Cells were seeded in 96-well plates, in 6 well replicates (3×10^3 cells/well). After 24 hours the media was removed and replaced with glucose- and glutamine- free media (Gibco) supplemented with 5mM glucose, 1mM glutamine and/or 150 μ M palmitate, conjugated to 2% (wt/vol) FA-free BSA. For glucose-deprived conditions DMEM no glucose, no glutamine media was supplemented with 1mM glutamine and 150 μ M palmitate; for glucose and glutamine-deprived conditions media was supplemented with 150 μ M palmitate only. Media (5mM glucose, 1mM glutamine, 150 μ M palmitate) supplemented with 10% FBS was used as a control. At 0, 6, 12 and 24 hours, 50 μ L of MTT reagent was added to wells. MTT reactions were incubated at 37°C and 5% CO₂ for 4 hours, and then stopped by replacing media with 100 μ L DMSO (Sigma–Aldrich) per well. Absorbances were read at 540 nm using a TECAN infinite M1000 pro plate reader. For live cell counts, cells were seeded in 6-well plates at 5×10^5 cells/well. After 24 hours, media was replaced as described for the MTT assay, and cells counted by Trypan blue (Gibco) exclusion at 6 and 24 hours.

U-¹³C stable isotope tracing

Cells were seeded in 6-well plates (5×10^5) for 2D culture, or as above for 3D spheroid models. After 24 hours, wells were washed with warm PBS and media replaced with 600 μ L of DMEM no glucose (0mM), no glutamine (0mM) media (Gibco), supplemented with 5mM glucose, 1mM glutamine and 150 μ M FAs replaced with their respective [U-¹³C] forms (Palmitate (16:0), Oleate (18:0), Stearate (18:1), Linoleate (18:2), or Linolenate (18:3)). For glucose-deprived conditions, DMEM no glucose, no glutamine media was supplemented with 1mM glutamine and [U-¹³C]-150 μ M palmitate; for glucose and glutamine-deprived conditions, media was supplemented with [U-¹³C]-150 μ M palmitate only. FAs were conjugated to 2% (wt/vol) FA-free BSA containing media for 24 hours at 37°C prior to treating cells. Cells were incubated in ¹³C containing medium for 6 hours. For spheroid experiments, 200 μ L of

spheroids was pooled and collected and centrifuged at 1400 rpm for 5min at room temperature. The supernatant medium was collected and used for further extracellular substrate experiments. The remaining cell pellet was washed with 2mL cold PBS and centrifuged again. The cell pellet was collected for subsequent LC-MS analysis.

Extracellular substrate sampling

Cells were seeded in 6-well plates (5×10^5) for 2D culture of as above for 3D spheroid models. After 24 hours, wells were washed with warm PBS and media replaced with 1mL or 2mL of DMEM no glucose, no glutamine media (Gibco) supplemented with 5mM glucose, 1mM glutamine and 150 μ M FAs. 100 μ L of extracellular media were collected from wells at 3, 6, 12, 24 hour timepoints for cells incubated in 1mL of media, and 12, 24 hours for cells incubated in 2mL of media. For spheroid experiments, ^{13}C labelled medium was collected from cells at 0 and 6 hours timepoints. Media samples were centrifuged at 16, 000xg for 5min at 4°C and the supernatant collected for subsequent LC-MS analysis.

Cell lysate extraction for LC-MS

Media was aspirated from plates and the cells were washed with 2mL ice-cold 0.9% (w/v) NaCl. Cells were scraped in 300 μ L of extraction buffer, EB, (1:1 LC/MS methanol:water (Optima) + 0.1x internal standards (non-endogenous polar metabolites) and transferred to a 1.5mL microcentrifuge tube. A further 300 μ L of EB was added to the cells and combined in the tube. 600 μ L Chloroform (Honeywell) was added before vortexing and incubating on ice for 10min. Tubes were vortexed briefly and centrifuged at 15, 000g for 10min at 4°C. The aqueous upper layer was collected and dried without heat, using a Savant SpeedVac (Thermo Fisher) for metabolomics LC-MS analysis. The lower layer was collected and dried under nitrogen flow for lipidomics LC-MS analysis.

Measuring stable-isotope labelled metabolites by LC-MS

Dried aqueous upper phase samples were resuspended in 40 μ L Amide buffer A (20mM ammonium acetate, 20mM ammonium hydroxide, 95:5 HPLC H_2O : Acetonitrile (v/v)) and vortexed and

centrifuged at 15,000g for 5min at 4°C. 20µL of supernatant was transferred to HPLC vials containing 20µL acetonitrile for LCMS analysis of amino acids and glutamine metabolites. The remaining 20µL of resuspended sample was transferred to HPLC vials containing 20µL LC-MS H₂O for LCMS analysis of glycolytic, pentose phosphate pathway (PPP), and TCA cycle metabolites. Amino acids and glutamine metabolites were measured using Vanquish-TSQ Altis (Thermo) LC-MS/MS system. Analyte separation was achieved using a Poroshell 120 HILIC-Z Column (2.1x150mm, 2.7µm) (Agilent) at ambient temperature. The pair of buffers used were Amide buffer A and 100% acetonitrile (Buffer B), flowed at 200µL/min; injection volume of 5µL. Glycolytic, PPP and TCA cycle metabolites were measured using 1260 Infinity (Agilent)-QTRAP6500+ (AB Sciex) LC-MS/MS system. Analyte separation was achieved using a Synergi 2.5µm Hydro-RP 100A LC Column (100x2mm) at ambient temperature. The pair of buffers used were 95:5 (v/v) water:acetonitrile containing 10mM tributylamine and 15mM acetic acid (Buffer A) and 100% acetonitrile (Buffer B), flowed at 200µL/min; injection volume of 5µL. Raw data from both LC-MS/MS systems were extracted using MSConvert⁶³ and in-house MATLAB scripts. The fractional contributions of labelled substrates to metabolites were calculated as the weighted sum by the number of labelled carbons²⁶.

Measuring extracellular substrates by LC-MS

20µL of collected extracellular media was mixed with 80µL water, vortexed. 10µL of diluted media was mixed with 90µL of extraction buffer containing 1:1 (v/v) acetonitrile and methanol + 1x internal standards (non-endogenous standards) at -30°C. The mixture was centrifuged at 12,000xg for 5min at 4°C and transferred into HPLC vials for LC-MS analysis measured using the Vanquish-TSQ Altis as described above. Raw data from both LC-MS/MS systems were extracted using MSConvert⁶³ and in-house MATLAB scripts.

Measuring stable isotope labelled lipids by LC-MS

Dried lower phase samples were resuspended in 100 μ L of 4:2:1 (v/v) isopropanol:methanol:chloroform containing 7.5mM ammonium formate. Cardiolipin and acyl-carnitine lipids were measured using a Thermo Scientific Q-Exactive-HF-X Hybrid Quadrupole Orbitrap LC-MS/MS system.

For cardiolipins, analyte separation was achieved using an Agilent Poroshell 120, EC-C18, 2.1x150mm, 2.7 μ m column. The pair of buffers used were 60:40 acetonitrile:water (v/v) with 10mM ammonium formate and 0.1% formic acid (mobile phase A), and 90:10 isopropanol:acetonitrile (v/v) with 10mM ammonium formate and 0.1% formic acid (mobile phase B), flowed at 200 μ L/min on negative mode. MS1 data was acquired with the following settings: 3.5kV, capillarity temperature 300°C, 120 000, injection time 100ms, AGC 1x10⁶, scan range 1100-1650. For ddMS2 data acquirement, the following settings were used: top 20, resolution 30,000, 200-2000, isolation 1.0m/z, nce 30, AGC target 1x10³, intensity threshold 5x10³, dynamic exclusion 20s. Raw data from both LC-MS/MS systems were extracted using MSConvert⁶³, in-house MATLAB scripts, and MSDial.

For acyl-carnitines, analyte separation was achieved using an Agilent Poroshell 120, HILIC, 2.1x100mm, 2.7 μ m column. The pair of buffers used were 0.1(v/v)% formic acid and 10mM ammonium formate in H₂O and 0.1(v/v)% formic acid in acetonitrile, flowed at 200 μ L/min on positive mode. MS1 data were acquired with the following settings: 3.5kV, capillarity temperature 300°C, 120 000, injection time 100ms, AGC 1x10⁶, scan range 200-1000. For ddMS2 data acquirement, the following settings were used: top 5, resolution 30,000, 200-2000, isolation 1.0m/z, nce 30, AGC target 1x10³, intensity threshold 5x10³, dynamic exclusion 20s. Raw data from both LC-MS/MS systems were extracted using MSConvert⁶³ and in-house MATLAB scripts.

Quantification and statistical analysis

Student's t-test, one- or two-way ANOVA with Dunnett's or Tukey's multiple comparisons, and linear regression statistical tests were performed with GraphPad Prism 9.0 (GraphPad Software). $P < 0.05$ was considered significant. Data are reported as mean $\pm$ standard error of the mean (SEM), standard deviation (SD), or min to max values of at least three independent determinations (N=3 as indicated in

figure legends) with at least 3 technical replicates each. Schematic diagrams were created with Biorender.com.

Supplementary information

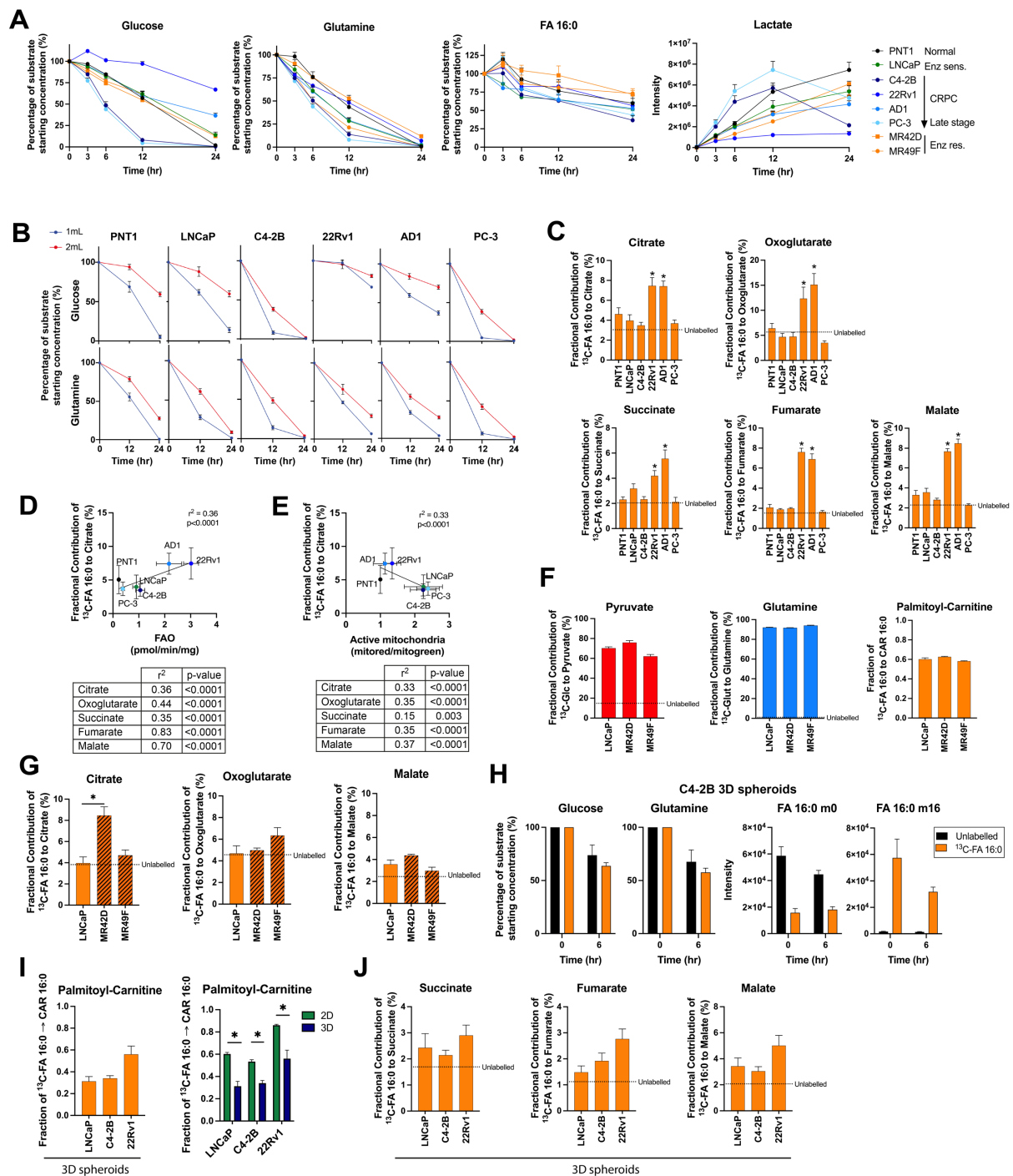


Figure S1, related to figure 1. Palmitate is a minor carbon source to the TCA cycle.

- (A) Percentage of glucose, glutamine and FA 16:0 relative to starting amount, and amount of lactate in culture media over 24 hours in 1mL culture medium. N=3 per cell line.
- (B) Percentage of substrate starting concentration of glucose or glutamine in 1mL (blue) or 2mL (red) culture medium. N=3 per cell line.

- (C) Fractional contribution of [U-¹³C]-FA16:0 to citrate, oxoglutarate, succinate, fumarate, and malate. N=3 per cell line. PCa cells vs PNT1 cells are shown.
- (D) Correlations of [U-¹³C]-FA 16:0 to citrate (plotted), oxoglutarate, succinate, fumarate, and malate to FAO. Error bars represent $\pm$ SD. P and r^2 values by linear regression.
- (E) Correlations of [U-¹³C]-FA 16:0 to citrate (plotted), oxoglutarate, succinate, fumarate, and malate to active mitochondria content. Error bars represent $\pm$ SD. P and r^2 values by linear regression.
- (F) Fractional contribution of [U-¹³C]-glucose to pyruvate (red), [U-¹³C]-glutamine to intracellular glutamine (blue), [U-¹³C]-FA 16:0 to palmitoyl-carnitine (orange) after 6 hours of labelling in LNCaP, MR42D and MR49F cells. N=3 per cell line.
- (G) Fractional contributions of [U-¹³C]-glucose, glutamine, or FA 16:0 to citrate, oxoglutarate and malate. N=3 per cell line.
- (H) Percentage of substrate starting concentration of glucose, glutamine, and FA 16:0 m0 and m16 comparing unlabelled to [U-¹³C]-FA 16:0 containing medium at 0 and 6 hours in representative C4-2B spheroids. N=3 per group.
- (I) Fractional contribution of [U-¹³C]-FA 16:0 to palmitoyl-carnitine (orange) in 3D spheroid LNCaP, C4-2B, and 22RV1 models. Comparison of [U-¹³C]-FA 16:0 to palmitoyl-carnitine in 2D (green) and 3D (blue) models. Statistically significant differences to 2D cells are shown (multiple unpaired student t-tests).
- (J) Fractional contribution of [U-¹³C]-FA 16:0 derived succinate, fumarate, and malate in 3D spheroid models. N=3 per cell line.

Graphs show mean $\pm$ SEM unless stated otherwise. * $p < 0.05$ by One-way ANOVA with Dunnett's multiple comparisons test, unless state otherwise.

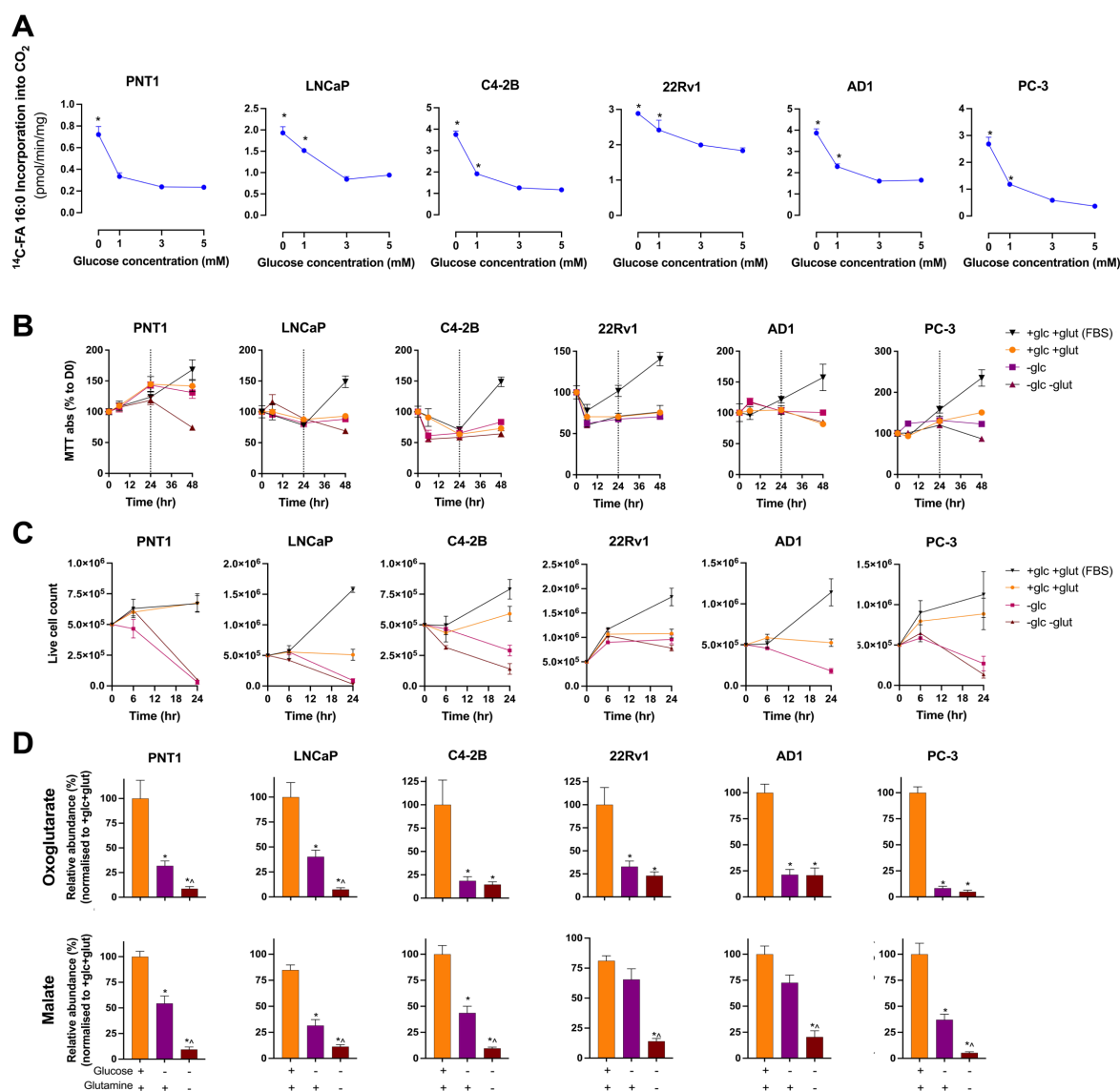


Figure S2, related to figure 2. Palmitate cannot maintain TCA cycle activity under glucose and glutamine deprivation.

- (A) [^{14}C]-FA 16:0 oxidation rates in the presence of 5mM, 3mM, 1mM, or 0mM glucose. N=3 per cell line. Statistically significant differences to 5mM glucose shown.
- (B) Impact of glucose- or glucose and glutamine- deprivation on metabolic activity assessed by MTT assay absorbance to 48 hours, normalised to day 0 results. Dotted line at 24 hours indicates replenishment of media.
- (C) Impact of glucose- or glucose and glutamine- deprivation on cell viability assessed by live cell counts at 0, 6 and 24 hours normalised to day 0 results.
- (D) Relative abundance (normalised to +glucose/+glutamine) of [^{13}C]-FA 16:0 to oxoglutarate or malate. N=3 per cell line.

Graphs show mean $\pm$ SEM. *compared to +glucose/+glutamine, ^compared to -glucose/+glutamine, $p < 0.05$ by One-way ANOVA with Dunnett's multiple comparisons test.

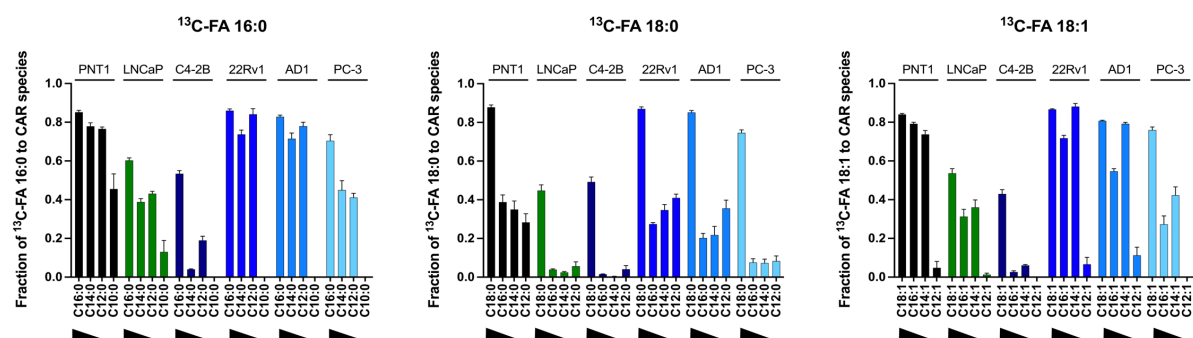


Figure S3, related to figure 3. LCFAs undergo incomplete FAO in prostate cells.

Fractional contributions of [U-¹³C]-FA 16:0, 18:0, 18:2 to shorter chain acyl-carnitine species. N=3 per cell line. Graphs show mean $\pm$ SEM.

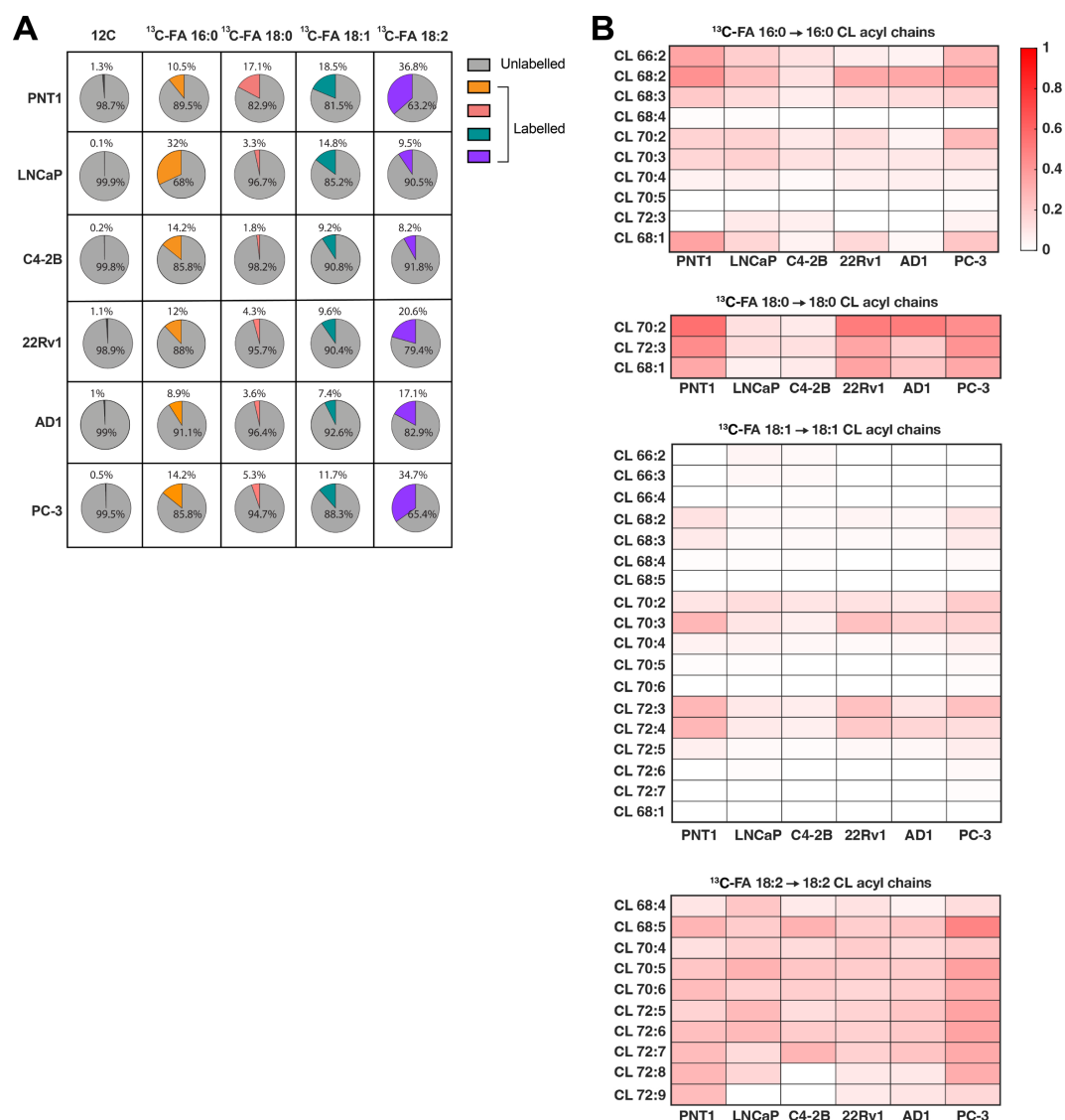


Figure S4, related to figure 4. FA 18:2 predominantly enriches CL pools compared to other FAs.

- (A) Percentage of labelled CL pools from [U-¹³C]-FA 16:0, 18:0, 18:1, 18:2 and unlabelled 12C controls. N=3 per cell line.
- (B) Fractional enrichment of [U-¹³C]-FA 16:0, 18:0, 18:1, 18:2 (150μM) to directly labelled acyl chains of CL species for 6hours. N=3 per cell line.

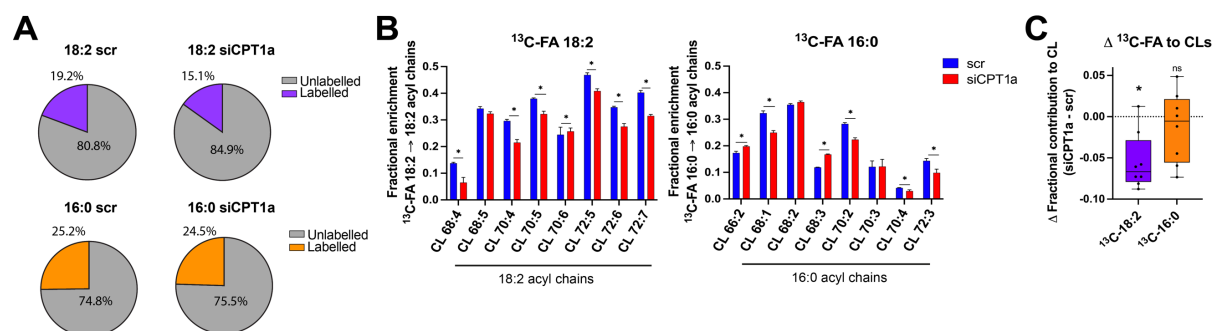


Figure S5, related to figure 5. CPT1a^{KD} reduces FA 18:2 incorporation to CLs.

- (A) Percentage of labelled to unlabelled (grey) CL pools from [U-¹³C]-FA 18:2 or 16:0 scrambled (scr) and siCPT1a cells. N=3 per group.
- (B) Fractional contribution of [U-¹³C]-FA 18:2 or 16:0 to 18:2 or 16:0 acyl chains of CL species. N=3 per group.
- (C) Delta differences between fractional contribution of [U-¹³C]-FA 18:2 or 16:0 to acyl chains of CL species. N=3 per group. Statistically significant differences within [U-¹³C]-FA 18:2 or 16:0 groups to 0 difference. Error bars represent $\pm$ min to max values.

Graphs show mean $\pm$ SEM unless stated otherwise. ns not significant, * p <0.05 by multiple unpaired t-tests between siCPT1a and scr control groups unless stated otherwise.

Acknowledgements

N.T.S. is supported by the Australian Rotary Health/Rotary Club of Blacktown City ‘Mel Grey’ PhD scholarship. L.M.B. and A.J.H. acknowledge grant support from The Movember Foundation/Prostate Cancer Foundation of Australia (MRTA3). A.J.H. is supported by a Robinson Fellowship from the University of Sydney and funding from the University of Sydney. L.M.B. is supported by a Principal Cancer Research Fellowship produced with the financial and other support of Cancer Council SA’s Beat Cancer Project on behalf of its donors and the State Government of South Australia through the Department of Health and was supported by a Future Fellowship from the Australian Research Council (FT130101004). We thank the Sydney Mass Spectrometry facility for access to LC-MS instruments; Julia Scott and Dr Zeyad Nassar in the Butler lab (University of Adelaide) for assistance with 3D spheroid and mitotracker experiment development; Dr Helen McGuire and the Sydney Flow Cytometry facility for assistance with mitotracker experiments; and Prof. Lisa Horvath and members of the Butler lab for scientific discussions.

Author contributions

Conceptualisation by A.J.H. and L.E.Q. Project administration, visualisation, writing-original draft by N.T.S. Investigation by N.T.S., M.H.S., and A.W. Methodology by L.E.Q. Software by N.T.S. and L.E.Q. Formal analysis by N.T.S. and L.E.Q. Writing-editing & reviewing by N.T.S., L.E.Q., A.J.H., and L.M.B. Funding acquisition, resources by A.J.H. Supervision by A.J.H., L.E.Q., and L.M.B.

Declaration of interests

The authors declare that they have no competing interests.

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Chapter 4

Fatty acids are not a significant contributor to the TCA cycle in cancer cell lines.

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Summary

Fatty acid (FA) oxidation (FAO) is upregulated in many cancers, but whether this correlates with a greater bioenergetic contribution, relative to glucose and glutamine, remains unclear. Here, we set out to determine how much carbon FAO contributes to the TCA cycle. We profiled the baseline FAO rates and capacities of 27 cancer cell lines from 10 tissue origins, and then selected 6 cancer cell lines that ranged from high-to-low FAO capacity. Despite the range of FAO rates, we still consistently found exogenous long chain FAs (LCFAs) to be a minor (<10%) TCA cycle substrate in all cells. Glucose withdrawal significantly increased FAO as measured by $^{14}\text{CO}_2$, while glucose and/or glutamine deprivation only marginally increased conversion of ^{13}C -palmitate into TCA cycle metabolites. Palmitate alone modestly assisted cataplerosis but did not sustain TCA cycle fluxes. Instead of FAO, we found rapid cardiolipin synthesis and turnover to be a significant consumer of exogenous LCFAs. Overall, our results provide major insights into the contributions of intra-mitochondrial LCFAs to cardiolipins in multiple cancer types independent to FAO activity.

Keywords

Mitochondria, fatty acid oxidation, parallel stable isotope tracing, citrate, cardiolipin, metabolomics

Introduction

The overexpression of fatty acid (FA) oxidation (FAO) enzymes plays a role in tumour growth and is widely associated with poor prognosis in cancers including glioma¹, prostate² and lung cancers³. Drugs targeting FAO reactions are effective against particular cancers and have been reported to reduce ATP production in pancreatic ductal adenocarcinoma⁴ and sensitise leukemia cells to proapoptotic effects by increasing membrane permeability⁵. Of these, the inhibition of carnitine palmitoyltransferase 1 (CPT1)^{6,7}, a well-known gatekeeper of FAO⁸, directly impacted ATP production leading to reduced cell viability in various cancers (glioblastoma⁹, prostate cancer¹⁰, myeloma¹¹ and glioma¹²), or indirectly by perturbing mitochondria structure or morphology first before impacting respiration^{13,14}. By shedding light on the gap between FAO and respiration, we stand a better chance at understanding the mechanisms behind FAO targeting drugs.

We would often link FAO to respiration by default, since NADH, FADH₂ and acetyl-CoA generated by FAO are directed into pathways producing ATP^{15,16}. In other words, we assume that FAs have a greater contribution to TCA cycle fluxes when FAO is increased. One way to verify this assumption is to compare the magnitude of FAO relative to the oxidation of other TCA cycle carbon sources, namely glucose and glutamine^{17,18}. To this end, our group established in prostate cancers (PCa) that exogenous long chain FAs (LCFAs) are minor carbon sources entering the TCA cycle compared to glucose and glutamine (Santiappillai *et al*, unpublished), despite PCa known to possess high FAO rates¹⁹. We set out to examine FAO contribution to TCA cycle fluxes by characterising a panel of cancer cells from different lineages.

Results

Fatty acid oxidation in cancer cells showed diverse rates and capacity.

To have adequate representation across the spectrum of FAO phenotypes, we screened 27 cancer cells from 10 different origin sites (Figure 1A) for their FAO rates using [1-¹⁴C]-palmitate labelled CO₂ production. We saw obvious differences in baseline FAO rates within and between cancer types (Figure 1B). Notably PCa cells in general had the highest FAO rates compared to other cancer types, with the exception of the MDA-MB-468 (breast) and HEPG2 (liver) cells (Figure 1B).

FAO rates increase in response to the inhibition of glycolysis or low glucose uptake, such as in glioblastoma and breast cancers^{20,21}. We also wanted to have sufficient coverage of cells in terms of FAO capacity, and so, we quantified FAO rates in glucose-free conditions. All 27 cancer cell lines substantially increased FAO rates, but the magnitude of increases was variable (1- to 8- fold) and did not correlate with baseline values (Figure 1C and S1A). We found FAO rates were suppressed by glucose in media; FAO only increased when media glucose was 1mM or below (Figure S1B and S1C), and that FAO was not dependent on nutrient gradients since a 5-fold increase in media palmitate did not increase FAO rate (Figure S1D).

From our FAO screening, we selected 6 cancer cell lines that represented the spectrum of high- to low-FAO capacity. They were ranked in terms of increasing fold changes (FC) of FAO rate (-glucose to + glucose): MDA-MB-231 and MCF-7 (breast), MIA PaCa-2 (pancreatic), DO4 (melanoma), HEPG2 (liver), and HT-29 (colorectal) (Figure 1D and S1A). Among these cell lines, CPT1a protein expression spanned 0.1-to-2.3-fold compared to MDA-MB-231 cells (Figure 1E), but unexpectedly correlated negatively with FAO FC (Figure 1F). There was no correlation of CPT1a protein expression with baseline (+ glucose) or maximal FAO (-glucose) (data not shown). Similarly, FAO (basal, maximal and FC) could not be explained by the cellular amount of active mitochondria (data not shown). Collectively, these results show that glucose availability had the greatest influence on FAO rates, followed by factors intrinsic to the cell lines rather than tissue types.

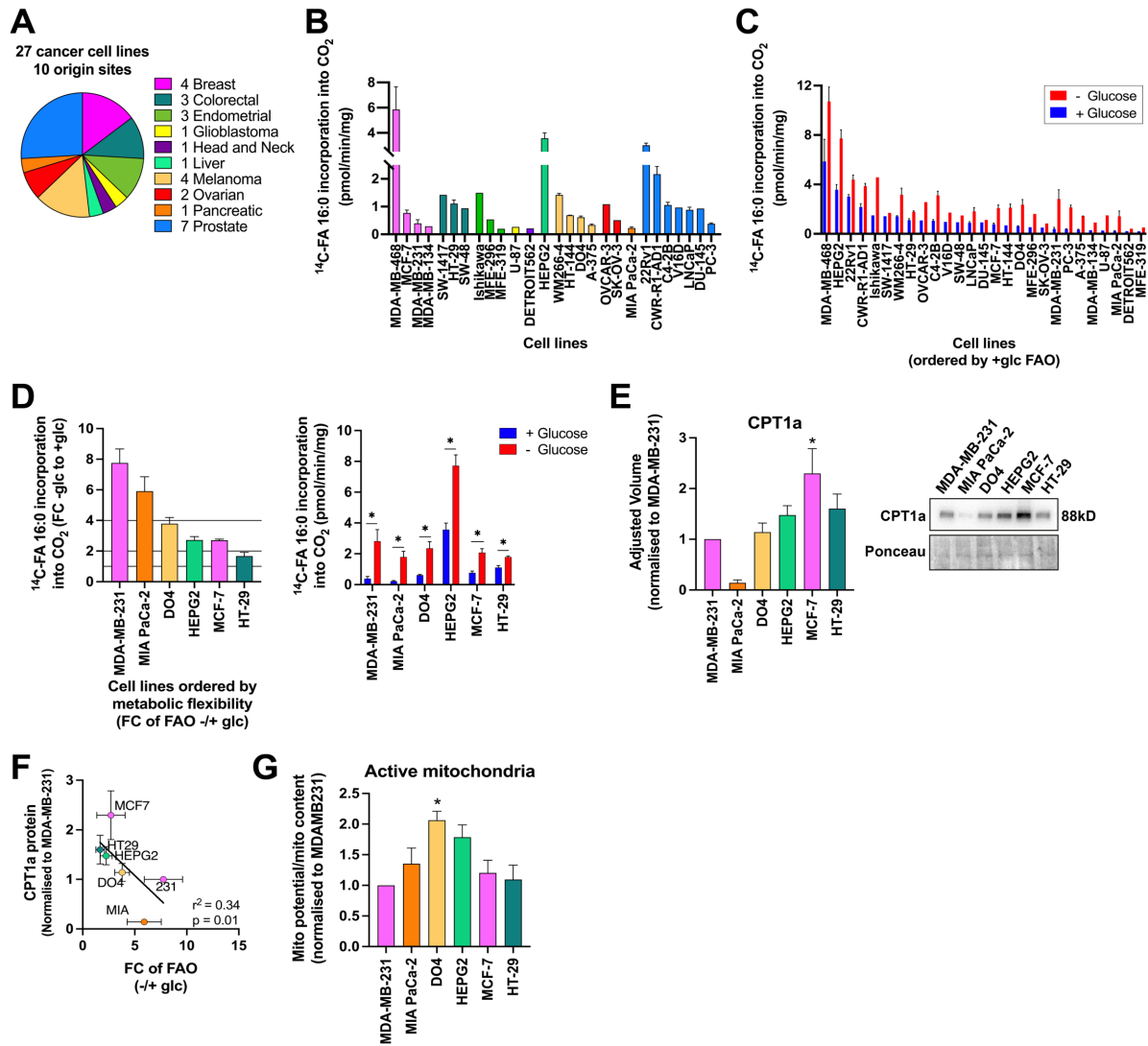


Figure 1 FAO in a panel of heterogeneous cancer cells from different tissue origin sites.

- (A) Panel of 27 cancer cell lines from 10 tissue origin sites measured for FAO capacity.
- (B) Basal $[1-^{14}\text{C}]$ -FA 16:0 oxidation rates in the presence of glucose. Cells ordered by cancer origin site. N=1-5 per cell line.
- (C) $[1-^{14}\text{C}]$ -FA 16:0 oxidation rates in the presence (blue) or absence (red) of glucose. N=1-5 per cell line.
- (D) Selection of 6 cell lines based on a range of fold change (FC) differences (<2 to >7, left) of FAO -/+ glucose (right). N=3-4 per cell line.
- (E) Quantification of CPT1a protein expression normalised to MDA-MB-231 cells. One of three immunoblot experiments is shown.
- (F) Correlation between CPT1a protein levels and the fold changes of FAO -/+ glucose. Error bars represent $\pm$ SD. P and r^2 values by linear regression.
- (G) Active mitochondria as a fraction of mitochondrial potential to mitochondrial content normalised to MDA-MB-231 cells. N=3-4 per cell line.

Graphs show mean $\pm$ SEM unless stated otherwise. * $p < 0.05$ by One-way ANOVA with Dunnett's multiple comparisons test, unless stated otherwise. See also Figure S1.

Palmitate is a minor TCA cycle carbon source compared to glucose and glutamine.

FAO is commonly assumed to be a major contributing source to the TCA cycle as its end-product acetyl-CoA can supply carbons to the TCA cycle and ATP production^{15,16}. We showed cancer cell lines span a spectrum of high-to-low FAO activity (Figure 1) and next questioned if these rates correlated with the amount of carbons arriving at the TCA cycle compared to glucose and glutamine. We utilised a parallel tracing strategy that consisted of feeding cells uniformly labelled ¹³C glucose, glutamine, or palmitate (FA 16:0) and quantifying the enrichment of TCA cycle intermediates in our panel of 6 cancer cell lines (Figure 2A). We first identified that 6 hours was the optimal tracing duration as media substrate levels were not fully depleted nor was there evidence of impaired substrate uptake (Figure S2A). Moreover, the depletion of glucose at 12 and 24 hours that can impact FAO rates (Figure 1) can be somewhat avoided by doubling the media volume (Figure S2B).

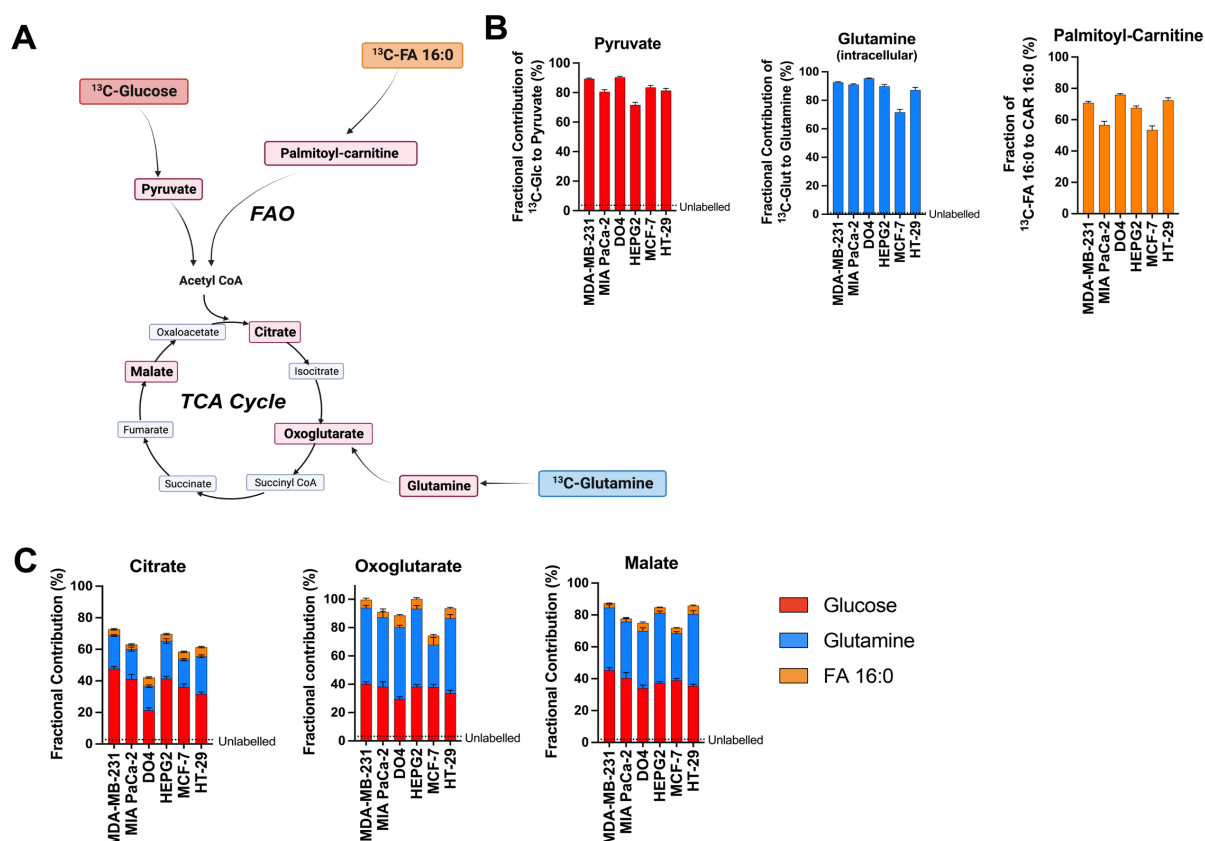


Figure 2 Palmitate is a minor contributing substrate source to the TCA cycle compared to glucose and glutamine.

- (A) Schematic of $[\text{U-}^{13}\text{C}]$ -glucose (5mM), glutamine (1mM), or FA 16:0 (150 μM) tracing into the TCA cycle and precursor metabolites for 6hours.
- (B) Fractional contribution of $[\text{U-}^{13}\text{C}]$ -glucose to pyruvate (red), $[\text{U-}^{13}\text{C}]$ -glutamine to intracellular glutamine (blue), $[\text{U-}^{13}\text{C}]$ -FA 16:0 to palmitoyl-carnitine (orange) after 6 hours of labelling. N=3 per cell line. Level of natural enrichment of metabolite standards indicated.
- (C) Fractional contributions of $[\text{U-}^{13}\text{C}]$ -glucose, glutamine, or FA 16:0 to citrate, oxoglutarate and malate. N=3 per cell line.

Graphs show mean $\pm$ SEM unless stated otherwise. See also Figure S2.

Following confirmation that a 6-hour endpoint for tracing experiments avoided substrate depletion, we quantified the relative contributions of exogenous palmitate, glucose, and glutamine to TCA cycle metabolites. We observed significant enrichments of key precursor pools; >80% enrichment of pyruvate from ^{13}C -glucose, >60% enrichment of intracellular glutamine from extracellular ^{13}C -glutamine, and >56% enrichment of palmitoyl-carnitine from ^{13}C -palmitate (Figure 2B). Despite being taken up and activated by cells, palmitate minimally contributed (<6%) to TCA cycle metabolites citrate, oxoglutarate and malate, compared to glucose (30-50%) and glutamine (20-45%) (Figure 2C).

Strikingly, this pattern was observed in all cell lines, and there was no distinction between cells with high (MDA-MB-231, MIA PaCa-2) or low (MCF-7, HT-29) FAO activity (Figure 1D). Therefore, these results show that exogenous palmitate is a minor contributing substrate to the TCA cycle in the presence of physiological glucose and glutamine, irrespective of cellular FAO activity as measured by $^{14}\text{CO}_2$ production.

Exogenous palmitate is insufficient to maintain TCA cycle activity in the absence of glucose and glutamine.

Several studies have shown that inhibiting FAO in glucose-deprived conditions or co-targeting with glycolysis inhibitors decreases respiration, cancer cell viability and tumour volume^{20,22}. Since we observed that FAO rates increase in glucose-deprived conditions (Figure 1C and 1D), we reasoned that FAO becomes the primary carbon source for the TCA cycle in these settings. We next set out to determine the maximal contribution of FAO to the TCA cycle. Firstly, we observed increased ^{13}C -palmitate enrichment into citrate in glucose-free, and glucose-/glutamine-free conditions in all cells except for the DO4 cells (Figure 3A and 3B). However, the contribution of palmitate to citrate remained <13%. This increased fuelling of the TCA cycle by FAO in glucose-/ glutamine free conditions was also associated with other changes in metabolism. Specifically, we observed increased m+2 and, to a greater extent, m+4 labelling of citrate that correspond to the 1st and 2nd turns of the TCA cycle, respectively (Figure 3C). These results suggest that palmitate-derived carbons go through multiple rounds of the TCA cycle when glucose, or both glucose and glutamine are absent in culture media.

Another striking shift in palmitate metabolism under glucose-/ glutamine-free media conditions was the enrichment of the cataplerosis metabolites glutamate and aspartate (Figure 3A). Under glucose and glutamine replete conditions, the conversion of palmitate carbon into glutamate and aspartate was minimal (~2-6%) (Figure 3D). However, a shift in palmitate carbon utilisation was observed in glucose and glutamine-deprived medium (~10-19%) and to a lesser extent in glucose-deprived medium (~5-14%) (Figure 3D). Overall, these results indicate that cancer cells have the capacity to increase the

catabolic conversion of palmitate into TCA cycle metabolites and amino acids, but this only occurs in extreme conditions when glucose and/or glutamine are unavailable.

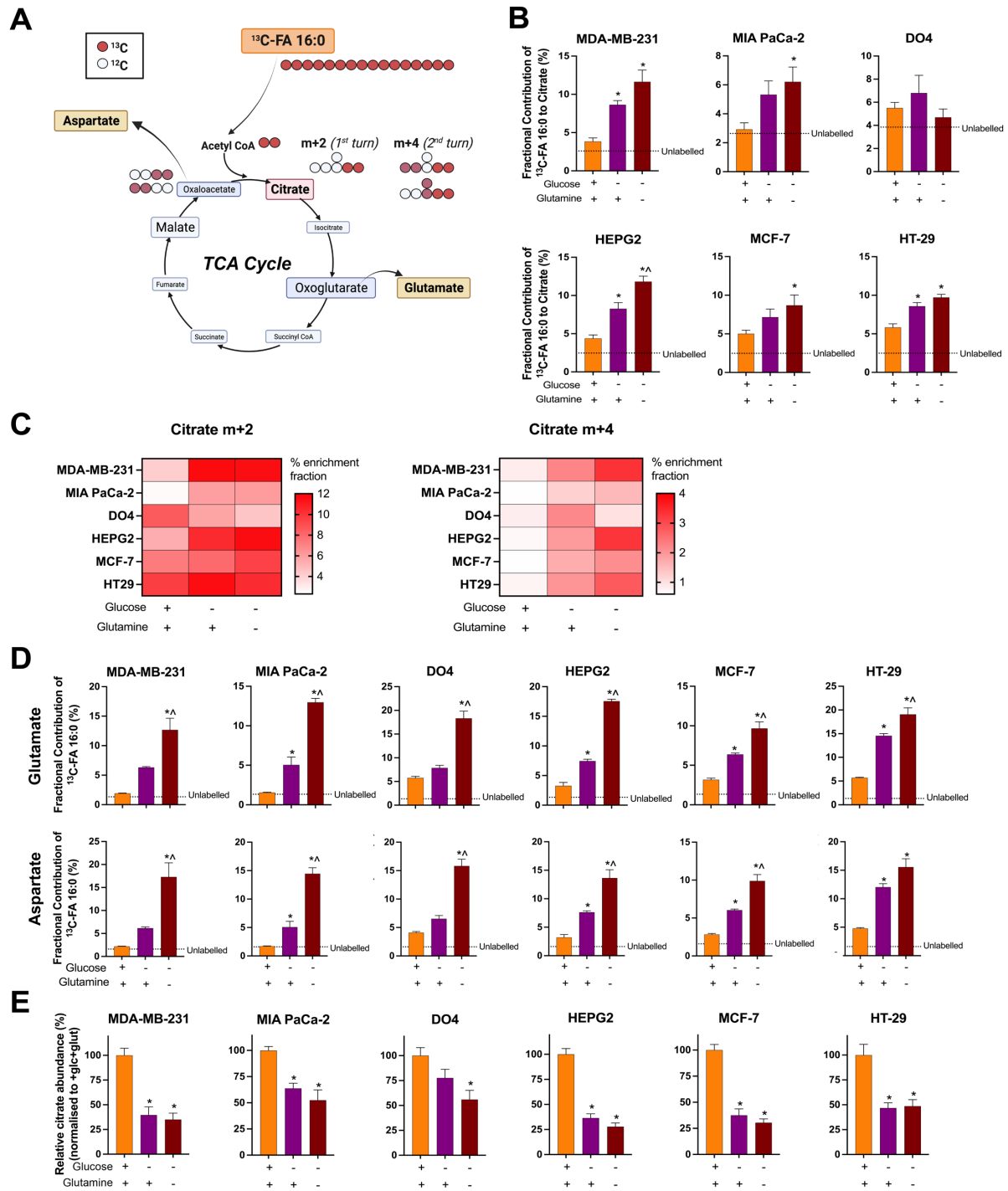


Figure 3 Palmitate alone cannot maintain TCA cycle activity.

- (A) Schematic tracing conversion of [U-¹³C]-FA 16:0 to citrate m+2 and m+4, glutamate, aspartate.
- (B) Fractional contribution of [U-¹³C]-FA 16:0 to citrate +glucose/+glutamine, -glucose/+glutamine, or -glucose/-glutamine. N=3 per cell line.
- (C) Enrichment fraction (%) of [U-¹³C]-FA 16:0 to m+2 and m+4 citrate + glucose/+glutamine, -glucose/+glutamine, and -glucose/-glutamine. N=3 per cell line.
- (D) Fractional contribution of [U-¹³C]-FA 16:0 to glutamate and aspartate +glucose/+glutamine, -glucose/+glutamine, or -glucose/-glutamine. N=3 per cell line.
- (E) Relative abundance (normalised to +glucose/+glutamine) of [U-¹³C]-FA 16:0 to citrate. N=3 per cell line.

Graphs show mean $\pm$ SEM. $P < 0.05$ by One-way ANOVA with Dunnett's multiple comparisons test, *compared to +glucose/+glutamine, ^compared to -glucose/+glutamine, See also Figure S3.

We observed increased incorporations of palmitate-derived carbons into TCA cycle metabolites in glucose-/ glutamine-free settings, and next questioned whether this was sufficient to maintain TCA cycle activity. By contrast to enrichment results, the relative abundance of glucose-/ glutamine-deprived citrate, oxoglutarate, and malate compared to nutrient-replete conditions significantly reduced (Figure 3E and S3B). These changes preceded the decreased cell viability that occurs between 6 to 24 hours (Figure S3A). Taken together, these findings demonstrate that despite the dynamic shift in FAO activity of cancer cells upon glucose-/glutamine-free conditions, palmitate remained insufficient to sustain TCA cycle metabolite levels and activity and cell viability.

Exogenous LCFAs are not major TCA cycle substrates.

One possible explanation for the minor contribution of palmitate-derived carbons to TCA cycle metabolites is that there are differences in the utilisation of FAs based upon saturation (double bonds) and carbon chain lengths. Palmitate (FA 16:0) was chosen first because it is the most abundant circulating FA in humans²³. Thus, we set out to test other LCFAs in order to generalise our findings. Cells were incubated in media containing physiological levels of glucose and glutamine, supplemented with [U-¹³C]-palmitate (FA 16:0), stearate (FA 18:0), oleate (FA 18:1), linoleate (FA 18:2), or linolenate (FA 18:3). Each ¹³C-FA enriched their direct acyl-carnitine counterpart species by a fractional ratio of 0.5-0.95 (Figure 4A). Consistent with the results of our experiments using palmitate, exogenous LCFAs contributed <10% of labelled carbons to citrate, oxoglutarate, or malate (Figure 4B). Interestingly, cell lines with the smallest FAO shift in activity when deprived of glucose (DO4, HEPG2,

MCF-7, and HT-29 cells) had increased enrichments across all labelled FAs to TCA cycle metabolites compared to an unlabelled control (Figure 4B). However, these enrichment patterns (<10%) from LCFAs were substantially less than glucose (30-50%) and glutamine (20-45%) (Figure 1). Collectively, these data further demonstrate that exogenous LCFAs of different saturation and chain lengths are minor carbon sources to the TCA cycle.

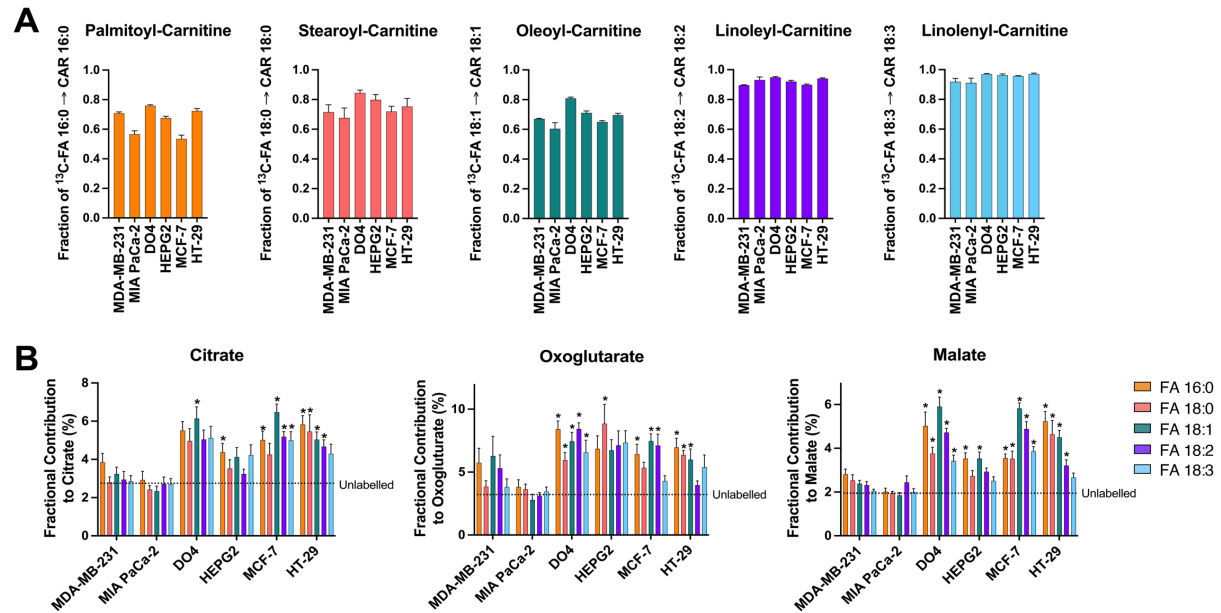


Figure 4 Extracellular LCFAs are minor contributing fuel sources to the TCA cycle in a pan-cancer setting.

- (A) Fractional contribution of acyl-carnitines associated with the $[\text{U-}^{13}\text{C}]$ - 16:0, 18:0, 18:1, 18:2, 18:3 substrates. N=3 per cell line.
- (B) Fractional contribution of $[\text{U-}^{13}\text{C}]$ -FA to citrate, oxoglutarate, malate. N=3 per cell line.

Graphs show mean $\pm$ SEM unless stated otherwise. $P < 0.05$ by One-way ANOVA with Dunnett's multiple comparisons test, *compared to unlabelled control (dotted line) See also Figure S4.

We observed substantial labelling from exogenous LCFAs directly into acyl-carnitines but a minimal utilisation by the TCA cycle, and next questioned if this could be explained by FAO not completely oxidising LCFAs to acetyl-CoA. Our results support this by showing FA 16:0, 18:0, and 18:1 greatly enrich shortened acyl-carnitine species (Figure S4), a known read-out of incomplete FAO^{24,25}. Specifically, in all cancer cells, FA 16:0 enriched CAR 14:0 to CAR 12:0, FA 18:0 enriched CAR 16:0 to CAR 12:0, and FA 18:1 enriched CAR 16:1 to CAR 12:1 (Figure S4). Together, these data provide evidence that LCFAs are not completely oxidised to the maximal amount of acetyl-CoA for the TCA

cycle, and this could explain the minimal utilisation of LCFA-derived carbons to the TCA cycle in cancer cells.

LCFAs substantially enrich cardiolipin pools in cancer cells.

We have comprehensively shown that exogenous LCFAs are not completely oxidised to acetyl-CoA and are minor contributing substrates to the TCA cycle in a panel of cancer cell lines that exhibit low-to-high FAO activity. Inhibition of CPT1a, the gatekeeper of mitochondrial LCFA entry, leads to reduced cell viability and respiration^{9-12,26}, and altered mitochondrial morphology^{3,14} in multiple cancers. As such, we hypothesised that intra-mitochondrial LCFA-CoAs (i.e., downstream of CPT1-CPT2) are not exclusively partitioned into beta-oxidation and may serve as substrates for other processes vital to mitochondrial function. We turned our attention to membrane lipid homeostasis, focusing on cardiolipins (CLs) as they are exclusively located in the inner mitochondrial membrane (IMM)²⁷, remodelled using LCFA-CoAs^{28,29}, and along with other mitochondrial membrane lipids, are reduced in CPT1 loss-of-function settings^{13,30}.

We first quantified the contribution of extracellular LCFAs, including FA 16:0, 18:0, 18:1 and 18:2, to a range of CL species. Notably, all LCFAs had substantial fractional enrichment into CL species (up to 0.6) following 6 hours of incubation (Figure 5A). FA 18:2 had on average a 2-fold-greater labelling pattern to a range of CL species compared to other LCFAs in all cancer cells (Figure S5A). The LCFA enrichment patterns into CLs could be explained by a substantial proportion of direct labelling from FAs 16:0, 18:0 and 18:2 into their counter-part CL 16:0, 18:0, and 18:2 acyl-chains (Figure S5B). By contrast to these LCFAs, FA 18:1 minimally incorporated into CL 18:1 acyl-chains (Figure S5B). Specifically, CL 68:2 is a species which consists of 16:0 and 18:1 acyl-chains and it was substantially labelled by exogenous FA 16:0. Extracellular FA 18:2 significantly enriched CL species that had 18:2 acyl-chains such as CL 72:6, CL 72:4 and CL 70:2 (Figure 5B). Furthermore, our data revealed only subtle differences between cell lines with no distinguishing patterns (Figure 5B). Taken together, CLs were highly enriched by extracellular LCFAs in a substrate dependent manner based on acyl-chain composition in a diverse range of cancer cells.

Mitochondrial CL remodelling specifically re-acylate nascent CLs with FA 18:2 over FA 16:0³¹. As such, we predicted that upon limiting CPT1a and thereby the entry of FA 18:2 into mitochondria, that there will be less incorporation of FA 18:2 into CLs. Conversely, we expected FA 16:0 incorporation to be unaffected by CPT1a^{KD} as it will likely be incorporated into CLs via the synthesis pathways. This is because *de novo* CL synthesis involves the endoplasmic reticulum-derived phosphatidic acid and conversion to phosphatidylglycerol (PG) in the mitochondria that have no specific affinity for FAs incorporated into CL acyl-chains³². Counter to our expectations, there was no difference in the turnover of CL pools from FA 18:2 between scrambled control and CPT1a^{KD} in MCF-7 cells (Figure 6E and S6A). Although we observed some minor differences in the contribution of FA 18:2 and FA 16:0 to PGs and CLs between the control and CPT1a^{KD} cells (Figure 6F and S6C), there was no overall effect observed (Figure S6B). Combined, these results suggest that CPT1a activity does not impact the incorporation of exogenous FAs into CLs by the remodelling pathways.

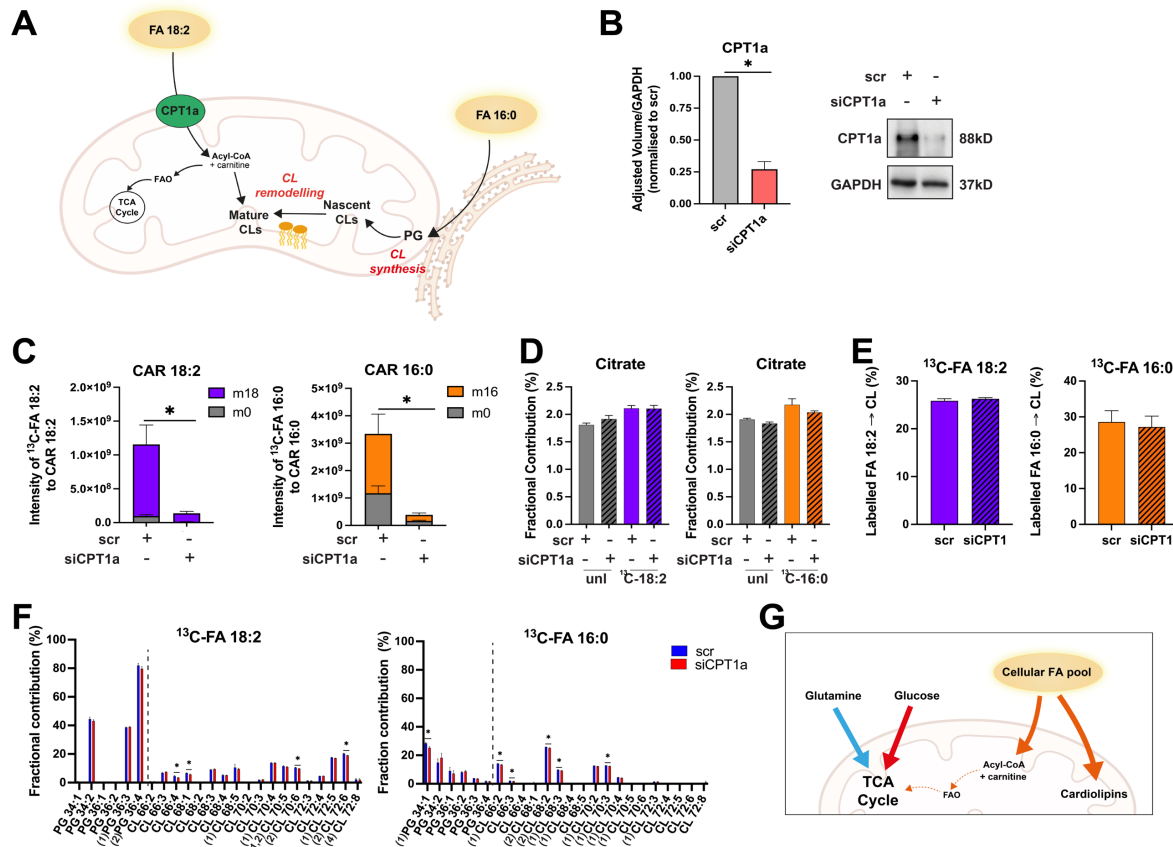


Figure 6 Levels of LCFA derived CLs are maintained in CPT1A^{KD} cells.

- (A) Schematic depicting expected FA 18:2 to CL remodelling and FA 16:0 to CL synthesis pathways.
- (B) CPT1a knockdown by reverse siRNA transfection in MCF-7 cells for 48 hours quantified by immunoblot. One of three experiments shown. N=3 per group.
- (C) Intensity of [U-¹³C]-FA 18:2 or 16:0 to carnitine species. N=3 per group. Statistically significant differences between labelled (m16 or m18) shown.
- (D) Fractional contribution of unlabelled or [U-¹³C]-FA 18:2 or 16:0 to citrate in scrambled (scr) or siCPT1a MCF-7 cells. N=3 per group.
- (E) Percentage of labelled CL pools from [U-¹³C]-FA 18:2 or 16:0. N=3 per group. Statistically significant differences between scr and siCPT1a groups shown.
- (F) Fractional contribution of [U-¹³C]-FA 18:2 or 16:0 to PG and CL species. Annotated n values on x-axis PG and CL species indicate the number of containing 18:2 or 16:0 acyl-chains. N=3 per group. Multiple unpaired t-tests.
- (G) Schematic depicting major carbon contributions from glucose and glutamine to the TCA cycle compared to LCFAs via FAO, and substantial contribution from LCFAs to acyl-carnitines and cardiolipins.

Graphs show mean ± SEM unless stated otherwise. *p<0.05 between siCPT1a and scr control groups by student's t-test unless stated otherwise. See also Figure S6.

Discussion

Although the convergence of glucose and glutamine at the TCA cycle has been a significant focus of the mitochondrial metabolism field in cancer, the contribution of FAO to the TCA cycle remains underappreciated. Here we show that exogenous LCFAs were not completely oxidised and were minor carbon sources to the TCA cycle compared to glucose and glutamine, irrespective of the FAO capacity of cancer cells. Moreover, CPT1a knockdown reduced acyl-carnitine levels, but this did not affect the contributions of FAs fuelling the TCA cycle. We also observed a substantial turnover of CLs from LCFAs in cancers, however, CPT1a depletion did not impact CL remodelling pathways in MCF-7 cells (Figure 6G). Together, our findings show a substantial role of LCFAs to CLs in cancers spanning a diverse spectrum of FAO activity. These novel insights could broaden our understanding to target CL-associated mitochondrial bioenergetics in cancers.

Cancer cell metabolism is highly adaptable under nutrient-deprived conditions and can switch to alternative fuel sources to maintain tumour survival³³. Notably, previous studies observed highly glycolytic cells can also upregulate FAO as a partial compensatory measure when glucose uptake is limited or deprived to maintain OXPHOS and ATP production^{20,22}. Our results support these phenomena as we showed that cancer cells undergo a metabolic shift to increase FAO rates and the contribution of palmitate to the TCA cycle and cataplerosis metabolites when deprived of glucose and glutamine. These results were in striking contrast to the minor carbon fuelling from LCFAs to the TCA cycle in glucose and glutamine replete conditions. However, this adaptive role in fuelling the TCA cycle could not meet the levels sustained by glucose or glutamine, and most importantly, the TCA cycle metabolite pool sizes were markedly reduced in substrate-deprived settings. These results show that FAO is an insufficient carbon source to maintain TCA cycle activity.

Others have shown that inhibiting CPT1a, the initiating step for LCFA entry to the mitochondria, leads to decreased respiration and cell viability^{9-12,26}. The primary assumption made by these studies is that mitochondrial LCFAs are solely destined to undergo complete oxidation within the mitochondria and

provide acetyl-CoA for the TCA cycle, and subsequent ATP production to support tumour growth. These studies presumptively couple CPT1a activity to FAO and bioenergetics. A key example is a study that combined glycolysis and CPT1a inhibitors that synergistically improved survival in a glioblastoma model and concluded that co-targeting of glycolysis and FAO is a new therapeutic strategy²⁰. Our findings demonstrated here and in our recent study (Santiappillai *et al.*, unpublished), directly challenge these assumptions by showing that LCFAs are a minor carbon source to the TCA cycle and that this was unaffected by CPT1a knockdown. A logical explanation for this lack of effect was that LCFAs entering the mitochondria via CPT1a are not completely oxidised for the TCA cycle, nor are the sole intra-mitochondrial fate as evidenced by enriched shortened acyl-carnitines from LCFAs and the decreased acyl-carnitine levels after CPT1a knockdown.

CLs provided a reasonable mitochondrial fate for exogenous LCFAs to investigate as they play significant roles in ETC complex structure, bioenergetics, and maintaining mitochondrial integrity^{34,35}, and are dominated by long chain fatty acyl chains²⁸. Our data supported our previous findings (Santiappillai *et al.*, 2018) that CLs are a major sink for exogenous LCFAs. Previous studies have shown that CL levels are reduced when CPT1 activity is limited^{13,30}. As such, CPT1a may regulate the assembly of CL acyl chains from LCFA-CoAs by CL remodelling pathways that undergo specific 18:2 donation³¹. By contrast to our previous study in C4-2B PCa cells (Santiappillai *et al.*, 2018), we discovered that CPT1a^{KD} had no effect on CL remodelling pathways in MCF-7 breast cancer cells. This could result from MCF-7 cells acquiring acyl-CoAs from sources outside the mitochondria as a compensatory measure when CPT1a activity is limited to maintain CL levels and functions. For example, MCF-7 cells may have shifted to a reliance on Tafazzin-dependent remodelling, which requires mitochondrial independent PC mediated acyl-transfers³⁶, instead of MLCLAT1 enzymes that require intra-mitochondrial acyl-CoA donation³⁷. Additionally, MCF-7 cells could shift from mitochondrial remodelling to ER-derived synthesis pathways with no intrinsic acyl-CoA preference when CPT1a activity is limited³². Future experiments could investigate the role of Tafazzin remodelling pathways, or CL synthesis pathways, to uncover novel dynamic shifts in LCFA utilisation for CLs to maintain cancer growth.

A limitation of these studies was that we only quantified the contribution of exogenous LCFAs, glucose, and glutamine which totalled 40-80% of the TCA metabolite pools, but other contributing carbon sources were likely at play. We could rule out the potential incorporation of free fatty acids and lipids as we incubated our cells in serum-free medium. However, endogenous FA sources, or amino acids in DMEM tracing medium, like branched chain amino acids, can also contribute carbons to the TCA cycle³⁸⁻⁴⁰. Another limitation of our study was that the CPT1a knockdown could only reduce MCF-7 protein levels by 73% when we tried to determine the role of CPT1a to CL remodelling. It is conceivable that this was insufficient to limit CL turnover from FA 18:2. Therefore, future experiments could utilise stable knockdowns for a more successful impairment of CPT1a activity, balanced with the possibility of adaptive mechanisms to overcome loss-of-functions.

Our study in multiple cancer cells provides quantitative evidence that LCFAs are minor carbons sources to the TCA cycle compared to glucose and glutamine, irrespective of intrinsic FAO activity. Fundamentally, we propose a new model for CPT1a to FAO and the TCA cycle by challenging the assumption that FAO is the sole fate of LCFAs entering the mitochondria in cancer cells. We provide evidence that CLs are a significant sink for exogenous LCFAs in cancer cells. We propose that CL pathways are adaptive and may compensate for impaired CPT1a activity to sustain CL levels. Together, this study provides the grounding to investigate the role of CLs in cancer cells for mitochondrial bioenergetics and a solid rationale to uncouple the therapeutic effects of CPT1a inhibitors and the TCA cycle.

Methods

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-CPT1a	Abcam	Cat#Ab128568; RRID: AB_11141632
Rabbit monoclonal anti-GAPDH	Cell Signalling Technology	Cat#2118; RRID: AB_561053
Chemicals, peptides, and recombinant proteins		
Enzalutamide	Selleckchem	Cat#S1250
Etomoxir	Sigma-Aldrich	Cat#C986W52
Lipofectamine RNAiMAX transfection reagent	Thermo Fisher Scientific	Cat#13778075
[1- ¹⁴ C] Palmitic acid	Perkin Elmer	Cat#NEC075H250UC
Palmitic acid	Sigma-Aldrich	Cat#P0500
D-Glucose	Sigma-Aldrich	Cat#273053
L-Glutamine	Gibco	Cat#25030081
Fatty acid free BSA	Bovogen	Cat#BSAS0.10
L-carnitine hydrochloride	Sigma-Aldrich	Cat#C0283
Mitotracker Green	Thermo Fisher Scientific	Cat#M7514
Mitotracker Red	Thermo Fisher Scientific	Cat#M7512
FCCP	Sigma-Aldrich	Cat#SML2959
DTT	Sigma-Aldrich	Cat#D0632
Protease and Phosphatase Inhibitor Cocktail, EDTA free	Astral Scientific	Cat#T-2496
Thiazolyl blue tetrazolium bromide MTT reagent	Sigma-Aldrich	Cat#M2128
Linoleic acid	Sigma Aldrich	Cat#L1012
D-Glucose (U-13C6, 99%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-1396

L-Glutamine (U-13C5, 99%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-1822-H
Palmitic acid (U-13C16, 98%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-409
Stearic acid (U-13C18, 98%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-6990
Linoleic acid (U-13C18, 98%)	Cambridge Isotope Laboratories, Inc.	Cat#CLM-10487
Linolenic acid (U-13C18, 98%)	Cambridge Isotope Laboratories, Inc.	Cat#8386
Critical commercial assays		
Pierce BCA Protein assay kit	Thermo Fisher Scientific	Cat#23225
Immobilon Crescendo Western HRP substrate	Merck Millipore	Cat#WBLUR0500
Experimental models: Cell lines		
MDA-MB-231	ATCC	ATCC HTB-26; RRID: CVCL_0062
MDA-MB-468	ATCC	ATCC HTB-132
MCF7	ATCC	ATCC HTB-22
MDA-MB-134	ATCC	ATCC HTB-23
C4-2B	ATCC	ATCC CRL-3315
PC-3	ATCC	ATCC CRL-1435
22Rv1	ATCC	ATCC CRL-2505; RRID: CVCL_1045
LNCaP	ATCC	ATCC CRL-1740; RRID: CVCL_1379
DU145	A/Prof. Luke Selth, Flinders University	ATCC HTB-81
CWR-AD1	A/Prof. Luke Selth, Flinders University	Nyquist 2013 ⁴¹ , Li 2013 ⁴²
MR42D	Prof. Lisa Butler, SAHMRI	Bishop 2017 ⁴³
HEPG2	A/Prof. Susan McLennan, University of Sydney	Nagarajan 2019 ⁴⁴
Ishikawa	Prof. Kyle Hoehn, UNSW	RRID: CVCL_2529

MFE296	Prof. Kyle Hoehn, UNSW	RRID: CVCL_1406
MFE319	Prof. Kyle Hoehn, UNSW	RRID: CVCL_2112
U87	Prof. Lenka Munoz, University of Sydney	RRID: CVCL_3429
MIAPACA-2	A/Prof Thomas Grewal, University of Sydney	RRID: CVCL_0428
OVCAR3	A/Prof. David Croucher, Garvan Institute	RRID: CVCL_0465
SKOV3	A/Prof. David Croucher, Garvan Institute	RRID: CVCL_0532
A375	Dr Lorey Smith, Peter MacCallum Cancer Centre	ATCC CRL-1619; RRID: CVCL_0132
HT144	Dr Lorey Smith, Peter MacCallum Cancer Centre	ATCC HTB-63; RRID: CVCL_0318
WM266.4	Dr Lorey Smith, Peter MacCallum Cancer Centre	ATCC CRL-1676; RRID: CVCL_2765
DO4-M1	Dr Lorey Smith, Peter MacCallum Cancer Centre	Parmenter 2014 ⁴⁵
Detroit562	A/Prof Thomas Grewal, University of Sydney	RRID: CVCL_1171
HT29	A/Prof. Kellie Charles, University of Sydney	RRID: CVCL_A8EZ
SW-48	A/Prof. Kellie Charles, University of Sydney	RRID: CVCL_1724
SW-1417	A/Prof. Kellie Charles, University of Sydney	RRID: CVCL_1717
Oligonucleotides		
ON-TARGETplus Human CPT1A siRNA SMARTpool	Horizon Discovery	Cat#L-009749-00-0005

ON-TARGETplus Non-targeting Control pool	Horizon Discovery	Cat# D-001810-10-05
Software and algorithms		
GraphPad Prism V9	GraphPad Software	https://www.graphpad.com/
Image Lab 6.1	Bio-Rad	https://www.bio-rad.com/en-au/product/image-lab-software
FlowJo 2	FlowJo LLC	https://www.flowjo.com/
MATLAB	Mathworks	https://au.mathworks.com/products/matlab.html
MSConvert	N/A	Chambers 2012 ⁴⁶
MSDial	Riken	http://prime.psc.riken.jp/compms/msdial/main.html
Biorender	Biorender	https://biorender.com/

Method details

Cell lines and culture conditions

A panel of 27 adherent cancer cell lines from 10 different origins were used in this study. Cell lines were obtained both from vendors and kindly provided by academic labs. Details of culturing media (Gibco) can be found in Supp Table 1. All culturing media were supplemented with 10% fetal bovine serum (Cytiva Hyclone) and 1% penicillin/streptomycin (Gibco). Cells were incubated at 37°C and 5% CO₂.

Cell Transfection

MCF-7 cells were transfected for 48 hours using RNAiMAX transfection reagent and 25 pmol pooled CPT1a siRNA or non-targeting scrambled control, according to the manufacturer's instructions. After 48 hours, cells were used for subsequent radiolabelling, western immunoblotting, and ¹³C-labelling experiments.

¹⁴C-Radiolabelling

Cells were washed in warm PBS and incubated in 600µL media containing 0.1mmol/L cold palmitate, [¹⁴C] palmitate (0.1µCi/mL; Perkin Elmer) conjugated to 2% (wt/vol) FA-free BSA and 1mmol/L - carnitine in RPMI or DMEM glucose-free medium (Gibco) for 4 hours. Fatty acid oxidation rates were determined from CO₂ production as previously described¹⁹ and supplemented 0mM or 5mM glucose to measure metabolic flexibility. Protein content was measured by BCA protein assay and absorbances read at 540nm using a Tecan infinite M1000 pro plate reader. Based on the fold changes of FAO rates +/- 5mM glucose, a curated selection of 6 cell lines were selected ranging from high (above 4-fold), mid (2-4-fold) and low (0-2-fold) ranges of fold changes for subsequent LC-MS.

Radiolabelled glucose titration

MDA-MB-231 cells were treated as above for radiolabelled experiments, supplemented with 0, 1, 3, 5, 7, 9 11, 25mM glucose +/- 10µM etomoxir. In the selected panel of 6 cell lines, cells were treated as above for radiolabelled experiments, supplemented with 0, 1, 3, 5mM glucose.

Mitotracker

Cells were seeded in 12-well plates at a density of 2×10^5 cells/well in RPMI or DMEM HG medium. After 24 hours, cells were washed in warm PBS and obtained by trypsinisation and collected in serum-free RPMI medium. The cell suspension was centrifuged at 1500 rpm for 5min in fluorescence-activated cell sorting (FACS) tubes. Cells were subsequently stained with 200nM Mitotracker Red and Mitotracker Green at 37°C for 30mins. Additionally, unstained, red only, green only, and 2.5µM FCCP treated cells were included as experimental controls. Cells were centrifuged at 1500 rpm for 5min and fixed with 4% paraformaldehyde at 37°C for 10min. Cells were washed with warm PBS and centrifuged at 1500 rpm for 5min and resuspended in 150µL PBS before the samples were read on a BD LSR Fortessa flow cytometer. Data analysis was performed using FlowJo software.

Immunoblotting

Cells were seeded in 6-well plates (5×10^5). After 24 hours, cells were lysed in RIPA buffer containing 1% DTT and 0.1% protease and phosphatase inhibitor cocktail. Protein content was measured by BCA protein assay, and absorbances were read at 540 nm using a TECAN infinite M1000 pro plate reader. Cell lysates were subjected to SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore). Ponceau staining or GAPDH was performed on membranes as a loading control, and then membranes were immunoblotted for CPT1a. Chemiluminescence was performed using Luminata Crescendo Western HRP Substrate and imaged using the Bio-Rad ChemiDoc MP Imaging System and Image Lab software 4.1 (Bio-Rad Laboratories).

Cell viability

Cells were seeded in 96-well plates in 6 well replicates (3×10^3 cells/well). After 24 hours the media was removed and replaced with Glucose- and Glutamine free media (Gibco) supplemented with 5mM glucose, 1mM glutamine and/or 150 μ M FAs, conjugated to 2% (wt/vol) FA-free BSA. For glucose-deprived conditions, DMEM no glucose, no glutamine media was supplemented with 1mM glutamine and 150 μ M palmitate; for glucose and glutamine-deprived conditions, media was supplemented with 150 μ M palmitate only. Media supplemented with 10% FBS was used as a control. At 6, 12 and 24 hours, 50 μ L of MTT reagent was added to wells. MTT reactions were incubated at 37°C and 5% CO₂ for 4 hours, and then stopped by replacing media with 100 μ L DMSO (Sigma–Aldrich) per well. Absorbances were read at 540 nm using a TECAN infinite M1000 pro plate reader. Cells were seeded in 6-well plates at 5×10^5 cells/well for live cell counts. After 24 hours, media was replaced as described for the MTT assay, and cells counted by Trypan blue (Gibco) exclusion at 6 and 24 hours.

[U-¹³C]-stable isotope tracing

Cells were seeded in 6-well plates (5×10^5). After 24 hours, plates were washed with warm PBS and media replaced with 600 μ L of DMEM no glucose, no glutamine media (Gibco) supplemented with

5mM glucose, 1mM glutamine and/or 150 μ M FAs, replaced with their respective [U-¹³]-C forms. [U-¹³C]-FAs (Palmitate (16:0), Oleate (18:0), Stearate (18:1), Linoleate (18:2), or Linolenate (18:3). For glucose-deprived conditions DMEM no glucose, no glutamine media was supplemented with 1mM glutamine and [U-¹³C]-150 μ M palmitate; for glucose and glutamine-deprived conditions media was supplemented with [U-¹³C]-150 μ M palmitate only. FAs were conjugated to 2% (wt/vol) FA-free BSA-containing media for 24 hours at 37°C before treating cells. Cells were incubated in ¹³C containing medium for 6 hours.

Extracellular substrate sampling

Cells were seeded in 6-well plates (5x10⁵). After 24 hours, wells were washed with warm PBS and media replaced with 1mL or 2mL of DMEM with no glucose, no glutamine media supplemented with 5mM glucose, 1mM glutamine, and 150 μ M FAs, conjugated to 2% (wt/vol) FA-free BSA. 100 μ L of extracellular media were collected from wells at 3, 6, 12, 24 hour timepoints for cells incubated in 1mL of media, and 12, 24 hours for cells incubated in 2mL of media. Media samples were centrifuged at 16,000 x g for 5min at 4°C, and the supernatant collected for subsequent LC-MS analysis.

Cell lysate extraction for LC-MS

Media was aspirated from plates and the cells washed with 2mL ice-cold 0.9% (w/v) NaCl. Cells were scraped with 300 μ L of extraction buffer, EB, (1:1 LC/MS methanol:water (Optima) + 0.1x internal standards (non-endogenous polar metabolites) and transferred to a 1.5mL microcentrifuge tube. A further 300 μ L of EB was added to the cells and combined in the tube. 600 μ L Chloroform (Honeywell) was added before vortexing and incubating on ice for 10 min. Tubes were vortexed briefly and centrifuged at 15,000g for 10min at 4°C. The aqueous upper layer was collected and dried without heat using a Savant SpeedVac (Thermo Fisher) for metabolomics LC-MS analysis. The lower layer was collected and dried under nitrogen flow for lipidomics LC-MS analysis.

Measuring stable-isotope labelled metabolites by LC-MS

Dried aqueous upper phase samples were resuspended in 40µL Amide buffer A (20mM ammonium acetate, 20mM ammonium hydroxide, 95:5 HPLC H₂O: Acetonitrile (v/v)) and vortexed and centrifuged at 15,000g for 5min at 4°C. 20µL of supernatant was transferred to HPLC vials containing 20µL acetonitrile for LCMS analysis of amino acids and glutamine metabolites. The remaining 20µL of the resuspended sample was transferred to HPLC vials containing 20µL LC-MS H₂O for LCMS analysis of glycolytic, pentose phosphate pathway (PPP), and TCA cycle metabolites. Amino acids and glutamine metabolites were measured using Vanquish-TSQ Altis (Thermo) LC-MS/MS system. Analyte separation was achieved using a Poroshell 120 HILIC-Z Column (2.1x150mm, 2.7µm) (Agilent) at ambient temperature. The pair of buffers used were Amide buffer A and 100% acetonitrile (Buffer B), flowed at 200µL/min; injection volume of 5µL. Glycolytic, PPP and TCA cycle metabolites were measured using 1260 Infinity (Agilent)-QTRAP6500+ (AB Sciex) LC-MS/MS system. Analyte separation was achieved using a Synergi 2.5µm Hydro-RP 100A LC Column (100x2mm) at ambient temperature. The pair of buffers used were 95:5 (v/v) water:acetonitrile containing 10mM tributylamine and 15mM acetic acid (Buffer A) and 100% acetonitrile (Buffer B), flowed at 200µL/min; injection volume of 5µL. Raw data from both LC-MS/MS systems were extracted using MSConvert⁴⁶ and in-house MATLAB scripts. The fractional contributions of labelled substrates to metabolites were calculated as the weighted sum by the number of labelled carbons⁴⁷.

Measuring extracellular substrates by LC-MS

20µL of collected extracellular media was mixed with 80µL water, vortexed. 10µL of diluted media was mixed with 90µL of extraction buffer containing 1:1 (v/v) acetonitrile and methanol + 1x internal standards (non-endogenous standards) at -30°C. The mixture was centrifuged at 12,000xg for 5min at 4°C and transferred into HPLC vials for LC-MS analysis measured using the Vanquish-TSQ Altis as described above. Raw data from both LC-MS/MS systems were extracted using MSConvert⁴⁶ and in-house MATLAB scripts.

Measuring stable isotope labelled lipids by LC-MS

Dried lower phase samples were resuspended in 100 μ L 4:2:1 (v/v) isopropanol:methanol:chloroform containing 7.5mM ammonium formate. Cardiolipin and acyl-carnitine lipids were measured using a Thermo Scientific Q-Exactive-HF-X Hybrid Quadrupole Orbitrap LC-MS/MS system.

For cardiolipins, analyte separation was achieved using an Agilent Poroshell 120, EC-C18, 2.1x150mm, 2.7 μ m column. The pair of buffers used were 60:40 acetonitrile:water (v/v) with 10mM ammonium formate and 0.1% formic acid (mobile phase A), and 90:10 isopropanol:acetonitrile (v/v) with 10mM ammonium formate and 0.1% formic acid (mobile phase B), flowed at 200 μ L/min on negative mode. MS1 data was acquired with the following settings: 3.5kV, capillarity temperature 300°C 120 000, injection time 100ms, AGC 1x10⁶, scan range 1100-1650. For ddMS2 data acquirement, the following settings were used: top 20, resolution 30,000, 200-2000, isolation 1.0m/z, nce 30, AGC target 1x10³, intensity threshold 5x10³, dynamic exclusion 20s. Raw data from both LC-MS/MS systems were extracted using MSConvert⁴⁶ and in-house MATLAB scripts and MSDial.

For acyl-carnitines, analyte separation was achieved using an Agilent Poroshell 120, HILIC, 2.1x100mm, 2.7 μ m column. The pair of buffers used were 0.1(v/v)% formic acid and 10mM ammonium formate in H₂O and 0.1(v/v)% formic acid in acetonitrile, flowed at 200 μ L/min on positive mode. MS1 data was acquired with the following settings: 3.5kV, capillarity temperature 300°C, 120 000, injection time 100ms, AGC 1x10⁶, scan range 200-1000. For ddMS2 data acquirement, the following settings were used: top 5, resolution 30, 000, 200-2000, isolation 1.0m/z, nce 30, AGC target 1x10³, intensity threshold 5x10³, dynamic exclusion 20s. Raw data from both LC-MS/MS systems were extracted using MSConvert⁴⁶ and in-house MATLAB scripts.

Quantification and statistical analysis

Student's t-test, one- or two-way ANOVA with Dunnett's or Tukey's multiple comparisons, and linear regression statistical tests were performed with GraphPad Prism 9.0 (GraphPad Software). $P < 0.05$ was considered significant. Data are reported as mean $\pm$ standard error of the mean (SEM), standard

deviation (SD), or min-max values of at least three independent determinations (N=3 as indicated in figure legends) with at least 3 technical replicates each. Radiolabelling experiments for the expansive cell line panel were of at least one independent determination (N=1-x as indicated in the respective figure legend) with at least 3 technical replicates each. Schematic diagrams were created with Biorender.com.

Supplementary information

Supplementary Table 1: Cell line details

Cell line	Cancer type	Culture media (Gibco)
MDA-MB-231	Breast	DMEM HG
MDA-MB-468		RPMI
MCF7		DMEM HG
MDA-MB-134		RPMI
C4-2B	Prostate	RPMI
PC-3		RPMI
22Rv1		RPMI
LNCaP		RPMI
DU145		RPMI
CWR-AD1		RPMI
MR42D		RPMI + 10 μ M enzalutamide
HEPG2	Liver	DMEM HG
Ishikawa	Endometrial	DMEM HG
MFE296		DMEM HG
MFE319		DMEM HG
U87	Glioblastoma	DMEM HG
MIAPACA-2	Pancreatic	DMEM HG
OVCAR3	Ovarian	RPMI
SKOV3		RPMI
A375	Melanoma	RPMI
HT144		RPMI
WM266.4		RPMI
DO4-M1		RPMI
Detroit562	Head and Neck	DMEM/F12
HT29	Colorectal	DMEM HG

SW-48		DMEM HG
SW-1417		DMEM HG

Supplementary figures

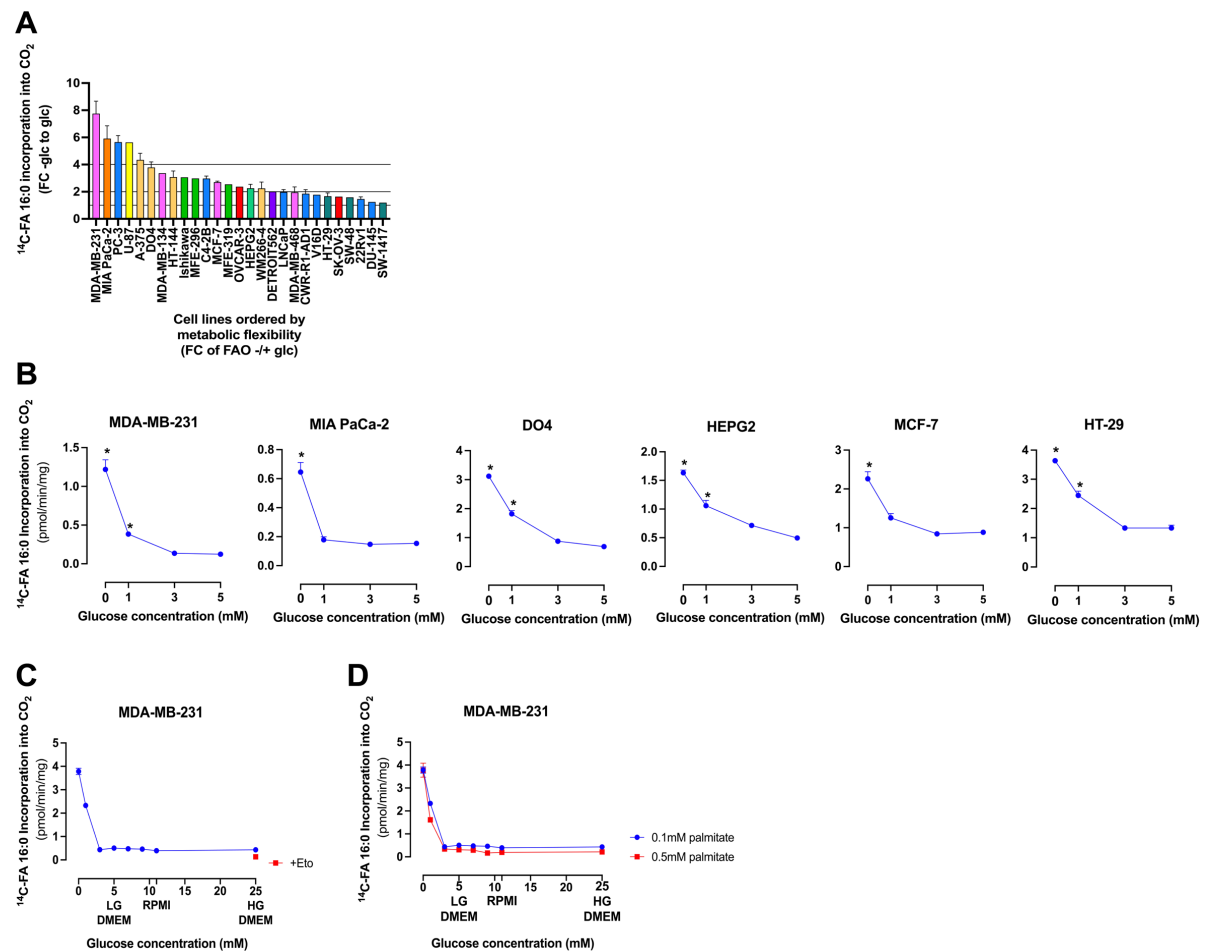


Figure S1, related to figure 1. FAO increases most dramatically under complete glucose deprivation.

- (A) Fold change (FC) difference of ^{14}C -FA 16:0 oxidation rates $\rightarrow$ glucose. Cell lines ranked by fold change difference magnitude. N=1-5 per cell line.
- (B) [^{14}C]-FA 16:0 oxidation rates in the presence of 5mM, 3mM, 1mM, or 0mM glucose. N=3 per cell line. Statistically significant differences to ^{14}C -FA 16:0 oxidation experiments in 5mM glucose shown.
- (C-D) [^{14}C]-FA 16:0 oxidation rates in MDA-MB-231 cells in the presence of 0 to 25mM glucose $\rightarrow$ etomoxir in 0.1mM ^{14}C -FA 16:0 (C) and compared to 0.5mM ^{14}C -FA 16:0 N=1, 3 technical replicates.

Graphs show mean $\pm$ SEM. * $p < 0.05$ by One-way ANOVA with Dunnett's multiple comparisons test.

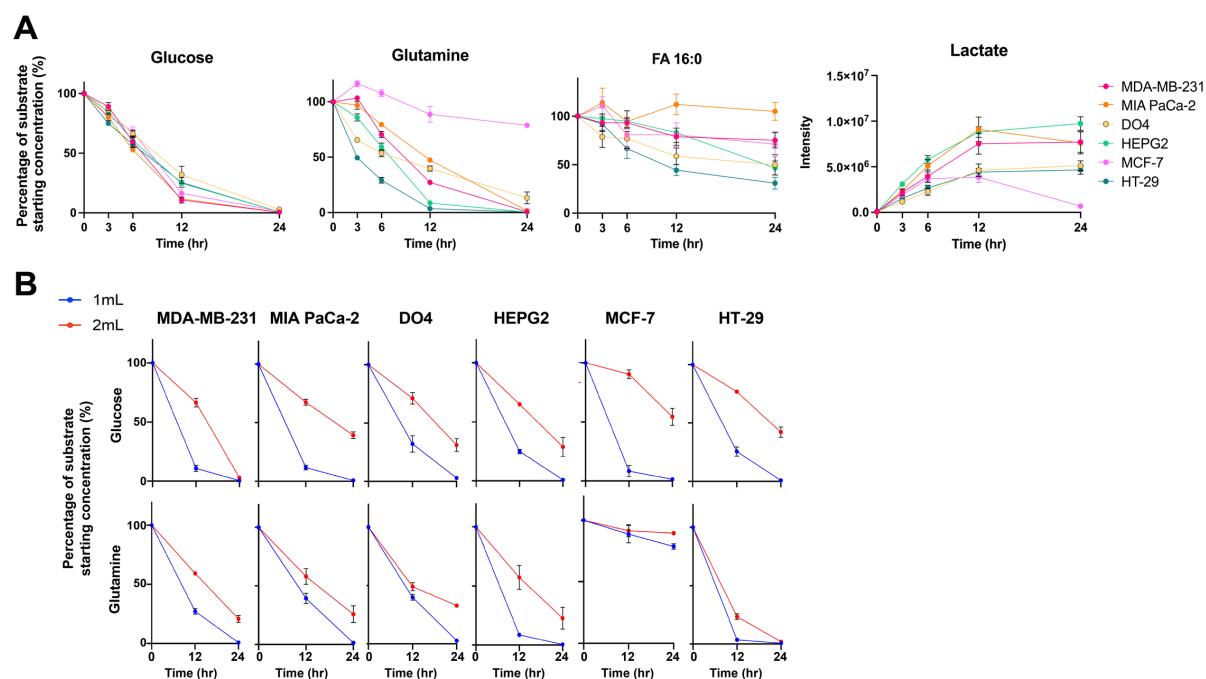


Figure S2, related to figure 2. Substrate uptake and production of cancer cells over 24 hours.

- (A) Percentage of substrate starting concentration glucose, glutamine, and FA 16:0, and intensity of lactate uptake over 24 hours. N=3 per cell line.
- (B) Percentage of substrate starting concentration glucose or glutamine at 1mL (blue) or 2mL (red) medium. N=3 per cell line.

Graphs show mean $\pm$ SEM.

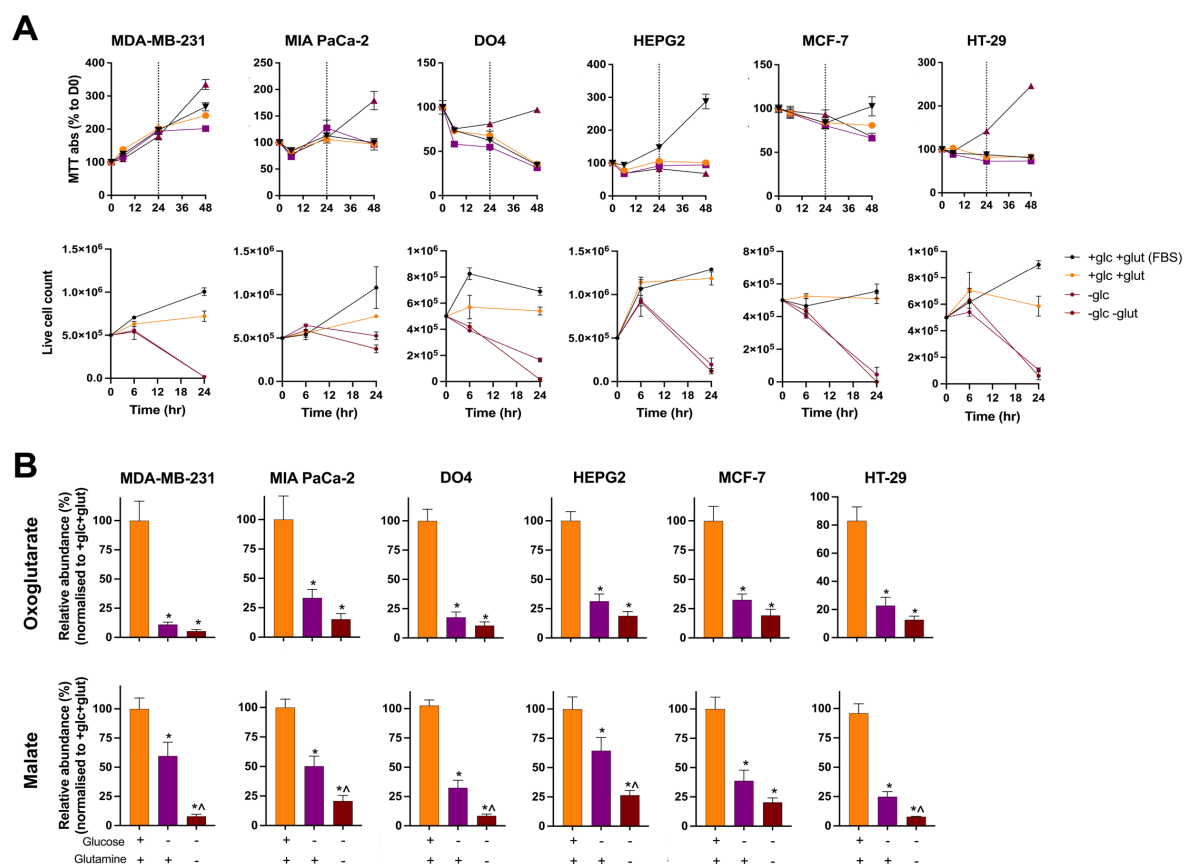


Figure S3, related to figure 3. Palmitate cannot completely maintain TCA cycle activity under glucose and glutamine deprivation.

(A) Impact of glucose- or glucose and glutamine- deprivation on metabolic activity (top) assessed by MTT assay absorbance results to 48hours normalised to day 0 results (Dotted line at 24 hours indicates replenishment of media); or Cell viability (bottom) assessed by live cell counts at 0, 6 and 24 hours normalised to day 0 results.

(B) Relative abundance (normalised to +glucose/+glutamine) of [U-¹³C]-FA 16:0 to oxoglutarate or malate. N=3 per cell line.

Graphs show mean $\pm$ SEM. $P < 0.05$ by One-way ANOVA with Dunnett's multiple comparisons test. *compared to +glucose/+glutamine, ^compared to -glucose/+glutamine.

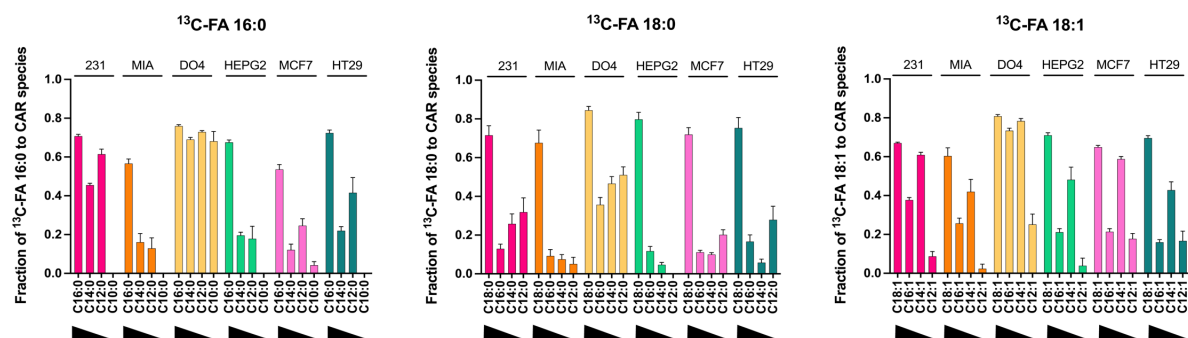


Figure S4, related to figure 4. LCFAs undergo incomplete FAO in cancer cells.

Fractional contributions of [U-¹³C]-FA 16:0, 18:0, 18:2 to shorter chain acyl-carnitine species. N=3 per cell line. Graphs show mean $\pm$ SEM.

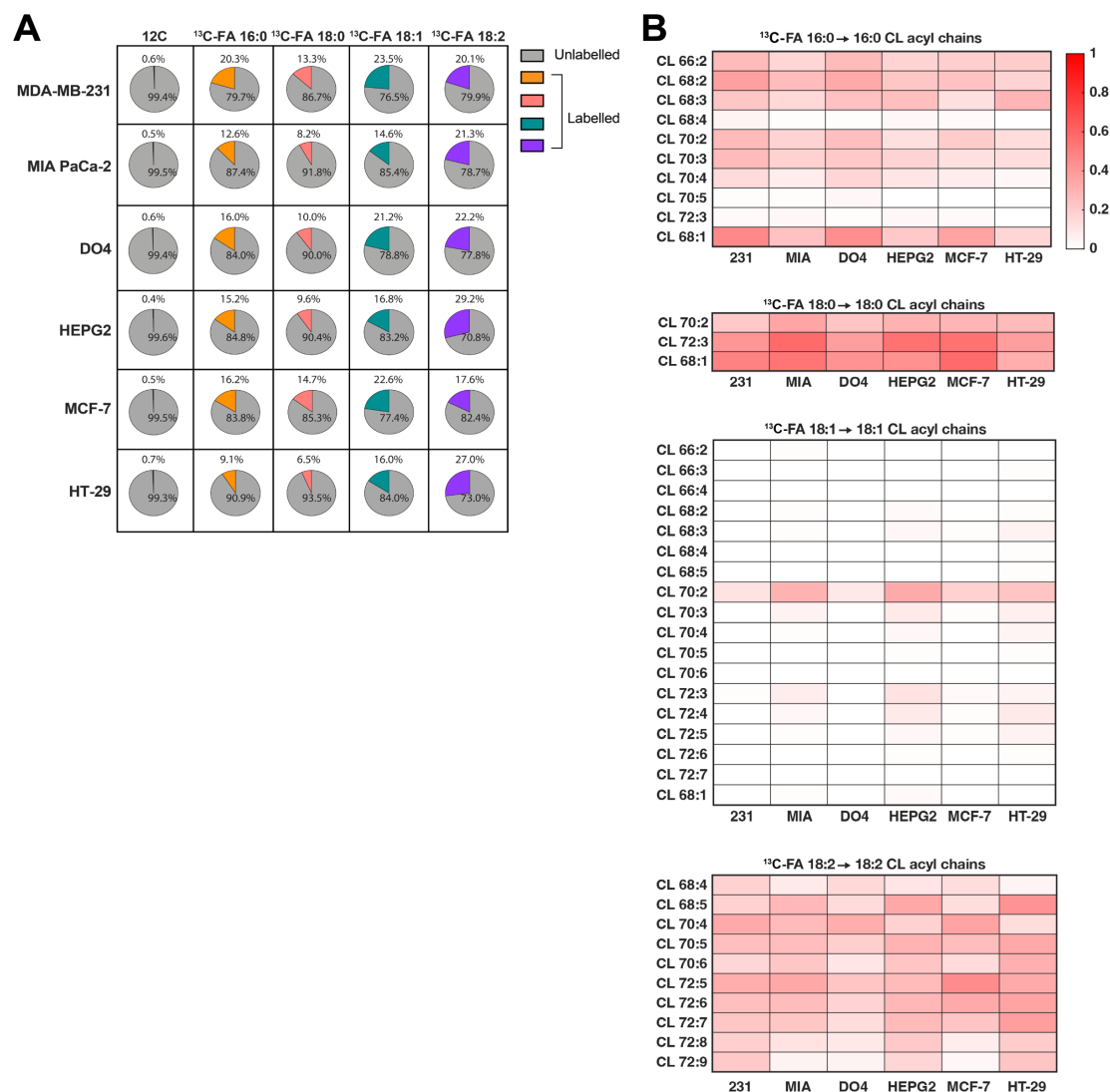


Figure S5, related to figure 5. FA 18:2 predominantly enriches CL pools compared to other FAs across cancer cells.

- (A) Percentage of labelled CL pools from $[\text{U-}^{13}\text{C}]$ -FA 16:0, 18:0, 18:1, 18:2 and unlabelled ^{12}C controls. N=3 per cell line.
- (B) Fractional enrichment of $[\text{U-}^{13}\text{C}]$ - FA 16:0, 18:0, 18:1, 18:2 (150 μM) to directly labelled acyl chains of CL species for 6hours. N=3 per cell line.

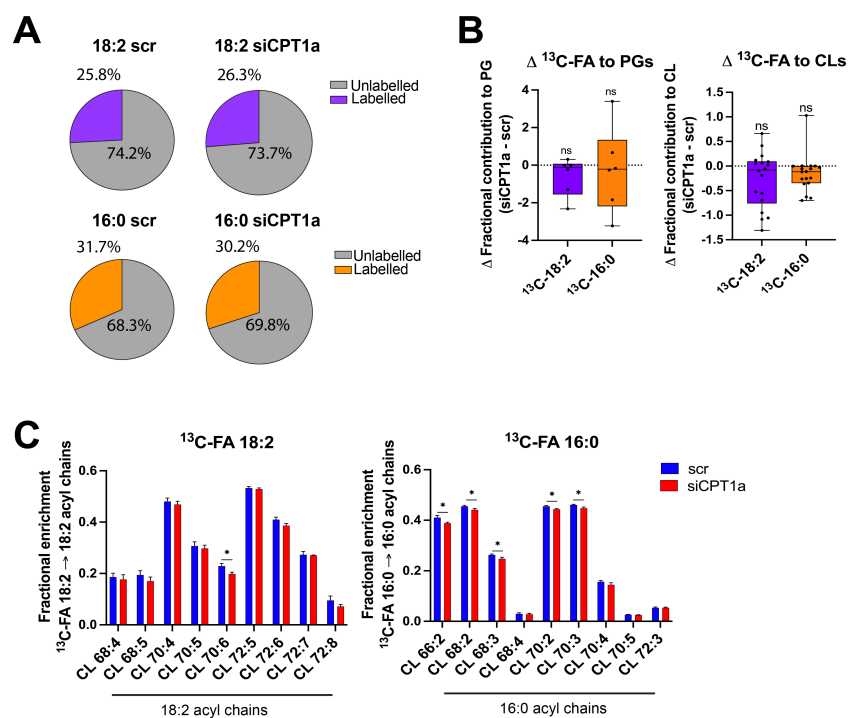


Figure S6, related to figure 6. CPT1a^{KD} does not affect FAs 18:2 or 16:0 incorporation to CLs.

- (A) Percentage of labelled to unlabelled (grey) CL pools from [U-¹³C]-FA 18:2 or 16:0 scrambled (scr) and siCPT1a cells. N=3 per group.
- (B) Delta differences between the fractional contribution of [U-¹³C]-FA 18:2 or 16:0 to PG and CL species. N=3 per group. Statistically significant differences within [U-¹³C]-FA 18:2 or 16:0 groups to 0 difference. Error bars represent $\pm$ min to max values.
- (C) Fractional contribution of [U-¹³C]-FA 18:2 or 16:0 to 18:2 or 16:0 acyl chains of CL species. N=3 per group.

Graphs show mean $\pm$ SEM unless stated otherwise. ns not significant, *p<0.05 by multiple unpaired t-tests between siCPT1a and scr control groups unless stated otherwise.

Acknowledgements

N.T.S. is supported by the Australian Rotary Health/Rotary Club of Blacktown City ‘Mel Grey’ PhD scholarship. A.J.H. is supported by a Robinson Fellowship from the University of Sydney. This work was supported by funding from The University of Sydney. We thank the Sydney Mass Spectrometry facility for access to LC-MS instruments; Dr Helen McGuire and the Sydney Cytometry core facility for assistance with mitotracker experiments; and all collaborators who kindly donated cell lines specified in the key resources table.

Author contributions

Conceptualisation by A.J.H. and L.E.Q. Project administration, visualisation, writing-original draft by N.T.S. Investigation by N.T.S., M.H.S., M.G., and A.W. Methodology by L.E.Q. Software by N.T.S., and L.E.Q. Formal analysis by N.T.S and L.E.Q. Writing-editing & reviewing by N.T.S., L.E.Q., and A.J.H. Funding acquisition, resources by A.J.H. Supervision by A.J.H., and L.E.Q.

Declaration of interests

The authors declare that they have no competing interests.

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5. Discussion

5.1 Overview

Understanding the roles of diet, the tumour microenvironment, and patient physiology in cancer progression has expanded our knowledge of how cancers rewire their metabolism and the reasoning behind developing metabolically targeted therapies (Altea-Manzano et al., 2020, Fendt et al., 2020, Garcia-Bermudez et al., 2020, Pavlova et al., 2022, Stine et al., 2022). Investigations of cancer metabolism have often been cancer-type-centric (Hensley et al., 2016, Kamphorst et al., 2015, Maher et al., 2012), but there is growing evidence that the changes in metabolism that sustain tumourigenesis are shared between cancer types. For example, lung (Momcilovic et al., 2018) and colorectal (Reckzeh et al., 2019) cancers, depend on glycolysis but, when inhibited, can be metabolically rewired to upregulate glutamine metabolism to maintain the metabolic needs for cancer survival. The studies described in this thesis have made new insights into the metabolism shared by cancer cells from a range of tissue lineages. This knowledge could identify new opportunities to target common metabolic vulnerabilities in subsets of diverse cancer types and improve clinical outcomes. Furthermore, many studies have described how cancer cells metabolise glucose and glutamine but have failed to account for any interactions to FA metabolic pathways that are also essential for cancer progression (Dueregger et al., 2015, Mashimo et al., 2014, Vriens et al., 2019). The studies within this thesis have comprehensively characterised the interactions of glucose, glutamine, and FA metabolism within the mitochondria of cancer cells and their relationships between the TCA cycle, beta-oxidation and cardiolipin homeostasis. Combined, this knowledge challenges the existing paradigm of how mitochondria metabolise fatty acids and how it likely supports cancer cell growth.

5.2 Common and distinct patterns of glucose and glutamine metabolism in cancer cells

The influence of increased glucose and glutamine uptake and utilisation by the TCA cycle to support tumourigenesis and bioenergetics is well established (Hensley et al., 2013, Jiang et al., 2016). Studies

of the interplay between glucose and glutamine as substrates for the TCA cycle have propelled our understanding of the dynamic substrate switching that can occur under therapeutic interventions to maintain cancer growth (Cheng et al., 2011, Reckzeh et al., 2019, Yang et al., 2014). For example, colorectal cancer and glioblastoma cells increase the use of glutamine as a TCA cycle substrate in glucose-restricted conditions to maintain TCA cycle metabolite levels and cancer growth (Reckzeh et al., 2019, Yang et al., 2009). The studies reported in this thesis have provided new insights into the interactions between glucose and glutamine as the major TCA cycle substrates.

One of the significant findings presented in Chapter 3 was that glucose and glutamine were the primary carbon sources used by the TCA cycle compared to FAs in prostate cancer (PCa) cells, irrespective of pathophysiological stage. These results were surprising as early-stage PCa is characterised as lipid reliant and “non-Warburg” and only shifts to a glucose-reliant phenotype at later disease stages (Dueregger et al., 2015, Mah et al., 2020). The low rates of glucose metabolism and, in part, the slow-growing rates of tumours, are reasons why [^{18}F]-fluorodeoxyglucose positron emission tomography (FDG PET) that exploits substantial glucose uptake by tumours is not commonly used in the clinic for early-stage PCa patients (Jadvar, 2013). Further, progressive PCa tumours have been shown to utilise glucose to a greater extent than early-stage PCa and can be effectively imaged by FDG PET/CT scans for diagnosis (Jadvar, 2016). Together, our results in Chapter 3 showed that glucose is dominantly utilised by PCa cells irrespective of disease-stage or uptake rates. Interestingly, the dominant glucose and glutamine anaplerotic phenotype was also observed in a panel of cancer cells from different tissue lineages (Chapter 4). Further, we identified glucose- and glutamine-dominant utilisation signatures to OXPHOS, glutamine cataplerosis, and lactate production in a subset of cancer cells from different tissue lineages using a ratio-metric approach between pyruvate and TCA cycle metabolites (Chapter 2). Interestingly, this subset of cells did not have greater uptake rates of glucose or glutamine, which led us to conclude that there is a disconnect between the way cancers utilise and uptake substrates. This hypothesis was supported by the findings in Chapter 3 and 4 that showed glucose and glutamine were substantially utilised by the TCA cycle, irrespective of substrate uptake rates in PCa and diverse cancer types. Furthermore, this uncoupling of substrate uptake and turnover has been shown in a previous

study that directly compared FDG-PET/CT uptake measures to ^{13}C -glucose labelling in lung cancer patients and concluded that FDG uptake has no correlation to glycolytic flux (Kernstine et al., 2020). Taken together, our results suggest that cancers can metabolise glucose and glutamine to a significant extent and sheds light on FDG PET tools that do not fully capture the prevalence of glucose utilisation across the spectrum of PCa disease-stage or cancer types. Future studies should focus on defining the mechanisms that underpin the glucose and glutamine utilisation phenotypes observed in diverse cancer settings, and how these relate to substrate uptake rates.

It was important to understand how glucose and glutamine are predominantly utilised in cancers to help gain greater insights into therapeutic opportunities that could target this interplay of substrates. It is conceivable that the dominant glucose and glutamine utilisation signature observed at the TCA cycle in a cluster of cancer cells (Chapter 2) was linked to their increased lactate production from both glycolysis and gluconeogenesis, and increased respiration. Previous studies have shown that lung cancers can utilise lactate as a TCA cycle substrate itself (Chen et al., 2016, Faubert et al., 2017, Hui et al., 2017), and so it was possible that lactate produced from glutamine-derived gluconeogenesis fuels the TCA cycle and creates a dominant glucose and glutamine dominant signature within a cluster of diverse cancer cells to achieve greater rates of respiration. Further, it was plausible that this subset of cells with greater glucose and glutamine utilisation and lactate production was caused by an increased cellular demand for NAD^+ , which is a co-factor for LDH and supports oxidation once ATP turnover is exceeded (Luengo et al., 2021). Although we observed no difference of NAD^+/NADH ratios between cancer cells with high or low glucose and glutamine utilisation, it remains to be determined if differences in ATP metabolism can explain these phenotypes. Chapter 4 also provided evidence that glucose and glutamine are greatly utilised by the TCA cycle in a range of different cancer types. Therefore, it is conceivable that synergistic inhibition of glucose and glutamine metabolism that has been shown to impair colon (Reckzeh et al., 2019), lung (Momcilovic et al., 2018), glioblastoma (Yang et al., 2009), and liver (Méndez-Lucas et al., 2020) cancer cell viability may be applicable across a broader spectrum of cancers and thereby provide novel therapeutic opportunities to cancers historically characterised as “non-Warburg” like PCa.

Notably, the identification of glucose and glutamine signatures in Chapter 2 were identified by a ratio-metric approach between pyruvate and TCA cycle metabolites, as the TCA cycle is a central converging hub of glucose and glutamine. In comparison to linear central carbon and amino acid pathways covered in our metabolomics approach, the TCA cycle provided information to analyse the distinct uptake, production, and metabolism of glucose and glutamine. Further investigation is needed to assess whether metabolic signatures can be observed across other ‘hub’ pathways to study the interplay between different substrates. Nonetheless, the results presented in this thesis provide evidence to support the distinct ways glucose and glutamine are metabolised in cancers, and a new ratio-metric approach to identify metabolic signatures that converge at the TCA cycle. Our data showed that subsets of cancers dominantly utilise glucose and glutamine and that this occurs independent of substrate uptake rates, tissue lineage, or disease-stage.

5.3 The insufficient role of FAO to maintain the TCA cycle

Amongst the studied TCA cycle carbon sources, LCFAs remain a poorly appreciated substrate due to unvalidated assumptions surrounding the importance of FAO to bioenergetics (Liu, 2006). Although it is established that FAO is increased in many cancer types (Balaban et al., 2019, Lin et al., 2017, Linher-Melville et al., 2011), studies often assume this increase leads to enhanced acetyl-CoA production, thus fuelling greater TCA cycle flux and ATP production (Carracedo et al., 2013, Ma et al., 2018). Furthermore, complete palmitate-oxidation is known to produce approximately 4-times the ATP yield of glucose oxidation (Mookerjee et al., 2017). A significant limitation of this concept is the dogmatic and unquantified view that LCFAs entering the mitochondria via the carnitine shuttle system are solely partitioned to beta-oxidation and completely oxidised to its end-product acetyl-CoA that is a major substrate to the TCA cycle.

Chapter 3 reports the outcomes of experiments that assessed the contribution of exogenous glucose, glutamine and LCFAs to the TCA cycle in PCa cells that undergo impaired cancer progression when FAO is inhibited (Bramhecha et al., 2022, Nassar et al., 2020, Russo et al., 2023). Strikingly, the results in Chapter 3 showed that exogenous LCFAs are minor carbon sources for the TCA cycle, compared to

glucose and glutamine in PCa cells. Crucially, this minor usage of LCFA carbons to the TCA cycle relative to glucose and glutamine was also observed in a panel of cell lines from multiple cancer types that displayed a spectrum of FAO capacities (Chapter 4). Furthermore, limiting the mitochondrial entry of LCFAs at CPT1a had no impact on the enrichment of LCFAs to the TCA cycle in prostate and breast cancer cells (Chapter 3 and 4). These results came as a surprise as they contradicted the importance of mitochondrial FAO for bioenergetics (Pike et al., 2011, Schlaepfer et al., 2014, Tirado-Vélez et al., 2012). One explanation for the minor sourcing of FA carbons to the TCA cycle was that FAO could not supply enough acetyl-CoA to fuel the TCA cycle compared to glycolysis and glutamine anaplerosis. This was supported by results in Chapters 3 and 4 that revealed palmitate oxidation, in the absence of major carbon-donating glucose and/or glutamine, could not maintain TCA cycle metabolite levels. To consolidate the high FAO rates observed in PCa and other cancers, and the low carbon donation from FAO-derived acetyl-CoA to the TCA cycle, we proposed that LCFAs were not completely oxidised and thus a less than predicted amount of LCFA carbons arrived at the TCA cycle. The results in Chapters 3 and 4 supported this hypothesis by revealing exogenous LCFAs enrich shortened acyl-carnitines that would not occur if FAs were completely oxidised to acetyl-CoA. These results are the first to show in cancer cells that exogenous LCFAs are minor TCA cycle carbon sources compared to glucose and glutamine, and that they are not completely oxidised to acetyl-CoA within the mitochondria.

5.4 Resolving the mitochondrial fates of LCFAs: The TCA Cycle vs. Cardiolipins

LCFAs are essential for maintaining mitochondrial bioenergetics and membrane homeostasis as inhibiting CPT1a leads to cell death, disrupted mitochondrial morphology, and reduced ATP production (Pike et al., 2011, Schlaepfer et al., 2014, Tirado-Vélez et al., 2012, van Meer et al., 2008). Although CPT1a has been viewed as the rate-limiting enzyme of FAO, our results in Chapters 3 and 4 showed that FAO is not a major bioenergetic substrate to the TCA cycle nor is affected by impaired CPT1a activity. It was conceivable that CPT1a may facilitate the entry of LCFAs to the mitochondria for other essential bioenergetic and structural purposes, and that this may explain the cellular disruptions observed when CPT1a is inhibited. CLs influence mitochondrial bioenergetics as they provide the

platform for ETC complex formation (Paradies et al., 2014), and mitochondrial morphology due to their unique four acyl-chain conical structure (Falabella et al., 2021). The outcomes from the experiments reported in Chapter 3 (prostate cancer) and 4 (a range of cancers) demonstrate that CLs are a major mitochondrial sink for extracellular LCFA carbons, compared to the TCA cycle (Chapter 3, 4). Interestingly, we also discovered that the magnitude of LCFA incorporation into CLs after 6 hours in a diverse panel of cancer cells was substantial and occurred rapidly unlike previous studies that established CLs were slow to turnover in HeLa cell lines and cardiac tissues (Oemer et al., 2021, Schlame et al., 2000, Wahjudi et al., 2011). It is understandable that terminally differentiated cardiac tissues would have a slow turnover of phospholipid pools compared to proliferating cancer cell lines, as both our studies in multiple cancer cell lines (Chapter 3,4) and in HeLa cells by Oemer et al. (2021) showed substantial labelling from exogenous LCFAs at 6 hours. Together, these results showed that after 6 hours of labelling, LCFAs enriched CLs to a greater magnitude than TCA cycle metabolites.

As results in Chapters 3 and 4 showed CLs are substantially turned over by LCFAs, it was conceivable that CPT1a may regulate the level of LCFA incorporation into CLs. This hypothesis was supported by emerging evidence that has implicated CPT1 in the regulation of CL levels and mitochondrial membrane homeostasis (Yao et al., 2018, Zhang et al., 2021). Mitochondrial CL remodelling pathways preferentially use 18:2 acyl-CoAs to form mature CLs, while the enzymes in the CL synthesis pathway have no reported substrate preference for particular FA species (Hostetler et al., 1975, Taylor and Hatch, 2009). As such, CPT1a could regulate the intra-mitochondrial partitioning of FA 18:2 for the remodelling of CLs. In line with this view, knockdown of CPT1a in PCa cells reduced the incorporation of extracellular FA 18:2 into mitochondrial CL, likely via the remodelling pathways (Chapter 3). Combined with the observations that LCFAs are minor carbon sources for citrate production (Chapter 3, 4) and reduced CPT1a protein levels selectively impaired LCFA incorporation into CLs (Chapter 3), we proposed that CPT1a is also a rate-limiting enzyme for mitochondrial CL remodelling via MLCLAT1. In light of this, previous studies that established CPT1a as a rate-limiting enzyme for FAO by the disruption of ATP production and mitochondria structure were most likely observing the effects from altered CL homeostasis that impacts ETC complex formation and mitochondria morphology (Pike

et al., 2011, Schlaepfer et al., 2014, Tirado-Vélez et al., 2012, van Meer et al., 2008). Processing these observations through this lens redefines the relationships between CPT1a, FAO, and mitochondrial metabolism in PCa (Bramhecha et al., 2022, Liu, 2006, Nassar et al., 2020, Russo et al., 2023).

Notably, the role of CPT1a in CL remodelling remains to be explored in cancers other than PCa, since observed no changes in CL labelling or remodelling pathways when CPT1a protein levels were reduced in MCF-7 breast cancer cells (Chapter 4). This lack of effect in MCF-7 cells may, in part, be explained by differential CPT1 isoform usage in cancers, as others have shown that knockdown of the CPT1c isoform reduced CL levels in PANC-1 pancreatic cancer cells, MDA-MB-231 breast cancer cells, HCT-116 colorectal cancer cells, and A549 lung cancer cells (Zhang et al., 2021). Therefore, it is conceivable that CPT1 isoforms can adaptively compensate for each other when one is inhibited to sustain the availability of LCFA-CoAs for CL homeostasis. Specifically, that MCF-7 cells could upregulate CPT1c utilisation when CPT1a activity is impaired. Another explanation for why CPT1a had no effect on CL labelling, is that these cells switch to a greater flux through the CL synthesis pathways that utilise ER-derived constituents and undergo acyl-CoA independent remodelling when mitochondrial CL remodelling is impaired.

Results reported in this thesis show that CLs are a major mitochondrial sink for exogenous LCFAs compared to the TCA cycle via FAO in a diverse panel of cancer cell lines. These observations expand the known role of CPT1a in regulating mitochondrial fatty acid metabolism, to include the availability of LCFAs for CL remodelling in PCa. Future studies could focus on exploring the adaptive measures of cancer cells to maintain CL homeostasis in response to impaired availability of LCFAs such as the loss of CPT1a and the effect on ETC function and respiration. In this setting, new insights could be provided for the role of mitochondria function in tumour growth and survival.

5.5 Advancing pan-cancer strategies to identify metabolic vulnerabilities

Genomic complexity and dynamic nutrient dependencies form the heterogeneous landscape of cancers both across disease states and tissue origin types (Altea-Manzano et al., 2020, Dagogo-Jack and Shaw, 2018, Fendt et al., 2020, Pavlova et al., 2022). Pan-cancer approaches have identified metabolic

phenotypes across the spectrum of cancer tissue origins that were not observed from cancer-type specific studies (Cherkaoui et al., 2022, Jain et al., 2012, Li et al., 2019, Ortmayr et al., 2019, Shorthouse et al., 2022). Studies including those that have used the formative NCI-60 (Ortmayr et al., 2019, Shoemaker, 2006) and CCLE panels (Barretina et al., 2012, Li et al., 2019), have provided a proof-of-concept that there are common vulnerabilities between cancer types that cancer-specific therapies have utilised. This thesis took pan-cancer approaches to identify metabolic phenotypes that were common in subsets of cells from a range of cancer types. In Chapter 2 we illustrated differing substrate utilisations of glucose and glutamine in a pan-cancer panel of cell lines that were identified using a ratio-metric metabolome approach. In Chapter 4 we identified a novel role for LCFAs in CL homeostasis among diverse cancer types that span the spectrum of FAO capacities. Taken together, and like previous pan-cancer studies, the diversity of cancers could be utilised to our advantage to identify common metabolic phenotypes similarly seen within cancer types (Chapter 3).

The applications of identifying metabolic phenotypes from a pan-cancer approach can uniquely lead to repurposing existing therapeutics generally used for specific cancer types. The PRISM drug-repurposing screen (578 cancer cell lines, 24 tissue types) (Corsello et al., 2020), GDSC2 (639 cancer cell lines, 16 tissue types) (Yang et al., 2012), NCI-60 (60 cell lines, 9 tissue types) (Shoemaker, 2006), and CCLE (479 cancer cell lines, 36 tissue types) (Barretina et al., 2012) drug screens identified multiple cancer types that are sensitive to common molecular targets not limited to cancer-targeting drugs. The study in Chapter 2 used the PRISM (Corsello et al., 2020), GDSC2 (Yang et al., 2012), and pan-cancer loss-of-function DEMETER (Tsherniak et al., 2017) and Project Score (Behan et al., 2019) screens to support the increased glucose and glutamine utilisation signature we observed in a subset of diverse cancer cells. In contrast to the expansive drug screens that aimed to identify vulnerabilities on a grand scale, we took a similar approach by Joly et al. (2021) to first narrow the scope of our database analyses to KEGG-defined targets of human metabolic pathways (Kanehisa and Goto, 2000). Interestingly, we observed greater sensitivities of cancer cells to lactate metabolism, OXPHOS, glutamine metabolism, and TCA cycle inhibition or loss-of-function, that taken together, support the identified distinct glucose and glutamine utilisation signature from our ratio-metric approach. By incorporating *in*

silico analysis of publicly available pharmacologic and loss-of-function screens, we identified specific metabolic vulnerabilities of high-flux pathways like glycolysis, TCA cycle, and glutamine metabolism that support cancer proliferation. Moreover, we provide evidence that therapeutic approaches can be utilised among diverse cancer types irrespective of genetic complexity, disease stage, or tissue origin. A fitting example is the observation of a common sensitivity to glutaminase (GLS) inhibition in different cancer types. Our results complemented previous cancer-type specific GLSi studies in breast, non-small lung, and head and neck cancers that explored its vulnerability as an effective therapeutic approach to reduce cancer growth (Gross et al., 2014, van den Heuvel et al., 2012, Wicker et al., 2021). Taken together, the parallel analyses of pan-cancer drug sensitivity and loss-of-function databases, alongside our ratio-metric approach to identify metabolic signatures in subsets of multiple cancer types, may expedite the identification of common metabolome-based vulnerabilities that can be deployed for clinical use to repurpose existing therapeutics in diverse cancer settings.

Although ours and other pan-cancer studies have identified clusters of cells that exhibited distinctive metabolic phenotypes between broad screens of cancer cell lines, several limitations of *in vitro* cell models warrant mentioning. Unlike cell lines, *in vivo* models provide greater understandings of drug clearance, mechanisms of the surrounding tumour microenvironments, and nutrient gradients, which are essential to develop pre-clinical therapeutic opportunities and the translation of drugs into the clinic (Elia and Haigis, 2021, Yadav et al., 2021). Another limitation of cell line models is the interpretation of metabolic phenotypes irrespective of tissue origin. Whilst the proliferating cell lines derived from tissue sites provide an informative insight into metabolic preferences, studies from *in vivo* mouse models have shown tissue-specific levels of substrate metabolism within the TCA cycle (Hui et al., 2020, Neinast et al., 2019). For example, branch-chained amino acid carbons contributed ~20% to the TCA cycle in the pancreas compared to 1-6% in other mouse tissue sites including the heart and kidney (Neinast et al., 2019). Similarly, glucose was the predominant carbon source to the TCA cycle in brain tissues compared to other mouse tissue sites including the pancreas and kidney (Hui et al., 2020). Therefore, it remains to be seen whether metabolic phenotypes in subsets of cancers from different tissue origins, such as our observed dominant labelling from glucose and glutamine to TCA cycle

metabolites (Chapter 4) and drug responses (Chapter 2) hold true in *in vivo* models. Further investigations could explore this gap in *in vitro* studies to quantify the identification and nuances of metabolic phenotypes irrespective of tissue origins among cancer types *in vivo*. Together, these studies could lead to highly informative and captivating results that could significantly propel the use of pan-cancer approaches for cancer drug development in the future.

The studies in this thesis utilised parallel stable isotope tracing strategies to validate the metabolic signatures and evaluate the metabolic fates of supplemented substrates, that fundamentally could not be observed from metabolite abundances alone (Chapter 2). There are however limitations to this method including the time at which steady state of isotopic tracing is met, which can differ greatly by individual substrate turnover, or the dilution of enriched metabolite pools by unlabelled carbon sourcing substrates (Antoniewicz, 2018). Comprehensive metabolic flux analysis provides a significantly greater understanding of how substrates, like glucose and glutamine, are used following uptake over time (Antoniewicz, 2018, Quek et al., 2020). As our labelling studies only used a single time-point of 6 hours, future studies could implement parallel tracing of other TCA cycle substrates with metabolic flux analysis to overcome these limitations.

5.6 Conclusion

In conclusion, the results presented in this thesis build a clearer picture of how cancer cells metabolise exogenous LCFAs in relation to the major substrates glucose and glutamine in the mitochondria. Focusing in on mitochondrial metabolism provided new insights into the relationships of glucose and glutamine metabolism that converge at the TCA cycle in different cancer types. Importantly, this thesis redefines the role of FAO in cancer cells by revealing that LCFAs are minor carbon sources to the TCA cycle compared to glycolysis and glutamine anaplerosis in cancer cell lines spanning the spectrum of FAO activity. This led to the investigation of other significant mitochondrial fates of LCFAs following CPT1a-mediated entry. CLs were identified as the major sink for mitochondrial LCFAs in multiple cancer types, and this was regulated by CPT1a in PCa. Collectively, these findings overcame the genetic heterogeneity of diverse cancer types to reveal unifying metabolic signatures in subsets of cells that

explained how major substrates are metabolised in the mitochondria. Overall, these new insights lay the foundation to identify vulnerabilities common in different cancers and repurpose traditionally cancer-type specific therapeutics to more diverse cancer settings.

6. References

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