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Title:

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Date:

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**INVESTIGATING THE
METABOLIC CONSEQUENCES
RESULTING FROM THE ONCOGENIC ACTIVATION
OF THE PI3K-AKT PATHWAY**

Presented by

Sabrina Caiazzo

Submitted in total fulfilment of the requirements of the degree of

Doctor of Philosophy

The Sir Peter MacCallum Department of Oncology

The University of Melbourne

September, 2020

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ABBREVIATIONS

Abbreviation	Name
14C	Carbon-14
18F	Fluorine-18
2DG	2-Deoxy-D-glucose
2H	Deuterium
2H2O	Heavy water
4EBPs	Binding proteins
α- KG	α - Ketoglutarate
AA	Antimycin
ABCD	ATP Binding Cassette Subfamily D
ACC	Acetyl-CoA carboxylase
ACLY	ATP-citrate lyase
ADP	Adenosine di-phosphate
AgRP	Agouti-Related Protein
APC	Anaphase-promoting complex
ARC	Arcuate nucleus
ATF	Active transcription factor
ATGL	Adipose Triglyceride Lipase
ATP	Adenosine triphosphate
ATP5A	ATP synthase subunit alpha
ATP5Q1	ATP synthase subunit gamma
β-HB	Beta Hydroxybutrate
BAT	Brown adipose tissue
BBB	Blood–brain barrier
BH	Breakpoint cluster homology domain
BL6	Black 6
BP	Bisphosphate
BSA	Bovine serum albumin
C/EBPα/β	CCAAT/enhancer-binding proteins alpha and beta

C2	Protein-kinase-C homology-2 domain C2
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and Amphetamine-Regulated Transcript
CASP9	Caspase 9
CD36	Cluster of differentiation 36
Cdk	Cyclin-dependent kinases
CE	Ceramide
CHO	Cholesterol
CK	Casein kinase
	Congenital Lipomatous asymmetric Overgrowth of the trunk with lymphatic, capillary, venous, and combined-type Vascular malformations, Epidermal naevi, Skeletal and
CLOVES	spinal anomalies
CNS	Central nervous system
CO2	Carbon dioxide
CoA	Coenzyme A
CPM	Counts per minutes or counts per million
CRC	Colorectal cancer
CREB	cAMP response element-binding protein
Cyc	Cytochrome c complex
DAG	Diacylglycerides
DEPTOR	DEP-domain containing mTOR interacting protein
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DXA	Dual energy X ray absorptiometry
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetracetic acid
EE	Energy expenditure
EGF	Epidermal growth factor
EGFR	Epithelial growth factor receptor
EGSEA	Gene Set Enrichment Analyses
eIF4E	Eukaryotic translation initiation factor 4E

ELISA	Enzyme-linked immunoassay
FA	Fatty acid
FADH	Fumarylacetoacetate Hydrolase Domain Containing 1
FADH2	Flavin adenine dinucleotide
FASN	Enzyme fatty acid synthase
FAT	FRAP-ATM-TRRAP
FATC	FAT C-terminus
FCCP	Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone
FDG	Fluorodeoxyglucose
FFA	Free fatty acid
FGF	Fibroblast growth factor
FOXO	Forkhead box subfamily
FRB	FKBP-rapamycin-binding
G6PC	Glucose-6-phosphatase
G6PDH	Glucose-6-phosphate dehydrogenase
GLP	Glucagon-like peptide
GLS	Glutaminase
GLUT	Glucose transporter
GPT	Gutamate pyruvate transaminase
GSK3	Glycogen synthase kinase-3
GTT	Glucose tolerance test
HDB	High-dimensional biology
HEAT	Huntington, Elongation Factor 3, PR65/A, TOR
HGP	Hepatic glucose production
HIF-1α	Hypoxia inducible factor 1 alpha
HISAT	Hierarchical indexing for spliced alignment of transcripts
HK	Hexokinase
HSL	Hormone-sensitive lipase
HSP	Heat shock protein
I.P.	Intraperitoneal
I.V.	Intravenous injection
IAA	Iodioacetamide

iAUC	Integrate area under the curve
ID	Injected dose
IGF	Insulin-like growth factor
IGFR	IGF receptor
INSIG	Insulin induced gene
INSIG2	Insulin induced gene 2
IR	Insulin receptor
ISH2	Inter- Src-homology 2 domain
IVD	Isovaleryl-CoA dehydrogenase
KATP	ATP-sensitive K ⁺ -calcium
KRAS	Kirsten rat sarcoma 2 viral oncogene homolog
KRB	Krebs-ringer modified buffer
LDH1	Lactate dehydrogenase 1
LY6E	Lymphocyte antigen 6 family member E
MAOA	Monoamine oxidase A
MAPK	Mitogen activate protein kinases
MCA	Multiple Correspondence Analysis
Mdh2	Malate dehydrogenase
MDM2	Murine double minute 2
MLEM	Maximum-likelihood expectation maximization
mLST8	Mammalian lethal with sec-13 protein 8
mRNA	Messenger RNA
MS-GC	Mass Spectroscopy-Gas chromatography
MSIN	stress-activated protein kinase-interacting protein
MT-CO	Mitochondrially Encoded Cytochrome C Oxidase
mTOR	Mammalian target of rapamycin
N/G	Nystatin-Gentamicin
NADPH	Nicotinamide adenine dinucleotide phosphate
NBF	Neutral buffered formalin
NDUFB	NADH:Ubiquinone Oxidoreductase Subunit B
NDUFS	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit
NF-κB	Nuclear factor-kappa B

NGS	Next generation sequencing
NPY	Neuropeptide Y
NRD	Negative regulatory domain
NSDHL	NAD(P)d steroid dehydrogenase-like
NSPC	Neural stem/progenitor cells
O2	Oxygen
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
P	Proline
PBS	Phosphate-buffered saline
PCK	Phosphoenolpyruvate carboxykinase
PDH	Pyruvate dehydrogenase
PDK	phosphoinositide-dependent kinases
PDK1	Pyruvate dehydrogenase kinase 1
PET-CT	Positron emission tomography-Computed Tomography
PFK	Phosphofructokinase
PFKBP	6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase
PGAM1	phosphoglycerate mutase-1
PGC	Peroxisome proliferator-activated receptor-gamma coactivator
PH	Pleckstrin homology
PHLPP	PH Domain And Leucine Rich Repeat Protein Phosphatase
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-kinase
PIP	Phosphatidylinositol-3-phosphate
PIP2	Phosphatidylinositol-4-5-phosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PL	Phospholipids
POMC	Pro-opiomelanocortin
PP	Protein phosphatase
PPAR	Peroxisome proliferation-activator receptor

PPP	Pentose phosphate pathway
PRAS	Proline-rich Akt substrate
PROS	PIK3CA-related overgrowth spectrum
PTEN	Phosphatase and tensin homolog
PTT	pyruvate tolerance test
PX	Phox homology domain
q-RT-PCR	Real-Time quantitative reverse transcription PCR
RAPTOR	Regulatory associated protein of Mtor
RBD	RAS-binding domain
RER	Respiratory exchange rate
RICTOR	mTOR, rapamycin insensitive companion of mTOR
RNA	Ribonucleic acid
ROCK	Rho-associated, coiled-coil containing protein kinase
ROS	Reactive species of oxygen
ROT	Rotenone
RQ	Respiratory quotient
RTK	Receptor tyrosine kinases
S6K	p70 ribosomal protein S6 kinase
SDHB	Succinate dehydrogenase complex iron sulfur subunit B
SDS	Sodium dodecyl sulfate
SGK	Serum/Glucocorticoid Regulated Kinase
SH2	Src-homology 2 domain
SH3	Src-homology 3 domain
SLC	Solute carriers
SLC1A3	Excitatory amino acid transporter 1
SLC27A	Fatty acid transporter solute carrier family 27 member
SLC7A5	Alanine, serine, cysteine-preferring transporter 2
SREBP	Sterol regulatory element-binding protein
T1DM	Type I diabetes mellitus
T2DM	Type II diabetes mellitus
TAG	Triacylglyceride
TALDO	Transaldolase 1

TAM	Tamoxifen
TBA	Tris-buffered saline
TBX	Factor T-box
TCA	Tricarboxylic acid
TCEP-DTT	Tris-2-carboxyethyl-phosphine-Dithiothreitol
TD-NMR	Time Domain Nuclear Magnetic Resonance
TEAB	Triethylammonium bicarbonate
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TKT	Transketolase
TLC	Thin layer chromatography
TMEM26	Transmembrane protein 26
TXNIP	Thioredoxin-interacting protein
UBC	Human ubiquitin C
UCP1	Uncoupling Protein 1
UQCRC	Ubiquinol-cytochrome-c reductase core protein
UV	Ultraviolet (radiation)
VCO2	Volume CO2
VHL	von Hippel-Lindau
VO2	Volume O2
VPS	Vacuolar protein-sorting
WAT	White adipose tissue
WD40	W-D dipeptide
xCT	Cysteine/glutamate antiporter
XF	Extracellular flux

ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge and express my sincerest gratitude to my supervisor, Professor Wayne Phillips. Thank you for believing in me and for giving me the chance to join your fantastic laboratory. Thank you for the guidance and support that you have given me over the past four years. Thank you for teaching me that science is not only based on competition or the run to success, but it's founded on passion for what we do and care for others. Joining your team has changed me both personally and professionally for the better and I will never be grateful enough for that.

To my co-supervisors Dr. Camilla Mitchell and Dr. Lorey Smith, thank you for your professional help and for your support every time I felt down. Thank for encouraging me to continue in this career. I have been blessed to have had the opportunity to learn from you.

A special acknowledgment goes to Professor Matthew Watt and Dr. Garron Dodd for their essential help in this project. I am grateful to have had the chance to collaborate with you and to learn from you. Although you were not my supervisors, that's how I will remember you. Your kindness and patient in helping me through my PhD, has made me feel as one of your student and I sincerely appreciate it.

To my mentor Professor Rick Pearson and my PhD committee, in particular Professor Rob Ramsey for encouraging me when things didn't go as planned and to direct me towards the right direction.

To Karen Montgomery for teaching me everything I know about mice, to be the person I could always go to when I felt down or insecure and for all laughs.

To the rest of my lab all, in particular to Mariana Corrales and Swati Dawar for always listening to me and cheering me up, and Kenji Fujihara for his professional advice and suggestions for my job interviews. I have been so lucky to have people like you to talk to and to always find support from. I have learned a lot from such a professional environment. You have been like surrogate families, who I shared unforgettable moments with.

Thank you to all my friends and housemates, who have been celebrating with me/cheered me up with a glass of wine for all of my accomplishments/failure. I never felt alone or alienated in this city and that's thanks to you all.

Last but not least I thank my family who have always encouraged me to follow my dreams, even when it meant to move 14,373 km away. It hasn't been easy for us to stay apart all these years but they believe in me and support me, and always will.

ABSTRACT

The phosphoinositide 3-kinase (PI3K) pathway is one of the most commonly activated pathways in a variety of cancers and it has recently been highlighted as one of the primary modulators of cell metabolism. This has opened promises and challenges for the development of therapeutic strategies to target metabolism in cancer cells harbouring mutations in the PI3K pathway. Through an inducible “exon switch” approach, our laboratory has previously generated mice ubiquitously expressing a mutation in *Pik3ca*, the gene coding for the subunit p110 α of PI3K. By using this mouse model (*UbCre^{ER}Pik3ca^{H1047R}*) our laboratory has shown that mutations in the PI3K pathway lead to dramatic severe defects in glucose homeostasis resulting in hypoglycaemia and hypoinsulinemia.

In this thesis the causes responsible for the metabolic dysfunction observed in these mutant mice are investigated. This thesis provides evidence that mutations in the PI3K pathway lead to increased glucose uptake by the tissues, inhibition of hepatic gluconeogenesis and inhibition of insulin release from the pancreas.

Previous studies performed on the *UbCre^{ER}Pik3ca^{H1047R}* mouse model have also shown increased body weight and organomegaly. This thesis demonstrates that the increase in body weight, resulting from the activation of the *Pik3ca^{H1047R}* mutation was not associated with adiposity. On the contrary, mutations in the PI3K pathway determine loss of body fat and increased lipolysis in the adipose tissue of mice, whilst the tissue growth is associated to hypertrophy or hyperplasia.

Furthermore, oncogenic activation of the *Pik3ca^{H1047R}* mutation *in vivo* leads to alteration of the respiratory exchange rate and energy expenditure of the mice and stimulates browning of the adipose tissue.

This thesis also shows that activation of the PI3K pathway alters the expression of genes and proteins involved in metabolic pathways, and that these alterations are organ-specific, therefore opening promises for customising treatments to individual patients.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any institute.

All the material previously published or written by any other person is appropriately referenced in this thesis.

RNA sequencing was performed by the genetic core at the Peter MacCallum Cancer Centre. Monitoring and data collection of the metabolic cages was performed by the Melbourne mouse metabolic phenotyping platform (MMMP) coordinator Vanessa Haynes, at the animal house of the University of Melbourne. Isolation and culturing of the pancreatic islets was performed by Prof. Helen Tomas' lab at the St. Vincent's Institute.

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September 2020

CHAPTER 1

INTRODUCTION

1.1 Cancer

The term cancer refers to a group of diseases that have several common characteristics. Cancers develop when the normal cells of the body acquire uncontrolled ability to proliferate and evade the physiologic mechanisms of programmed cell death, such as apoptosis or necrosis. Most cancers start in a particular organ defined as the primary site or primary tumour. When the tumour is limited to its original location and does not spread outside its normal boundaries to other parts of the body, it is defined as benign tumour. However, if a benign tumour continues to grow at the original site and damages nearby organs, it becomes malignant and it is defined as cancer. If not treated at an early stage, cancer can break away from the primary tumour, enter the bloodstream or lymphatic system, and travel to a new organ to form secondary tumours, also called metastasis. Tumorigenesis is, therefore, defined as a multistep process in which each step corresponds to a genetic alteration. Each genetic change confers a specific growth advantage that drives the progressive transformation from normal to malignant cells (Rubin, 1994).

Genetic alterations can result in the chronic expression of genes that support cell growth and block apoptosis, indicated as pro-oncogenes, or in the loss of genes responsible for slowing cell division, repairing DNA and inducing programmed cell death, known as tumour-suppressor genes or anti-oncogenes. Both increased activity of pro-oncogenes and the loss of tumour-suppressor genes give a proliferative advantage to the cells. Cancers can develop in any part of the body and are usually categorised according to the tissues of origin as:

Carcinoma: when the site of origin is the skin or the tissues that cover internal organs;

Sarcoma: when the primary tumour forms in the bone, muscle, blood vessel, adipose or connective tissues;

Leukaemia: cancer that begins in the bone marrow or in other tissues that generate blood cells;

Lymphoma and myeloma: cancer that form in the cells of the immune system;

Central nervous system cancer: cancer that originates in the brain or spinal cord.

Despite the wide heterogeneity of genetic changes and the different cellular localisation of the tumours, there are at least six capabilities shared in common by most human tumours: evading apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, unlimited replicative potential and tissue invasion and metastasis (Hanahan and Weinberg, 2000). These characteristics are nowadays defined as the “six hallmarks of cancer” and are largely used to categorise different tumours. However, subsequent research in the field has led to the introduction of new hallmarks of cancers, particularly the role of genomic instability, inflammation and the reprogramming of the energetic metabolism of cancer cells (Hanahan and Weinberg, 2011).

Biological or environmental factors can be responsible for the development of cancer. Biological or internal factors include age, gender, inherited genetic defects and skin type. Environmental factors include exposure to UV, viruses such as the human papilloma virus, exposure to asbestos and excessive exposure to some chemicals. Behavioural factors include smoking, use of alcohol and excessive sun exposure. An unhealthy diet and lack of exercise can also increase the risk of cancer.

Various approaches are currently used for the treatment of cancer patients. The most common treatments available are chemotherapy or immunotherapy, which are based on the use of drugs that destroy or slow the growth of cancer cells, radiotherapy which consists of the use of high energy radiation, or surgery. Surgery can also be used to remove hormone producing glands to control cancer growth (hormone therapy).

Although our knowledge of cancer has increased in the last decade, cancer still poses one of the biggest burdens on health care in the world. Cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths in 2018 (World Health Organisation). Whilst the risk of contracting lung, prostate, colorectal, stomach and liver cancers is higher in men than women, breast, colorectal, lung, cervical and thyroid cancers typically affect more women than men (World Health Organisation). Modifying behaviours that increase the risk of cancer can reduce cancer burden. Early detection of cancer and improved management of patients who develop cancer can also increase the survival of cancer patients.

1.2 Dysregulated metabolism contributes to oncogenesis

1.2.1 Aerobic glycolysis in cancer

The peculiarity of cancer cells is their ability to undergo chronic and uncontrolled cell growth and division. In order to meet the increased bioenergetics and biosynthetic demand, tumour cells reprogram pathways of nutrient acquisition, nutrient metabolism and response to oxidative stress. The ability of highly proliferative cells to reprogram their metabolism has been recognized as one of the hallmarks of cancer (Hanahan and Weinberg, 2011).

The concept of cancer metabolism was first introduced in the 1920s by Otto Warburg (Warburg, 1956). Under aerobic conditions normal cells produce chemical energy in the form of adenosine triphosphate (ATP) by oxidating pyruvate through the tricarboxylic acid (TCA) cycle (also known as the Krebs cycle). In hypoxic conditions, cells switch to glycolysis for ATP production by converting pyruvate to lactic acid. This process is catalysed by the enzyme lactate dehydrogenase 1 (LDH1) (Rogatzki et al., 2015), and releases only 2 ATP molecules versus 36 ATP molecules per cycle obtained through the TCA cycle. Otto Warburg observed that rapidly proliferating tumour cells can reprogram their metabolism, by limiting their energy production largely to glycolysis. The conversion of glucose to lactate, which can occur in hypoxic normal cells, persists in cancer tissues, despite the presence of oxygen. This phenomenon has been termed “aerobic glycolysis” or “Warburg effect” (Vander Heiden et al., 2009). A high rate of aerobic glycolysis is thought to be advantageous for highly proliferating tumour cells because glucose is the most abundant extracellular nutrient. Although the amount of ATP per glucose consumed is low, if the glycolytic flux is high enough, the percentage of cellular ATP produced from glycolysis can exceed that produced from oxidative phosphorylation (DeBerardinis et al., 2008). Furthermore, the degradation of glucose provides molecular intermediates needed to generate cellular biomass, such as nucleotides, lipids and nicotinamide adenine dinucleotide phosphate (NADPH). Additionally, aerobic glycolysis offers the advantage to engage metabolite-mediated autocrine and paracrine signals that do not occur in normal tissues (Vander Heiden et al., 2009). Lactate, for instance, acts on the tumour microenvironment determining inflammation and angiogenesis (Rawat et al., 2019).

The shift to aerobic glycolysis is driven by multiple oncogenic signalling pathways, such as the Phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signalling. The best-studied effector downstream of PI3K is AKT1, also known as protein kinase B (PKB). AKT1 drives tumour glycolytic phenotype by increasing the expression and membrane translocation of glucose transporters (Fang et al., 2015) and by phosphorylating key glycolytic enzymes such as hexokinases (Roberts et al., 2013) and phosphofructokinase 2 (Novellademunt et al., 2013). Furthermore, AKT1 phosphorylates forkhead box subfamily O (FOXO), resulting in increased glycolytic capacity (Nakae et al., 2008).

A common characteristic of many tumours is that they reside in a low-oxygen environment (hypoxia) ranging from 0 to 2% of oxygen (DeBerardinis and Chandel, 2016). In order to increase the access to oxygen and nutrients, hypoxia inducible factor 1 alpha (HIF-1 α) up-regulates angiogenic growth factors such as the vascular endothelial growth factor and epidermal growth factor through transcriptional activation (Singh et al., 2017). During normoxia, HIF-1 α is associated to the von Hippel-Lindau (VHL) tumour suppressor, which targets HIF-1 α for ubiquitination and degradation. During hypoxia, the association to VHL is inhibited resulting in stabilization of the HIF-1 α protein and transcriptional activity of the HIF-1 complex (DeBerardinis et al., 2008).

mTOR, one of the downstream effectors of AKT1, can stabilize HIF-1 α , which, in turn, increases the expression of the glucose transporter 1 (GLUT1), glycolytic enzymes and pyruvate dehydrogenase (PDH) (Nagao et al., 2019), which blocks the entry of pyruvate into the TCA cycle. Constitutive cellular stabilization of HIF-1 α during normoxia can also occur in tumours as a result of mutations in the tumour suppressor VHL or in the enzymes succinate dehydrogenase (Selak et al., 2005) and fumarate hydrogenase (Isaacs et al., 2005), therefore contributing to aerobic glycolysis. As part of the adaption to hypoxia, HIF1 α also activates the expression of LDH1 and pyruvate dehydrogenase kinase 1 (PDK1), which together function to channel pyruvate to lactate and away from its oxidation into acetyl-CoA for entry into the mitochondrial TCA cycle (Nagao et al., 2019).

Mutations in the kirsten rat sarcoma 2 viral oncogene homolog (KRAS) and v-raf murine sarcoma viral oncogene homolog B1 (Kumar et al., 2007b) have also been found to regulate the hypoxic induction of HIF-1 α and HIF-2 α . In colon cancer mutant KRAS enhances HIF-

1 α by regulating its translation through the PI3K pathway, whilst mutant BRAF enhances mRNA expression and translation of HIF-1 α and HIF-2 α (Kikuchi et al., 2009). Effects of KRAS on HIF-1 α and HIF-2 α synergise with the PI3K/AKT signalling pathway also in breast cancer cell lines (Blancher et al., 2001). A mutation in the gene *BRAF* (V600E) increases HIF-1 α expression and cell survival under hypoxic conditions in melanoma (Kumar et al., 2007a), whilst HIF-1 α expression is altered by inhibitors of BRAF (Parmenter et al., 2014).

Another commonly deregulated pathway in cancer is the MYC signalling pathway, which, when upregulated, increases the expression of many genes that support anabolic growth, including transporters and enzymes involved in glycolysis (Goetzman and Prochownik, 2018). Additionally, MYC post-transcriptionally regulates HIF1 α expression (Doe et al., 2012). Increased glycolytic flux allows glycolytic intermediates to supply carbon for the TCA cycle, which in proliferative cells is used to fuel biosynthetic pathways.

1.2.2 The TCA cycle in cancer

The TCA cycle is the second stage of cellular respiration, the process by which cells break down organic fuel molecules in the presence of oxygen to obtain the energy needed to sustain growth and division. The TCA cycle is carried out in the mitochondrial matrix and its intermediates are used as precursors for macromolecule synthesis. The utilization of TCA intermediates in biosynthetic pathways requires carbon to continuously be supplied to the cycle so that intermediate pools are maintained.

Cancer cells require a large amount of lipids as well as nucleotides and amino acids during cell division. Therefore, intermediates of the TCA cycle need to be continuously replenished through a process called anaplerosis. The two main anaplerotic pathways in cancer cells are glutaminolysis, which produces α -ketoglutarate from glutamine, and pyruvate carboxylation, which produces oxaloacetate from glucose and/or pyruvate. Oxidation of the branched-chain amino acids isoleucine and valine can also provide some tissues with anaplerotic intermediates (DeBerardinis and Chandel, 2016). Real time ^{14}C -nuclear magnetic resonance (NMR) analysis has shown that the oxidation of glutamine leads to the conversion of

glutamine carbon into lactic acid in a manner analogous to the partial oxidation of glucose during aerobic glycolysis (Reitzer et al., 1979). By providing oxaloacetate, glutamine maintains the mitochondrial membrane potential and acts as a fuel for the synthesis of nucleotides, proteins and lipids (DeBerardinis et al., 2008). The ability of glutamine to donate its nitrogen and carbon into several growth promoting pathways, determine its importance as a nutrient in cancer. On the other hand, glutamine contributes to amino acid biosynthesis and mTORC1-dependent mRNA translation (Wise and Thompson, 2010).

Glutamine is imported into the cells through the activation of transporters such as the alanine, serine, cysteine-preferring transporter 2, also called SLC7A5 (Cormerais et al., 2018). The activity of the SLC7A5 transporter is controlled by c-MYC or the transcription factor E2F and is highly expressed in cancers (Altman et al., 2016). Once in the cytosol, glutamine is converted to glutamate by glutaminase, and subsequently metabolized to α -ketoglutarate by either glutamate dehydrogenase or glutamate-oxaloacetate transaminase.

α -Ketoglutarate is further oxidized in the TCA cycle to generate ATP or aspartate, which is a key substrate for nucleotide biosynthesis (DeBerardinis and Chandel, 2016). In the cytosol glutamine can either be converted to glutamate by cytosolic glutaminase or exported through the antiporter SLC7A5, in exchange for branched-chain amino acids such as leucine (Nicklin et al., 2009). There are two isoforms of glutaminase encoded by two different genes, *GLS1* and *GLS2*. *GLS1* is the main isoform expressed in cancer cells and has been shown to be upregulated in a wide variety of cancers, including breast, lung, cervix and brain (Altman et al., 2016; Saha et al., 2019).

Cells lacking glutaminase activate a compensatory anaplerotic mechanism catalysed by the enzyme pyruvate carboxylase. Pyruvate carboxylase allows the cells to use glucose-derived pyruvate rather than glutamine for anaplerosis (Cheng et al., 2011). Pyruvate carboxylase has been shown to be upregulated in breast cancer (Phannasil et al., 2015), whilst its knockdown showed a marked reduction in viability and proliferation rates, suggesting that the perturbation of the pyruvate carboxylase increases cancer aggressiveness (Phannasil et al., 2017). The existence of a compensatory mechanism between glutaminase and pyruvate carboxylase, also suggests that cancer cells need to sustain the TCA cycle to proliferate.

During the rapid proliferation of cancer cells, glutamine replenishes the biosynthetic precursors required for fatty acid synthesis (DeBerardinis et al., 2007). In glioblastoma cells, glutamine-derived oxaloacetate accounts for a high fraction of citrate synthesis (DeBerardinis et al., 2007). Furthermore, glutamine provides carbons for the formation of acetyl-coenzyme A (CoA) that is required for lipid synthesis via reductive carboxylation under conditions of hypoxia or mitochondrial dysfunction (Metallo et al., 2011).

Due to its role in the uptake of essential amino acids and in maintaining the mitochondrial membrane potential and integrity, many cancers depend on glutamine for their continued growth and survival (Wise and Thompson, 2010). For instance, some c-MYC-transformed cells require the presence of glutamine in order to maintain viability and the withdrawal of glutamine from these cells results in depletion of TCA cycle intermediates (Yuneva et al., 2007). Glutamine metabolism also plays a major role in the anti-oxidative capacity of the cells. Glutamine-derived glutamate is used in the synthesis of the anti-oxidant glutathione, through the condensation with cysteine and glycine. Cystine is an extracellular source of cysteine, which enters the cells via the cysteine/glutamate antiporter (xCT), in exchange with glutamate. xCT has been found to be altered in many cancers (Ji et al., 2018; Koppula et al., 2017; Koppula et al., 2018), whilst its pharmacological inhibition increases reactive oxygen species (ROS) levels (due to reduced glutathione levels) and suppresses tumour growth (Liu et al., 2017; Timmerman et al., 2013; Tsuchihashi et al., 2016). Furthermore, glutamine oxidation supports redox homeostasis producing NADPH, which, in turn, is used for the reduction of oxidized glutathione, protecting the cells from oxidative stress (Choi and Park, 2018). The tumour suppressor p53 has been found to induce the expression of GLS2, therefore increasing the levels of glutamate, α -ketoglutarate, and mitochondrial respiration rate, and decreasing ROS levels in cells (Hu et al., 2010). Mutant p53 accumulation has also been shown to suppress the xCT/glutathione axis, therefore diminishing glutathione synthesis. This process renders mutant-p53 tumours susceptible to oxidative stress-induced cell death (Liu et al., 2017). Interestingly, some cancer cells have the capacity to adapt to the lack of glutamine for survival and growth. Cancers harbouring p53 activation, indeed, are able to survive upon glutamine deprivation through upregulation of the arginine transporter solute carrier family 7 member 3, which increase arginine uptake. Arginine induces p53 activation to promote cell growth in response to glutamine starvation (Lowman et al., 2019).

Cancer cells carrying a mutation in the *PIK3CA* gene have also been described as being dependent on glutamine due to their ability to reprogram glutamine metabolism as described in section 1.4.2.

1.2.3 The Pentose Phosphate Pathway (PPP) in cancer

The pentose phosphate pathway (PPP) branches from the first step of glycolysis, which is catalysed by hexokinase and uses glucose-6-phosphate as substrate. It is composed of two branches: the oxidative branch, which generates NADPH and ribonucleotides and the non-oxidative branch, which converts glycolytic intermediates, such as fructose-6-phosphate and glyceraldehyde-3-phosphate into pentose phosphates and vice versa (Patra and Hay, 2014). Due to its role in generating pentose phosphates to supply their high rate of nucleic acid synthesis and NADPH, which is required for both the synthesis of fatty acids and cell survival, the PPP is particularly important for cancer cells. NADPH is required not only for fatty acid and cholesterol biosynthesis but also for the generation of reduced glutathione, a major scavenger of reactive oxygen species (Nathan and Ding, 2010). The rate-limiting step in the oxidative branch of PPP, which generates the first molecule of NADPH, is catalysed by the glucose-6-phosphate dehydrogenase (G6PDH). Therefore, the expression and activity of this gene are tightly regulated. The activity of G6PDH is mainly regulated by the $\text{NADP}^+/\text{NADPH}$ ratio. NADPH negatively regulates the activity of G6PDH, whereas NADP^+ is required for its enzymatic activity and for its proper conformation. However, G6PDH is also regulated by pro-oncogenic signalling pathways, including PI3K and RAS which induce the release of bound G6PDH to a soluble fraction, therefore increasing its activity (Stanton et al., 1991). Similarly, the expression of the enzymes involved in the non-oxidative branch of the PPP, as the transaldolase 1 (TALDO) and the transketolase (TKT), which divert fructose-6-phosphate and glyceraldehyde-3-phosphate from glycolysis to the PPP, are altered in cancer cells (Basta et al., 2008; Chung et al., 2004; Xu et al., 2016). Increasing the glycolytic flux in cancer cells might also indirectly impact the non-oxidative PPP. The loss of the tumour suppressor p53, for instance, increases glucose uptake in cancer cells (Schwartzberg-Bar-Yoseph et al., 2004), which supports both glycolysis and the PPP. Furthermore, p53 indirectly inhibits the oxidative PPP through the suppression the glycolytic enzyme phosphoglycerate mutase-1 (PGAM1), which in normal cells increases the oxidative

PPP by decreasing the level of glyceraldehyde-3-phosphate. The p53-induced glycolysis and apoptosis regulator TIGAR also inhibits glycolysis, redirecting the metabolites to the PPP (Bensaad et al., 2006). Another p53 target, the pro-apoptotic protein NOXA has been found to promote cell survival of myeloid and lymphoid cancer cells by facilitating the first step in the PPP, thus accelerating the generation of NADPH (Lowman et al., 2010). Additionally, the ataxia telangiectasia mutated kinase activates the PPP, independently of p53, by mediating the interaction between heat shock protein 27 (Hsp27) and G6PDH, therefore increasing the activity of G6PDH and the level of NADPH and nucleotides (Cosentino et al., 2011). Another oncogene responsible for increased activation of the non-oxidative PPP is KRAS. In pancreatic cancer KRAS activates the hexokinase 2 (HK2), consequently increasing glucose flux, which is required to generate glucose-6-phosphate (Patra et al., 2013). mTORC1 is also known to induce upregulation of the oxidative PPP by elevating the activity of the sterol regulatory element-binding proteins (SREBPs), which in turn stimulate the transcription of the gene encoding G6PDH (Duvel et al., 2010).

1.2.4 Lipid metabolism in cancer

In addition to glucose and glutamine metabolism, the increased biosynthesis of lipids has been recognized as an important component of the metabolic reprogramming in cancer cells. In 1953, Medes and colleagues observed for the first time that neoplastic tissues have aberrant activation of *de novo* lipogenesis (G Medes, 1953). Although the exact role of lipid synthesis in cancer is not fully understood, it is likely that *de novo* lipogenesis contributes to the generation of structural lipids. Sterols and phosphoglycerides, indeed, are required for the generation of biological membranes as well as cholesterol and steroids (Baenke et al., 2013). Lipids such as triacylglycerides (TAGs) are instead used as energy storage. Some lipids can also act as secondary messengers and could contribute to signalling processes in cancer cells. For instance, the lysophosphatidic acid stimulates colorectal cancer cell proliferation and survival by inducing dysregulation of transcription factors, activation of mitogen-activated protein kinases, pro-inflammatory cytokines, and angiogenic factors (Lee and Yun, 2010). The first step of lipid synthesis is the conversion of citrate to acetyl-CoA by the enzyme ATP-citrate lyase (ACLY). Acetyl-CoA is then converted to malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC). Most of the acetyl-CoA, used for the *de novo* synthesis of

fatty acids (FAs) and cholesterol is generated from glucose via the conversion of pyruvate to citrate in the TCA cycle. However, cancer cells are also able to generate citrate for the FA biosynthesis through the reductive metabolism of glutamine (Vander Heiden et al., 2009). Acetyl-CoA and malonyl-CoA are then coupled to the enzyme fatty acid synthase (FASN). Repeated condensations of acetyl groups generate a basic 16-carbon saturated FA: palmitic acid, which is then further elongated and desaturated to generate all the diverse FAs (Baenke et al., 2013). FAs are finally used to generate diacylglycerides (DAGs) and TAGs, cholesterol and cholesteryl-esters (Baenke et al., 2013). The biogenesis of FAs and cholesterol is regulated by the SREBPs, a family of transcription factors that are crucial for maintaining cellular lipid homeostasis (Horton et al., 2002). Aberrant activation of SREBPs can contribute to obesity, fatty liver disease, insulin resistance and cancer development (Shao and Espenshade, 2012). SREBP is regulated by PI3K–AKT–mTORC1-dependent mechanisms. AKT promotes the stability of mature SREBP1 by inhibiting glycogen synthase kinase-3 (GSK3), which phosphorylates and promotes SREBP1 ubiquitination and proteasome-dependent degradation (Dong et al., 2015). AKT-dependent activation of SREBP-1 and SREBP-2 leads to the induction of key enzymes of the cholesterol and fatty acid biosynthesis pathways (Porstmann et al., 2008). Additionally, the inhibition of the downstream target of AKT, mTORC1, results in reduced expression of key lipogenic enzymes, such as FASN, ACC1 and ACLY (Porstmann et al., 2008). mTORC1 also regulates the expression of lipogenic genes independently of SREBPs by increasing the mRNA splicing of genes involved in *de novo* lipogenesis (Lee et al., 2017). On the other hand, the combination of proteomic, lipidomic, and metabolomic analyses in the liver revealed that mTORC2 also promotes *de novo* fatty acid and lipid synthesis, leading to tumour development (Guri et al., 2017).

Tumour suppressor can also regulate lipogenesis. For instance, mutant forms of the p53 tumour suppressor have been shown to induce SREBP-dependent expression of enzymes involved in the cholesterol biosynthesis (Freed-Pastor et al., 2012). Furthermore, high expression of the epithelial growth factor receptor (EGFR) has been associated with high levels of nuclear SREBP1 in glioblastoma multiforme (Guo et al., 2009). In agreement with this, treating glioblastoma multiforme cells that express EGFR with inhibitors of FA and cholesterol biosynthesis reduced xenograft tumour formation (Guo et al., 2009).

Although cancer cells obtain lipids preferentially through *de novo* synthesis, using glucose and glutamine (G Medes, 1953), FAs can also be obtained through direct exogenous uptake from the surrounding microenvironment (G Medes, 1953). Specialised transporters are required to facilitate efficient movement of lipids across the plasma membrane. The best characterised lipid transporter is the cluster of differentiation 36 (CD36). High expression of CD36 has been correlated with poor prognosis across several tumour types, including breast, ovarian, gastric and prostate (Calvo et al., 1998; Ladanyi et al., 2018). Pascual *et al.* showed that high levels of CD36 and lipid metabolism genes are essential to initiate metastasis and the use of anti-CD36 neutralizing antibodies completely inhibits metastasis initiation (Pascual et al., 2017). Silencing *CD36* in human prostate cancer cells reduces fatty acid uptake and cell proliferation. Likewise, deletion of *Cd36* in the prostate of *Pten*^{-/-} mice reduced fatty acid uptake and slowed cancer progression (Watt et al., 2019).

1.3 PI3K signalling

The PI3K signalling pathway is one of the most important intracellular pathways, which regulates a wide range of cellular functions such as cell growth and proliferation, differentiation, motility, survival, angiogenesis and metabolism (Yang et al., 2019). Alterations in the PI3K/AKT/mTOR pathway occur in a variety of human cancers including breast cancer (31%), endometrial cancer (37%), cervical cancer (29%), bladder cancer (22%), anal cancer (27%), colorectal cancer (17%) and head and neck squamous cell carcinoma (14%) (Yang et al., 2019). Genetic events leading to activation of the PI3K/AKT/mTOR pathway in cancer include two hotspots of mutation in *PIK3CA*, E542, and E545 in the helical domain, and H1047 and G1049 in the kinase domain (Thorpe et al., 2015). Somatic mutations in *PIK3R1* and *PIK3R2* have been identified in a number of different cancers, the majority of which are substitutions or in-frame insertions or deletions in the inter-SH2 (iSH2) domain of p85 α (Cizkova et al., 2013; Jaiswal et al., 2009; Urick et al., 2011). Mutations in other regulators of the PI3K pathway occur in a variety of cancers. In particular, *PTEN* is one of the most commonly mutated cancer genes overall (Keniry and Parsons, 2008). Mutations of *PTEN* are genomic deletion and small point mutations that inactivate its function (Keniry and Parsons, 2008). Other alterations include mutations in *AKT1* (E17K) (Zhang et al., 2017),

inactivating mutations in both *TSC1* and *TSC2* (Kwiatkowski et al., 2016) and activating mutations in *MTOR* (Kwiatkowski et al., 2016).

Using multiple molecular profiling platforms, Zhang and colleagues examined the entire PI3K/AKT/mTOR pathway and its components in over 10,000 human cancers and 32 cancer types profiled by TCGA (Zhang et al., 2017). Their analysis revealed the presence of somatic alteration for key genes. In particular, *PIK3CA* was 14% mutated and 6% amplified across all cancers; 9% of cancers had mutated *PTEN*, whilst 7% of cancers showed deletion or two-hit loss of *PTEN*. Other mutations involving *PIK3R1* (4% mutated), *PPP2R1A* (2% mutated), *AKT1* (1% mutated), *AKT1* (3% amplified), *TSC1* (2% mutated), *STK11* (2% mutated; 1% deletion or two-hit loss), *RICTOR* (3% amplified), and *MTOR* (4% amplified) were also characterised.

PI3Ks are a group of plasma membrane-associated lipid kinases, which are activated by phosphorylating phosphatidylinositol (PI) membrane lipids at the 3-hydroxyl group of their inositol ring. The PI3K activity was first described in the mid-1980s, as associated with viral-oncogene-encoded tyrosine kinases (Sugimoto et al., 1984; Whitman et al., 1985). Further studies led to the general adoption of the term PI3K to refer to any activity capable of phosphorylating the 3-OH group of the inositol ring of lipids (Vanhaesebroeck et al., 2012). Through their phosphorylating capacity, PI3Ks generate a range of intracellular lipid second messengers. For example, PI3K phosphorylates PI to generate phosphatidylinositol-3-phosphate (PIP), phosphatidylinositol-4-phosphate to form phosphatidylinositol-3, 4-bisphosphate and phosphatidylinositol-4-5-phosphate (PIP₂) to produce phosphatidylinositol-3,4,5-trisphosphate (PIP₃) (Yu and Cui, 2016). The phosphatase and tensin homolog (PTEN) is a 3-phosphatase that can terminate the PI3K signalling by dephosphorylating PIP₃ back to PIP₂. PIP₃ can also be dephosphorylated by the 4- and 5- phosphatases to produce phosphatidylinositol-3,5-biphosphate and phosphatidylinositol-3,4-biphosphate (Vanhaesebroeck et al., 2012).

1.3.1 Classes of PI3Ks

PI3Ks are divided into 3 classes (classes I, II, III; Figure 1.1) with different structures, specific substrates (Thorpe et al., 2015), cellular localisation and upstream activators (José María Rojo, 2014). Class I PI3Ks are further classified into class IA and class IB.

Class IA PI3Ks are heterodimers consisting of a p85 regulatory subunit and p110 catalytic subunit. The p110 catalytic subunit of IA PI3K consists of 3 isoforms, p110 α , p110 β and p110 δ , encoded by the genes *PIK3CA*, *PIK3CB* and *PIK3CD*, respectively. Each isoform contains several domains: p85 binding domain, RAS-binding domain (RBD), protein-kinase-C homology-2 domain (C2), a helical domain and a catalytic domain (Yang et al., 2019).

The isoforms p110 α and p110 β are ubiquitously expressed, whilst p110 δ expression is largely restricted to leukocytes (Ross and Cantrell, 2018). The p85 regulatory subunit of IA PI3K also has multiple isoforms: p85 (α and β), p55 (α and γ) and p50 α . The p85 α , p55 α and p50 α isoforms are encoded by the *PIK3R1* gene, p85 β is encoded by the *PIK3R2* gene, whilst the *PIK3R3* encodes for the p55 γ isoform. All the p85 regulatory isoforms possess an inter-SH2 (iSH2) domain that binds the catalytic subunits, flanked by SH2 domains. The isoforms p85 α and p85 β possess an additional N-terminal SH3 domain and the breakpoint cluster homology (BH) domain (Thorpe et al., 2015). The interaction between the regulatory and catalytic subunit of PI3K is essential to achieve maximal enzymatic activity (Bi et al., 1999).

Class IB PI3K consists of only one isoform of the catalytic domain, p110 γ , produced by the *PIK3CG* gene. Since the catalytic domain of class IB PI3K does not contain the p85 binding domain, the p110 γ catalytic subunit cannot bind the p85 regulatory subunit, and instead associates with the p101 regulatory subunit or to the more recently discovered, p84/p87^{PIKAP}, regulatory subunit (Voigt et al., 2006). The *PIK3R5/6* gene encodes for the p101 subunit, whilst the *PIK3R5/6* gene encodes for the p84/p87 subunit (Jean and Kiger, 2014). The major substrate for class I (A and B) PI3Ks is PI (4,5) P₂, but they can also act on PI.

Class II PI3Ks is a monomer with three isoforms, PI3K-C2 α , PI3K-C2 β and PI3K-C2 γ . The structure of these enzymes is similar to the class I PI3K, with RBD, helical and kinase domains, but they also have an additional C2 domain and a C-terminal Phox homology (PX) domain. The major substrate for class II PI3Ks is PIP, but they can also act on PI.

Class III PI3K is a heterodimeric protein consisting of a catalytic and a regulatory domain. The catalytic domain, named vacuolar protein-sorting 34 (VPS34), is composed of C2, helical and kinase domains. The regulatory subunit, named p150, consists of a kinase domain along with a HEAT domain (a repeat structure found in Huntingon Elongation factor 3, a subunit of protein phosphatase-2A and TOR1 proteins) and WD40 domain (a series of approximately 40 amino acids which terminate in a tryptophan-aspartic acid (W-D) dipeptide). Class III PI3K specifically phosphorylates PI.

The activation of the class IA PI3K is dependent upon receptor tyrosine kinases (RTKs). In the absence of activating signals, the p85 regulatory subunit interacts with catalytic domain of p110 and inhibits its kinase activity (Vadas et al., 2011). In the presence of activating signals the p85 regulatory subunit binds to the phosphorylated tyrosine residues on the RTKs, through its iSH2 domains, leading to the translocation of PI3K to the membrane. Once in the membrane, the p85 regulatory domain loses its inhibitory effect on p110, which becomes available to phosphorylate PIP_2 to PIP_3 (Vadas et al., 2011) (Figure 1.2). Class IA PI3K can also be activated by direct interaction with RAS GTPase (Thorpe et al., 2015).

The activation of the class IB PI3K occurs upon binding with the G-protein-coupled receptors (Thorpe et al., 2015). The activation of class II and III PI3Ks are not well understood. The class II may be activated by hormones, growth factors, chemokines, cytokines, phospholipids, and calcium (Ca^{2+}), whilst the class III is thought to be activated by nutrients such as amino acids and glucose (Thorpe et al., 2015).

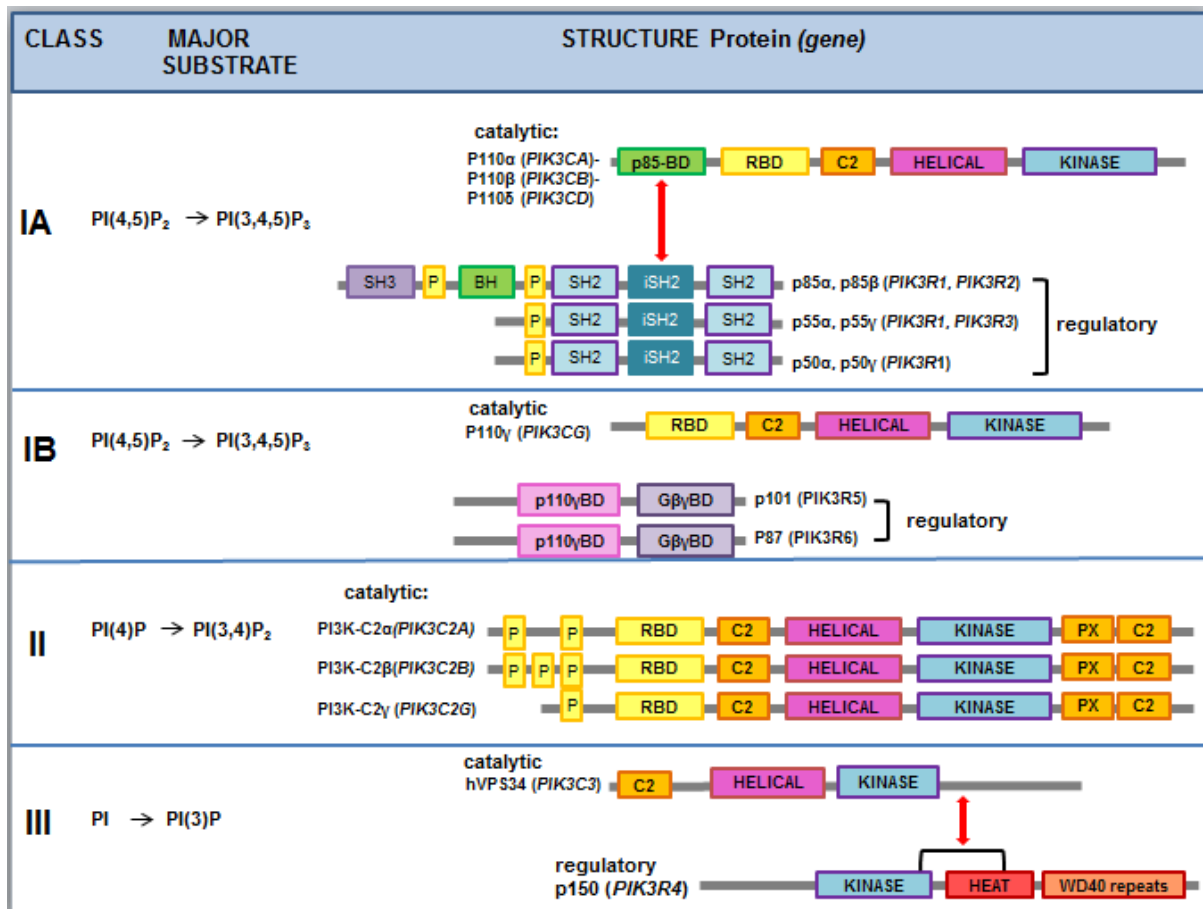


Figure 1. 1: PI3K family classes and isoforms:

Shown are the substrates, genes and domain structures of the various isoforms of each of the four classes of PI3Ks. PI3Ks $PI(4,5)P_2$ = phosphatidylinositol-4,5-bisphosphate; $PI(3,4,5)P_3$ = phosphatidylinositol-3,4,5-trisphosphate; $PI(\text{Muhammet F Gulen et al.})P$ = phosphatidylinositol-4-phosphate; $PI(3,4)P_2$ = phosphatidylinositol-4,5-bisphosphate; $PI(3)P$ = phosphatidylinositol-3-phosphate. BD = binding domain; RBD = RAS-binding domain; C2 = protein-kinase-C homology-2 domain. SH2 = Src-homology 2 domain; SH3 = Src-homology 3 domain; Ish2 = inter- Src-homology 2 domain, BH = breakpoint cluster homology domain; PX= Phox homology domain. VPS34 = vacuolar protein-sorting 34; HEAT= Huntington, Elongation Factor 3, PR65/A, TOR domain, WD40 domain (W-D) dipeptide domain. P = proline-rich domain. Figure modified from Thorpe *et al.* (Thorpe et al., 2015).

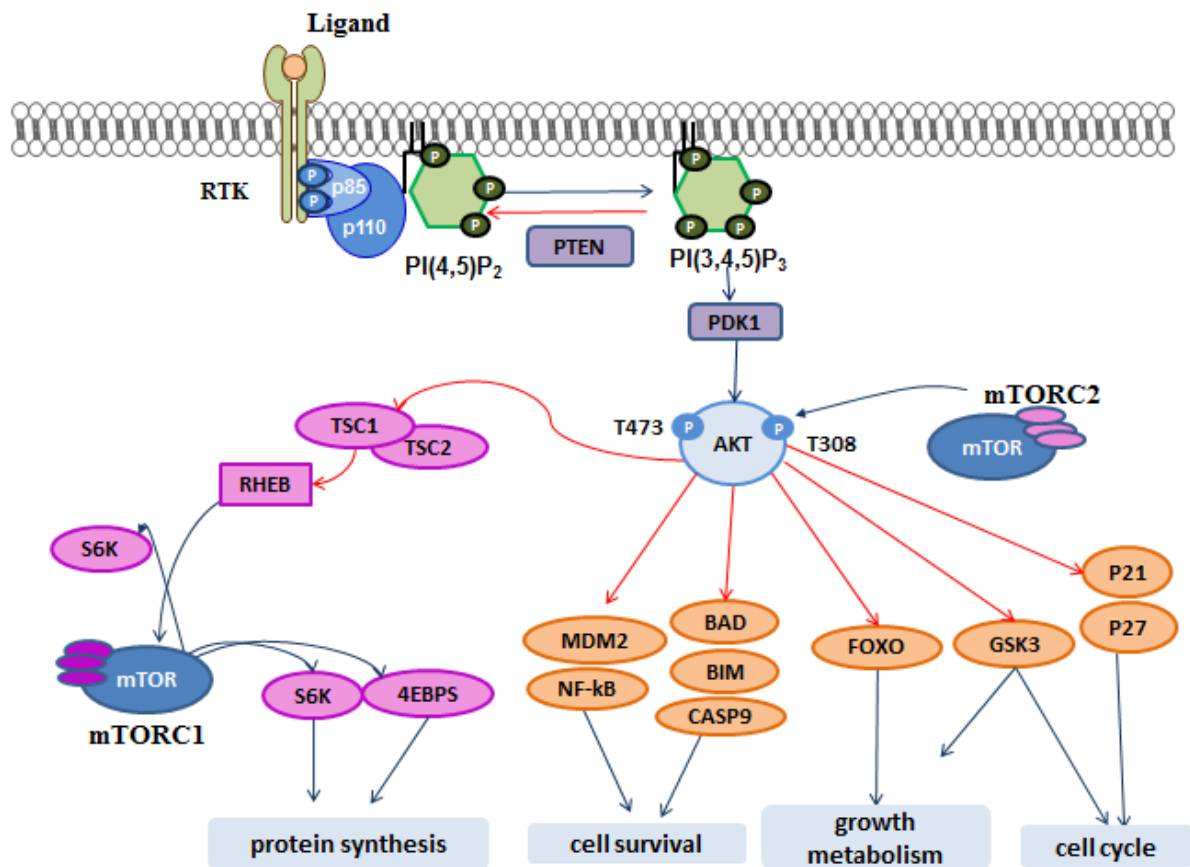


Figure 1. 2: Class I PI3K signalling pathway and downstream effects on cellular functions. PIP₃ generated by PI3Ks activates the kinases PDK1 and AKT. AKT is also activated by mTORC2.

AKT promotes cell survival by activating MDM2 and NF-κB and the pro-apoptotic factor BAD, BIM, caspase 9 (CASP9). AKT promotes growth, metabolism, and tumorigenesis by inhibiting the FOXO family of transcription factors and GSK-3. AKT promotes cell cycle progression by activating the cell cycle regulators p21 and p27 and inhibiting GSK-3. AKT promotes mTORC1 activity by phosphorylating and inhibiting TSC1 and 2, thereby enabling the GTPase Rheb to activate mTORC1. p70 ribosomal protein S6 kinase 1 (S6K1) is phosphorylated by mTORC1. mTORC1 promotes protein synthesis by phosphorylating translational regulators S6K1 and binding protein 1 and 2 (4EBP1 and 4EBP2).

1.3.2. AKT signalling

AKT is a 57-kDa serine/threonine kinase, originally identified as an inhibitor of GSK3 β in response to the insulin-like growth factor (IGF) (Bellacosa et al., 1991). Subsequently, Woodgett and Coffey (Coffey and Woodgett, 1991), identified a protein kinase that they named PKB, due to the similarity with protein kinases A and C (Alessi et al., 1996). There are three AKT/PKB isoforms conserved in mammalian genomes, AKT1 (PKB α), AKT2 (PKB β) and AKT3 (PKB γ) (Manning and Toker, 2017), which are 80% identical in amino acid sequence, and all contain an N-terminal pleckstrin homology (PH) domain, that regulates the membrane translocation of AKT and a C-terminal regulatory domain (Hill and Hemmings, 2002). AKT1 is the only isoform expressed ubiquitously, whilst AKT2 is mainly expressed in insulin-sensitive tissues, such as skeletal muscle, adipose tissues and liver. AKT3 is only expressed in testes and brain (Easton et al., 2005).

PIP₃ produced by the class I PI3K acts as a secondary messenger by providing docking sites for 3-phosphoinositide-dependent kinases, PDK1 and PDK2. PDK1 phosphorylates AKT at Thr-308 leading to its partial activation (Alessi et al., 1997). However, a second phosphorylation event at Ser-473, mediated by PDK2, is necessary to fully activate AKT (Feng et al., 2004; Sarbassov et al., 2005). Similar phosphorylation events are also observed in AKT2 (T309 and S474) and AKT3 (T305 and S472) (Manning and Toker, 2017). PDK2, also known as RICTOR associated serine/threonine kinase mTORC2 (Figure 1.2) (Vadas et al., 2011), is one of the downstream targets of AKT defining the existence of a positive feedback loop between AKT and mTORC2 (Yang et al., 2015).

Downstream effectors of AKT are regulated through serine and/or threonine phosphorylation by AKT. Those effectors share a common minimal sequence motif, Arg-Xaa-Arg-Yaa-Zaa-Ser-Hyd (Alessi et al., 1996). Although the 2 events of phosphorylation at Thr-308 and Ser-473 are obligatory for the maximal AKT activation, many other post-translational modifications also regulate the function of AKT. For instance, the constitutive phosphorylation of Thr-450 is required for proper folding of the nascent AKT polypeptide (Facchinetti et al., 2008). Furthermore, the phosphorylation of Ser-129 (Di Maira et al., 2005) has been shown to increase the catalytic activity of AKT, whereas the phosphorylation of Thr-312 (Muhammet F Gulen et al., 2012) attenuates its activity. Beyond phosphorylation,

various other post-translational modifications of AKT have been identified, such as acetylation, oxidation, glycosylation and ubiquitination (Manning and Toker, 2017). The major downstream target of AKT is the RAPTOR associated serine/threonine kinase mTORC1. AKT activates mTORC1 by phosphorylating and inhibiting the tuberous sclerosis proteins 1 and 2 (TSC1 and TSC2). The phosphorylation of cytoplasmic proteins by AKT leads to inhibition of apoptosis and activation of signalling that mediate cell growth and survival. The main targets inhibited by AKT are GSK-3 β , the pro-apoptotic proteins Bad, Bim and caspase 9, the cell cycle regulators p21 and p27, and the FOXO family transcription factors. On the contrary, the nuclear factor-kappa B (NF- κ B) regulator IKK- α , the p53 inhibitor murine double minute 2 (MDM2), the genetic stability guardian telomerase reverse transcriptase and the cyclic adenosine monophosphate (cAMP) responsive element binding protein 1 (CREB) transcription factor are activated by AKT (Ersahin et al., 2015) (Figure 1.2)

1.3.3 mTOR signalling

mTOR is a 289 kDa ubiquitous serine/threonine protein kinase activated by phosphorylation in response to growth factors and hormones (Chong et al., 2010), whose action is inhibited by the antifungal metabolite Rapamycin, produced by *Streptomyces hygroscopicus* (Li et al., 2014a). mTOR contains several conserved functional domains: the N-terminal domain possesses 20 tandem HEAT repeats, which serve for protein-protein interaction, whilst the C-terminal region contains a kinase domain, a catalytic domain containing an FKBP-rapamycin-binding (FRB) domain, a FRAP-ATM-TRRAP (FAT) domain, a FAT C-terminus (FATC) domain and a putative negative regulatory domain (NRD) (Li et al., 2014b).

The association of mTOR to regulatory proteins generates the complexes mTORC1 and mTORC2 with different substrate specificity and cellular function. mTORC1 consists of mTOR, mammalian lethal with sec-13 protein 8 (mLST8), regulatory associated protein of mTOR (RAPTOR), DEP-domain containing mTOR interacting protein (DEPTOR) and Proline-rich Akt substrate 40kDa (PRAS40). mTORC2 consists of mTOR, rapamycin insensitive companion of mTOR (RICTOR), stress-activated protein kinase-interacting protein 1 (mSIN1) and mLST8 (Li et al., 2014b).

Unlike mTORC2, which responds only to growth factors, mTORC1 responds to many extracellular signals such as growth factors, oxygen, energy levels and nutrients. The action of mTORC2 is limited to the organisation of the cytoskeleton and cell survival, whilst mTORC1 promotes a vast range of cellular processes such as growth and proliferation, proteins, nucleotides and lipids synthesis, glucose and glutamine metabolism and autophagy (Li et al., 2014b). Downstream targets of mTORC2 include AKT, the Serum/Glucocorticoid Regulated Kinase 1 (SGK1), and various protein kinase C members. The best characterized effectors of mTORC1 are the p70 ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor 4E (eIF4E) - 4E-BP1. The two complexes show different sensitivity to rapamycin. Whilst rapamycin acutely inhibits mTORC1, the chronic exposure to rapamycin is necessary to inhibit mTORC2 (Li et al., 2014a).

1.3.4 GSK3 signalling

GSK3 is an unconventional kinase, which is constitutively active in the absence of an external signal and becomes inhibited in response to signals from membrane-bound receptors, such as insulin and WNT signalling (Beurel et al., 2015). GSK3 exists in two forms, GSK3 α and GSK3 β , encoded by separate genes, *GSK3A* and *GSK3B* (Hermida et al., 2017). Both GSK3 α and GSK3 β exhibit strong preferences for substrates that have already been primed through phosphorylation by other kinases, such as the casein kinase-1 (CK1) and the mitogen activate protein kinases (MAPK) (McCubrey et al., 2014). The most common target for GSK3 is the sequence S/T-X-X-X-S/T(P), where GSK3 phosphorylates serine/threonine four residues N-terminal to a pre-phosphorylated serine/threonine (Beurel et al., 2015).

GSK3 activity is modulated by exposure to a variety of growth factors including insulin and AKT. AKT inhibits GSK3 through phosphorylation of its Ser-9 (GSK3 β) or Ser-2 (GSK3 α) (McCubrey et al., 2014). The GSK3 kinases phosphorylate greater than 40 proteins including over 12 transcription factors (McCubrey et al., 2014). Furthermore, GSK3 has been shown to phosphorylate several upstream and downstream components of the PI3K/AKT/mTOR signalling network, including AKT itself, RICTOR, TSC1 and 2, PTEN and the insulin receptor substrate 1 and 2 (IRS1 and 2), thus generating feedback control mechanisms within the network (Hermida et al., 2017).

1.3.5 FOXO signalling

FOXO proteins belong to the Forkhead family of transcription factors, which is characterized by a conserved DNA-binding domain, the 'Forkhead box' and comprises more than 100 members in humans, classified from FOXA to FOXR (Wang et al., 2014b). The class "O" consists of four members, namely FOXO1, FOXO3a, FOXO4 and FOXO6. FOXOs function to maintain cellular homeostasis in response to internal and external environmental changes (Tsuchiya and Ogawa, 2017).

FOXO are regulated downstream of the insulin/PI3K/AKT signalling. AKT-mediated FOXO phosphorylation increases its association with the chaperone protein 14-3-3 in the nucleus. The binding to the protein 14-3-3 results in the translocation of FOXO proteins from the nucleus to the cytoplasm, leading to their transcriptional inactivation (Wang et al., 2014b). However, Ni and colleagues reported the existence of a feedback network by which sustained activation of FOXO proteins basal levels increases AKT phosphorylation and kinase activity by suppressing its interaction with the protein phosphatase 2A (PP2A) (Ni et al., 2007). The resulting increase in AKT activity leads to diminished insulin signalling, reduced membrane translocation of GLUT4, and decreased insulin-dependent glucose uptake.

This founding highlighted a new mechanism through which FOXO controls insulin sensitivity and glucose metabolism. FOXOs have also additional important roles in metabolism, cellular proliferation, stress resistance, and apoptosis and their activity is tightly regulated by post-translational modification, including phosphorylation, acetylation, and ubiquitylation (Wang et al., 2014b).

1.3.6 Regulation of PI3K-AKT pathway by negative and positive feedback mechanisms

Negative regulation of the PI3K-AKT pathway can be achieved by dephosphorylation of PIP₃ to PIP₂ or through inactivation of the AKT protein. PTEN is the main negative regulator protein which converts PIP₃ to PIP₂. PP2A (Andjelkovic et al., 1996) and PH Domain And Leucine Rich Repeat Protein Phosphatase (PHLPP) (Brognard et al., 2007) negatively regulate AKT through dephosphorylation at Thr-308 and at Ser-473, respectively (Figure 1.3). The pathway can also regulate itself through positive or negative feedback mechanisms.

For instance, NF- κ B, activated by AKT, represses PTEN (Vasudevan et al., 2004) expression as a positive feedback, whilst GSK3 phosphorylates and activates AKT. In contrast, mTORC1-S6K1 phosphorylates IRS-1 at multiple serine residues, preventing binding to RTKs, therefore resulting in the suppression of PI3K activity. Furthermore the inhibition of FOXO by AKT leads to transcriptional inactivation of RTK1 and inhibition of the PI3K pathway (Faes and Dormond, 2015) (Figure 1.3).

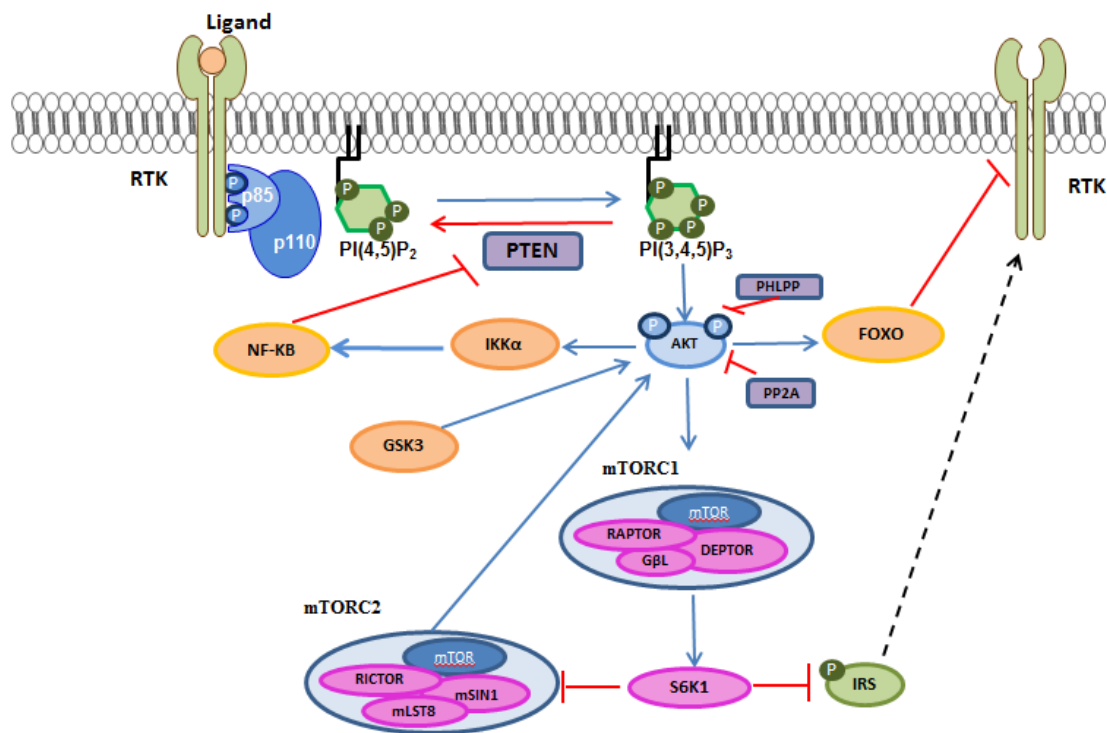


Figure 1. 3: Regulation of PI3K-AKT pathway by negative and positive feedback mechanism.

Negative regulation of PI3K-AKT pathway is achieved by dephosphorylation of PIP₃ to PIP₂ by PTEN. PP2A and PHLPP negatively regulate AKT through dephosphorylation at Thr308 and at Ser473, respectively. mTORC1-S6K1 phosphorylate IRS-1, preventing its binding to RTKs, therefore resulting in the suppression of PI3K. Furthermore the inhibition of FOXO by AKT leads to transcriptional inactivation of RTK1 and inhibition of the PI3K pathway. Positive regulation of PI3K-AKT pathway is achieved by the action of NF-κB, which represses PTEN, whilst GSK3 phosphorylates and activates AKT. mLST8: mammalian lethal with sec-13 protein 8, RAPTOR: regulatory associated protein of mTOR, DEPTOR: DEP-domain containing mTOR interacting protein, PRAS40: Proline-rich Akt substrate 40kDa. RICTOR: rapamycin insensitive companion of mTOR, mSIN1: stress-activated protein kinase-interacting protein 1.

1.3.7 PTEN signalling

The tumour suppressor function of PTEN was described in 1997 by three independent laboratories (J Li 1997) (Li and Sun, 1997) (Steck et al., 1997). Subsequent studies established its role as negative regulator of the PI3K/AKT signalling pathway (Downes et al., 2007) (Stiles et al., 2004). PTEN is a dual lipid and protein phosphatase made of 403 amino acids. The C2 domain of PTEN mediates the affinity for membrane phospholipids, whilst the phosphatase domain contains the signature CX5R (HCKAGKGR) p-loop structure for phosphatases (Chen et al., 2018b). The main biological effect of PTEN is to act as a negative regulator of the PI3K signalling pathway by dephosphorylating its lipid substrate PIP₃ to PIP₂.

A wide number of transcriptional factors control the expression of *PTEN*, including the tumour suppressor p53, the early growth response protein 1 (EGR-1), the peroxisome proliferation-activator receptor γ (PPAR γ) and the active transcription factor 2 (ATF2) (Chen et al., 2018b). *PTEN* is also transcriptionally repressed by SNAIL and SLUG (Uygur et al., 2015) (Escriva et al., 2008), which compete with p53 for *PTEN* promoter binding. Other factors can also bind the *PTEN* promoter, such as c-JUN and NF- κ B (Oliva-González et al., 2017), which negatively regulate its expression. The NOTCH1 targets MYC and HES1 were also shown to transcriptionally induce and inhibit *PTEN*, respectively (Correia et al., 2014).

PTEN can also be post-translationally regulated by several mechanisms, including ubiquitination, acetylation, oxidation and phosphorylation (Chen et al., 2018b). Hyper methylation of CpG dinucleotide-rich regions of PTEN is commonly implicated in cancer, including breast cancer, hepatocellular carcinoma and thyroid cancers (Correia et al., 2014).

The effects of PTEN in cell growth, survival, cell migration and differentiation, and cell and organ size control, as well as metabolism are principally regulated by AKT. However, PTEN is also reported to play important roles in chromosome stability, DNA repair and cell cycle regulation, independently of AKT. For instance, PTEN promotes the stability and transcriptional activity of the tumour suppressor p53 by direct binding (Freeman DJ, 2003) (Wen Hong Shen 2007) (Tang and Eng, 2006). PTEN can also bind the centromere specific binding protein C leading to premature centromere separation (Shen et al., 2007). In addition,

PTEN has been found to collaborate with E2F to enhance DNA repair (Shen et al., 2007), and with the anaphase-promoting complex (APC) to promote the proteolysis of mitotic cyclins (Chen et al., 2018b).

1.4 Metabolic reprogramming induced by PI3K signalling in cancer

PI3K is the most commonly activated pathway in human cancer. Oncogenic activation of the pathway reprograms cellular metabolism by increasing nutrient transport and by regulating the activity of metabolic enzymes. By inducing metabolic reprogramming, PI3K supports the anabolic demand of cancer cells. These metabolic adaptations of cancer cells could reveal metabolic vulnerabilities and help the development of new therapeutic strategies.

1.4.1 PI3K promotes aerobic glycolysis in cancer

The PI3K/AKT signalling network modulates the activity of diverse downstream effects on cellular metabolism, directly through the regulation of nutrient transporters and metabolic enzymes or indirectly by controlling the expression of transcription factors that, in turn, regulate the expression of key components of metabolic pathways. Recent studies have demonstrated specific functions for p110 α subunit of class I PI3Ks in growth factor and insulin signalling. Mice heterozygous for a mutation in the p110 α (D933A) subunit displayed attenuated insulin signal associated with reduction of IRS proteins levels (Foukas et al., 2006), whilst mice with chronic partial inactivation of p110 α were protected from age-related reduction in insulin sensitivity, glucose tolerance and fat accumulation (Foukas et al., 2013; Foukas et al., 2006). Similarly, ablation of p110 β in mice, impairs insulin sensitivity and glucose homeostasis, in an Akt-independent fashion (Jia et al., 2016).

The effects of PI3K on glucose metabolism are mediated by its downstream target AKT. Of the 3 AKT isoforms, AKT2 is strongly correlated with the regulation of glucose homeostasis and is the predominantly expressed in insulin-responsive tissues. Mice lacking Akt2 and Akt3 were glucose and insulin intolerant and exhibited an approximately 25% reduction in body weight compared to wild-type mice (Dummler et al., 2006). Cho and colleagues showed

that, in contrast to *Akt2* null mice, *Akt1* -deficient mice are normal with regard to glucose tolerance and insulin-stimulated disposal of blood glucose, thus revealing the non-redundant functions of *Akt1* and *Akt2* genes with respect to organismal growth and insulin-regulated glucose metabolism (Cho et al., 2001). Similarly, *Akt3* was observed to influence postnatal brain development but not glucose homeostasis (Tschopp et al., 2005). Furthermore, a work from Eva Gonzalez, and Timothy E McGraw, (Gonzalez and McGraw, 2009) established that *Akt2* has a crucial role in the maintenance of glucose homeostasis. Their work showed that targeted deletion of *Akt2* in mice, but not *Akt1* or *Akt3*, results in fasting hyperglycaemia, hyperinsulinemia, glucose intolerance and impaired glucose uptake by fat and muscle cells.

The best characterised metabolic role of PI3K/AKT on cancer cells is its ability to potentiate aerobic glycolysis. The expression of constitutively active AKT is sufficient to promote glucose uptake and aerobic glycolysis (Elstrom et al., 2004).

AKT modulates glycolysis by promoting glucose uptake both through GLUT1, the main glucose transporter upregulated in cancer, and GLUT4. A major mechanism by which AKT potentiate GLUT4 activity is through phosphorylation and inhibition of its downstream target AS160 (Eguez et al., 2005), which promotes GLUT4 trafficking, but does not have any effect on GLUT1. Thioredoxin-interacting protein (TXNIP) has, instead, been found to mediate the regulation of both GLUT1 and GLUT4 trafficking, downstream to AKT (Waldhart et al., 2017). AKT phosphorylates and inhibits TXNIP, therefore removing its inhibitory effect on GLUT1.

In addition to glucose uptake, AKT controls key steps in glycolysis through the phosphorylation and activation of specific glycolytic enzymes. Following its transport into cells, glucose gets activated by hexokinases that phosphorylate glucose to form glucose-6-phosphate, which cannot be transported out of the cell. AKT promotes HK2 activity by increasing its association with a voltage-dependent anion channel at the outer mitochondrial membrane, therefore coupling glucose metabolism to oxidative phosphorylation (Gottlob et al., 2001).

HK2 expression is elevated in many human cancers (Liu et al., 2016), including prostate (Wang et al., 2014a), pancreatic (Anderson et al., 2017), liver cancer (DeWaal et al., 2018) and glioblastoma (Wolf et al., 2011). AKT also phosphorylates and activates the glycolytic

enzyme 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase (PFKFBP) (Deprez et al., 1997), which catalyses the interconversion of fructose-6-phosphate and fructose-2, 6-bisphosphate, therefore leading to enhanced glycolytic flux. Furthermore, the PI3K signalling can potentiate the glycolytic flux in an AKT-independent fashion through mobilization of aldolase A from the actin of the cytoskeleton. This event leads to activation of aldolase A and potentiates its effect on glycolysis (Hu et al., 2016). High aldolase A expression in cancer is correlated with poor patient prognosis (Jiang et al., 2018b) (Dai et al., 2018).

In hypoxia AKT stabilises HIF1 α (Semenza, 2003), and phosphorylates and increases the activity of phosphofructokinase 1 (PFK1), which inhibits PDH (Chae et al., 2016). HIF1 α also activates LDH, thus facilitating a switch towards glycolysis to support cancer cell proliferation under hypoxic conditions (Marín-Hernández et al., 2009). Furthermore, AKT promotes glycolysis through the stabilization of the transcription factor (Gregory et al., 2003), which phosphorylates MYC and targets it for proteasomal degradation. MYC induces expression of the major glucose transporters and most glycolytic enzymes and can drive aerobic glycolysis (Stine et al., 2015). All the above described effects of AKT in induction of aerobic glycolysis can render cancer cells dependent on glucose for survival (Granja et al., 2015) and it has been shown that the dependence on glucose resulting from AKT activation in cancer can be reverted by pharmacologic activation of FA- β -oxidation. The effect of the PI3K/AKT pathway on aerobic glycolysis is summarised in figure 1.4.

1.4.2 PI3K sustains the TCA cycle in cancer

PIK3CA mutations reprogram glutamine metabolism by upregulating glutamate pyruvate transaminase 2 (GPT2) in colorectal cancer (CRC) cells, making them more dependent on glutamine (Hao et al., 2016). Indeed, treatment with a glutamate pyruvate transaminase 2 inhibitor suppressed *in vivo* growth of *PIK3CA* mutant, but not wild-type, colorectal cancers (Feng et al., 2016). mTORC has also been found to regulate glutamine metabolism by inhibiting the activity of the cystine/glutamate antiporter, xCT, through phosphorylation of its light chain (solute carrier family 7 member 11, also called SLC7A11) at serine 26 in the cytosolic N terminus, in response to the nutritional state of the cells (Gu et al., 2017). Cancer

cells often require glutamine for growth, thereby distinguishing them from most normal cells. The effects of the PI3K/AKT pathway on the TCA cycle are summarised in figure 1.4.

1.4.3 PI3K promotes *de novo* lipid synthesis in cancer

The PI3K–AKT pathway induces *de novo* lipid synthesis through both post-translational and transcriptional mechanisms. AKT can initiate *de novo* lipid synthesis by directly phosphorylating ACLY (Berwick et al., 2002), with consequent increased production of acetyl-CoA, which is used for sterol and fatty acid synthesis. AKT can also promote *de novo* lipid synthesis through activation of the SREBP family of transcription factors, which induce the expression of enzymes responsible for fatty acid and sterol synthesis, including ACLY (Porstmann et al., 2005). AKT activates SREBP through at least two downstream branches: 1) by activating mTORC, which activates SREBP (Duvel et al., 2010) (Owen et al., 2012; Ricoult et al., 2016; Yecies et al., 2011), and 2) by inhibiting GSK3, which targets the mature active form of SREBP for ubiquitin-dependent degradation (Sundqvist et al., 2005). SREBPs have been found to be elevated in diverse cancer lineages, and these remain potential targets of interest for cancer therapy (Rohrig and Schulze, 2016). The effects of the PI3K/AKT pathway on lipid synthesis are summarised in figure 1.4.

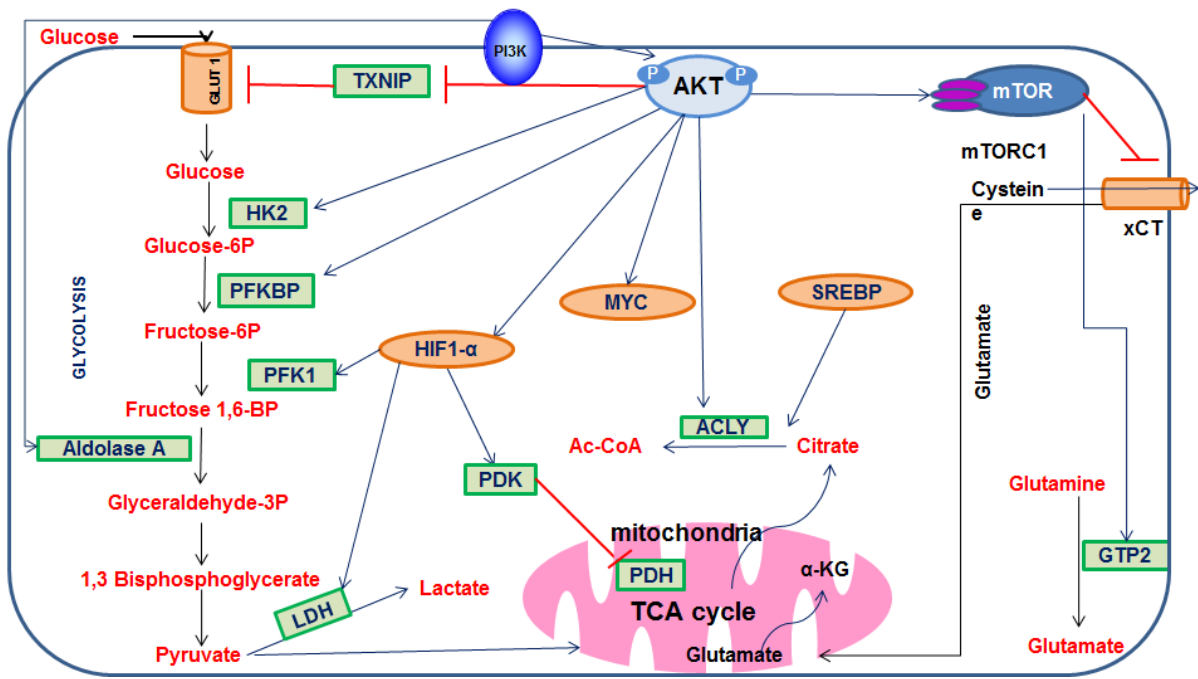


Figure 1. 4: The PI3K/AKT pathway in glycolysis, TCA cycle and lipids biosynthesis.

PI3K/AKT promotes aerobic glycolysis by promoting plasma membrane localization GLUT1 by directly phosphorylating and inhibiting TXNIP. AKT phosphorylates and activates HK2. AKT phosphorylates and activates PFKBP, which produces fructose-2,6-bisphosphate (fructose-2,6BP), an allosteric activator of PFK1. PI3K activates Aldolase A in an AKT-independent manner. Pyruvate, the end product of glycolysis, is either converted to lactate by LDH or can enter the mitochondria and the TCA cycle for oxidation initiated by PDH. AKT phosphorylates PDK1, therefore promoting inhibition of PDH, whilst activating LDH. AKT stimulates the entry of glutamate into the TCA cycle by activating GTP2 and by blocking the cysteine-glutamate antiporter xCT system. AKT regulates lipid synthesis through direct phosphorylation of ACLY, or indirectly, phosphorylating SREBP. ACLY generates acetyl-CoA in the cytosol from the TCA cycle-derived citrate. α-KG = α- Ketoglutarate. Figure modified from (Hoxhaj and Manning, 2020).

1.4.4 PI3K promotes nucleotide synthesis in cancer

The PI3K–AKT–mTORC1 network promotes glucose carbon flux into both the oxidative and non-oxidative branches of the PPP, thus producing ribose for nucleotide synthesis. The PI3K–AKT pathway modulates the oxidative PPP pathway through mTORC1-mediated activation of SREBP and its transcriptional induction of glucose-6-phosphate dehydrogenase (G6PD) expression (Duvel et al., 2010). AKT has also been found to directly activate TKT (Saha et al., 2014), a key enzyme in the non-oxidative PPP. Altered activity of enzymes belonging to both branches of the PPP, including G6PD and TKT, are overexpressed in cancer cells (Cascante et al., 2000; Ju et al., 2017; Kowalik et al., 2017; Patra and Hay, 2014).

At the transcriptional level, AKT controls nucleotide synthesis, in part, through its regulation of MYC, which directly binds and activate genes involved in purine and pyrimidine biosynthesis (Liu et al., 2008). As a downstream effector of PI3K–AKT signalling, mTORC1 has also emerged as a major driver of de novo nucleotide synthesis, which it regulates through both post-translational and transcriptional mechanisms. mTORC1 acutely stimulates pyrimidine synthesis through the S6K-mediated phosphorylation. The mTORC1 signalling also induces de novo purine synthesis through transcriptional mechanisms involving MYC, SREBP and ATF4 (Ben-Sahra et al., 2016). The effects of the PI3K/AKT pathway on nucleotide synthesis are summarised in figure 1.5.

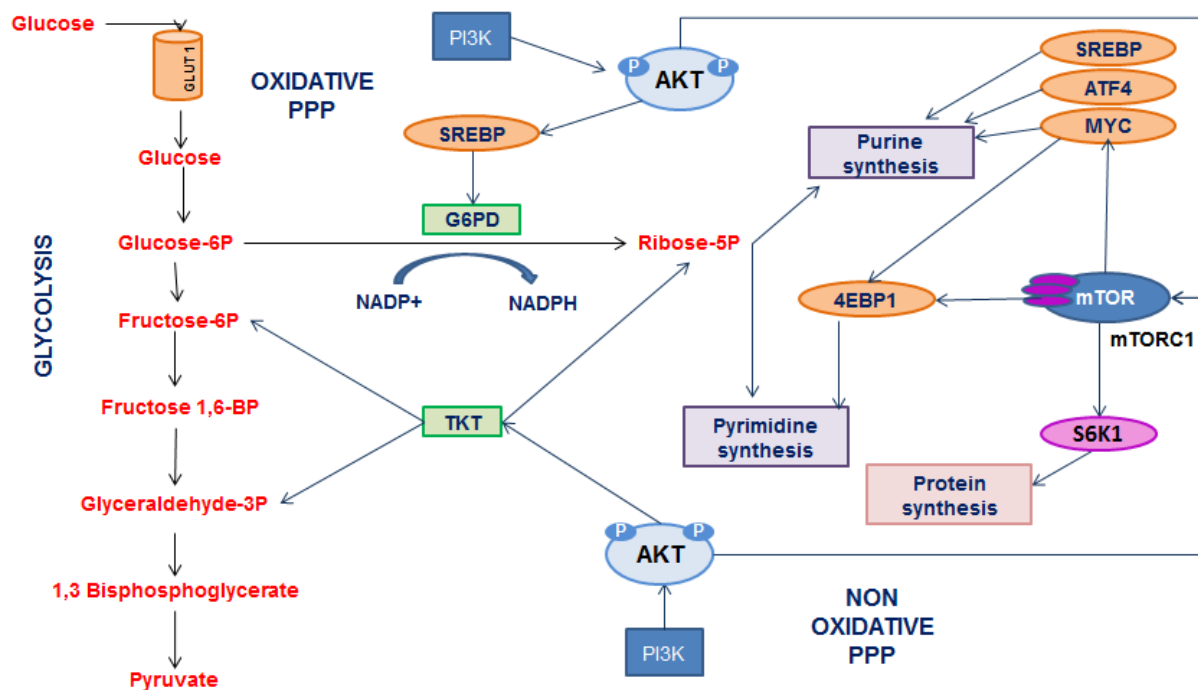


Figure 1. 5: The PI3K/AKT pathway in PPP, nucleotide and protein synthesis.

AKT stimulates oxidative PPP by SREBP mediated activation of G6PD. NADP⁺ additionally activates G6PD. AKT stimulates non-oxidative PPP by activating TKT. AKT induces *de novo* pyrimidine synthesis through activation of mTORC1-S6K1 and by activating the non-oxidative PPP enzyme, TKT. TKT diverts fructose-6-phosphate and glyceraldehyde-3-phosphate from glycolysis to the PPP. AKT promotes purine synthesis through activation of mTORC1 which induces SREBP, ATF4 and MYC. AKT induces protein synthesis through activation of mTORC1-4EBP. Figure modified from (Hoxhaj and Manning, 2020)

1.4.5 PI3K promotes protein synthesis in cancer

The PI3K/AKT pathway induces protein synthesis through the activation of the downstream target mTORC1, whilst MYC transcriptionally induces the expression of multiple components of the protein synthesis machinery (van Riggelen et al., 2010). Pourdehnad and colleagues uncovered a functional link between MYC and mTOR-dependent phosphorylation of 4EBP1, for the regulation of protein synthesis control. They found that mTOR-dependent phosphorylation of 4EBP1 is required for cancer cell survival in MYC-dependent tumour initiation and maintenance (Pourdehnad et al., 2013). The effects of the PI3K/AKT pathway on protein synthesis are summarised in figure 1.5.

1.4.6 Targeting PI3K in cancer

Due to its role in cancer development and progression PI3K has become an attractive therapeutic target in the treatment of cancer. A large number of drugs targeting PI3K have been developed and are currently in clinical trials (Yang et al., 2019). PI3K inhibitors include dual PI3K/mTOR inhibitors, pan-inhibitors and isoform-specific inhibitors. The limited efficacy of these inhibitors is related to the existence of feedback loops and compensatory pathway that, once activated, induce drug resistance. Several mechanisms have been associated with the development of acquired resistance to PI3K inhibitors in cancer. Re-activation of the PI3K pathway is a common mechanism of drug resistance. Mutations and activation of *PIK3CA* and *PIK3CB*, for instance, has been found to induce drug resistance (Huw et al., 2013) (Nakanishi et al., 2016), as well as phosphorylation of the regulatory subunit, p85 (Han et al., 2018). Loss of *PTEN* gene (Juric et al., 2015) and activation of mTOR (Elkabets et al., 2013) signalling have also been proposed to induce resistance to PI3K inhibitors. Activation of alternative pathways, such as the RAS-RAF-MEK-ERK pathway (Ruicci et al., 2018) (Chakrabarty et al., 2012; Serra et al., 2011) and the MYC pathway (Okada et al., 2018) (Dey et al., 2015) are also found in resistant cancers. Additionally, upregulation of IGF1R confers resistance to isoform-specific inhibitors of PI3K in ovarian cancer (Zorea et al., 2018). The microenvironment of the tumours also influences the activity of drugs targeting PI3K. Dual inhibition of PI3K/mTOR, in fact, increases the activity of

JAK-STAT5 with consequent increase of interleukin-8, which has been associated to cancer resistance (Britschgi et al., 2012).

Due to the ability of cancer cells to adapt to the presence of the drug, combination therapies are under investigation for the treatment of cancer patients harbouring the PI3K mutation. Some evidence supports a beneficial role of dietary restriction in combination with doxorubicin and cyclophosphamide for the treatment of tumours (Lee et al., 2012a). In agreement with this, Hopkins and colleagues showed that the use of a ketogenic diet suppresses the insulin feedback resulting from the use of PI3K inhibitors (Hopkins et al., 2018). Therefore, the use of PI3K inhibitors combined with diet manipulation may be useful in treating cancer with reduced metabolic side effects.

1.5 Systemic metabolism and cancer.

Tumours are initiated by gene mutations. However, the context within which the cells reside determines whether these tumours progress to malignant cancers. The availability of metabolic fuels and the metabolic signals in the microenvironment is critically important for the progression of cancer. The tumour microenvironment comprises a multitude of cell types (cancer-associated fibroblasts, macrophages, dendritic cells, lymphocytes) in various metabolic states. The uptake and export of metabolites from and by the cells in the tumour microenvironment determine the availability of each metabolite for the tumour (Coleman et al., 2020). For instance, while it is well known that proliferating tumour cells upregulate glucose consumption, activated cytotoxic T cells also rely on aerobic glycolysis and enhanced glucose uptake. Therefore T cells compete with the tumour for the availability of glucose (Palmer et al., 2015) (Pearce, 2010). High concentrations of lactate, an end product of glycolysis, are critical regulators of the tumour microenvironment. Tumour growth is also closely related to cancer-associated fibroblasts present within its microenvironment. Tumour cells, indeed, switch their metabolic state between glycolysis and oxidative phosphorylation through metabolic interplay with cancer-associated fibroblasts (Wanandi et al., 2018).

Obesity and type II diabetes mellitus (T2DM) also impact cancers progression by altering the context in which the cancer cells are developing (Holly et al., 2019). This is realised by

altering the availability of nutrients and by altering intracellular and inter-tissues signals. Obesity and TDM2 are, therefore, a great example of how the systemic metabolism influences cancer development and progression. In obesity and diabetes, the availability of metabolites, especially carbohydrates, fatty acids and lipids, are altered (Al-Goblan et al., 2014); the activity of many metabolic regulators is also perturbed, including insulin and IGFs. Furthermore, diabetes and obesity are associated with a chronic low inflammatory state and the levels of many inflammatory cytokines are also affected (Ellulu et al., 2017). Additionally, by increasing the availability of lipids and cholesterol, obesity and TDM2 offer substrates for the production of steroids that may be important for driving some cancers. Obesity can also directly damage some tissues. For instance, it can damage the oesophagus due to chronic acid reflux (Long and Beales, 2014), drive development of gallstones in the gallbladder (Stinton and Shaffer, 2012) and induce liver damage due to fatty acid infiltration (Fabbrini et al., 2010).

Additionally, in obesity, the excess of macronutrients in the adipose tissues stimulates them to release inflammatory mediators such as tumour necrosis factor α and interleukin 6 (Ellulu et al., 2017). Increased levels of interleukin 6 stimulate the liver to synthesize and secrete C-reactive protein, which is characteristic of systemic inflammation. The consequent reduction of adiponectin, leads to altered insulin sensitivity, metabolic abnormalities, and increases the risk of cancer in the tissue (Ellulu et al., 2017).

Altered levels of insulin can also affect sex steroid production contributing to an increased risk of cancers that are affected by sex hormone levels, particularly cancers of the breast, endometrium and ovary (Joung et al., 2015).

1.6 The insulin–PI3K signalling axis

Insulin is an anabolic peptide hormone secreted by the β -cells of the pancreas, which acts through a RTK located in the membrane of target cells. Insulin is synthesised as a single-chain precursor, preproinsulin (Steiner et al., 1967), which is processed via cutting of the signal fragment to form the intermediate proinsulin. Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle,

known as the C peptide. Within the endoplasmic reticulum, proinsulin is exposed to several specific endopeptidases which excise the C peptide. The final mature form of insulin is, therefore, composed of two chains, chain A made of 21 amino acids, and chain B made of 30 amino acids, connected by two disulphide bonds (Dodson and Steiner, 1998). Insulin and free C peptide then accumulate in the cytoplasm inside secretory granules, ready to be released when the β -cell is stimulated.

Once released, insulin binds insulin receptors (IRs) on the cell surface of metabolically active tissues such as muscle, liver, and fat. IRs are heterotetrameric glycoproteins containing two extracellular (α) and two intracellular (β) subunits. The binding of insulin to the extracellular subunits leads to IR dimerization and activation (Perlman et al., 1989). In 1985, Ullrich and Ebina (Ebina et al., 1985; Ullrich et al., 1985) established that, not only the ligand, but also the IR itself is a tyrosine kinase, which catalyses the transfer of one phosphate of ATP to tyrosine residues on protein substrates. The binding of the ligand, activates the protein-tyrosine kinase domain of the IR and allows its auto-phosphorylation. Unlike other RTKs, IRs do not bind signalling proteins directly, but instead bind to the insulin receptor substrate (IRS), as well as the adaptor SHC (SRC homology 2 domain containing) (Boucher et al., 2014), finally resulting in the activation of the intracellular signalling cascade.

One of the primary steps in the insulin signalling pathway is the activation of the class IA PI3K (Shepherd et al., 1998), achieved through binding of SH2-domains of adaptor subunits to phosphorylated YMXM motifs in IRS-proteins (Myers and White, 1993). On the contrary, there is currently no evidence that class IB PI3Ks are regulated by insulin.

1.6.1 The role of insulin in metabolism

One of the principal functions of insulin is the regulation of glucose homeostasis. Glucose homeostasis is defined as the balance between the rate of glucose consumed with a meal, the rate of glucose absorbed by the intestine during the fed state, the amount of glucose produced by the breakdown of glycogen (glycogenolysis) and the rate of glucose formed from glucose precursors, mostly lactate, pyruvate and amino acids, through gluconeogenesis. Once inside the cells, glucose undergoes glycolysis, thereby generating ATP, resulting in an increased

ATP/ADP ratio. The increased ATP/ADP ratio leads to the closure of ATP-sensitive K^+ -channels (K_{ATP} -channels), which drives depolarization of the membrane. This depolarization allows the opening of voltage-dependent calcium-channels with a consequent increase in intracellular calcium concentrations. The elevated concentration of calcium triggers the fusion of insulin-containing granules with the cellular membrane and the subsequent release of their content (Roder et al., 2016).

Glycogenolysis and gluconeogenesis are partly under the control of glucagon, a hormone produced in the α -cells of the pancreas. In short fasting conditions glycogenolysis is the primary mechanism by which glucose is made available, whilst over longer periods of fasting, the glucose is produced by gluconeogenesis in the liver and released into the blood stream. In conditions of high level of blood glucose, insulin helps to clear up the glucose from the blood in three ways (Hughes et al., 2018). Firstly, insulin stimulates insulin-sensitive peripheral tissues, primarily skeletal muscle, hepatocytes and adipocytes to increase their glucose uptake, by stimulating the translocation of the insulin-dependent glucose carrier, glucose transporter type 4 (GLUT4). Secondly, insulin acts on the liver to promote glycogenesis. Finally, insulin inhibits glucagon secretion from pancreatic α -cells, thus signalling the liver to stop producing glucose via glycogenolysis and gluconeogenesis. The action of insulin on glucose homeostasis is mediated by AKT, which phosphorylates AS160 substrate to stimulate the translocation of GLUT4 to the cell membrane surface. AKT also stimulates the expression of hexokinases and regulates the expression of glycolytic enzymes, as above described (Section 1.2.1).

Glucose is not the only agent responsible for the control of insulin release. Long-chain free fatty acids (FFAs) and short-chain FFAs have also been found to modulate insulin release (Roder et al., 2016). Whilst long-chain FFAs triggers insulin secretion, short-chain FFAs inhibit glucose-stimulated insulin secretion, due to decreased glucose oxidation and the subsequent decreased ATP/ADP ratio (Roder et al., 2016). Despite its well-known role in glucose metabolism, the insulin-AKT axis is also known to regulate the expression of a variety of genes responsible for gluconeogenesis and lipogenesis. For instance, the expression of genes that induce gluconeogenesis, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6PC) are inhibited by AKT (Zhang et al., 2011). Furthermore,

insulin increases the expression levels of SREBPs, which, in turn, increases cholesterol and fatty acid accumulation (Huang et al., 2018).

1.6.2 Insulin-PI3K signalling maintains the balance between metabolism and diseases.

As described above, the PI3K/AKT signalling pathway is required for normal metabolism. AKT phosphorylates and inhibits AS160 allowing the migration of intracellular vesicles containing GLUT4 to the plasma membrane. Increased expression of GLUT4 leads to enhanced glucose uptake into the tissues. This process restores the physiological levels of glucose within minutes of serum insulin elevation. An imbalance of the PI3K/AKT signalling leads to insulin resistance, followed by obesity and T2DM (Fruman et al., 2017). However, the maintenance of the insulin/PI3K/AKT pathway is a complicated mechanism that involves the integration of signals from various tissues, including pancreas, liver, skeletal muscle, adipose tissue and brain (Huang et al., 2018).

1.6.3 The insulin-PI3K pathway in the pancreas.

The pancreas is an organ located in the abdomen, which has two main functions: an exocrine function that helps in digestion and an endocrine function that regulates blood sugar. The endocrine component of the pancreas consists of islet cells (within the islets of Langerhans) that create and release hormones directly into the bloodstream. The two main hormones produced in the pancreatic islets are glucagon, secreted by the alpha cells (α -cells) and insulin secreted by the beta cells (β -cells) (Da Silva Xavier, 2018). After a meal, glucose is transported inside the pancreas via GLUT2, where it is metabolized via glycolysis and the TCA cycle, leading to an increase in cellular ATP/ADP ratio. The increased ATP/ADP ratio leads to the closure of ATP-sensitive K^+ -calcium (K_{ATP} -channels), which drives depolarization of the membrane. This depolarization allows the opening of voltage-dependent calcium-channels with a consequent increase in intracellular calcium concentrations. The elevated concentration of calcium triggers the fusion of insulin-containing granules with the cellular membrane and the subsequent release of their content (Roder et al., 2016).

Kahn and colleagues defined a directly proportional relationship between insulin sensitivity and the activity of β -cells, showing that insulin resistant humans have higher activity of β -cells, necessary to release additional insulin and maintain normal glucose tolerance. In these patients, the high expression of β -cells results in the development of hyperinsulinemia (Kahn et al., 1993). A dysfunction of the β -cells in insulin resistance leads to impaired glucose tolerance and development of T2DM (Oliveira et al., 2014). Constitutive activation of AKT1, as well as mutations in its downstream targets, FOXO1, GSK3 and mTOR have been shown to regulate the proliferation of β -cells (Jiang, 2018; Tuttle et al., 2001). According with this, in obesity and T2DM, the insulin-mediated PI3K/AKT pathway is blocked, which reduces insulin secretion and β -cell function (Tuttle et al., 2001). Furthermore, the excess levels of circulating FFAs in obese subjects have been shown to impair the function of β -cells (Huang et al., 2018). Moreover, calcium homeostasis, growth factors and hormones have a modulatory effect on the β -cells function and proliferation (Jiang, 2018). The insulin-PI3K pathway in the pancreas is shown in figure 1.6 A.

1.6.4 The insulin-PI3K pathway in the skeletal muscle

Skeletal muscle is the major site of insulin-dependent glucose uptake (Kraegen et al., 1985; Thiebaud et al., 1982; Zhang and Liu, 2014). In the muscle AKT increases glucose uptake through GLUT4 and stimulates glycogen synthesis by inducing the glycogen synthase to generate glycogen from glucose-6-phosphate. When the insulin response is no longer efficient, the activity of glucose transporters and key intracellular enzymes, involved in glucose metabolism, become depressed. Defects in glucose transport in skeletal muscle result in impaired whole body glucose uptake in T2DM patients (Zierath et al., 1996). Additionally, AKT stimulates protein synthesis and mRNA translation by regulating mTORC1 and its downstream effectors such as p70-S6K1, eIF4E and 4E-BP1 (Huang et al., 2018). AKT can also control lipid utilization and storage, as well as stimulating FFA acid uptake in skeletal muscle and increasing the activity of key enzymes involved in muscle fat oxidation (Zhang and Liu, 2014). The observation that diabetic patients have elevated concentrations of fasting plasma FFA (Oliveira et al., 2014) led to the discovery that muscle lipid accumulation plays a central role in the initiation of muscle insulin resistance (Fruman et al., 2017). For instance,

ceramide leads to reduction of glucose utilization in the muscle (Huang et al., 2018). The insulin-PI3K pathway in the skeletal muscle is shown in figure 1.6 B.

1.6.5 The insulin-PI3K pathway in the adipose tissue

Adipose tissue plays important roles in the maintenance of energy homeostasis, including insulation and protection of tissues and organs from heat and cold, thermogenesis, adipogenesis, and the production of adipokines (Zhang and Liu, 2014). Adipose tissue accounts for approximately 10% of insulin-dependent glucose uptake (Huang et al., 2018). Insulin-AKT signalling regulates the metabolism of adipose tissues by promoting glucose uptake, through the glucose transporter GLUT4 and glucose utilization, protein synthesis and fatty acid uptake from the circulating plasma (Huang et al., 2018; Zhang and Liu, 2014). Furthermore, the PI3K/AKT signalling pathway promotes *de novo* lipid biosynthesis and inhibits lipolysis in the adipose tissue by regulating the activity of SREBP and FOXO1. In the fasting state, lipolysis is induced by β -adrenergic signalling, which induces cAMP accumulation and subsequent PKA-mediated phosphorylation of hormone sensitive lipase (HSL) and perilipin (Duncan et al., 2007). In the fed state, PI3K/AKT inhibits PKA and thus suppresses lipolysis (Choi et al., 2010). Interestingly, abnormal glucose metabolism caused by insulin resistance in adipose tissues impacts other tissues. Specific reduction of GLUT4 in adipose tissues, for instance, results in insulin resistance in skeletal muscle and liver (Abel et al., 2001). On the other hand, an excessive release of FFAs, due to decreased lipogenesis and increased lipolysis, leads to ectopic non-physiologic accumulation of lipids in other parts of the body (Bays et al., 2004) The insulin-PI3K pathway in the adipose tissue is shown in figure 1.6 C.

1.6.6 The insulin-PI3K pathway in the liver

The liver acutely responds to insulin by reducing glucose levels. Whilst in the fasting state, glucose is primarily generated in the liver by gluconeogenesis and glycogenolysis, in the fed state, hepatic glucose production (HGP) and glycogenolysis are suppressed in response to PI3K/AKT signalling. AKT suppresses enzymes responsible for the induction of

gluconeogenesis, PEPCCK and G6PC (Zhang and Liu, 2014), inhibits lipolysis and induces FA synthesis by activating FA synthetic enzymes expression through SREBP1 (Lewis et al., 1997). AKT suppresses the expression of the insulin induced gene 2 (INSIG2), a liver-specific transcript encoding the SREBP-1c inhibitor, through an mTORC1-independent pathway (Yecies et al., 2011). In obesity, the decreased FFA uptake and glucose utilization in the adipose tissue results in ectopic accumulation in other tissues, including the liver (Duncan et al., 2007). Accumulation of lipids in the liver is highly correlated with insulin resistance and non-alcoholic fatty liver disease (Utzschneider and Kahn, 2006). Meanwhile, excessive oxidation of FFAs increases the hepatic acetyl-CoA content and thus activates pyruvate carboxylase, which is required for gluconeogenesis (Huang et al., 2018). The insulin-PI3K pathway in the liver is shown in figure 1.6 D.

1.6.7 The insulin-PI3K pathway in the brain

The brain has only recently been reported to be an insulin-sensitive organ, and its function in glucose metabolism and energy regulation has been characterized (Roh et al., 2016). Insulin enters the central nervous system (CNS) through the blood–brain barrier (BBB) by receptor-mediated transport (Plum et al., 2005). Once inside the brain insulin regulates food intake, sympathetic activity and inhibits hepatic gluconeogenesis (Obici et al., 2002) and neuronal apoptosis (Plum et al., 2005). The CNS, mainly the hypothalamus, has a pivotal role in integrating signals from peripheral tissues, such as the pancreas, white adipose tissue and the gut, to regulate energy homeostasis (Varela and Horvath, 2012).

Scherer and colleagues showed that mice lacking the neuronal IR exhibit increased lipolysis and decreased *de novo* lipogenesis in white adipose tissue (WAT) (Scherer et al., 2011), highlighting the role of neuronal insulin in lipids metabolism. Furthermore, experiments performed in dogs and baboons showed that intracerebroventricular injection of insulin increases pancreatic insulin secretion (Chen et al., 1975) and decreases food intake and body weight (Woods et al., 1979).

The basal part of the hypothalamus, the arcuate nucleus (ARC) contains a special modification in the BBB which allows for the entry of nutrients, hormones and other

molecules from the blood. The ARC contains important populations of neurons, Pro-opiomelanocortin / Cocaine- and Amphetamine-Regulated Transcript (POMC/CART) and Agouti-Related Protein / Neuropeptide Y (AgRP/NPY), which responds to signals from hormones and nutrients. POMC and CART neurons secrete neuropeptides that decrease food intake and body weight, whilst AgRP and NPY neurons increase food intake. In fasting conditions, the expression of AgRP/NPY is increased and POMC/CART expression is decreased, whilst during a state of energy surplus, AgRP/NPY levels are diminished and POMC/CART levels are elevated. Both neuropeptides are important to maintain normal lipid and glucose homeostasis in peripheral tissues, such as the liver, muscles and the pancreas (Varela and Horvath, 2012).

Leptin and insulin mediate the effects of these neuropeptides on glucose and energy metabolism in the POMC and AgRP neurons (Varela and Horvath, 2012). The actions of leptin and insulin are mediated by the leptin receptor-b and IR receptors, respectively, expressed in the hypothalamus. Leptin and insulin induce two different pathways: the Janus Kinase 2- Signal transducer and activator of transcription 3 and PI3K–AKT pathways, respectively, in hypothalamic neurons (Thon et al., 2016). However, the PI3K signalling pathway acts to integrate leptin and insulin signals (Belgardt et al., 2008; Cota et al., 2006; Hill et al., 2008; Kitamura et al., 2006; Morrison et al., 2005). The action of both leptin and insulin in the hypothalamus induce expression of POMC/CART genes and inhibit expression of the NPY and AgRP genes (Varela and Horvath, 2012). Insulin resistance in the brain is followed by disorders of glucose metabolism in other insulin-sensitive organs, eventually leading to systemic insulin resistance (Huang et al., 2018). The insulin-PI3K pathway in the brain is shown in figure 1.6 E.

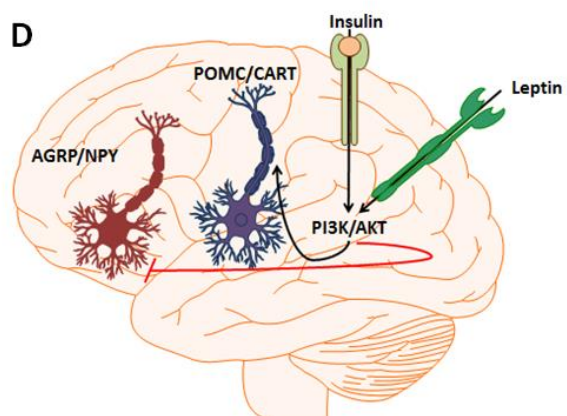
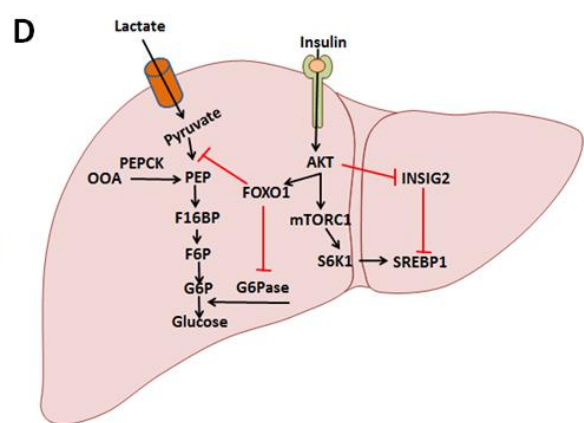
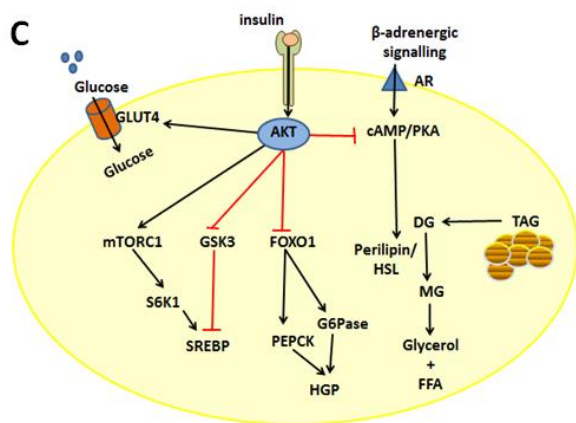
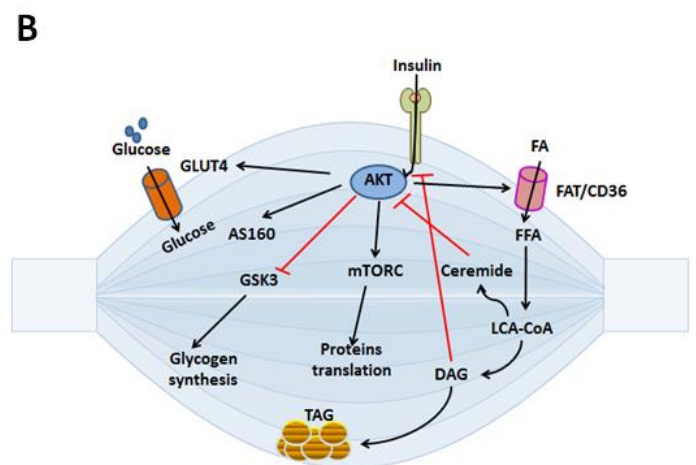
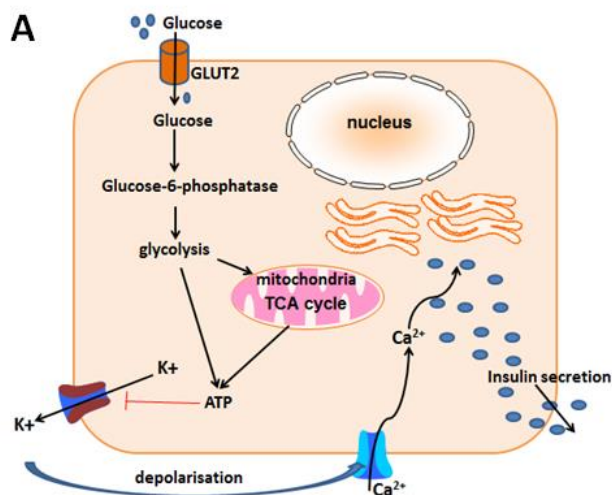


Figure 1. 6: Regulation of the insulin/PI3K/AKT pathway in various organs.

A) Schematic representation of the insulin pathway in the pancreas: glucose is transported inside the pancreas via GLUT2, where it is metabolized via glycolysis and the TCA cycle, leading to an increase in cellular ATP/ADP ratio. The increased ATP/ADP ratio leads to the closure of K⁺-ATP-channels, depolarization of the membrane, opening of voltage-dependent Ca⁺-channels, increase in intracellular calcium concentrations and consequent release of insulin. B) Schematic representation of the insulin pathway in the skeletal muscle: AKT increases glucose uptake through GLUT4, stimulates protein synthesis and mRNA translation by regulating mTORC1 and its downstream effector S6K1, blocks glycogen synthesis by inhibiting GSK3 and increases FA uptake and storage. Increased ceramide inhibits AKT. C) Schematic representation of the insulin pathway in the adipose tissue: AKT promotes glucose uptake, through GLUT4 and glucose utilization, promotes *de novo* lipid biosynthesis by activating m-TORC-SREBP and by inhibiting FOXO1 mediated hepatic glucose production (HGP), inhibits lipolysis by inhibiting PKA-mediated phosphorylation, HSL and perilipin. D) Schematic representation of the insulin pathway in the liver: AKT suppresses PEPCK and G6PC and induces fatty acid synthesis by activating SREBP1 and suppressing the expression of INSIG2, through an mTORC1-independent pathway E) Schematic representation of the insulin pathway in the brain: leptin and insulin converge on the PI3K pathway to induce expression of *POMC/CART* genes and inhibit expression of the *NPY/AgRP* genes. Red arrows indicate inhibition, whilst black arrows indicate activation.

1.7 Insulin- PI3K signalling maintains the balance between metabolism and cancer

Considerable evidence supports a synergistic relationship between obesity-related insulin resistance, T2DM and cancer. Insulin and IGF1 mediate their intracellular effects via the activation of the IR and the IGF1 receptor (IGF1R), respectively, and both activate the downstream PIK-AKT-mTOR pathway (Mirdamadi et al., 2017). Several studies have shown an association between the presence of T2DM and cancer incidence, including colorectal, hepatic, pancreatic, breast, endometria, and urinary tract cancers (Orgel and Mittelman, 2013). Moreover, metabolic syndromes, which are characterised by elevated insulin levels due to insulin resistance have been associated with the increased incidence of mortality from site-specific cancers (Orgel and Mittelman, 2013).

Tumour initiation and progression are dependent upon the ability of tumour cells to sustain malignant growth through the acquisition of sufficient nutrients for energy and biomass production. One of the key abilities of the tumour is the shift from oxidative phosphorylation to aerobic glycolysis (Vander Heiden et al., 2009), with consequent increase of glucose uptake. The increase in glucose flux is possible due to genetic alterations that evolve within tumours, such as mutations in the *PIK3CA* gene. Thus, insulin signalling can be seen as enabling tumour development by providing a mechanism for PI3K activation to drive enhanced glucose uptake (Hopkins et al., 2020). Mutations in the *PIK3CA* gene, which encodes for the p110 α isoform of PI3K, are associated with human diseases, including overgrowth syndromes such as CLOVES (Congenital Lipomatous asymmetric Overgrowth of the trunk with lymphatic, capillary, venous, and combined-type Vascular malformations, Epidermal naevi, Skeletal and spinal anomalies) (Martinez-Lopez et al., 2017) and human cancers (Hopkins et al., 2020). Conversely, a reduction of PI3K activity is found in patients with SHORT syndrome (Dymant et al., 2013), a rare condition characterized by delayed growth and features resembling type I diabetes mellitus (T1DM) (Hopkins et al., 2020).

Similarly mice harbouring mutations in the *Pik3ca* gene have been shown to have severe defect in glucose metabolism, associated to hypoinsulinemia, hypoglycaemia and organomegaly (Kinross et al., 2015). Studies on the involvement of insulin signalling in cancer pathogenesis have led to the observation that proteins involved in the insulin signalling are poor prognostic markers of cancer (Kim et al., 2012). For instance, insulin

receptor expression is an independent predictor of decreased overall survival in non-small cell lung cancer (Kim et al., 2012). Interestingly, long-term low-protein, low-calorie diet and endurance exercise, which lower insulin levels, modulate metabolic factors associated with cancer risk (Fontana et al., 2006). Increased circulating IGF1 levels have also been associated with an increased risk for prostate (Wolk et al., 1998) and colorectal (Muc-Wierzgon et al., 2014) cancer development. In agreement with this, an epidemiological study evaluating pancreatic cancer risk factors has revealed that T2DM is the third major risk factor for this disease (Li, 2012).

Data from population-based studies have consistently suggested a strong link between antidiabetic treatment and a decrease in cancer incidence and mortality (Bowker et al., 2006; Currie et al., 2009). However, patients with T2DM treated with insulin have been shown to increase cancer-related mortality (Bowker et al., 2006). In contrast, epidemiological studies have suggested a protective role for metformin in cancer development. Studies on patients with T2DM on metformin have demonstrated a lower risk of cancer (Currie et al., 2009; Evans et al., 2005). Newer therapies targeting these systems are being studied and show great promise as cancer treatments (Cohen and LeRoith, 2012). However, Hopkins et al. have shown that the use of PI3K inhibitors for the treatment of several model tumours in mice is limited by the fact that the presence of inhibitors initiates a systemic glucose–insulin feedback, which is sufficient to re-activate PI3K signalling (Hopkins et al., 2018). This suggests that in order to increase the treatment efficacy of cancers with mutations in the PI3K pathway, it is fundamental to account for the systemic effect of PI3K on systemic metabolism.

1.8 Mouse models of insulin resistance

Over the past years rodents have been used to study insulin resistance and diabetes using several approaches, including genetic, pharmacological, surgical, and dietary inductions (Islam and Loots du, 2009; Wong et al., 2016). The first insulin signalling knockout mouse studies were published about 25 years ago (Araki et al., 1994; Tamemoto et al., 1994). Since then many mouse models have been characterised, but have been unsuccessful for the discovery of new drugs targeting insulin resistance in T2DM. Most of the mouse models with

insulin pathway induction or deregulation are used for the study of insulin resistance-related diabetes. Tissue-specific knockouts of IRS have been proven to be particularly useful for this purpose. Mouse models with disrupted *Irs-1* gene were resistant to the glucose-lowering effects of insulin and had impaired glucose tolerance and decreased glucose uptake (Tamemoto et al., 1994). Moreover, *Irs-2*-deficient mice showed insulin resistance in the liver and skeletal muscle resulting in deterioration of glucose homeostasis (Withers et al., 1998).

However, in 2014, Bunner and colleagues described a list of limitations of the mouse models used to study insulin resistance, including physiological, genetic, and metabolic differences between mice and humans, such as the different glucose disposal and glycogen storage patterns (Bunner et al., 2014). The same work also pointed out the technical challenges of the experiments performed to investigate mouse metabolism (Bunner et al., 2014). Insulin sensitivity, for instance, is often measured using a hyperinsulinemic-euglycemic clamp test, which involves either implanted arterial catheters or repeated blood sampling. The results of this test are dependent on a number of experimental factors which are not standardized between laboratories, including fasting time and use of anaesthesia (Bunner et al., 2014). Furthermore, the specific inbred strain, genetic background, housing conditions, and diet were described as important factors in determining the outcome of the experiments (Bunner et al., 2014). Kulkarni and colleagues generated transgenic mice that are double heterozygous for knockout of the *Ir* and *Irs-1*, on three genetic backgrounds (C57BL/6 [B6], 129Sv, and DBA) further highlighting the influence of genetic background on the outcome of the experiments (Kulkarni et al., 2003). Indeed, BL6 double heterozygous mice exhibited more marked hyperinsulinemia, a higher degree of islet hyperplasia and faster development of hyperglycaemia than the double heterozygous mice. On the other hand, DBA mice had intermediate insulin levels, and evidence of islet degeneration, with 64% developing diabetes. Activation of the p110 α subunit of PI3K has also been associated to insulin resistance. Mice carrying a knockin mutation (D933A), that abrogates p110 α kinase activity, had reduced somatic growth, hyperinsulinemia, glucose intolerance, hyperphagia and increased adiposity associated to altered IRS proteins signal (Foukas et al., 2006)). Conversely, long term p110 α inactivation protects from insulin resistance, glucose tolerance and increased fat accumulation, in mice, by suppressing the down-regulation of insulin

receptor substrate (IRS) protein levels and enhancing insulin-induced Akt signalling (Foukas et al., 2013).

1.9 The insulin pathway in *in vivo* cancer mouse models

Hyperinsulinemia, increased expression of the insulin receptor and the IGF-1R are important factors linking diabetes, obesity, and cancer. A number of mouse models have been developed in order to understand the role of insulin, IGF-1, and their receptor signalling in tumour development. Transgenic mice expressing human IGF-1 in basal prostate epithelial cells have been shown to have increased expression of IGF-IR and development of early prostate cancer (DiGiovanni et al., 2000). Transgenic mice carrying transgenic expression of a fusion receptor that is constitutively activated by homodimerization developed salivary and mammary adenocarcinomas at 8 weeks of age (Carboni et al., 2005). On the contrary, the expression of dominant-negative mutations of *Igf-ir* or dysfunctional *IGF-IR* inhibited the growth of lung cancer cell lines (Lee et al., 2003) and decreased proliferation in mammary glands in mice (Gallagher and LeRoith, 2010), respectively. Interestingly, the expression of a dominant-negative, kinase-dead human IGF-IR exclusively in skeletal muscle led to accelerated mammary gland development and enhanced phosphorylation of IR/IGF-IR and AKT in mammary tissue (Novosyadlyy et al., 2010).

1.10 The *Pten/ Pi3k/ Akt* pathway in *in vivo* cancer mouse models

Most mutations in the PI3K pathway occur in the *PIK3CA* and *PTEN* genes. A number of mouse models have been developed in order to understand the role of these two genes in tumour development.

1.10.1 *Pi3k* mouse models

Activating mutations in *PIK3CA* have been reported in many cancer types including breast, colon, endometrial, glioblastoma, ovarian and hepatocellular cancers (Mitchell and Phillips, 2019). The mutations *E542K* and *E545K* in the helical domain, and *H1047R* in the catalytic domain, are the three most frequent activating mutations (Yuan and Cantley, 2008). Whilst mutations in the kinase domain result in enhanced enzymatic activity, mutations in the helical domain de-repress the inhibitory effect of p85 on the p110 α catalytic subunit, thus enhancing the catalytic activity of PI3K (Zhao and Vogt, 2008). Transgenic mice, carrying a constitutively active form of *Pik3ca* induced by myristoylation of p110 α (MYR-p110 α), developed alveolar hyperplasia and intraductal neoplasia with a small percentage of mammary tumours. The combination of MYR-p110 α with a cyclin-dependent kinases (*Cdk4*) activating mutation (*R24C*) led to increased tumorigenesis, emphasizing the interaction of the CDK4/Rb/E2F cascade and the PI3K signalling in human cancers (Renner et al., 2008). In a subsequent study the myristoylated form of p110 α was inserted into the Rosa26 locus and conditionally expressed upon the activation of Cre-recombinase (Sheen et al., 2016). Although the expression of the MYR-p110 α in the mammary gland had minimal transforming activity by itself, it accelerated mammary tumour initiation when induced in an oncogenic mouse model harbouring homozygous *p53* deletion and a *Kras*^{G12D} missense mutation (Sheen et al., 2016).

In order to characterise the effects of specific *Pik3ca* mutations, conditional *_Pik3ca*^{H1047R} and *Pik3ca*^{E545K} knock-in mice were generated by cloning mutant *Pik3ca* cDNAs into the Rosa26 locus (Mitchell and Phillips, 2019). When the expression of these transgenic alleles was induced by Cre recombinase driven by the mammary restricted promotor MMTV or by the WAPi promoter (Whey Acidic Protein; active in mammary alveolar progenitor cells and differentiated secretory luminal cells) the mice developed mammary tumours (Mitchell and Phillips, 2019). However, the mice models mentioned above failed to express the *Pik3ca* mutations at endogenous levels. The advantage of knock-in mouse models is to allow for specific mutations to be introduced into endogenous genes and maintained through the germline so that the expression of the mutant is under control of the endogenous promoter (Mitchell and Phillips, 2019).

The most widely used mouse model with specific mutations introduced into the endogenous *Pik3ca* gene is the model using a novel ‘exon-swap’ strategy generated by Kinross *et al.* (Kinross et al., 2012). This model takes advantage of the fact that the H1047R mutation occurs in the final exon of the *Pik3ca* gene (exon 20). LoxP sites flanking the wild type exon 20 were inserted into one allele of *Pik3ca* and a tandem copy of exon 20 containing the H1047R mutation was placed downstream of the wild type exon. The action of Cre on LoxP sites results in the deletion of the wild type exon 20 and its replacement with the exon 20 harbouring the H1047R mutation. As a result the *Pik3ca*^{H1047R} mutation is expressed at physiological levels and only in cells that would normally express *Pik3ca*. Therefore, the mouse model accurately reproduces the scenario of a heterozygous somatic mutation in the endogenous gene, as occurs in human tumours (Kinross et al., 2012). By using this mouse model, Kinross *et al.* first showed that the *Pik3ca*^{H1047R} mutation alone is not responsible for tumour formation (Kinross et al., 2012). This was then validated by subsequent studies obtained by using a wide range of Cre recombinase transgenes in combination with this model to target *Pik3ca*^{H1047R} expression in a tissue-specific fashion (Mitchell and Phillips, 2019).

In most of these studies, *Pik3ca*^{H1047R} alone was not able to induce tumours when expressed at endogenous levels, as opposed to transgenic mouse models driven by exogenous promoters, where *Pik3ca* mutation alone was able to induce lung (Engelman et al., 2008), mammary (Liu et al., 2011) (Meyer et al., 2011) and colon tumours (Leystra et al., 2012). Other knock-in models, generated using a similar ‘exon-swap’ approach to Kinross *et al.*, further confirmed that *Pik3ca*^{H1047R} requires an additional ‘second hit’ mutation in another gene in order to initiate tumorigenesis (Stratikopoulos et al., 2015; Yuan et al., 2013).

1.10.2 *Pten* Models

One of the most common genetic alterations in the PI3K pathway is inactivation of the *PTEN* gene. The expression of *PTEN* can be altered through several mechanisms: loss of heterozygosity (LOH), point mutations, homozygous deletion or promoter methylation, which have been found in many tumours, especially metastatic human cancers (Inoue et al., 2013). Germline mutations in *PTEN* have been identified in familial cancer predisposition

syndromes, such as Cowden and Bannayan–Riley–Ruvalcaba syndromes (Yehia et al., 2018). Mutations in the *PTEN* gene that results in a loss of its function are commonly found in glioblastoma, melanoma, prostate, breast, ovarian, endometrial, thyroid, lymphoid, and colorectal cancer, and in hepatocellular and renal-cell carcinoma (Dillon and Miller, 2014). The use of *Pten* knock-out mice models has shown that whilst the homozygous loss of *Pten* is embryonic lethal (Suzuki et al., 1998), heterozygous *Pten*^{+/-} mice develop tumours in multiple tissues, similarly to the cancer predisposition pattern in human Cowden syndrome (Carnero and Paramio, 2014).

Mouse models with mutations in the *Pten* gene have shown that a loss of *Pten* expression is essential for initiating prostate cancer (Di Cristofano et al., 1998) (Chen et al., 2005). Loss of *Pten* also accelerates tumorigenesis in prostate cancer harbouring mutations in other genes, such as *p27* and *p53* (Chen et al., 2005) (Di Cristofano et al., 2001). In advanced prostate cancer, the Transforming Growth Factor Beta 1 / SMAD Family Member 4 (TGF-β-SMAD4) signalling pathway is activated upon the loss of *Pten* expression (Ding et al., 2011). In agreement with this, prostate-specific *Pten* and *Smad4* double knock-out results in the development of metastatic prostate cancer (Ding et al., 2011). An increase in the onset of prostate cancer is also observed when *Pten* expression is lost in combination with another oncogenic change in genes such as *Her2*, *Erg*, *K-Ras*, *Sox9*, and *Bmi* (Carnero and Paramio, 2014).

Tissue-specific *Pten*-deletion models, generated using LoxP/CRE technology, have been shown to induce development of specific tumours. For instance, transgenic mice with conditional *Pten* knock-out in the epidermis, developed carcinomas associated with an increase in AKT and changes in ERK activity (Denggao Yao 2006). Likewise, conditional *Pten* knock-out in the mammary glands forms basal-like mammary tumours (Saal et al., 2008).

Mice carrying additional genomic copies of *Pten* (*Pten(tg)*) are protected from cancer and present a significant extension of life span (Ortega-Molina and Serrano, 2013). Interestingly, these mice showed increased energy expenditure and favoured oxidative phosphorylation upon glycolysis, thus preventing the metabolic reprogramming characteristic of cancer cells. Furthermore, *Pten (tg)* mice had elevated uncoupling Protein 1 (*Ucp1*) expression in brown

adipocytes with a consequent decreased adiposity. This mouse model could be relevant for future studies on cancer, obesity, diabetes and aging.

1.10.3 *Akt* Model

Activating mutations in *AKT* have been reported to occur in cancers at a low frequency. Somatic mutations in *AKT1* were discovered in breast, colorectal, ovarian, lung cancers and leukaemia (Carnero and Paramio, 2014). The most common mutation (*E17K*) alters the lipid binding site of *AKT1*, causing constant membrane localization, independently of the signal from PIP_3 (Carnero and Paramio, 2014). Somatic mutations of *AKT2* and *AKT3* have also been reported in different tumour types (Carnero and Paramio, 2014).

Some tissues are more susceptible to tumorigenesis upon *AKT* activation than others. However, the expression of activated *Akt* alone does not induce tumorigenesis. For instance, *AKT* activation is essential, but not sufficient, in the development of skin carcinogenesis (Segrelles et al., 2002). Additionally, activation of *Akt* in the pancreas was found to increase the proliferative capacity of prostate cells, which resulted in prostate intraepithelial neoplasia (Majumder et al., 2003), but did not induce tumour formation. Similarly, constitutively active *AKT* in the mammary gland using an epithelial-specific MMTV promoter causes delayed post lactational involution mammary gland, but no neoplastic transformation (Ackler et al., 2002).

Mammary-specific expression of myr*AKT1* in MMTV-myr*Akt1* transgenic mice delays post-lactation involution (Schwertfeger et al., 2001) and increases susceptibility of mice to induction of mammary tumours by the carcinogen 9,10-dimethyl-1,2 benzantracene (DMBA) (Blanco-Aparicio et al., 2007). Whilst, ablation of *Akt1* inhibits the development of mammary tumours in MMTV-Neu and MMTV-polyoma middle T (MMTV-PyMT) transgenic mice, *Akt2* ablation accelerates tumorigenesis in these models (Maroulakou et al., 2007).

Although activation of *AKT* alone does not induce tumour formation, *AKT* is required for the development of tumours in combination with mutations in other genes. For example, the coexpression of activated *Ras* and activated *Akt* causes glioblastoma multiforme in mice,

which is not observed in mice when these oncogenes are expressed alone (Qiao et al., 2012). Additionally, mice expressing a constitutively active *Akt* in combination with loss of *p53* expression in the stratified epithelia develop oral cavity tumours that are similar to human head and neck squamous cell carcinomas (Moral et al., 2009).

1.10.4 Mouse models of non-malignant *PIK3CA*-related conditions

While the majority of mouse models of *Pik3ca* mutation have focused on tumorigenesis, these models also allow for the study of other pathogenic effects of *Pik3ca* mutation that are not related to cancer. Indeed, activating mutations in *Pik3ca* have been linked to a spectrum of noncancerous overgrowth disorders, collectively known as *PIK3CA*-related overgrowth spectrum (PROS) and vascular malformations. Earliest mouse models of a constitutive active *PIK3CA* were performed by using tissue-specific expression of an activated form of p110 α (Shioi et al., 2000).

The first mouse model generated by Shioi *et al.* induced cardiac- specific expression of constitutively active p110 α , and resulted in an increase in heart size but did not induce tumorigenesis. By using an inducible constitutively active form of PI3K, in which the Cre recombinase activates the expression of a myristoylated form of p110 α Sheen *et al.* showed that constitutive activation of PI3K leads to undefined blood vessels, haemorrhage, lack of normal blood circulation and defective angiogenesis (Sheen et al., 2015).

Kirnoss *et al.* used the exon-swap *Pik3ca*^{H1047R} knock-in mouse crossed with a mouse that carries a tamoxifen-inducible Cre recombinase driven by the ubiquitin C promoter (*UCre*^{ERT2}) to induce widespread expression of *Pik3ca*^{H1047R} in adult mice. This led to an unexpected early death, coinciding with widespread increase in organ size, hypoglycaemia, intolerance to fasting and increased glucose turnover (Kinross et al., 2015). Together these phenotypes highlighted the critical role of *PIK3CA* in metabolism and growth. In another study *LSL-Pik3ca*^{H1047R} mice expressing the *Pik3ca*^{H1047R} mutation in all the tissues had early death attributed to vascular defects (Castel et al., 2016).

To better represent the segmental overgrowth characteristic of PROS, Castillo *et al.* used a tamoxifen-inducible Cre recombinase under the control of the Brachyury transcription factor

promoter ($T\text{-}Cre^{\text{ERT2}}$) to activate $Pik3ca^{H1047R}$ in the embryonic mesoderm between embryonic days 7.5-20.5. In order to activate the mutations in a mosaic-fashion to prevent embryonic lethality, they used low doses of 4-hydroxy-tamoxifen. These mice were born with subcutaneous vascular malformations in different body locations however no overgrowth developed (Castillo et al., 2016).

Recently, Venot *et al.* generated a mouse model of PROS using a chimeric $Pik3ca$ transgene ($R26\text{StopFLP110}$) with $CAGG\text{-}Cre^{\text{ER}}$ to generate mice that ubiquitously express a dominant active $Pik3ca$ upon tamoxifen administration (Venot et al., 2018). When treated with a low dose of tamoxifen (4 mg kg^{-1}) the mice developed multiple phenotypic abnormalities, including organomegaly, asymmetrical overgrowth of extremities, disseminated voluminous tumours and visible subcutaneous vascular abnormalities, similar to the lesions observed in patients with PROS.

1.11. Research project aims

Cancer cells alter their metabolic programme to facilitate their proliferation and survival and become independent from exogenous growth stimuli. To meet their metabolic demand, cancer cells switch to be heavily dependent upon glucose-dependent metabolism. The PI3K/AKT pathway has a wide range of effects on cellular metabolism and its aberrant activation induces growth and survival independent of exogenous growth factors-independent growth and survival. Aside from its role in maintaining glucose homeostasis PI3K has been described as one of the principal modulators of aerobic glycolysis, TCA cycle, lipid, nucleotides and proteins synthesis in cancer. This makes the PI3K pathway an attractive target for therapeutic intervention. However, the use of PI3K inhibitors is limited by the presence of feedback loops and the activation of alternative pathways. Characterising the effects of mutations in the insulin-PI3K pathway on the metabolism of normal cells might be fundamental to understanding its role in cancer development. The aim of this thesis is, therefore, to investigate the effect of mutations in the insulin-PI3K pathway on the metabolism program of normal tissues. Furthermore, this thesis aims to characterise how the systemic metabolism affects the metabolic reprogramming resulting from mutations in the PI3K pathway in order to help design combination therapies that target PI3K in combination

with drugs that target metabolism. The specific aims are as follows:

Aim 1: To characterise the role of mutations in the PI3K signalling pathway on glucose homeostasis, *in vivo*.

Aim 2: To characterise the role of mutations in the PI3K signalling pathway on systemic metabolism, *in vivo*.

Aim 3: To characterise the role of mutations in the PI3K signalling pathway on lipid metabolism *ex-vivo*.

Aim 4: To characterise changes in mRNA and protein expression of metabolic enzymes in the intestine, liver and brain of mice harbouring both the *Pik3ca*^{H1047R} mutation and *Pten* loss.

Aim 5: To characterise the metabolic profile of the intestine and brain of mice harbouring the *Pik3ca*^{H1047R} mutation through *in vitro* studies on intestinal organoids and neuro-spheres.

Hypothesis

The effects of mutations in the PI3K/AKT/mTOR pathway are not limited to glucose metabolism, but affect systemic metabolism at multiple sites.

CHAPTER 2

MATERIAL and METHODS

2.1 Xenograft techniques

2.1.1 Mouse strains

All mice used are on the black 6 (BL6) background:

- *Pik3ca*^{lat-H1047R/lat-H1047R} mice (Kinross et al., 2012) were created by Ozgene for the Surgical Oncology Laboratory, Peter MacCallum Cancer Centre.
- *UCreER* mice (*UCreER*^{T2}; BL6; Tg (UBC-cre/ESR1)1Ejb/J were obtained from The Jackson Laboratory, Bar Harbor, ME, USA (Ruzankina et al., 2007).
- *Pten*^{fl/fl} mice BL6; *Pten*^{tm1Hwu}
- were obtained from The Jackson Laboratory, Bar Harbor, ME, USA (Groszer et al., 2001).

Pik3ca^{lat-H1047R/lat-H1047R} mice and *Pten*^{fl/fl} mice were crossed with heterozygous Ubiquitin Cre-expressing mice to generate mice expressing the heterozygous *Pik3ca*^{H1047R/WT} mutation or the homozygous *Pten*^{-/-} mutation, in every organ, following tamoxifen administration. Breeding strategies are shown in Figure 2.1.

The mice had access to standard chow diet (irradiated Barastoc mouse cubes; RidleyAgriProducts, Melbourne, VIC, Australia) with calculated digestible energy of 14.2 MJ / Kg and water for the whole duration of the experiments. All animal experiments were approved by the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee.

Mice were monitored 2-3 times a week and sacrificed by cervical dislocation or carbon dioxide at specific end points or if showing signs of distress such as lethargy, deformed skull, recessed eyes, slow movements, fuzzy fur and shallow rapid breathing.

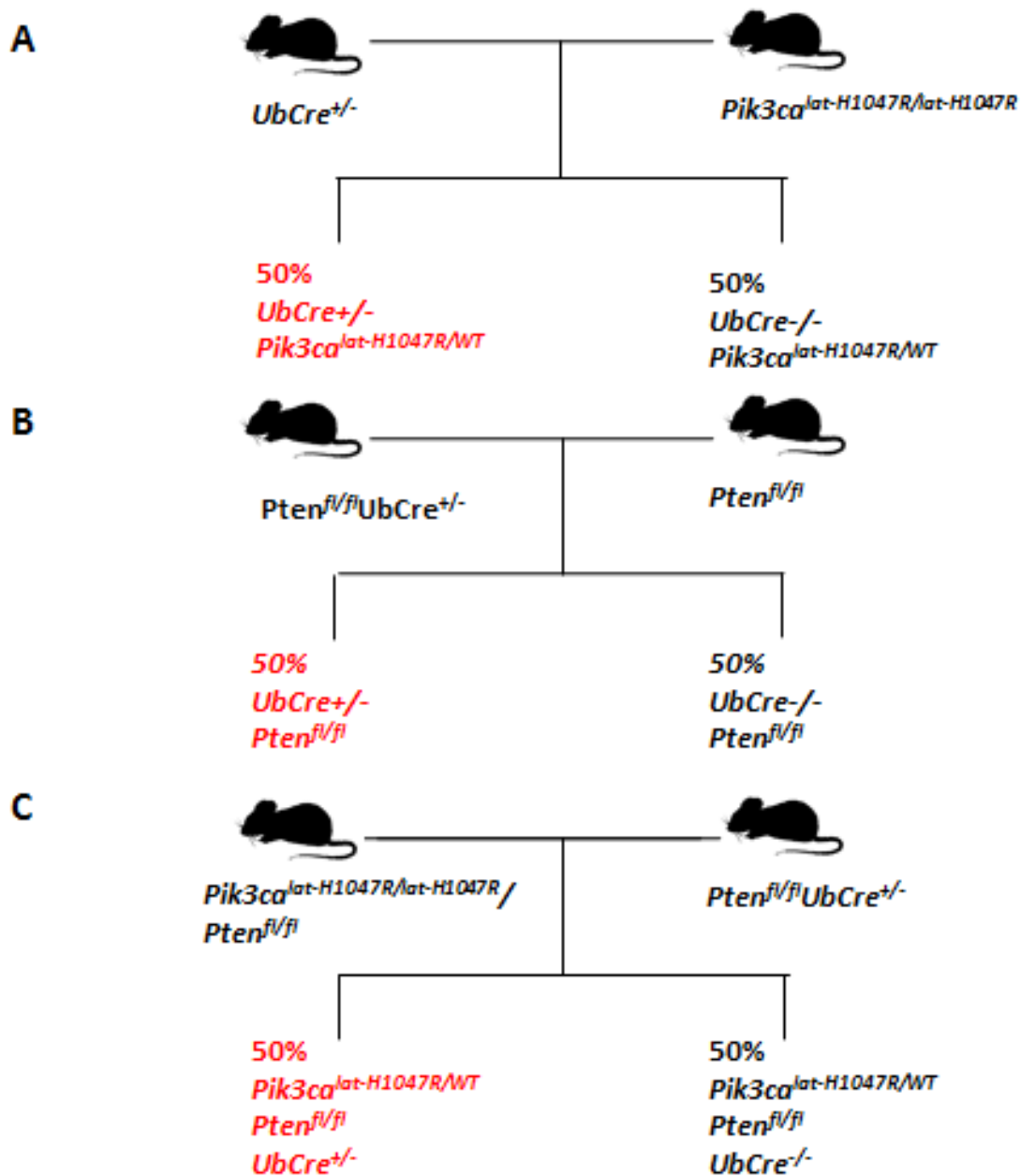


Figure 2. 1: Breeding schemes and expected genotypes of mice.

A) Breeding scheme for the generation of $Pik3ca^{lat-H1047R/WT} UbCre$ mice by crossing a homozygous $Pik3ca^{lat-H1047R/lat-H1047R}$ mouse with a heterozygous $UbiquitinCre$ -expressing mouse. B) Breeding scheme for the generation of $Pten^{-/-} UbCre$ mice by crossing a homozygous $Pten^{fl/fl}$ mouse with an $UbiquitinCre^{+/-}/Pten^{fl/fl}$ mouse. C) Breeding scheme for the generation of double mutant $Pik3ca^{lat-H1047R/WT}/Pten^{-/-} UbCre$ mice by crossing a $Pik3ca^{lat-H1047R/lat-H1047R}/Pten^{fl/fl}$ -expressing mouse with an $UbiquitinCre^{+/-}/Pten^{fl/fl}$ mouse. All the crosses gave 50% mutant (in red) and 50% wild type (black) mice.

2.1.2 Mouse genotyping

Pik3ca genotyping was performed by RT-PCR. Genomic DNA (0.5µl) extracted from mice was mixed with 10uM of each of primers 19F, 20F and 20R, 2.5mM dNTPs and 0.05µl Gotaq polymerase (Promega Corporation Alexandria NSW 2015, AU) in a total of 10µl Gotaq buffer and amplified at 95°C for 10min followed by 95°C for 30sec, 55°C for 30sec, 72°C for 40sec for 35 cycles and then 72°C for 5min. The amplified product was then run on 2% agarose gel (150V, 70min) and stained with ethidium bromide. Shown are the results from 8 representative mice. Primer sequences:

19F (5'-TTGGTTC- CAGCCTGAATAAAGC-3'),

20F (5'-TCCACACCATCAAGCAGCA-3')

20R (5'-GTCCAAGGC- TAGAGTCTTTTCGG-3').

Pten and *Cre* genotyping was performed by RT-PCR. Genomic DNA (0.5µl) extracted from mice was mixed with 10uM of each of primers, 2.5mM dNTPs and 0.05µl Gotaq polymerase (Promega Corporation) in a total of 10µl Gotaq buffer and amplified at 94°C for 3min followed by 94°C for 30sec, 60°C for 30sec, 72°C for 40sec for 35 cycles and then 72°C for 10min, 4°C for 5 min. The amplified product was then run on 2% agarose gel (150V, 70min) and stained with ethidium bromide. Shown are the results from 8 representative mice. Primer sequences:

Pten F (5'-CAAGCACTCTGCGAACTGAG-3'),

Pten R (5'-AAGTTTTTGAAGGCAAGATGC-3'),

CRE F: 5'-GGGATTGCTTATAACACCCTGTTACG-3',

CRE R: 5'-TATTCGGATCATCAGCTACACCAGAG-3'

2.1.3 Drug administration

In vivo expression of the Ubiquitin-Cre recombinase was induced by administration of tamoxifen (Tam; T5648, Sigma Aldrich, Castle Hill NSW 2154, AU) solubilized at 30 mg/ml in a mixture of 98% sunflower oil [(sunflower seed oil from *Helianthus Annuus* (Sigma Aldrich Pty Ltd, Castle Hill NSW 1765 AU)] and 2% ethyl alcohol (Sigma Aldrich, Castle Hill NSW 2154, AU). Tamoxifen was administered by oral gavage using a 20G x 33mm flexible gavage needle (Instech Laboratories, Currambine 6028, Western Australia) in a dose of 200 mg/kg, on 2 consecutive days. This dose was previously proven to activate the mutations in every tissue of the mouse (Kinross et al., 2015). BYL-719 [Alpelisib (MedChemExpress St Lucia, QLD 4067, AU)] was administered by oral gavage, using a 20G x 33mm flexible gavage needle once or twice per day at a dose of 50 mg/kg, until the mice were sacrificed.

2.1.4 Tail arterial bleeds

Tail arterial bleeds were used for blood collection. For the initial bleed, the tail tip was removed (no more than 2mm) using clippers and the tail milked for a drop of blood. Subsequent bleeds were performed by removal of the scab formed on the tip of the tail.

2.1.5 Glucose Tolerance Test

Mice were fasted for 4 hours before to deliver glucose (2g/kg) by oral gavage. Blood glucose levels were measured using a hand held glucometer (Accu-Check Performa; Roche Diagnostics, Castle Hill, NSW, Australia) after 0, 15, 30, 45, 60, 90 and 120 minutes and expressed in mmol/l. The incremental area under the curve was calculated using Excel (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA). The mice were returned to a standard chow diet at the end of the assay.

2.1.6 Pyruvate Tolerance Test

Mice were fasted for 3 hours before delivery of pyruvate (2g/kg) by intraperitoneal injection (i.p). Blood glucose levels were measured, using a hand held glucometer (Accu-Check Performa; Roche Diagnostics, Castle Hill, NSW, Australia) after 0, 15, 30, 45, 60, 90 and 120 minutes and expressed in mmol/l. The incremental area under the curve was calculated using Excel (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA). Mice were returned to a standard chow diet at the end of the assay.

2.1.7 Promethion metabolic cages

Pik3ca^{H1047R/WT}*UbCre* mice were certified pathogen-free following specialised faecal pathogen testing and transferred to the University of Melbourne Biomedical Science Animal Facility (BSAF, Parkville, Victoria 3010, AU). Mice were housed in pathogen-free laboratory cages for 1 week in the BSAF, to allow acclimation. Mice had access to standard chow diet and water throughout the experiment. Tamoxifen was administered by oral gavage 6 days before to transfer the mice to the Promethion cages (Promethion®, Sable Systems, LasVegas, NV), where mice were housed individually for a period of 2.5 days. The first 12 hours in the metabolic cages were necessary for acclimation of the mouse to the new cage, and the subsequent 24 hours were required in order to obtain stable values of respiratory exchange rate (RER), energy expenditure (EE), food and water intake and physical activity during night time and day time. The last 24 hours a running wheel (4.5" (11.5 cm) diameter, MiniMitter, Bend, OR) was add to each cage. During the 2.5 days mice were monitored daily for signs of distress, abnormal feeding and drinking behaviour and changes in body mass. Water bottles and food hoppers were connected to load cells for food and water intake monitoring. Ambulatory activity and position of the mice in the cage were detected with XYZ beam arrays (BXYZ-R, Sable Systems, LasVegas, NV). Integrated fuel cell oxygen analyser, spectrophotometric CO₂ analyser and capacitive water vapour partial pressure analyser (GA3, Sable Systems, Las Vegas, NV) were connected to the cages to measure oxygen consumption and carbon dioxide production for each mouse at 5-min intervals (Kaiyala et al., 2012). Respiratory exchange

rate (RER) was calculated as the ratio of CO₂ production over O₂ consumption. Energy expenditure measures were obtained using a computer controlled indirect calorimetry system (Promethion®, Sable Systems, LasVegas, NV) and calculated as Kcal/hr using the Weir equation (Weir, 1949). Data were analysed using Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA). Measurements were analysed on Prism-GraphPad using two-ways anova. Figure 2.2 shows the timeline of the experimental protocol.

	Mice transferred to BSAF. Allowed to 1 to 2 weeks to acclimate	Mutations induced with oral gavage of tamoxifen	Mutations induced with oral gavage of tamoxifen	Monitoring	Monitoring	Monitoring	Minispec measurement of body composition. Moved to Promethion metabolic cages for	Monitoring. Addition of a running wheel to each cage	Monitoring	Monitoring	Removed from Promethion	Endpoint: sacrifice mice and dissection
DAY	0	1	2	3	4	5	6	7	8	9	10	

Figure 2. 2: Panel displaying the timeline for the Promethion metabolic cages analysis

Pik3ca^{H1047R} and respective control mice were transferred from the breeding facility of Peter MacCallum Cancer Centre (Melbourne, VIC 3000, AU) to the animal facility at the School of Biomedical Sciences (BSAF) (The University of Melbourne, Parkville, VIC 3010, AU). On day 0 and 1 mice were injected with tamoxifen (200 mg/kg) and monitored onwards. On day 5 the body composition of the each mouse was measured using Time Domain Nuclear Magnetic Resonance (TD-NMR) and moved to the Promethion metabolic cages for 2.5 days. At day 6 a running wheel was added to each cage. Mice were monitored at any time. On day 8 the mice were returned to their original cages. Mice were sacrificed and organs were collected for *ex vivo* analysis.

2.1.8 Whole body composition analysis

Pik3ca^{H1047R/WT}*UbCre* mice were injected with tamoxifen. Six days post tamoxifen administration mice were guided into the MiniSpec restrainer (LF50, Bruker Analyzer, Billerica, Massachusetts, United States), to measure fat mass, lean mass and free fluid through Time Domain Nuclear Magnetic Resonance (TD-NMR). Once the scan was complete (approximately 2 minutes) the restrainer was removed and mice were returned to their original cages. Data were exported on a Microsoft® Office Excel spreadsheet and expressed as % of fat, lean or fluids normalised on the body weight of the mice.

2.1.9 Plasma insulin

Mice were fasted for 3-4 hours before to deliver glucose (2g/kg) by oral gavage. Blood samples were collected by tail bleeding in collecting 1.5ml tubes containing 10mM Ethylenediaminetetracetic acid (EDTA) (Sarstedt Microvette CB300 EDTA 1000pcs). Samples were centrifuged at 1.500g for 10 minutes (5424R-Eppendorf). Supernatant representing the plasma fraction was collected and stored at -80°C until use. Each plasma sample (5µl) was used to measure the levels of insulin using a commercially available mouse insulin ELISA kit (Crystal Chem, Elk Grove Village, Illinois, US), following the instructions of the manufacturer. Plasma insulin concentration was interpolated using the standard curve and mean absorbance values for each sample. Absorbance was read at 450nm and 630nm ($A_{450} - A_{630}$) on the Cytation 3 Cell Imaging Multi-Mode Reader (Millennium Science Australia Pty Ltd, Mulgrave, Victoria, Australia 3170) and calculated as 450nm-630nm. Data were analysed using Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA).

2.1.10 [¹⁴C]-deoxy-D-glucose tracer uptake

Mice were fasted for 4 hours before delivery of glucose (2g/kg) by oral gavage. ¹⁴C-2-Deoxy-D-glucose tracer (10µCi/mouse; made in saline) (PerkinElmer, Melbourne, Victoria 3150, AU) was administered by i.p. injection immediately after administration of glucose. After 30

minutes mice were sacrificed by cervical dislocation. Brain, muscle, small and large intestine, pancreas, liver, lung, spleen, heart and kidney were harvested, snap frozen in liquid nitrogen and stored at -80°C until use. Plasma of each mouse (10µl) was added to 20µl 5% zinc sulfate (ZnSO₄) and 20µl saturated barium hydroxide (BaOH) and mixed up. Each supernatant (15µl) was transferred to a scintillation vial containing 85µl H₂O and 2ml Ultima Gold scintillation cocktail (PerkinElmer, Melbourne, Victoria 3150, AU). Counts in the plasma were used to build a disappearance curve using 1 minute as 100%. About 30mg of each tissue was homogenised in 1.4ml 2.75% ZnSO₄ at 13.000rpm for 10min. Part of the supernatant (400µl) was transferred into scintillation vial and topped up with 2ml scintillation cocktail (total counts). Another 400µl of the original supernatant were transferred into new epi containing 140µl saturated barium hydroxide (BaOH), mixed up, spun at 13.000 rpm for 10min and add to 2ml scintillation cocktail (free counts). Radioactivity was detected using a scintillation counter (Tri-Carb 4910TR liquid scintillation analyser, PerkinElmer, Melbourne, Victoria 3150, AU) and expressed as counts per minutes (CPM)/ mg tissue/tissue weight. Data were analysed using Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA).

2.1.11 Tracer [¹⁸F] Fluorodeoxyglucose (FDG) biodistribution

After 3 hours fasting, mice were anaesthetised with a mixture of isoflurane and oxygen. ¹⁸F-labelled glucose (PerkinElmer, Melbourne, VIC, AU) (~2-3MBq per mouse) was injected intravenously (i.v.). One hour after the injection, blood was collected through cardiac puncture. Mice were sacrificed by cervical dislocation and brain, muscle, small and large intestine, pancreas, liver, lung, spleen, heart and kidney were harvested and weighed. The radioactivity present in each tissue was measured using a γ-counter (PerkinElmer, Melbourne, VIC, AU) and expressed as CPM/tissue weight. Results were adjusted on the time of injection and on the decay activity of the original injection solution and listed as a percentage of the injected dose per gram of tissue mass (% ID/g). Data were analysed using Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA).

2.1.12 Positron emission tomography (PET)

After 3 hours fasting, mice were anaesthetised by isoflurane inhalation. Mice were intravenously injected with ~2-3 MBq ^{18}F -labelled glucose. PET was performed one hour post injection, using G8 acquisition software (version 2.0.0.10). During the acquisition (10 minutes) the mice were anesthetized with a mixture of isoflurane and oxygen. The images were reconstructed using the 3D maximum-likelihood expectation maximization (MLEM). Gauss post reconstruction filtering was performed using VivoQuant post-processing software (version 2.10, inviCRO Imaging Services and Software, Boston, U.S.A).

2.1.13 Deuterated Water enrichment

Deuterated water (containing 0.9% w/v NaCl) was delivered by i.p injection (20 μl /g of body weight), (Deuterium Oxide, Sigma Aldrich, Castle Hill NSW 2154). Mice had access to 5% (v/v) ^2H -labeled-drinking water during the whole duration of the test. 7 days post injection the mice were sacrificed and brain, liver, spleen and intestine were extracted, snap frozen in liquid nitrogen and stored at -80C. Plasma samples were collected through eye bleeding (~30 μl whole blood = ~10-15 μl plasma) into vacutainer tubes containing 0.1% EDTA, at day 1, 4 and 7 post injection and stored at -80C. Samples were transferred to the department of School of Exercise and Nutrition Sciences, Faculty of Health, Deakin University (Deakin University, 221 Burwood Hwy, Burwood, Victoria 3125, AU). DNA and proteins were extracted from the stored tissues and analysed by gas chromatography-mass spectroscopy (GC-MS), as previously described (Foletta et al., 2016). Data were analysed using Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA).

2.1.14 Lactate Production

Blood samples were collected by tail bleeding in 1.5ml collecting tubes containing 0.1% (v/v) EDTA (Sarstedt Microvette CB300 EDTA 1000pcs). Samples were centrifuged at 1.500g for 10 minutes (5424R-Eppendorf). Supernatant representing the plasma fraction was collected

and stored at -80°C until use. Plasma samples were serially diluted in phosphate-buffered saline (PBS) to ensure the readings were within the standard curve. Plasma lactate was measured using the L-Lactate assay kit (Jomar Life Research, Caribbean Park, Victoria 3179, AU) following the instruction of the manufacturer. A standard curve was prepared by adding 0.3-2 mM lactate standards in duplicate. Lactate reagent (15µl) (Jomar Life Research) was add to each well of a 96 wells plate and incubated in a CO₂ free incubator at 37°C for 45 minutes. Acetic acid (0.5mM) was add to each well (15µl per well) to stop the reaction. Absorbance was read at 490nm (A₄₉₀) using the Cytation 3 Cell Imaging Multi-Mode Reader (Millennium Science Australia Pty Ltd, Mulgrave, Victoria, Australia 3170). Plasma lactate concentration was interpolated using the standard curve and mean absorbance values for each sample. Data were analysed using Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA).

2.1.15 Beta Hydroxybutrate (β-HB) production

Blood samples were collected by tail bleeding in 1.5ml collecting tubes containing 0.1% (v/v) EDTA (Sarstedt Microvette CB300 EDTA 1000pcs). Samples were centrifuged at 1.500g for 10 minutes (5424R-Eppendorf). Supernatant representing the plasma fraction was collected and stored at -80°C until use. The levels of Beta Hydroxybutrate (β-HB) in the plasma of the mice were measured using the β-HB Assay Kit (Abcam, Melbourne, Victoria 3000, AU). Plasma samples (5µl) were added to each well of a 96 wells plate and the volume was adjusted to 50µl with the β-HB assay buffer. A standard curve was prepared by adding 0-10nM β-HB standard, in duplicate. Reaction mix (50µl) was added to each well and incubated for 30 minutes protected from light. Absorbance was read at 450nm (A₄₅₀) on the Cytation 3 Cell Imaging Multi-Mode Reader (Millennium Science Australia Pty Ltd, Mulgrave, Victoria, Australia 3170). Plasma β-HB concentration was interpolated using the standard curve and mean absorbance values for each sample. Data were analysed using Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA).

2.1.16 Tissues collection

Mice were sacrificed by cervical dislocation and the organs harvested. The weight of each organ was measured using an electronic balance (Australian Instrument Services Pty Ltd, Bayswater, Victoria, AU). Tissues were snap-frozen in liquid nitrogen and stored at -80°C until needed for RNA and proteins extraction or fixed in 10% neutral buffered formalin (NBF) for histologic analysis. After 24h NBF was replaced with 70% ethanol and kept at 4°C until use. If needed to generate intestinal organoids, the small intestine was used fresh.

2.1.17 Proteins extraction

Frozen mouse tissues were homogenised in 2ml microcentrifuge tubes (Corning Axygen® MCT-200-C MaxyClear™ Boil-Proof Snaplock Microcentrifuge Tube, 2ml, RNase/DNase-Free, Clear PP Homopolymer, Non-Sterile) containing porcelain magnetic beads and lysis RIPA buffer (1 mM EDTA; 1% (v/v) Nonidet P-40; 0.5% (w/v) sodium deoxychlorate; 0.1% sodium dodecyl sulfate (w/v); 50mM sodium fluoride; 1mM sodium pyrophosphate in PBS) supplemented with phosphatase and protease inhibitor cocktail (Roche Diagnostics, Castle Hill, NSW, Australia), using the Fast Prep-24 homogeniser (MP Biomedicals Seven Hills, NSW 2147, AU). The samples were centrifuged at 20.000g for 12 seconds in a refrigerated centrifuge (5424R-Eppendorf) and the supernatant was used to extract proteins. The proteins were quantified with the Bradford assay (Bradford, 1976) using BSA as standard and the Cytation 3 Cell Imaging Multi-Mode Reader (Millennium Science Australia Pty Ltd, Mulgrave, Victoria, Australia 3170).

2.1.18 Western Blotting

Proteins extracted from the tissues were added to Laemmli sample buffer [10% sodium dodecyl sulfate (SDS), 10% glycerol, 0.004% bromophenol blue, 0.125M Tris-Cl, pH 6.8, 10% 2-mercaptoethanol] and boiled at 95°C or heated at 50°C for 5 to 10 minutes before to be used for western blots. 10% in house made gels [1.5M Tris-HCl pH 8.8, 40% acrylamide, distilled water, 10% SDS, 10% Ammonium persulfate (APS), Tetramethylethylenediamine

(TEMED)] or commercial available precast 10%, 12% and 6-18% gels (Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad, Gladesville NSW, AU) were used, according to the size of the proteins. An equal amount of proteins was loaded into the wells of the SDS-PAGE gel, along with molecular weight marker (Spectra™ Multicolour Broad Range Protein Ladder). Gels were run at 80V for the first 20 minutes, to allow the proteins to pass through the stacking gel. Thus the voltage was increased to 120V for 1h (Bio-Rad Power pac 300). Proteins were transferred on nitrocellulose membranes, previously activated with pure methanol for 1 min and rinsed with transfer buffer. Transfer was performed by using the Trans-Blot® Turbo system, for 30 minutes at 25V (semi-dry transfer) or transferred for 1h at 4°C in transfer tanks (wet transfer), depending upon the size of the proteins. Transfer of proteins to the membranes was checked using Ponceau S. Membranes were incubated in blocking buffer [5% w/v skim milk, 0.1% v/v Tween 20 in Tris-buffered saline (TBS)] for 1hour at room temperature or overnight at 4°C and probed overnight with appropriate dilutions of primary antibody in dilution buffer (table 1) for 2 hour at room temperature or overnight at 4°C. Membranes were rinsed 3 times in 1% TBS-T (0.15 v/v Tween 20 in TBS, 5 minutes per wash) before to be incubated with appropriate dilutions of conjugated secondary antibody in dilution buffer, at room temperature for 1 hour. The signal was detected by using the Pierce™ develop ECL plus western blotting substrate kit (ThermoFisher scientific, Scoresby Victoria 3179, AU) or the ECL™ western Blotting detection reagents (GE Healthcare Life Science). Anti-β-actin or anti-vinculin antibodies were used as loading control. Chemiluminescence was detected using the darkroom development technique or the ChemiDoc™ touch imagine system (Bio-Rad, Gladesville NSW, AU). The antibodies used for this study are listed in table 2 (pag 73)

2.1.19 Immunohistochemistry

For inguinal WAT and interscapular BAT immunohistochemistry animals were sacrificed and tissues immediately dissected and fixed in buffered formalin solution for 24 hours. Tissues were embedded in paraffin and 4 µm sections of the entire block prepared. Every 13th section of the tissue was used to detect UCP-1 by immunohistochemistry. Slides were baked at 60°C for one hour in an environmental chamber (Labec Laboratory Equipment, NSW, AU). Thus, slides were dewaxed using an automatic slide stainer (Leica Biosystems,

Mount Waverley Victoria, AU). Sections were subjected to antigen retrieval in citrate acid buffer (pH 6.0) at 95°C for 20 min. Sections were rinsed in water (5 minutes) and TBS-T (5 minutes). Sections were blocked with 3% (v/v) Hydrogen peroxide (H₂O₂) in water. After washing with TBS-T for 5 minutes, sections were blocked with 10% Bovine Serum Albumin (BSA) (g/v) in 1% (v/v) TBS-T for 1 hour. Slides were incubated overnight (4°C) with the primary antibody (Table 2). Sections were washed three times in 0.1% (v/v) TBS-T. Sections were incubated with an anti-rabbit DAKO envision + system-HRP labelled polymer (DAKO, Agilent Technologies Australia Pty Ltd, Mulgrave VIC, AU) and then washed three times with 0.1% TBS-T. Sections were incubated for 1 to 2 minutes with DAKO liquid DAB + substrate chromogen system ((DAKO, Agilent Technologies Australia Pty Ltd) and finally washed with water for 5 minutes. Slides were counterstained for hematoxylin and eosin using the automatic slide stainer (Leica Biosystems, Mount Waverley Victoria, AU). A coverslip was added to each slide using a coverslipper (DAKO, Agilent Technologies Australia Pty Ltd). Images were acquired using the OLYMPUS VS120 slide scanner (Olympus Australia Pty Ltd, Notting Hill VIC, AU), and visualised using the OlyVIA software (Olympus Life Science) using 40X magnification.

2.1.20 RNA extraction

Frozen mice tissues were moved into 2ml tubes (Corning Axygen® MCT-200-C MaxyClear™ Boil-Proof Snaplock Microcentrifuge Tube, 2mL, RNase/DNase-Free, Clear PP Homopolymer, Non-Sterile) containing porcelain magnetic beads and NucleoSpin® RNA lysis buffer and homogenised using the Fast Prep-24 homogeniser (MP Biomedicals Seven Hills, NSW 2147, AU). Thus the samples were centrifuged for 30 seconds at rapid spin in a refrigerated centrifuge (5424R-Eppendorf) and the supernatant used for the RNA extraction. RNA was extracted using the NucleoSpin® RNA (Macherey Nagel, Western Australia 6065), following the instructions of the manufacturer. RNA was quantified at the spectrophotometer (NanoDrop ND1000, Thermo Fisher, Scoresby VIC 3179, AU).

2.1.21 Quantitative reverse transcription polymerase chain reaction (q-RT-PCR).

Complementary DNA (cDNA) was synthesized from the RNA extracted from mice tissues by using the transcription first strand cDNA synthesis kit (Roche Diagnostics, Castle Hill, NSW, Australia). The c-DNA was used as template for the q-RT-PCR. PCR was performed using SYBR Green qPCR Master Mix (LightCycler[®] 480 SYBR green, Roche Diagnostics, Castle Hill, NSW, Australia) at the LightCycler[®] 480 Instrument (Roche Diagnostics, Castle Hill, NSW, Australia) [95°C: 10 min pre-incubation, (95°C: 10 sec: 60°C:20 sec: 70°C:20 sec) X 40cycles: amplification, 95°C: 10 sec, 65°C: 60 sec: melting, 40°C: cooling]. Changes in gene expression were analysed using the $\Delta\Delta C_t$ method and normalised against β -actin. Primer sequences are listed in table 2 (page 74). Data were analysed using Microsoft[®] Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA).

2.1.22 Lipolysis

Mice were sacrificed by cervical dislocation. Epididymal fat (male mice) and periovarial fat (female mice) was extracted and cut into 3 pieces. Each piece of tissue was incubated in 1.5ml tube containing basal buffer (DMEM (Dulbecco's Modified Eagle Medium, Sigma-Aldrich, Castle Hill NSW 2154, AU) with 2% (w/v) BSA), forskolin buffer (DMEM containing 2% (w/v) BSA and 20 μ M forskolin) or isoproterenol buffer (DMEM containing 2% (w/v) BSA and 1 μ M isoproterenol). Tubes were incubated at 37°C in a water bath for 2 hours.

Adipose tissue was removed from each tube, rinsed in saline solution (0.9% NaCl), weighed and moved to a new 1.5ml tube. Tissues were snap frozen in liquid nitrogen and stored at -80°C until use. Lipids content was measured in the incubation buffer using free glycerol reagent (Sigma Aldrich, Castle Hill NSW 2154, AU). Standard curve (0-0.25 mg/ml Glycerol standard solution) was made to assure that each sample was in the range of detection. Standards (5 μ l) in duplicates and each sample in the appropriate volume were added to a 96 wells plate. Free glycerol reagent (220 μ l) (Sigma Aldrich, Castle Hill NSW 2154, AU) was added to each well. Plate was incubated at 37°C for 5 minutes. Absorbance was read at 540nm (A_{540}) using the Cytation 3 Cell Imaging Multi-Mode Reader (Millennium Science Australia Pty Ltd, Mulgrave, Victoria, Australia 3170). Free glycerol concentration was

interpolated using the standard curve and mean absorbance values for each sample. Data were analysed using Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA).

2.1.23 Fatty acids oxidation (FAO)

Mice were anaesthetised to remove the soleus muscle. Microtubes (1.0ml) containing 1M sodium hydroxide (NaOH) were inserted in 1.5ml microtubes and add to a 20 ml glass vial containing fatty acids oxidation buffer [low glucose-DMEM (Dulbecco's Modified Eagle Medium-Sigma-Aldrich-5mM glucose)] pre gassed for 10 minutes with 95% O₂-5% CO₂, 1% BSA, 100mM carnitine, 100 mM oleate, 1μCi/ml ¹⁴C-labelled oleate). Muscles were incubated in glass vials at 37°C in a water bath for 2 hours. Muscles were carefully removed from the glass vial and rinsed in saline solution (0.9% NaCl). Weight of each muscle was recorded. Muscles were frozen in liquid nitrogen and stored at -80°C until use. One millilitre of 1M Perchloric acid (HClO₄) was quickly added to each glass vial and incubated for 1 hour. Microtubes containing NaOH were moved to scintillation tubes containing 2ml Ultima Gold scintillation cocktail (PerkinElmer, Melbourne, Victoria 3150, AU). Radioactivity was detected using the scintillator and expressed as disintegration per minute (DPM)/mg tissue. Results correspond to the fraction of ¹⁴C-labeled oleate oxidized to CO₂ the TCA cycle. Muscles were transferred to 12X75mm glass test tubes containing 1.8 ml of 2:1 chloroform: methanol. Tissues were homogenised mechanically using a homogeniser. Saline solution (0.9% NaCl, 600μl) was added to each sample. Tubes were vortexed and incubated at room temperature for 30 minutes. Thus, tubes were spin for 10 minutes at 10,000g to separate the aqueous phase from the organic phase. The top layer of each sample, corresponding to the aqueous phase (800μl) was transferred to a scintillation tube containing 2ml Ultima Gold scintillation cocktail (PerkinElmer, Melbourne, Victoria 3150, AU). Radioactivity was detected using a scintillation counter (Tri-Carb 4910TR liquid scintillation analyser (PerkinElmer, Melbourne, Victoria 3150, AU) and expressed as disintegration per minute (DPM)/mg tissue. Counts correspond to the fraction of ¹⁴C-labeled acid soluble metabolites (Asmann et al.) which reflects incomplete oxidation of fatty acids. The bottom layer of each sample, corresponding to the organic phase (1000μl) was transferred to fresh glass test tubes and dried under nitrogen steam at 40°C for about 10 minutes. Organic phase was used to

visualise the ^{14}C -labeled lipids inside the muscle by thin layer chromatography (TLC). TLC was performed using 250 μm glass backed silica gel plates (20cm x 20cm). Guide lanes of 1.5mm width were scored onto the plate, allowing 1cm margin at each side. Standard mix was prepared (2.5mg/ml cholesterol linoleate ($\text{C}_{45}\text{H}_{76}\text{O}_2$), 1.75mg/ml glyceryl tripalmitate ($\text{C}_{51}\text{H}_{98}\text{O}_6$), 1.7mg/ml oleate ($\text{C}_{18}\text{H}_{33}\text{O}_2$), 0.875 mg/ml dipalmitin ($\text{C}_{35}\text{H}_{68}\text{O}_5$), 1mg/ml C-24-ceramide ($\text{C}_{42}\text{H}_{83}\text{NO}_3$), 2.5mg/ml L- α -phosphatidylcholine (L- α -PC) in 2:1 chloroform: methanol (CHCl_3 : CH_3OH)). Each sample was reconstituted in 30 μl of standard mix and spotted 2cm from the base of the plate in its respective lane, using a 100 μl Hamilton TLC syringe (Hamilton Medical, Hamilton VIC 3300). Standard mix was used as blank. Thus TLC was performed in 3 steps by running the plate once in a solution of chloroform: methanol: water (65:25:4), until the solvent has reached 50% of the plate, and twice in a solution of 75:35:1hexane: diethyl ether: acetic acid (C_6H_{14} : C_2H_5 : CH_3COOH) until the solvent has reached 90% of the plate. After each step the plate was dried for 10 minutes. Plates were sprayed with 0.02% (v/v) dichlorofluorescein ($\text{C}_{20}\text{H}_{10}\text{Cl}_2\text{O}_5$) in ethanol. Bands were visualised using a UV light. Each line was scraped off the plate and silica was collected in scintillation tubes containing 2ml Ultima Gold scintillation cocktail. Radioactivity was detected using the scintillator and expressed as disintegration per minute (DPM)/ mg tissue. Data were analysed using Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA).

2.1.24 Hepatic glucose production

Mice were sacrificed and liver was extracted. The liver was cut into 300 μm slices, using a Krumdieck Tissue Slicer (Alabama Research & Development, Munford, AL). The slices were incubate in M199 for 1 hour and then washed in PBS. Slices were incubate in high glucose DMEM for 1 hour and washed in PBS (3 times). Slices were incubated in low glucose DMEM supplemented with 10mM lactate and 10mM pyruvate with or without insulin (10nM). After 1 hour the liver slices were collected and weighed. Hepatic glucose production was assessed by using glucose oxidase assay for glucose concentrations on the secreted media. Glucose concentration was normalised on tissue weights.

2.1.25 ³I-RNA-sequencing

For RNA extraction, frozen tissues were crushed and parts of different regions of the tissue were collected and homogenised. RNAs extracted from brain, intestine and liver of mice control and mutant were checked for quality using a spectrophotometer (NanoDrop ND1000, Thermo Fisher, Scoresby VIC 3179, AU) and submitted to the Molecular Genomics core (Peter MacCallum Cancer centre, 3000, Victoria, Australia) for sequencing (³I-RNA sequencing). Differential expression output of each tissue types was filter based on p-value (≤ 0.05), and log fold change (≥ 0.5). Data analysis of the filtered list of genes was performed using Galaxy Europe platform. Ensemble of Gene Set Enrichment Analyses (EGSEA) algorithm was used to identify enriched or over-represented gene sets among the list of genes. The resulting gene sets were further filtered using the R/Bioconductor software package Limma and analysed based on the biological function using the Database for Annotation, Visualization and Integrated Discovery (DAVID) or visualised as a protein-protein interaction network using the functional protein association network String

2.1.26 Proteomic analysis

Frozen mouse tissues were homogenised and quantified as described in paragraph 2.3.7. The same concentration of protein from each sample (1mg/l) was transferred to 1.5 ml microtubes. Samples were precipitated in ice cold acetone overnight, at -20°C and centrifuged at 13,000rpm, 4°C, for 10 minutes (5424R-Eppendorf). Supernatant was removed and the pellet was rinsed in ice cold acetone. Samples were spun again at 13,000 rpm, 4°C, for 3 minutes. The supernatant was discarded and the pellet was resuspended in 100µl of 8M urea in 50mM Triethylammonium bicarbonate (TEAB), pH 8. The pellet was vortexed and incubated at 37°C on a shaker until completely solubilised. Protein concentration was quantified and optimised to make it constant between all the samples. Proteins were reduced with 500mM Tris-2-carboxyethyl-phosphine-Dithiothreitol (TCEP-DTT) to a final concentration of 10mM and incubated for 45 minutes at 37°C on a shaker. Proteins were alkylated by iodoacetamide (IAA). Proteins were diluted in 1M urea and digested with trypsin (1:50 enzyme-protein) (Pierce™ Trypsin Protease MS-Grade Thermo Fisher Scientific Australia Pty Ltd, Scoresby, Victoria 3179, AU) while shaking, at 37°C overnight.

Formic Acid (1% (v/v)) was added to each sample to acidify the solution. Oasis cartridge (Waters, Rydalmere, NSW 2116, AU) was first washed with 500µl 80% acetonitrile containing 0.1% (v/v) Trifluoroacetic acid (TFA) and then washed with 600µl 0.1% (v/v) TFA in water. Samples were loaded to the cartridge and then washed with 600µl 1% TFA. Thus samples were eluted with 400µl 80%ACN containing 0.1% TFA and collected in new 1.5ml microtubes. Samples were incubated in a vacuum centrifuge (Thermo Fisher Scientific, Savant® DNA120 SpeedVac® Scoresby, Victoria 3179, AU) to evaporate the supernatant down to 50µl. Samples were stored at 4°C until analysed by Mass Spectroscopy (MS) (Bio21 institute Parkville, Victoria 3052, AU).

Table 1: RT-PCR primer sequences

Gene	Forward (5'-3')	Reverse (5'-3')
<i>Actb</i>	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
<i>Agrp</i>	ATGCTGACTGCAATGTTGCTG	CAGACTTAGACCTGGGAACCTCT
<i>Atp5d</i>	CAGTGCCAACGTCAAGCAAG	CCCGGGCTGCCAAAGTA
<i>Atp5q1</i>	ATCCTGAGGGGCCTGACTTC	TAGGTTGGGTTTGGGGTTTTGG
<i>Cd137</i>	CGTGCAGAACTCCTGTGATAAC	GTCCACCTATGCTGGAGAAGG
<i>Cox7a2</i>	TACTCTACCGTGCCAGCTCT	AGGGTTGGAAGTTGCCTTTACA
<i>Cyc1</i>	CCCAACAGTAGCCTCGCTAT	CACAGGAAAGACCCCCAACT
<i>Hspa5</i>	CACTGGTCTCATCTGGCGAA	CCAGGCATGGTGGTTAGCA
<i>Ly6e</i>	CAACCCTGAGGCCCTTTTCC	GTTGGAGGGAGGGAAGATCG
<i>Mdh2</i>	CCTGCTTGGCTTTAGGGGAG	CCACATTCTAGCTTGGCAGC
<i>Ndufb8</i>	CCATCATCGGGGTATGGCTC	GTCCTGCGGCTTAGTCCTT
<i>Npy</i>	CAAGAGCAACAACCTCGGCATT	GAGAGGGACAGGTTGGCAATC
<i>Nsdhl</i>	CACCTTAGGATCCGCTGGGA	CAGGCATCCGCCCAATGTA
<i>Pomc</i>	ATGCCGAGATTCTGCTACAGT	TCCAGCGAGAGGTCGAGTTT
<i>Rela</i>	CCCTGTAGGACCAACTGAACC	CAGAACTCTGCAGGTGAGAC
<i>Tmem26</i>	ACCCTGTCATCCCACAGAG	TGTTTGGTGGAGTCCTAAGGTC
<i>Uqcr11</i>	GGCTGCCTAATCGTCCTTCT	GTGGGCCCTTGACTGACATC

Table 2: Antibodies for western blotting and immunohistochemistry

Antibody	Source	MW (kDa)	Applicati on	Dilution	Company	Catalogue number
AntiVinculin-HRP	Rabbit		WB	1/10.000	Cell Signal Technology	#2138
Anti-Total OXPHOS Cocktail	Mouse	20,27,35,50*	WB	1/1000	Abcam	ab110413
Anti-UCP1	Rabbit		IHC	1/1000	Abcam	ab10983
Anti-β-ACTIN-HRP	Mouse	42	WB	1/20.000	Sigma-Aldrich	a4854

* The OXPHOS cocktail contains 5 mouse mAbs: anti-CI subunit NDUF8 (ab110242), CII-30kDa (ab14714), CIII-Core protein 2 (ab14745), CIV subunit I (ab14705) and CV alpha subunit (ab14748) as an optimized premixed cocktail <https://www.abcam.com/total-oxphos-rodent-wb-antibody-cocktail-ab110413.html>

2.2 In vitro techniques

2.2.1 Crypts isolation

Mice were sacrificed by cervical dislocation. The large intestine was removed and rinsed with cold PBS containing Nystatin and Gentamicin (PBS-NG). Intestine was flushed with cold PBS-NG using a blunt ended needle attached to a 10ml syringe to remove faeces. The tissue was everted with a forceps starting at one extremity of the intestine and gently pulling it upward until completely everted. Excessive fat was removed by using a forceps. Intestine was placed in a 10cm petri dish and rinsed twice in cold PBS-NB for 15 minutes each wash, at 4°C, on an orbital shaker. The tissue was then cut into 5mm pieces and incubated in a 50 ml falcon tube containing 2mm EDTA (in PBS), on a roller for 40 minutes, at 4°C. Following incubation in PBS-EDTA, the tube containing the intestine was inverted vigorously 40 times to release crypts. Tissues fragment were let to settle and the supernatant, containing PBS-EDTA was discarded. Intestine was rinsed 4 times with 10ml PBS. Tubes were inverted 40 times to release crypts. After each wash the supernatant was collected in a new 50ml falcon tube. Each collected tissue fraction was filtered through a 70µm cells trainer to remove villi debris and collected in a common 50ml falcon tube. Tissues were centrifuged (200g, 3 min, 4°C) (5424R-Eppendorf) and rinsed in PBS for 3 times. Crypts were counted at the haemocytometer (Trajan Scientific and Medical) and used for organoid cultures.

2.2.2 Intestinal organoid culture

Intestinal crypts isolated as described in paragraph 2.4.1, were resuspended in Matrigel (Matrigel matrix Reconst Base Membrane, Life Technologies Pty Ltd, Woodend Victoria 3442, AU) and plated in a 48 wells plate (1000 crypts in 25ul Matrigel per well). Following 15 minutes incubation at 37°C in a CO₂ incubator, 250µl of complete organoid medium [advanced DMEM/F12 containing 10mM HEPES, 5mM glutamine, 1% (v/v) penicillin-streptomycin, 1mM N-acetyl-L-Cysteine, 50X B27 supplement (Life Technologies Pty Ltd, Woodend VIC 3442) 100X N2 supplement (Life Technologies Pty Ltd, Woodend VIC 3442), 50 ng/ml Epidermal Growth Factor (EGF) (Sigma-Aldrich, Castle Hill NSW 2154,AU), 10% r-spondin conditional media, 10µM of the Rho-associated, coiled-coil containing protein

kinase (ROCK) inhibitor, y-27632 (Sigma-Aldrich, Castle Hill NSW 2154,AU were add to each well. Plate was incubated at 37°C in a CO₂ incubator. After 3 days complete organoid growing medium was replaced with DMEM without y-27632 and further changed every 3-4 days

2.2.3 Intestinal organoid passaging

Matrigel was thawed on ice overnight or 2-3 hours before passaging organoids. Media was removed from each well using 1000µl filtered tips and replaced with 250µl cold PBS. Organoids were broken up by pipetting and scraping and collected in 15 ml falcon tubes (1 well per tube). Tubes containing organoids were centrifuged at 200g, 4°C, for 5 minutes (*Allegra X-12R*, Beckman Coulter, Mount Waverley ,Victoria 3149, AU). Supernatant was removed using 1000µl filtered tips and the organoids were resuspended in Matrigel. Each tube was split into 4 wells of a 48 wells plate (Corning® Costar® cell culture plates 48 well, flat bottom). Plate was incubated at 37°C in a CO₂ incubator for 10 minutes before 250ul/well of complete organoid growing medium without y-27632 were added. During single cells passaging, the organoids were collected into 15ml falcon tubes and resuspended in 1ml trypsin solution (trypsin-EDTA (0.25%), phenol red - Thermo Fisher Scientific Australia Pty Ltd, Scoresby, Victoria 3179, AU) to break up the tissues using a 1000µl filtered tip. Organoids growth medium was added to neutralise the action of trypsin. Tubes were spin at 4°C, 200g, for 3 minutes. Supernatant was removed and cellular pellet was resuspended in 1ml PBS. The process was repeated till all the Matrigel was removed and cells appeared as a clear pellet. Cells were counted at the hemocytometer (Trajan Scientific and Medical, Ringwood, Victoria 3134, AU), resuspended in Matrigel and seated at the desired density. Plate was incubated at 37°C in a CO₂ incubator for 10 minutes. Complete organoid growing medium without y-27632 was added to each well (250ul/well). Plate was incubated at 37°C in a CO₂ incubator.

2.2.4 Neurospheres culture

Neuronal spheroids were obtained from the Department of Microbiology and Immunology (DMI, U University of Melbourne, Parkville, Victoria 3010, AU) (Nguyen et al., 2018). Neurospheres were kept in 2ml/well complete growth medium-advanced DMEM/F12 containing 10mM HEPES, 2mM glutamine, 1% penicillin-streptomycin, 10ng/ml fibroblast growth factor (β -FGF) (Lonza Australia Pty Ltd), 20ng/ml EGF (Sigma-Aldrich, Castle Hill NSW 2154,AU) and 2% (v/v) B27 supplement (Life Technologies Pty Ltd), in ultra-low attachment 6 wells plates (Corning® Costar® Ultra-Low Attachment Multiple Well Plate, Sigma Aldrich, Castle Hill NSW 2154,AU). Half of the medium was changed every 2-3 days.

2.2.5 Neurospheres passaging

Growing medium containing neurospheres was collected in 15ml falcon tubes (1 tube each well) and centrifuges at 500rpm for 5 minutes (5424R-Eppendorf). Supernatant was removed and trypsin solution (trypsin-EDTA (0.25%), phenol red - Thermo Fisher Scientific Australia Pty Ltd, Scoresby, Victoria 3179, AU) was added to break up the tissues into single cells. 1 ml basal medium (advanced DMEM without growth factors) was added to neutralise the action of the trypsin. Thus tubes were centrifuged at 500 rpm for 5 minutes (5424R-Eppendorf). Supernatant was removed and cellular pellet was resuspended in PBS. Tubes were centrifuged at 500rpm for 5 minutes (5424R-Eppendorf). Supernatant was removed and cellular pellet was resuspended in complete growth medium. Each original well containing neurospheres was split into 3 wells of a six wells ultra-low attachment plate and topped up to 2ml complete growth medium. The plate was incubated at 37°C in a CO₂ incubator. During single cells passaging, neurospheres were collected into 15 ml falcon tubes and resuspended in 1ml trypsin solution to break up the tissues using a 1000µl filtered tip. Organoids growth medium was added to neutralise the action of the trypsin. Tubes were spin at 1500g, for 5 minutes (Allegra X-12R, Beckman Coulter, Mount Waverley, Victoria 3149, AU). Supernatant was removed and cellular pellet was resuspended in 1ml PBS. Tubes were spin at 1500g, for 5 minutes. Supernatant was removed and cellular pellet was resuspended complete growth medium. Cells were counted at the hemocytometer (Trajan Scientific and Medical, Ringwood, Victoria 3134, AU) and seated at the desired density.

2.2.6 Agilent Seahorse XF96 Metabolic Tests

Metabolic responses of intestinal organoids and neurospheres were measured using Agilent Seahorse XF96 Cell Mito Stress Test (In Vitro Technologies, Noble Park, Victoria 3174, AU). XF96 Seahorse sensor cartridge (In Vitro Technologies) was hydrated with Seahorse XF96 calibrant (In Vitro Technologies) and incubated overnight at 37°C in a CO₂ incubator. XF96 cell culture plate was coated with Cell-Tak solution (0.1M NaHCO₃, 1N NaOH, 22.4µg/ml Cell Tak, 10µl each well) for 20 minutes. Cell-Tak was removed and wells were rinsed with ultrafiltered water (20µl/well). Plate was dried for 2 hours inside a cells culture hood at room temperature. Organoids and neurospheres were counted at the haemocytometer. Pieces of organoids (10.000/well) and neurospheres (20.000/well) were resuspended in appropriate volume (180µl/well) of seahorse XF assay medium (basal XF medium, 10mM glucose, 200mM glutamax, 100mM sodium pyruvate) pre-warmed at 37°C in a water bath and corrected with 0.1N NaOH to make the pH=7.35±0.05. Pieces of organoids and neuro-spheres were seated in a XF96 cell culture plate. Plate was incubated in 37°C in a non CO₂ incubator for 1hour. Oxygen consumption rate was measured by loading the drug compounds Oligomycin (20µM), Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) (20µM), Rotenone (5µM) and Antimycin (5µM) to the sensor cartridge (Agilent Technologies). Extracellular acidification rate (ECAR) of intestinal organoids and neurospheres was measured by loading the drug compounds Rotenone (5µM) and Antimycin (5µM) (In Vitro Technologies) and 2-Deoxy-D-glucose (2DG) (Sigma-Aldrich, Castle Hill NSW 2154,AU) (200µM) to the sensor cartridge (Agilent Technologies). Seahorse XF assay medium not containing drugs was added to the resting ports of the sensor cartridge. Sensor cartridge was loaded to the instrument tray of the Seahorse machine to allow the calibration step (15- 30 minutes). Calibration plate was replaced with the XF96 cell culture microplate. The Seahorse XF Mito Stress Test Report Generator automatically calculated the Seahorse XF Cell Mito Stress Test parameters from Wave (Agilent Seahorse Wave Desktop software). Data were exported to a Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and analysed with Prism-GraphPad software (GraphPad Software, San Diego, CA).

2.2.7 Insulin secretion assay

Insulin secretion assay was performed by the Helen Thomas's laboratory at the St.Vincent hospital, following the following protocol: solution 1:13.5g NaCl in 500ml (460mM), solution 2 : 0.745g KCl (20mM), 4.03g NaHCO₃ (95.9mM), 0.407g MgCl₂.6H₂O (4mM) in 500ml, and solution 3:0.735g CaCl₂ dehydrate in 500ml (10mM) were prepared. Pancreatic islets were isolated. Pancreatic islets were washed in petri dish 3 times with

Krebs-Ringer Modified Buffer (KRB) (25ml solution 1, 25ml solution 2, 25ml solution 3, 5ml 0.5M HEPES, 20ml dH₂O, 54mg D-glucose (final concentration of 3mM). 20 islets were added to siliconized glass tubes and place in rack in water bath (37°C). 4 tubes of islets (20 islets per tube) per condition (3mM glucose and 20mM glucose) were prepared for a total of 8 tubes. The islets were incubated at 37°C in a water bath while shaking (shaking prevents the build-up of somatostatin which inhibits insulin secretion). KRB was removed from each tube and replaced with 200µl KRB with 3mM. Each tube was gassed with 95% air/5% CO₂. Each tube was incubated for 30 minutes at 37°C on a shaker. KRB (3mM glucose) was removed and replaced with 200µl of KRB containing 3mM or 20mM glucose. The tubes were gassed and incubated for 30 minutes at 37°C on a shaker. The supernatant was collected and used for the enzyme-linked immunoassay (ELISA) using a commercially available mouse insulin ELISA kit (Mercodia, Shapshire Bioscience, Redfern NSW 2016, AU), following the instructions of the manufacturer. Insulin concentration was interpolated using the standard curve and mean absorbance values for each sample. Absorbance was read at 450nm (A₄₅₀) on the Cytation 3 Cell Imaging Multi-Mode Reader (Millennium Science Australia Pty Ltd, Mulgrave, Victoria, Australia 3170). Data were analysed using Microsoft® Office Excel spreadsheet (Microsoft, Redmond, WA, USA) and Prism-GraphPad software (GraphPad Software, San Diego, CA).

CHAPTER 3

EXPLORING THE ROLE OF THE PI3K PATHWAY ON GLUCOSE HOMEOSTASIS IN MICE: COMPARING THE EFFECTS RESULTING FROM MUTATIONS IN THE *Pik3ca* AND *Pten* GENES

3.1 INTRODUCTION

The PI3K/AKT pathway orchestrates a myriad of cellular events, such as proliferation, cell survival, growth, angiogenesis and metabolism, in response to extracellular signals. As described in more detail in the introduction to this thesis (Chapter 1-Section 1.3), PI3K is activated downstream of several growth factors, including the insulin-like growth factor receptor, which stimulates growth and survival and the IR, which regulates metabolic homeostasis. The PI3K pathway is the most frequently activated pathway in a variety of human cancers, including breast cancer (Mukohara, 2015), endometrial cancer (Cheung et al., 2011), bladder cancer (Knowles et al., 2009), anal cancer (Shin et al., 2019), colorectal cancer (Cathomas, 2014) and head and neck squamous cell carcinoma (Qiu et al., 2006). Mutations in genes belonging to the PI3K/AKT pathway are oncogenic and increase the intrinsic activity of the pathway (Liu et al., 2009). The *PIK3CA* gene, which encodes the PI3K catalytic isoform p110 α is the second most frequently mutated oncogene in cancer (Fruman and Rommel, 2014). Several mouse models have been developed over the years to investigate the role of *Pik3ca* mutations in cancers and other diseases (Mitchell and Phillips, 2019).

3.1.1 Investigating the role of mutations in the PI3K pathway in metabolism.

Over the last decade the growing interest in cancer metabolism has highlighted PI3K as one of the major regulators of cancer metabolism. Previous analysis, performed in our laboratory, showed that the *Pik3ca*^{H1047R} mutation alone induces hypoglycaemia, hypoinsulinemia and organomegaly, in mice (Kinross et al., 2015). However, whether the metabolic changes were specific for the *Pik3ca*^{H1047R} mutation was not further investigated. Indeed, mutations in other

genes belonging to the PI3K pathway are also known to induce hyperactivation of PI3K, for instance the loss of the PI3K negative regulator, *Pten* (Liu et al., 2009).

Mutations in the *Pten* gene are often found in cancer and have been described as metabolic regulators in cancer progression (Ortega-Molina and Serrano, 2013; Zhou et al., 2019). They are also involved in the development of metabolic syndromes (Yehia and Eng, 2018). Furthermore, mutations in the *Pten* gene contribute to the malignant transformation in mice harbouring the *Pik3ca*^{H1047R} mutation (Kinross et al., 2012).

3.1.2 Investigating the role of mutations in the PI3K pathway in aerobic glycolysis.

It has been previously shown that *Pik3ca*^{H1047R} mutation induces hypoglycaemia (Kinross et al., 2015). One possible reason for the decrease in blood glucose levels is that the activation of the *Pik3ca*^{H1047R} mutation in every organ causes the tissues to increase their glucose uptake from the microenvironment.

One of the hallmarks of highly proliferative cells, indeed, is the increased dependence on glucose to fuel the production of cellular metabolites, required for the generation of new biomass. This phenomenon, first described by Otto Warburg in cancer cells, is known as aerobic glycolysis (Chapter 1-Section 1.2.1). This process allows the preservation of substrates for the synthesis of molecules involved in cellular division, such as lipids for membrane construction and nucleotides for DNA synthesis (Braccini et al., 2012).

The PI3K/AKT pathway drives the switch to aerobic glycolysis by increasing the activity of GLUTs and mTOR (section 1.3.2). Furthermore, AKT inhibits GSK-3 β and FOXO transcription factors (Chapter 1-Sections 1.3.3-1.3.5). Despite the well-known role of PI3K in cancer, the role of isolated mutations in the PI3K pathway on metabolism is still under investigation. A closer examination of the metabolic reprogramming resulting from mutations in the PI3K-AKT pathway can highlight gaps in our understanding of the role of PI3K in cancer development.

In this chapter two metabolic tracers: fluorine-18 (¹⁸F)-FDG and [¹⁴C]-2-deoxyglucose (2-DG) have been used to quantify the distribution of glucose in the animal tissues *ex-vivo*.

FDG is a glucose analogue where glucose has been modified with the radionuclide ^{18}F in place of the hydroxyl group on the 2nd carbon. As glucose, ^{18}F -FDG is transported inside highly proliferative cells by glucose transporters, GLUT 1-7. After being taken up into the cells, ^{18}F -FDG is phosphorylated by hexokinase. The resulting product, ^{18}F -FDG-6-phosphate lacks of the hydroxyl group, which prevents it from entering the next step in the glycolytic pathway, therefore trapping ^{18}F inside the cells (Cox et al., 2015). Since ^{18}F has a half-life of only about 1.8 hours, it is widely used in human studies.

The 2-DG is also a glucose analogue where the 2-hydroxyl group is replaced by hydrogen. Like glucose, the 2-DG is taken up by the cells via GLUTs and phosphorylated by hexokinases. The 2-DG-6-phosphate cannot be further metabolized and is trapped in cells (Bunivicius et al., 2013). Since the 2-DG is commonly marked with carbon-14 (^{14}C), which has a half-life of 5,730 years, its use for probing *in vivo* glucose metabolism is limited by its inapplicability for human studies.

Tumour cells that take up high amounts of glucose produce large volumes of lactate. For a long time, lactate was considered a “metabolic waste product” derived from aerobic glycolysis. However, it is now well known that lactate can be used in the TCA cycle to generate energy. Also, lactate acts as a carbon shuttle within and among cells, tissues and organs. This phenomenon is known as the “lactate shuttle theory” (Brooks, 2009) and is also observed in cancer. PI3K is known to be involved in lactate metabolic dysregulation by activating HIF-1 and c-Myc (Brooks, 2009; San-Millan and Brooks, 2017). In this chapter the levels of plasma lactate were measured in order to support the hypothesis that mutations in the PI3K pathway drive aerobic glycolysis.

3.1.3 Investigate the role of mutations in the PI3K pathway in hepatic gluconeogenesis.

In normal conditions the uptake of glucose into peripheral tissues is only part of a more complex mechanism that guarantees the maintenance of glucose homeostasis in the body. In order for blood glucose to remain in a range of 4-7 mM, liver, pancreas, intestine and peripheral tissues coordinate to maintain a fine balance between the glucose released into the

circulation, the glucose absorbed from the intestine, its storage as glycogen in the liver, and its uptake by peripheral tissues.

The liver plays a crucial role in maintaining glucose homeostasis. It stores glucose in the form of glycogen, and produces glucose by glycogenolysis and gluconeogenesis. During short-term fasting periods, the liver produces and releases glucose mainly from glycogen, (glycogenolysis). During prolonged fasting, instead, the liver undergoes *de novo* glucose synthesis from available precursors such as lactate, pyruvate, glycerol, and amino acids (gluconeogenesis). Abnormally increased rate of hepatic gluconeogenesis contributes to hyperglycaemia and diabetes, whilst abnormally decreased rate of hepatic gluconeogenesis contributes to hypoglycaemia and to a series of glycogen storage diseases.

Hepatic gluconeogenesis is induced by the expression of numerous transcription factors, including the cAMP response element-binding protein (CREB), FOXO1, and the CCAAT/enhancer-binding proteins alpha and beta (C/EBP α/β). Once active, these transcription factors stimulate the expression of phosphoenolpyruvate carboxykinase (*PEPCK*) and glucose-6-phosphatase (*G6PC*) (Rui, 2014), the two enzymes involved in the synthesis of glucose in the liver. In contrast, insulin potently suppresses gluconeogenesis (Chapter1-Section 1.4.2). Indeed, deletion of insulin receptors in mouse hepatocytes has been shown to markedly increase hepatic gluconeogenesis (Michael et al., 2000).

Additionally, liver-specific inhibition of the insulin receptor substrates *Irs1* and *Irs2* blocks hepatic insulin action, resulting in increased hepatic gluconeogenesis in mice (Dong et al., 2006). In contrast, liver-specific *FoxO1* deletion reduces the expression of *Pck1* (*PEPCK* in human) and *G6pc*, subsequently decreasing hepatic gluconeogenesis. This is more pronounced in double and triple liver-specific knock-outs lacking *FoxO1*, *FoxO3*, and *FoxO4* (Haeusler et al., 2010).

Furthermore, AKT regulates hepatic metabolism by phosphorylating the peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1 α and decreases the ability of PGC-1 α to activate gluconeogenic genes (Li et al., 2007).

3.1.4 Aims

The first aim of this chapter is to characterise the effects of mutations in the *Pten* gene - alone or in combination with mutations in the *Pik3ca* gene - on metabolic reprogramming *in vivo* in order to characterise whether the metabolic reprogramming previously observed in *Pik3ca*^{H1047R}*UbCre*⁺ mice is specific for a mutation occurring in the *Pik3ca* gene.

The second aim of this chapter is to characterise the causes responsible for hypoglycaemia and hypoinsulinemia in mice harbouring mutations in the PI3K pathway.

3.2 RESULTS

3.2.1 Mouse models for the conditional expression of mutations in the *Pik3ca* and *Pten* genes.

In order to investigate the effects of activation of the PI3K pathway on glucose utilization and metabolism two mouse models were used: conditional *Pik3ca* (*Pik3ca*^{H1047R/WT}) and/or *Pten* (*Pten*^{fl/fl}).

The *Pik3ca*^{H1047R/WT} mouse model was generated in our laboratory as previously described (Kinross et al., 2015). It consists of a knock-in of a single base change in exon 20 of the endogenous *Pik3ca* gene, leading to the expression of the protein with H1047R amino acid substitution. The mouse has loxP sites on both sides of the last exon (exon 20) of the endogenous *Pik3ca* gene and a tandem copy of exon 20 containing the *Pik3ca*^{H1047R} mutation downstream of the floxed wild type exon. Following the activation of the Cre-recombinase, the wild type exon 20 is excised and replaced by the mutant exon 20 (Figure 3.1 A). The mutant exon is thus ‘knocked-in’ to the endogenous gene and expressed at physiological levels in tissues that normally express the *Pik3ca* gene.

Pten^{fl/fl} mice (Lesche et al., 2002) were obtained from the Jackson Laboratory. These mice carry LoxP sites inserted on both sides of the exon 5 of the *Pten* gene. The mutation is latent before the activation of the Cre-recombinase *in vivo*. Once activated, the Cre recombinase induces the excision of exon 5 and generates a truncated, and inactive, form of the protein (Figure 3.1 B).

To express the heterozygous *Pik3ca*^{H1047R/WT} mutation across all mouse organs, homozygous *Pik3ca*^{lat-H1047R/lat-H1047R} mice were crossed with a B6 mouse expressing a tamoxifen-inducible Cre under the control of the human ubiquitin C (UBC) promoter (UbCreT2; Tg (UBC-cre/ESR1)1Ejb/J; The Jackson Laboratory, Bar Harbor, ME, USA).

UbCreT2 is a fusion protein composed of Cre recombinase and a mutant form of estrogen receptor that is selectively activated only in the presence of tamoxifen, but not estrogens (Lesche et al., 2002) (Figure 3.1C). To express the homozygous *Pten*^{-/-} mutation across all mouse organs, *Pten*^{fl/fl} mice were crossed with a B6 mouse that is heterozygous *UbCre*^{+/-} and homozygous for *Pten*^{-/-} (*Pten*^{-/-}*UbCre*^{+/-} mouse).

After administration of tamoxifen, *in vivo*, *UbCre* (active *UbCre*^{T2}) recombinase generates mice ubiquitously expressing the heterozygous *Pik3ca*^{H1047R/WT} or the *Pten*^{-/-} mutation, allowing us to investigate the consequences of the mutations in each organ of the mouse.

Finally, to express both the *Pik3ca*^{H1047R} mutation and *Pten* loss in all the organs of the mice we have crossed our *Pik3ca*^{lat-H1047R/lat-H1047R} mice with *Pten*^{fl/fl} mice. The resulting mouse was crossed with a *Pten*^{fl/fl}*UbCre*^{+/-} mouse to induce both the mutations in all the organs of the mouse.

In this thesis *Pik3ca*^{H1047R}*UbCre*⁺ mutant mice are indicated as “*Pik3ca*^{H1047R}”, *Pten*^{-/-}*UbCre*⁺ mutant mice are indicated as “*Pten*^{-/-}”, whilst double mutant mice are indicated as “*Pik3ca*^{H1047R}*Pten*^{-/-}” mice. *UbCre* negative control mice are used for each genotype (*Pik3ca*^{H1047R}*UbCre*⁻, *Pten*^{-/-}*UbCre*⁻, *Pik3ca*^{H1047R}*Pten*^{-/-} *UbCre*⁻) and indicated as control mice.

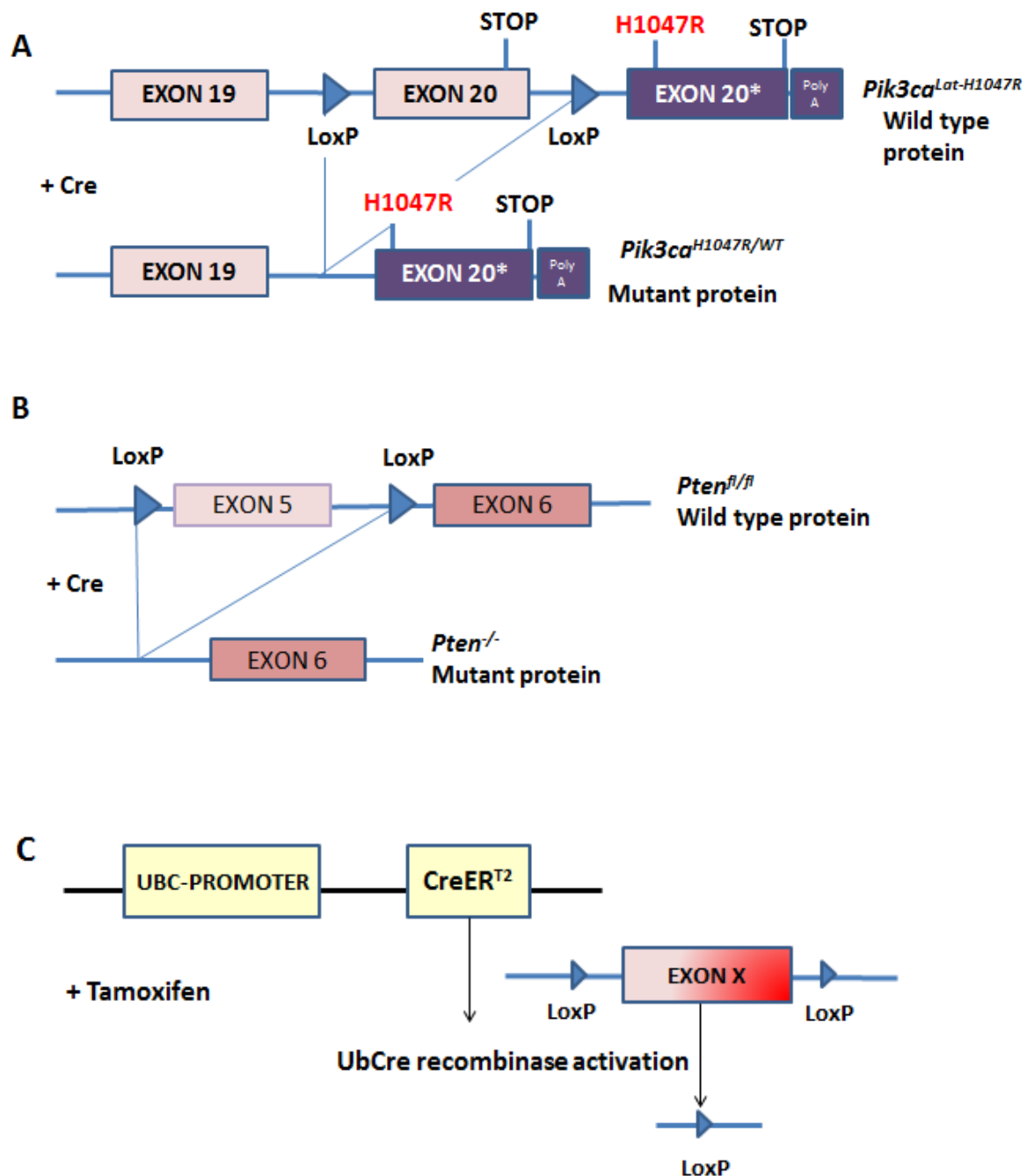


Figure 3. 1: Schematic representations of the latent and functional alleles of the *Pik3ca* and *Pten* genes.

A) A schematic of the *Pik3ca* gene showing the wild type exon 20 (pink), flanked by two loxP sites (triangles) and the tandem exon 20* (purple) containing the *Pik3ca*^{H1047R} mutation. Following Cre activation the wild type exon 20 is excised and replaced by the *Pik3ca*^{H1047R} mutant exon. **B)** A schematic of the *Pten* gene, showing the wild type exon 5, flanked by two loxP sites (triangles). Following Cre activation the wild type exon is excised, resulting in a truncated form of the protein. **C)** *CreER*^{T2} gene downstream to the ubiquitin C (UBC) promoter. After induction with tamoxifen the *CreER*^{T2} recombinase is activated. UbCre = active *CreER*^{T2}. Exon X represents a generic exon excised by the Cre recombinase. PolyA: 3' untranslated poly adenosine tail.

3.2.2. Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of the gene *Pten* lead to reduced survival.

A primary observation obtained from earlier studies performed on mice ubiquitously expressing the *Pik3ca*^{H1047R} mutation, was early death of the mutant mice, compared to control mice (Kinross et al., 2015). This observation was confirmed with *Pik3ca*^{H1047R} mice having a median survival of 25.5 days post tamoxifen treatment, compared to control mice, which were sacrificed 60 days post tamoxifen treatment with no pathologic phenotype (Figure 3.2). Interestingly, the survival rate of mice ubiquitously expressing the homozygous *Pten*^{-/-} mutation was similar to the survival rate observed for *Pik3ca*^{H1047R} mice with a median survival of 23 days post tamoxifen treatment, compared to control mice, which were sacrificed 60 days post tamoxifen administration (Figure 3.2). This observation suggests that ubiquitous hyper-activation of the PI3K pathway is lethal, independent of the mechanism by which the pathway is activated.

Since ubiquitous expression of the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations had similar effects on survival, it was hypothesised that the combination of the two mutations would cooperate, *in vivo*, further accelerating lethality of the mice. The combination of both mutations (*Pik3ca*^{H1047R}*Pten*^{-/-} mice) reduced survival to a median of just 4 days post tamoxifen treatment (Figure 3.2), supporting the hypothesis that the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations have synergistic effects when ubiquitously expressed *in vivo*. This is not surprising, because the loss of the negative regulator *Pten* would be anticipated to enhance the activation of the pathway by the *Pik3ca* mutation. Matched control mice were sacrificed 10 days post tamoxifen treatment without any sign of pathologic phenotype.

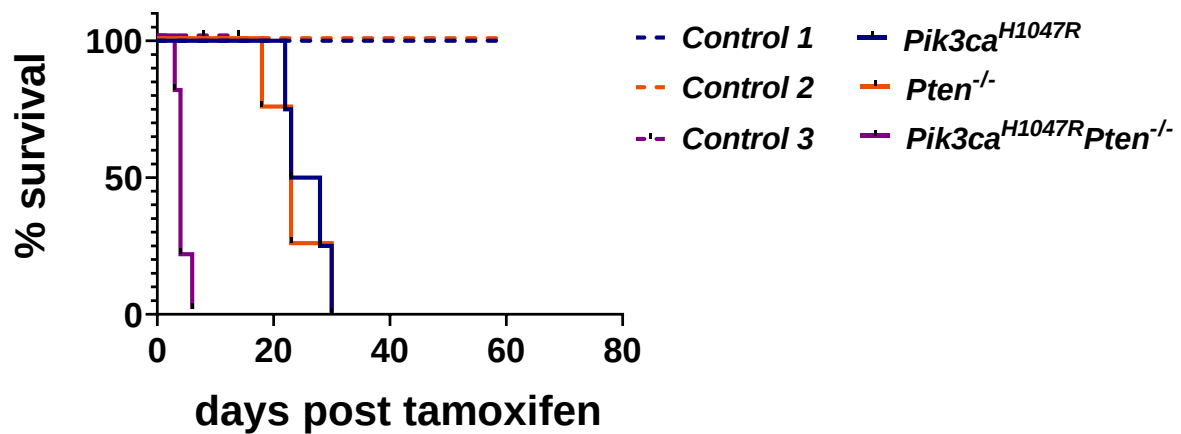


Figure 3. 2: Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of the gene *Pten* reduce mice survival. Kaplan-Meier survival curve of *Pik3ca*^{H1047R}, *Pten*^{-/-}, *Pik3ca*^{H1047R}*Pten*^{-/-} and matched control mice, indicated as Control 1, Control 2 and Control 3, respectively. Mice were administered tamoxifen (200mg/kg) on two consecutive days to activate the mutation and monitored onwards. Median survival of *Pik3ca*^{H1047R} (blue line), *Pten*^{-/-} (orange line) and *Pik3ca*^{H1047R}*Pten*^{-/-} (purple line) mice was 25.5, 23 and 4 days post tamoxifen, respectively. N = 4-6 in each cohort. Control mice are indicated by dashed lines. Matched control mice for *Pik3ca*^{H1047R}*Pten*^{-/-} mice were euthanized 60 days post tamoxifen administration. Matched control mice for *Pik3ca*^{H1047R}*Pten*^{-/-} mice were sacrificed 10 days post tamoxifen.

3.2.3. Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of the gene *Pten* lead to increased body weight and organomegaly.

One of the main consequences of ubiquitously expressing the PI3K pathway is a gain in body weight (Kinross et al., 2015). As previously observed, *Pik3ca*^{H1047R}-ubiquitously expressing mice had a marked increase in body weight 6 days post tamoxifen administration and the body size kept increasing throughout the life span of the mice (Figure 3.3). Similarly, mice ubiquitously expressing the *Pten*^{-/-} mutation, showed increased body weight 4 days post oral gavage of tamoxifen (Figure 3.3). Unlike single mutant mice, mice ubiquitously expressing both the mutations did not increase their body weight due to early death (Figure 3.3). To verify that the increase in body weight was associated with organomegaly, as previously observed, the mice were sacrificed at established time points and the tissues were harvested and weighed.

For this study, investigations were focused on the earliest time point at which metabolic alterations manifest. Therefore, *Pik3ca*^{H1047R} and *Pten*^{-/-} mice were sacrificed 7 days post tamoxifen as an increase in body weight was already detectable. *Pik3ca*^{H1047R}*Pten*^{-/-} mice, which only survived 4 days post induction of the mutations, were sacrificed 2 days post tamoxifen administration. At this time point the mutations were already active, but the mice were not yet showing signs of distress. Matched controls were sacrificed at the same time as the mutant mice.

A large number of tissues in ubiquitously expressing *Pik3ca*^{H1047R} mice increased in size, compared to control mice, particularly lungs, spleen, brain, intestine, stomach and pancreas (Figure 3.4 A). Interestingly, *Pten*^{-/-} mice had no increase in the size of any organ, 7 days post tamoxifen, compared to control mice (Figure 3.4 B). However, when the weight of the tissues was measured 10 days post tamoxifen treatment, the spleen and the small intestine of *Pten*^{-/-} mice gained weight, compared to control mice (Figure 3.4 C). This suggests that ubiquitous loss of the *Pten* gene leads to organomegaly, but the increase in tissue size occurs at a slower rate than *Pik3ca*^{H1047R} mice.

In contrast to mice harbouring the mutation in only one of the two genes, *Pik3ca* or *Pten*, the combination of the two mutations did not result in increased size of individual tissues (Figure 3.4D). However, since *Pik3ca*^{H1047R}*Pten*^{-/-} mice had an average survival of only 4 days, it is

not surprising that there was no increase in overall body weight or individual tissue weight because it is likely that the mice did not live long enough to manifest a detectable increase in size.

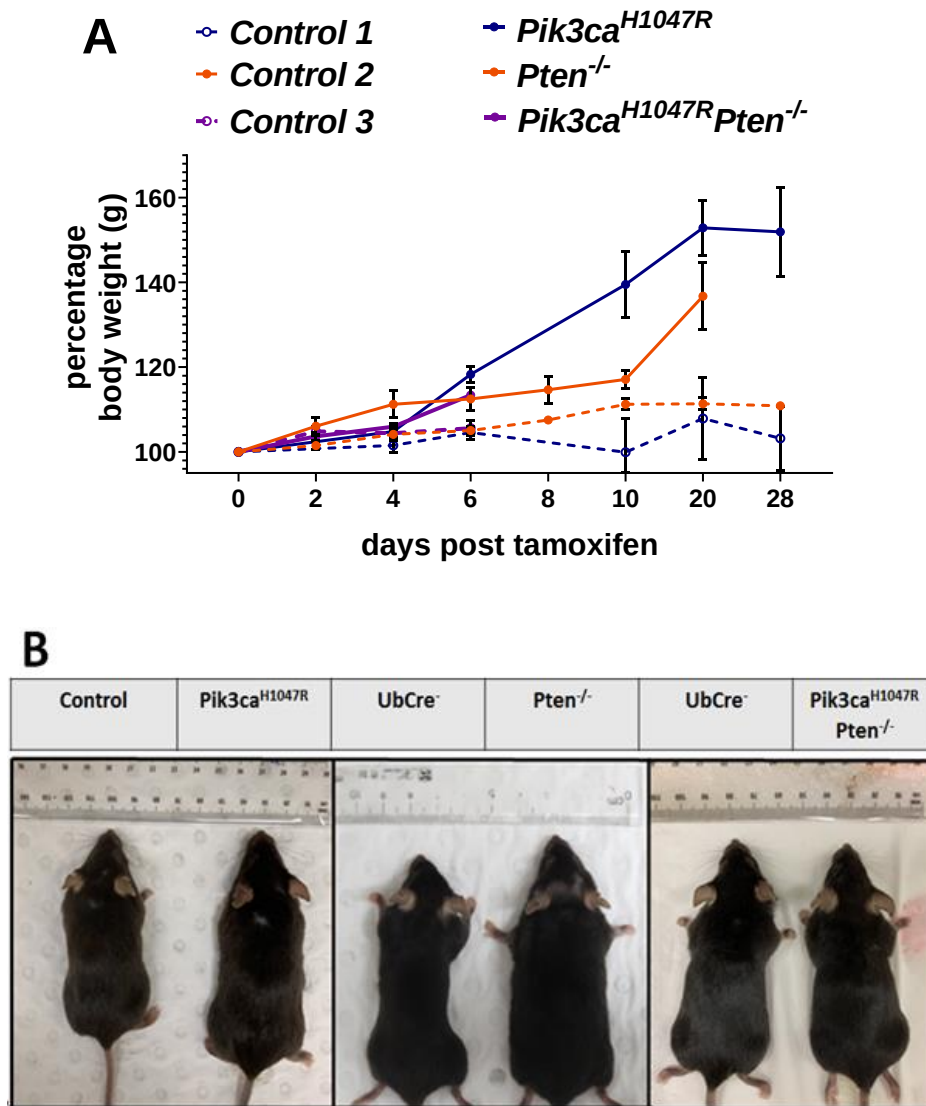


Figure 3. 3: Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of the gene *Pten* lead to increased body weight.

A) Body weight of *Pik3ca*^{H1047R} (blue), *Pten*^{-/-} (orange), *Pik3ca*^{H1047R}*Pten*^{-/-} (purple) and matched control mice, indicated as Control 1, Control 2 and Control 3, respectively. Mice were treated with tamoxifen (200mg/kg on two consecutive days). Body weight was monitored for the entire survival of the mice and expressed as percentage relative to the body weight prior to tamoxifen treatment. Control mice are indicated by the dashed line, whilst mutant mice are indicated by solid lines. N= 5-6 in each cohort. Error bars represent mean $\pm$ SEM.

B) Representative image of the body size of mutant mice vs control mice for each genotype at the end point.

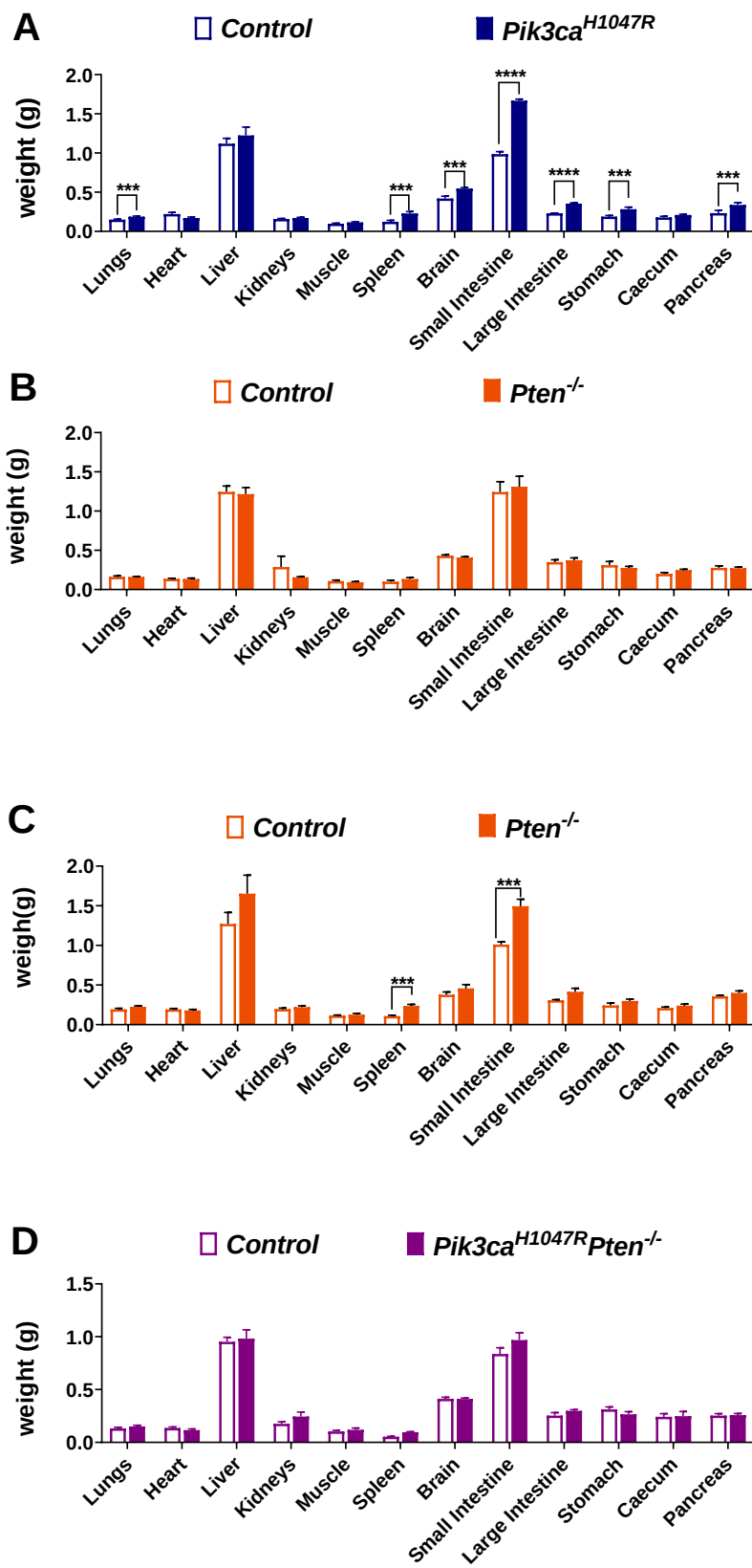


Figure 3. 4: Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of the gene *Pten* lead to increased organ size.

A) *Pik3ca*^{H1047R} mice and matched control mice were sacrificed 7 days post administration of tamoxifen (200mg/kg on two consecutive days). The tissues were then harvested and weighed (n= 7 control, 6 *Pik3ca*^{H1047R}). The content of the intestine was removed before to record its weight. *Pten*^{-/-} mice and matched control mice were sacrificed 7 days **(B)** and 10 days **(C)** post tamoxifen administration (n=4 control, 6-8 *Pten*^{-/-}) and thus, the tissues collected and weighed. **(D)** *Pik3ca*^{H1047R}*Pten*^{-/-} mice and matched control mice were sacrificed 2 days post treatment with tamoxifen (n = 6 control, 5 *Pik3ca*^{H1047R}*Pten*^{-/-}). Thus, the tissues were harvested and weighed. Control mice are indicated by empty bars, whilst mutant mice are indicated by solid bars. Error bars represent mean $\pm$ SEM. * = p.value $\leq$ 0.05, ** = p.value $\leq$ 0.01, *** = p.value $\leq$ 0.001.

3.2.4. Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of the gene *Pten* lead to severe effect on glucose homeostasis.

In agreement with the well-established role of PI3K in glucose metabolism, our laboratory has previously shown that mice ubiquitously expressing the *Pik3ca*^{H1047R} mutation develop early and severe hypoglycaemia (Kinross et al., 2015).

To further extend these observations, mice ubiquitously expressing *Pten*^{-/-} and/or double mutant mice were assessed in order to determine if similar effects were observed. Blood glucose levels of control and mutant mice were monitored over time, post tamoxifen treatment. Consistent with previous findings (Kinross et al., 2015), *Pik3ca*^{H1047R} mice had early and severe hypoglycaemia, 4 days post tamoxifen treatment (Figure 3.5 A), whilst the levels of glucose in the blood of *Pten*^{-/-} mutant mice, started to drop 10 days post tamoxifen administration (Figure 3.5 B). This observation, together with the delayed increase in the tissue size of *Pten*^{-/-}-expressing mice, compared to *Pik3ca*^{H1047R} mice (Section 3.2.2), suggests that a mutation in the *Pik3ca* gene leads to a more rapid and efficient hyperactivation of the PI3K/AKT pathway, compared to the loss of the negative regulator *Pten*. Interestingly, when induced together, the two mutations resulted in a more severe hypoglycaemia with a rapid drop of glucose levels within 2 days post tamoxifen administration (Figure 3.5 C). This observation further supports our hypothesis that the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations have synergistic effects, *in vivo*.

Due to the drop in blood glucose, it was hypothesised that mutant mice had defective glucose homeostasis associated with a rapid clearance of glucose from the blood stream, therefore lowering the levels of blood glucose. To verify this hypothesis, a glucose tolerance test (GTT) was performed. For the purpose of the test the mice were fasted for 4 hours prior to glucose administration through oral gavage (2g/kg) and the blood glucose was measured 0, 15, 30, 60, 90 and 120 minutes post glucose administration in mice *Pik3ca*^{H1047R}, double mutant mice and their matched control mice. The blood glucose of *Pten*^{-/-} and matched control mice was measure 0, 15, 45, 90 and 120 minutes post glucose administration. The blood was not collected at 30 and 60 minutes for these mice due to a delay in the tail bleeding occurred during the experiment. GTT results were expressed as both a time course of absolute blood glucose measurements and as the area under the curve (iAUC) (Figures 3.5 D-

G). Interestingly, *Pik3cd*^{H1047R} and *Pten*^{-/-} mice cleared the glucose at the same rate and no difference in the rate of glucose clearance between mutant and control mice was detected. Only when the two mutations occurred together, did mutant mice clear the glucose from the blood stream quicker than control mice, with a rapid return to baseline 15 minutes post glucose administration.

Overall, mutant mice, as well as control mice, did not display abnormal glucose tolerance as blood glucose levels rapidly increased within 15 minutes post glucose administration and then returned to basal levels within 1 hour post glucose oral gavage.

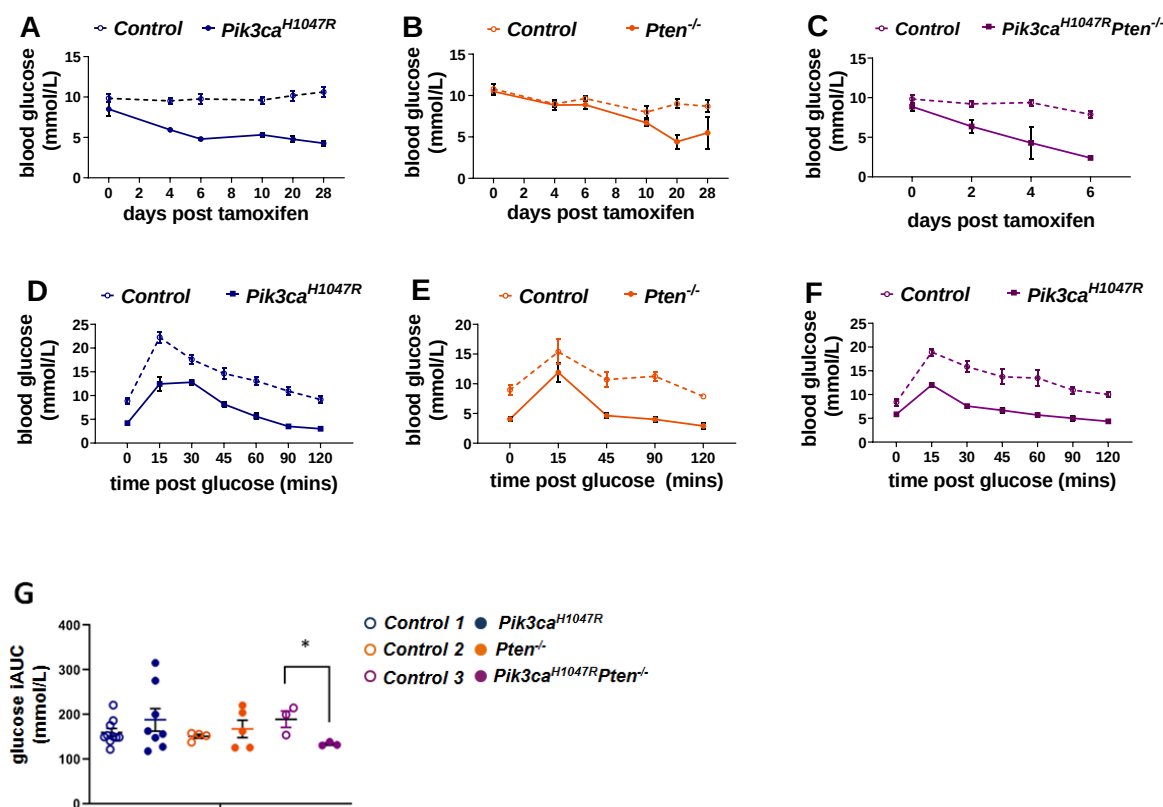


Figure 3. 5: Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of the gene *Pten* lead to severe effects on glucose homeostasis.

Graphs display blood glucose levels of **A)** *Pik3ca*^{H1047R}, **B)** *Pten*^{-/-}, **C)** *Pik3ca*^{H1047R}*Pten*^{-/-} mice and matched control mice, post treatment with tamoxifen (200mg/kg on two consecutive days). Blood glucose was measured daily for the lifespan of all the mice and expressed in mmol/L. Graphs show the GTT of **(D)** *Pik3ca*^{H1047R}, **(E)** *Pten*^{-/-}, **(F)** *Pik3ca*^{H1047R}*Pten*^{-/-} mice and matched control mice performed 7, 10 and 2 days post tamoxifen administration, respectively. Blood glucose measurements are expressed as absolute blood levels (mmol/L). Mice were fasted for 4 hours prior to glucose administration (2g/kg body weight) via oral gavage. Blood glucose was measured 0, 15, 30, 45, 60, 90 and 120 minutes post oral gavage in *Pik3ca*^{H1047R}, *Pik3ca*^{H1047R}*Pten*^{-/-} and matched control mice. Blood glucose was measured 0, 15, 45, 90 and 120 minutes post oral gavage in *Pten*^{-/-} and matched control mice. **(F)** The graph displays the level of blood glucose as the integrate area under the curve (iAUC) of *Pik3ca*^{H1047R} (blue), *Pten*^{-/-} (orange), *Pik3ca*^{H1047R}*Pten*^{-/-} (purple) mice and matched Control 1, Control 2 and Control 3 mice, respectively. N = 5-6 mice each genotype. Control mice are represented by dashed lines or empty dots, whilst mutant mice are represented by solid lines or solid dots. Error bars represent mean ± SEM. * = p.value ≤ 0.05.

3.2.5 Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of *Pten* lead to severe effects on insulin metabolism.

The results of the GTT, shown in Figure 3.5, demonstrated that the hypoglycaemia present in mutant mice was not associated with increased clearance of glucose from the blood. The GTT is the simplest and usually the first test applied to animal models but does not provide information on the mechanisms underlying the phenotype. To better characterise the effects of mutations in the PI3K pathway on glucose homeostasis, plasma insulin levels were measured in response to glucose administration. Mice were fasted for 4 hours prior to glucose administration by oral gavage (2g/kg). For each mouse, plasma samples were collected 0, 15 and 30 minutes post gavage of glucose, at exception of *Pten*^{-/-} and respective control mice, where plasma samples were collected 0, 15 and 45 minutes post glucose administration. This was due to a delay in the collection of plasma samples from the tail of *Pten*^{-/-} mice. An ELISA test was performed to quantify the levels of insulin in the plasma of the mice.

As expected, under normal physiological conditions the levels of insulin in the plasma of control mice increased within 15 minutes post glucose administration. As blood glucose was cleared from the blood stream, plasma insulin levels decreased, reaching almost basal levels 30 minutes post glucose administration (Figure 3.6 A). In contrast to control mice, *Pik3ca*^{H1047R} mice did not show glucose-dependent release of insulin in the plasma, indicated by the lack of increased levels of insulin post glucose administration. Interestingly, the levels of insulin in the plasma of mutant mice were below normal physiological levels even before administration of glucose (Figure 3.6 A). Similarly, double mutant mice had lower levels of plasma insulin than control mice, previous stimulation with glucose and had defective glucose-dependent insulin release (Figure 3.6 C). Interestingly, despite low basal insulin levels in the plasma of *Pten*^{-/-} mice, compared to control mice, the administration of glucose resulted in increased insulin release (Figure 3.6 B). This result might be associated to the low number of mice included in the experiment (the high variability between each mouse require the sample size to be bigger) or might be attributable to technical issues.

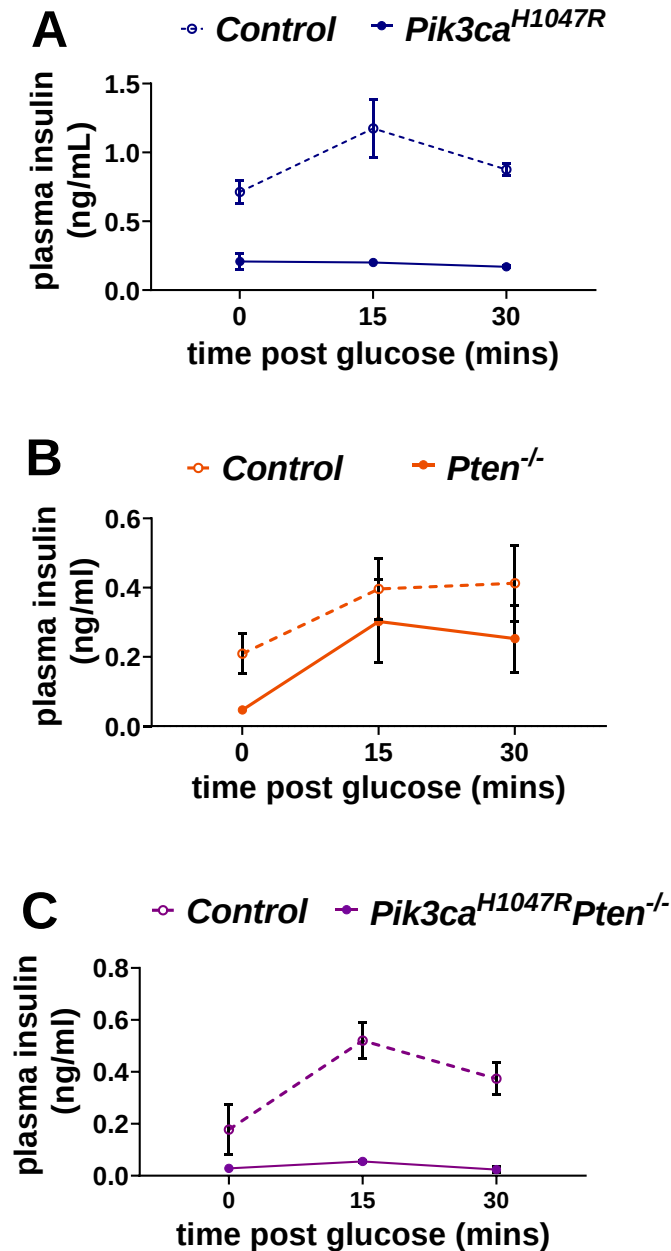


Figure 3. 6: Ubiquitous expression of Pik3ca^{H1047R} and/or loss of Pten lead to severe effects on insulin metabolism.

Graphs display the plasma insulin levels of **(A)** Pik3ca^{H1047R} , **(B)** $\text{Pten}^{-/-}$, **(C)** $\text{Pik3ca}^{H1047R} \text{Pten}^{-/-}$ and matched control mice treated with tamoxifen (200mg/kg on two consecutive days) and analysed 7,10 and 2 days after, respectively. Mice were fasted for 4 hours. Plasma samples of Pik3ca^{H1047R} , $\text{Pik3ca}^{H1047R} \text{Pten}^{-/-}$ and matched control were collected 0, 15 and 30 minutes post oral gavage of glucose (2g/kg body weight). Plasma levels of insulin were measured by ELISA and expressed as ng/ml. $n = 3-6$ mice each genotype. Control mice are represented by dashed lines, whilst mutant mice are represented by straight lines. Error bars represent mean $\pm$ SEM.

3.2.6 Ubiquitous expression of *Pik3ca*^{H1047R} and *Pik3ca*^{H1047R}*Pten*^{-/-} inhibits pancreatic release of insulin.

The previous section shows that mice harbouring the *Pik3ca*^{H1047R} mutation and/or loss of *Pten* had hypoinsulinemia and that their levels of plasmatic insulin did not increase in response to glucose stimulation. Since the *Pik3ca*^{H1047R} and/or *Pten*^{-/-} mutations are active in the pancreas of mutant mice, whether the hyperactivation of the PI3K pathway had direct inhibitory effect on the pancreatic islets, therefore stopping insulin release, was investigated. To assess this hypothesis mice harbouring the *Pik3ca*^{H1047R} mutation or both the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations were treated with tamoxifen for 7 and 2 days, respectively. Mice were then sacrificed and the pancreatic islet isolated. Thus, the islet cells were cultured in 3mM glucose, which represents the concentration of glucose sufficient to sustain the growth of the islets or in 20mM glucose which simulates glucose excess and induces rapid release of insulin from the islet cells. Levels of insulin in the medium were quantified using an ELISA test.

In the presence of 3mM glucose the islets isolated from control and *Pik3ca*^{H1047R} mutant mice released a similar amount of insulin into the medium. When stimulated with 20mM glucose the amount of insulin released by the islets of control mice increased, whilst the levels of insulin released by the islets of mutant mice remained unchanged (Figure 3.7 A). This suggests that the *Pik3ca*^{H1047R} mutation inhibits the release of insulin from the pancreatic islets. *Pik3ca*^{H1047R} *Pten*^{-/-} double mutant mice had similar insulin secretion to control mice at basal levels (3mM glucose), but the presence of 20mM glucose further stimulated insulin secretion in both control and mutant mice (Figure 3.7B).

To further validate whether the *Pik3ca*^{H1047R} mutation inhibits insulin release, *Pik3ca*^{H1047R} mice were treated with a PI3K inhibitor, BYL-719 (alpelisib-50mg/kg), 7 days post administration of tamoxifen. *Pik3ca*^{H1047R} mice treated with vehicle (0.5 % methylcellulose), instead of BYL-719, were used as control mice. Two hours post treatment with BYL-719, mice were stimulated with glucose and the plasma was collected. Basal levels of plasma insulin were measured using an ELISA test. Vehicle treated control *Pik3ca*^{H1047R} mice showed low levels of plasma insulin both at basal levels and after glucose stimulation, in agreement with previous observations. However, treatment with the PI3K inhibitor resulted

in increased levels of basal insulin release with a further slight increase post glucose stimulation (Figure 3.7 C). This data suggests that BYL-719 prevents the inhibitory effect of PI3K, allowing the pancreatic islets to release insulin.

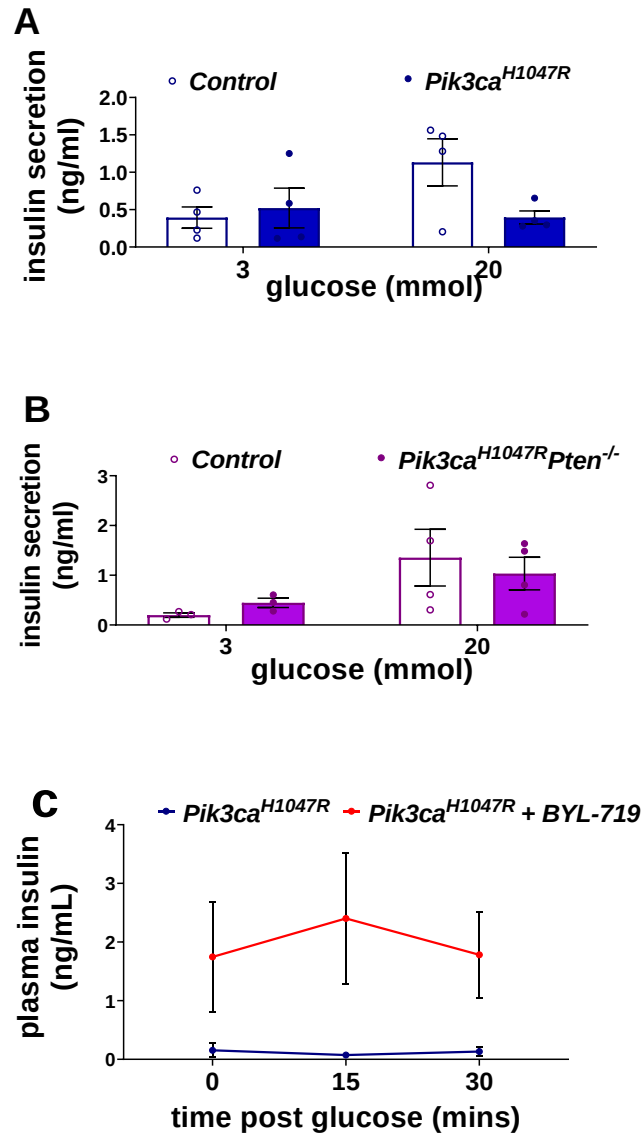


Figure 3. 7: Ubiquitous expression of $Pik3ca^{H1047R}$ inhibits pancreatic release of insulin.

Graphs display the insulin released into the medium by pancreatic islets isolated from (A) $Pik3ca^{H1047R}$ and (B) $Pik3ca^{H1047R} Pten^{-/-}$ mice, and matched control mice were treated with tamoxifen (200mg/kg on two consecutive days) 7, and 2 days before harvesting the islets, respectively. Islets were stimulated with 3mM and 20mM glucose and the medium collected. Insulin released into the medium was measured by ELISA and expressed as ng/ml. N= 4 pancreatic islets replicates. Control mice are represented by empty bars and empty dots, whilst mutant mice are represented by full bars and full dots. C) The graph displays the level of plasma insulin in $Pik3ca^{H1047R}$ mice treated with BYL-719 (50mg/kg) or an equivalent dose of 0.5% methylcellulose. Mice were administered tamoxifen 7 days prior to treatment with BYL-719. Two hours post BYL-719 administration mice were administered glucose (2g/kg body weight). Plasma was collected 0, 15 and 30 minutes post glucose stimulation. The blue line represents untreated mice, whilst the red line represents mice treated with BYL-719. Error bars represent mean $\pm$ SEM.

3.2.7 Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of *Pten* lead to increased glucose uptake in selective mouse tissues.

In the previous sections it was shown that mice carrying mutations in the PI3K pathway have severe hypoglycaemia and that the low levels of blood glucose are not dependent upon the action of insulin. A typical characteristic of highly proliferative cells is the switch to aerobic glycolysis and an associated increase in glucose uptake. Since mutant mice had a significant increase in organ size, it was hypothesised that the blood glucose was being taken up by the tissues in order to sustain the increased glycolytic metabolism. To assess this hypothesis, the uptake of two radio labelled glucose analogues, [¹⁸F]-fluoro-deoxyglucose (¹⁸F-FDG) and ¹⁴C-2-deoxy- D-glucose (2DG), were quantified in each individual tissue of the mice. Using *Pik3ca*^{H1047R} mice the two methods were compared. ¹⁴C-2DG was administrated by i.p. injection (2g/kg) and ¹⁸F-FDG was intravenously injected in the mouse tail (~2-3MBq per mouse) 7 days post tamoxifen administration, a time at which the drop in blood glucose and the increase in organs size were observed. It was found that the lungs, kidney and spleen of mutant mice took up more ¹⁸F-FDG tracer, compared to control mice. On the contrary, the muscle of *Pik3ca*^{H1047R} mice showed a slight drop in glucose uptake, compared to control mice (Figure 3.8B).

The uptake of ¹⁴C-2DG in individual tissues was highly variable between each mouse, therefore, the changes between the tissues of control and mutant mice were not statistically significant (Figure 3.8 A). Due to the high variability of the ¹⁴C-2DG uptake between tissues of different mice, the ¹⁸F-FDG tracer was chosen over the ¹⁴C-2DG tracer for the following investigation performed on *Pten*^{-/-} and *Pik3ca*^{H1047R}*Pten*^{-/-} mice.

To determine the destiny of glucose in *Pten*^{-/-} mice, ¹⁸F-FDG was injected 10 days post tamoxifen administration, a time point at which the glucose was cleared from the blood of these mice (Figure 3.5 B). Since double mutant mice do not survive longer than 4 days post tamoxifen administration, they were injected with the tracer 2 days post activation of the mutations with tamoxifen administration. Mice were sacrificed 1 hour after the tracer injection, tissue harvested and the distribution of ¹⁸F-FDG quantified using a scintillator counter.

Figure 3.8 C shows that *Pten*^{-/-} mice had increased ¹⁸F-FDG uptake in the large intestine and stomach, 10 days post tamoxifen administration, despite no increase in the size of these two tissues being observed at this time point (Figure 3.4 C).

In contrast to single mutant mice, none of the tissues of *Pik3ca*^{H1047R}*Pten*^{-/-} double mutant mice had altered glucose uptake, compared to control mice (Figure 3.8 D). Since double mutant mice did not show any increase in tissue size, compared to control mice (Figure 3.4 D), it was not surprising that their tissues did not uptake a higher amount of glucose, compared to control mice.

Figures 3.8 E and F show representative images of the PET scans performed on *Pik3ca*^{H1047R} and *Pik3ca*^{H1047R}*Pten*^{-/-} mice. PET-CT scans showed glucose uptake in the tail, heart and Harderian glands of control and mutant mice. However, it did not show any significant difference between *Pik3ca*^{H1047R} mutant and control mice, despite the changes observed in the bio distribution of the ¹⁸F-FDG tracer in individual tissues. This discrepancy might be explained by the fact that PET has a low intrinsic resolution which does not allow the characterisation of glucose distribution within the tissues. Due to this limitation, the scans were not repeated in *Pten*^{-/-} mice.

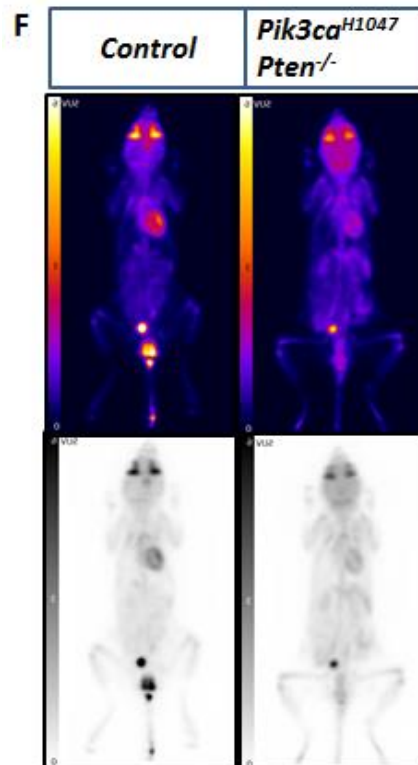
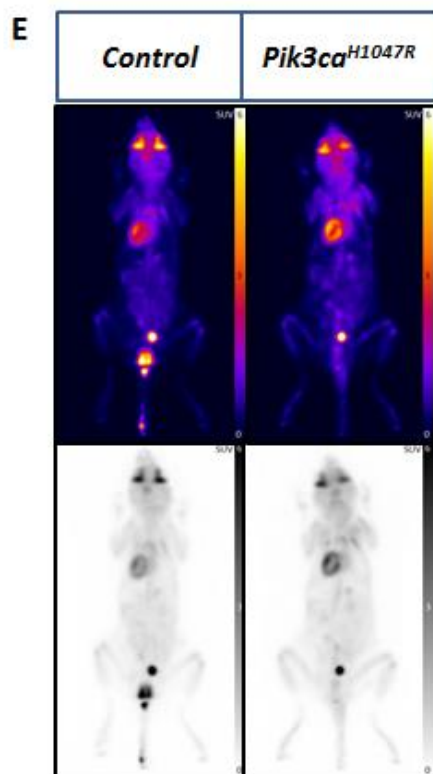
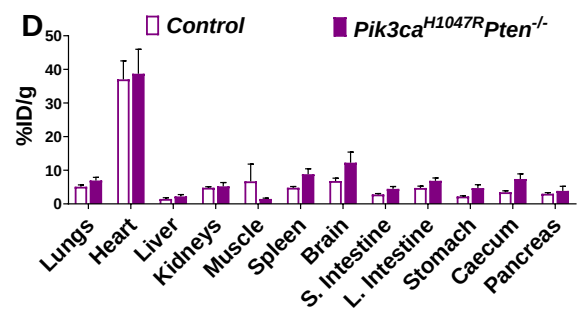
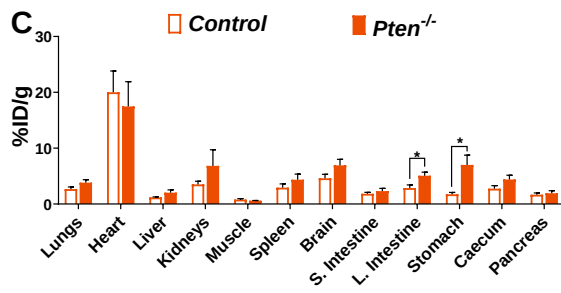
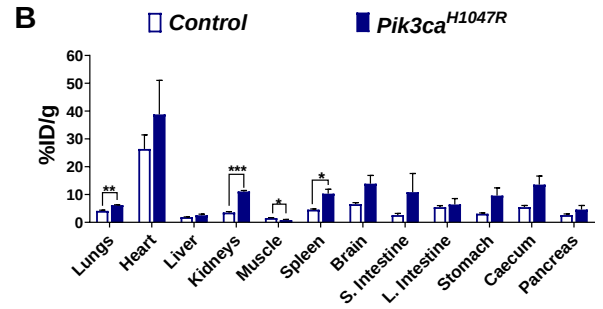
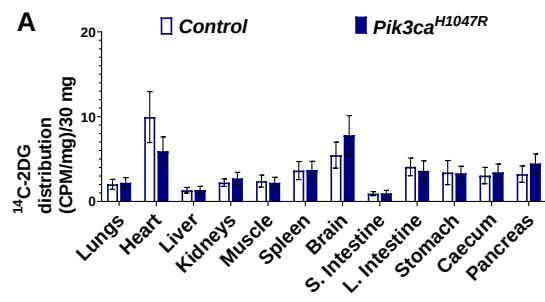


Figure 3. 8: Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of *Pten* lead to increased glucose uptake in selective mouse tissues.

A) Graph shows ¹⁴C-2DG bio-distribution in tissues of *Pik3ca*^{H1047R} and matched control mice. Mice were administered 2g/kg radioactive ¹⁴C-2DG by i.p. injection 7 days post treatment with tamoxifen (200mg/kg on two consecutive days). Mice were sacrificed 30 minutes post injection and 30 mg of each tissue was collected into scintillation tubes. Radioactivity was counted with a scintillation counter and expressed as CPM/g tissue weight (n=7 control, 6 *Pik3ca*^{H1047R}). Graphs display ¹⁸F-FDG bio-distribution in the tissues of **(A)** *Pik3ca*^{H1047R}, **(B)** *Pten*^{-/-} **(C)** and **(D)** *Pik3ca*^{H1047R}*Pten*^{-/-} mice. Mice were injected with ~2-3MBq radioactive ¹⁸F-FDG intravenously into the tail, 7, 10 and 2 days post tamoxifen administration, respectively. Mice were sacrificed 1 hour post injection and the tissues were collected into scintillation tubes. Radioactivity was measured using a γ-counter and expressed as CPM/tissue weight. Data were normalised to the time of injection and to the decay activity of the original injection solution and expressed as a percentage of the injected dose per gram of tissue mass (% ID/g). N=3-6 mice each genotype. Control mice are represented by empty bars, whilst mutant mice are represented by solid lines. Representative PET-CT scans of **(E)** *Pik3ca*^{H1047R} and matched control mice and **(F)** *Pik3ca*^{H1047R}*Pten*^{-/-} and matched control mice. Error bars represent mean ± SEM. * = p.value ≤ 0.05, *** = p.value ≤ 0.001.

3.2.8 Effect of *Pik3ca*^{H1047R} and/or *Pten*^{-/-} on lactate production.

One characteristic of highly proliferative cells is their reliance on aerobic glycolysis. The direct consequence of increased glycolytic metabolism is an increased need for glucose. Warburg observed that cancer cells tend to convert most glucose to lactate which is then partially used to generate NADPH and partially released into the blood stream where it acts as a paracrine signal (Vander Heiden et al., 2009). To assess if mice harbouring the *Pik3ca*^{H1047R} mutation had increased aerobic glycolysis, compared to control mice, the levels of lactate were measured in the plasma of mutant and control mice, using a commercial kit.

Mutant mice had similar levels of plasma lactate, independently of the genotype when measured at the end point (Figures 3.9 A-C). However, since plasma lactate can be shuffled inside and outside the tissues, the levels of plasma lactate can vary overtime. Therefore, plasma samples were collected from *Pik3ca*^{H1047R} and matched control mice 0, 4 and 7 post tamoxifen administration and plasma lactate concentration measured over time. Despite the fluctuation in levels of lactate in the plasma of control and mutant mice over time, no difference between control and mutant mice was observed (Figure 3.9 D).

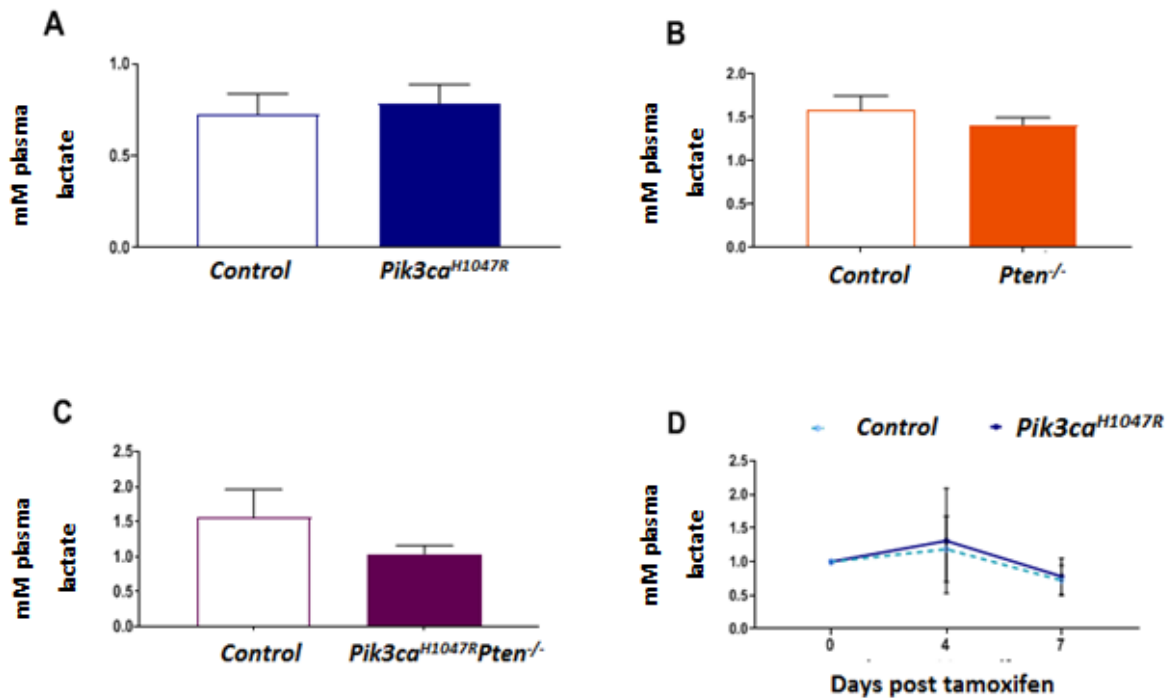


Figure 3. 9: Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of *Pten* do not alter plasma lactate levels.

Mice were treated with tamoxifen (200mg/kg) to activate the mutations respectively 7, 10 and 2 days before collection of plasma samples. Graphs display the plasma lactate levels of **(A)** *Pik3ca*^{H1047R}, **(B)** *Pten*^{-/-} and **(C)** *Pik3ca*^{H1047R}*Pten*^{-/-} and matched control mice. **D)** The graph shows the lactate concentration in the plasma of *Pik3ca*^{H1047R} and control mice 0, 4 and 7 days post tamoxifen administration. Plasma lactate was measured using a L-Lactate assay commercial kit (Jomar Life Research). Absorbance was measured at 490 nm (A_{490}). n= 3-7 mice of each genotype. Control mice are represented by empty bars or dashed lines, whilst mutant mice are represented by solid bars or solid lines. Error bars represent mean $\pm$ SEM.

3.2.9. Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of *Pten* block gluconeogenesis in the liver.

Many mechanisms control glucose homeostasis. Despite glucose uptake by metabolic active tissues, such as skeletal muscle and adipose tissue, glucose homeostasis is controlled by the activity of the liver which is responsible for glucose storage as well as endogenous glucose production. Impaired hepatic gluconeogenesis leads to homeostatic imbalance. For instance a drop in glucose production, in absence of nutrients, can cause hypoglycaemia. Since the hypoglycaemia observed in the mutant mice could not be explained by increased glucose uptake by the tissues, it was hypothesised that mutations in the *Pik3ca* and/or *Pten* genes induce hepatic dysfunction which leads to drop in gluconeogenesis and hypoglycaemia. This was tested using a pyruvate tolerance test (PTT) which utilises pyruvate as a precursor of glucose and assesses the ability of the liver to metabolise it to glucose, through gluconeogenesis. Mice were fasted for 3 hours prior to i.p. injection of pyruvate (2g/kg) and blood glucose levels measured 0, 15, 30, 60, and 90 minutes afterwards, to assess the rate at which pyruvate was metabolised to glucose. PTT results are expressed as time course of absolute blood glucose measurements (Figure 3.10). Before pyruvate administration the levels of glucose in the blood of *Pik3ca*^{H1047R}, *Pten*^{-/-} and *Pik3ca*^{H1047R}*Pten*^{-/-} mutant mice were already under physiological levels, consistent with the results shown above.

Whilst control mice produced glucose from pyruvate, as shown by the increase in blood glucose within 15 minutes post injection of pyruvate, no release of glucose into the blood stream was detected in response to pyruvate administration in the mutant mice. Indeed, the blood glucose of mutant mice remained under physiological levels (less than 5mmol/L) for the whole duration of the experiment, independently of the type of mutation (Figure 3.10 A-C). This result supported our hypothesis that the ubiquitous expression of mutations in the PI3K pathway blocks the ability of the liver to undergo gluconeogenesis.

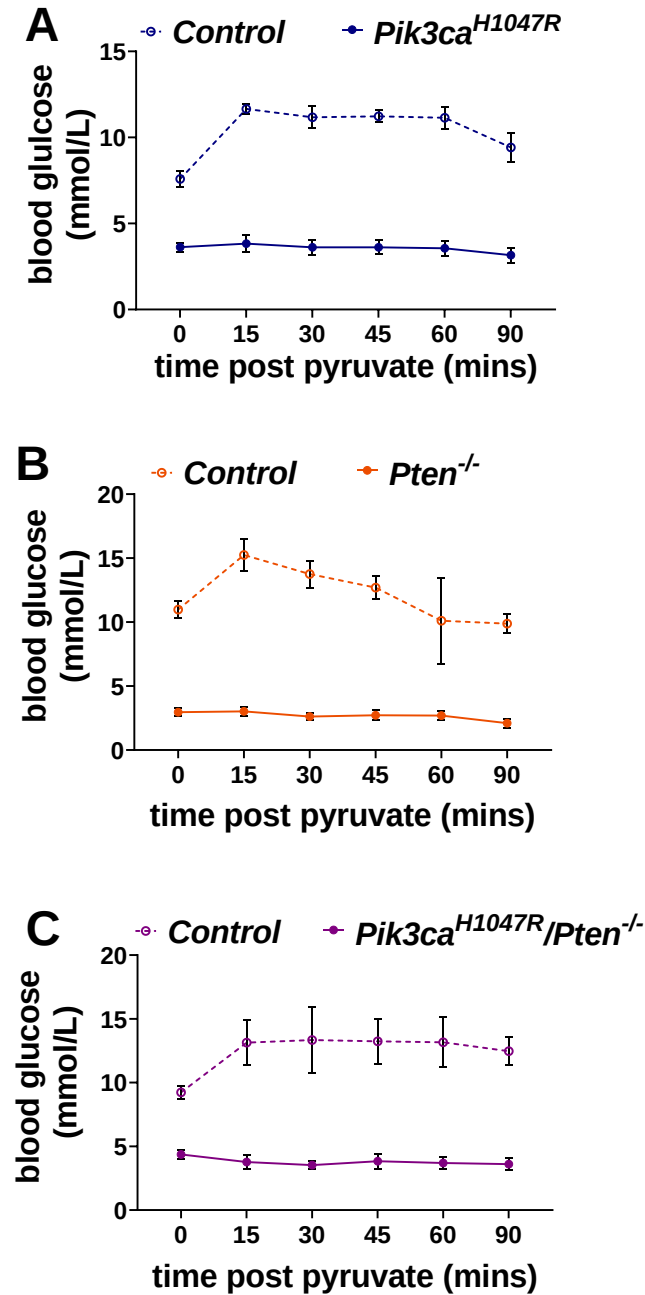


Figure 3. 10: Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of *Pten* block hepatic gluconeogenesis.

Graphs display the PTT of (A) *Pik3ca*^{H1047R}, (B) *Pten*^{-/-} and (C) *Pik3ca*^{H1047R}/*Pten*^{-/-} and matched control mice. Mice were treated with tamoxifen (200mg/kg) to activate the mutations respectively 7, 10 and 3 days before performing the PTT. A-C) Blood glucose measurement expressed as absolute blood levels (mmol/L). Mice were fasted for 3 hours prior to administration of pyruvate (2g/kg body weight) by i.p. injection. Blood glucose was measured 0, 15, 30, 45, 60 and 90 minutes post injection of pyruvate. n= 3-7 mice each genotype. Control mice are represented by dashed lines, whilst mutant mice are represented by solid lines. Error bars represent mean $\pm$ SEM.

3.2.10. Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of *Pten* reduce the expression of enzymes which catalyse hepatic gluconeogenesis.

Hepatic pyruvate metabolism is catalysed by the enzymes Pck1 and G6pc (Section 3.1.3). Since we have demonstrated that mutant mice had lost the ability to metabolise pyruvate to glucose, it was hypothesised that the expression of enzymes that catalyse gluconeogenesis might be reduced in mutant mice, compared to control mice. To assess this hypothesis a real-time q-RT-PCR (Real-Time quantitative reverse transcription PCR) was performed using RNA extracted from the liver of *Pik3ca*^{H1047R}*UbCre*, *Pten*^{-/-}*UbCre* and *Pik3ca*^{H1047R}/*Pten*^{-/-}*UbCre* mutant and respective control mice.

Figures 3.11 A-C show a reduction in the mRNA levels of the enzyme *G6pc* in all the mutant mice, compared to control mice, independently of the genotype. The mRNA levels of the enzyme *Pck1* had a tendency toward a decrease in each mutant mouse, compared to respective control mice, but for each genotype it was not statistically significant (p value was >0.05). This data suggests that mutations in the PI3K pathway alter the expression of glycolytic enzymes, further supporting the hypothesis of defective hepatic gluconeogenesis.

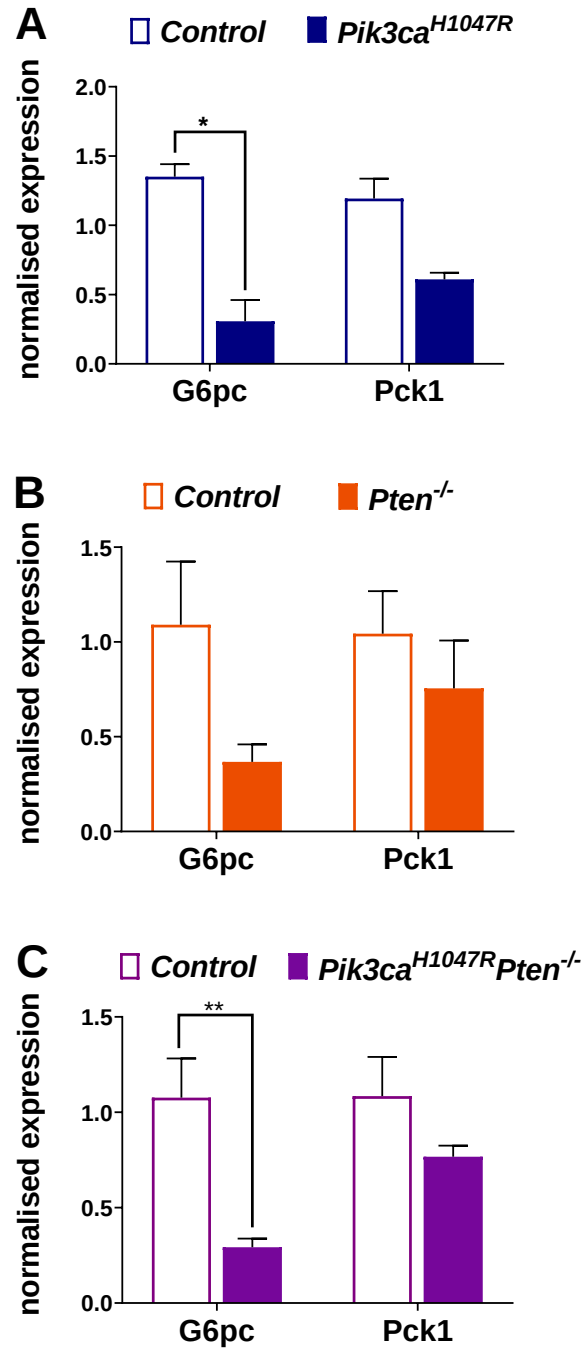


Figure 3. 11: Ubiquitous expression of *Pik3ca*^{H1047R} and/or loss of *Pten* block hepatic gluconeogenesis.

Graphs display the mRNA levels of *G6pc* and *Pck1* in (A) *Pik3ca*^{H1047R}, (B) *Pten*^{-/-} and (C) *Pik3ca*^{H1047R}*Pten*^{-/-} mice and matched control mice treated with tamoxifen 7, 10 and 3 days respectively before to be sacrificed. The liver was then harvested. Changes in gene expression are normalised against β -actin. Control mice are represented by empty bars, whilst mutant mice are represented by solid bars. N = 3 to 6 mice each genotype. Error bars represent mean $\pm$ SEM. * = p.value ≤ 0.05 , ** = p.value ≤ 0.01 .

3.2.11. Ubiquitous expression of the *Pik3ca*^{H1047R} mutation inhibits hepatic glucose release.

It has been demonstrated, in the previous two sections of this chapter, that mice harbouring mutations in the PI3K pathway have impaired gluconeogenesis. However, despite generating glucose by *de novo* glucose synthesis from available precursors (gluconeogenesis), the liver can also generate glucose by glycogen breakdown (glycogenolysis). To confirm that the glucose was not released from the liver, therefore resulting in hypoglycaemia, we performed a hepatic glucose production test (HGP) in the liver of control and mutant mice. For this test we used Infinity™ Glucose Oxidase Liquid Stable Reagent (Thermo Scientific), which detects the oxidation of glucose by glucose oxidase to gluconic acid and hydrogen peroxide. The hydrogen peroxide reacts in the presence of peroxidase with 4-hydroxybenzoic acid and 4- aminoantipyrine forming a red quinoneimine dye. The intensity of the colour formed is proportional to the glucose concentration and can be measured photometrically between 460 and 560 nm.

When nutrients are available, insulin is secreted from pancreatic β cells and promotes hepatic glycogen synthesis and lipogenesis (Sharabi et al., 2015). We reproduced this process by stimulating the cells with insulin and then measured the concentration of glucose found in the media, where the liver was incubated. As expected, the liver of control mice reduced glucose release in response to insulin stimulation. Interestingly, the liver of mutant mice released less glucose than control mice under basal conditions and stimulation with insulin did not lower it any further (Figure 3.12).

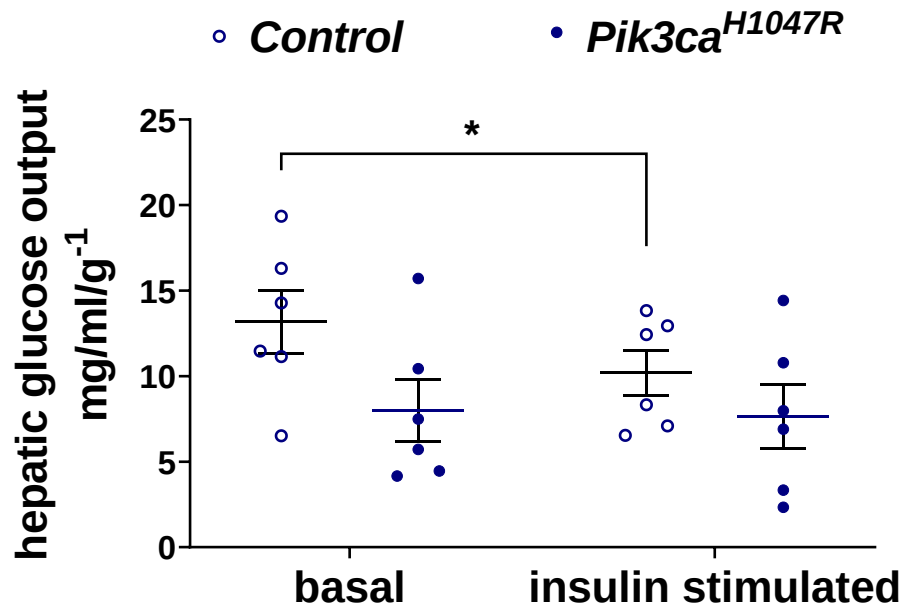


Figure 3. 12: Ubiquitous expression of *Pik3ca*^{H1047R} inhibits hepatic glucose output.

Graph shows the hepatic glucose production in *Pik3ca*^{H1047R} and matched control mice treated with tamoxifen for 7 days. The liver was harvested and cut into slices. Glucose production was assessed using a colorimetric assay, which accounts for the amount of glucose that is oxidized to produce hydrogen peroxide. The intensity of the colour formed is proportional to the glucose concentration and was measured photometrically at 500 nm. Error bars represent mean $\pm$ SEM. * = p.value $\leq$ 0.05.

3.3 DISCUSSION

Overall, this chapter showed that hyperactivation of the PI3K pathway leads to reduced survival, and metabolic dysfunctions in mice. Oncogenic activation of the PI3K pathway results in defective hepatic gluconeogenesis and loss ability of the pancreas to release insulin, therefore resulting in hypoglycaemia and hypoinsulinemia.

The data shown in this chapter demonstrate that the ubiquitous expression of mutations in the *Pik3ca* and *Pten* genes that activate the PI3K pathway are sufficient to induce early lethality in mice and that the two mutations synergize to drastically reduce the survival of the mice.

The *Pik3ca*^{H1047R} mouse model used in this study was previously generated in our laboratory, to express the heterozygous *Pik3ca*^{H1047R} mutation in every organ of the mouse (Kinross et al., 2015). Using this mouse model, our laboratory showed that a mutation in the *Pik3ca* gene reduced the survival of the mice, compared to control mice, with a median survival of 46.5 days post tamoxifen treatment (Kinross et al., 2015). However, when the analysis was repeated for the purposes of this project, survival of mutant mice was further reduced with a median of 25.5 days post tamoxifen treatment. This inconsistency in the survival rate is thought to be due to the different type of nutrition that the mice had access to. A common effect of the ubiquitous expression of the *Pik3ca*^{H1047R} mutation is the development of malocclusion occurring 3 weeks post treatment with tamoxifen. Mice developing malocclusion have difficulty in accessing the standard chow food and consequently reduce their food intake, further compromising the levels of blood glucose. To assure that the mice received the right amount of nutrients, previous investigators in our laboratory have fed the mice with Ensure nutritional powder, which is easy to chew and supplies all the nutrients contained in the standard chow diet. Alternatively the food was mashed to facilitate chewing. Those methods allowed extending the life span of the mice from 3 to 6 weeks post tamoxifen administration. However, since this project is focused on understanding the metabolic reprogramming occurring at the earliest time point at which the effects of the mutations *Pik3ca*^{H1047R} manifest (7 days post tamoxifen), it was unnecessary to extend the survival of the mice any further. Therefore, mice were kept on standard chow diet throughout the whole duration of the experiments.

In the same study performed on *Pik3ca*^{H1047R} mice, our laboratory showed that ubiquitous expression of the *Pik3ca*^{H1047R} mutation leads to increased body size associated with organomegaly. Interestingly, the increase in body weight was not associated to adiposity as no deposition of adipose tissue was observed (Kinross et al., 2015). Here we show that one week post treatment with tamoxifen several tissues of *Pik3ca*^{H1047R} mice, specifically lungs, spleen, brain, intestine, stomach and pancreas had increased in size (Kinross et al., 2015).

In contrast to the previous 3 week study (Kinross et al., 2012), no increase in the size of the liver was observed 7 days post tamoxifen treatment, suggesting that the liver requires a longer time to increase in size, compared to other tissues. The increase in tissues size observed in our mouse model is in agreement with the well characterised role of mutations in the *Pik3ca* gene as the underlying cause of several overgrowth syndromes. Indeed, Shioi et al. have also previously observed that constitutive activation of *Pik3ca* results in an increase in heart size (Shioi et al., 2000). Furthermore, *Pik3ca*^{H1047R} expression in neural progenitor results in brain enlargement, cortical malformation and epilepsy (Roy et al., 2015).

In 2018, Venot et al. published the first mouse model reproducing the typical characteristics of patients affected by non-cancerous overgrowth disorders known as *Pik3ca*-related overgrowth spectrum (PROS) (Keppler-Noreuil et al., 2014; Kurek et al., 2012; Lee et al., 2012b; Luks et al., 2015). Using a chimeric *Pik3ca* transgene (*R26StopFLP110**) with *CAGG-Cre*^{ER} to generate mice that ubiquitously express a dominant active *Pik3ca* upon tamoxifen administration, Venot et al. showed that mice harbouring *Pik3ca* mutations develop organomegaly, asymmetrical overgrowth of extremities, and subcutaneous vascular abnormalities (Venot et al., 2018). Whilst our mice were injected with a dose of 200mg/kg of tamoxifen, resulting in most of the organs having abnormal growth, Venot and colleagues used a dose of tamoxifen of 4 mg/kg, allowing only a mosaicism of *Pik3ca* mutations throughout the body, resulting in mosaic overgrowth.

This chapter also demonstrates that the ubiquitous expression of the *Pik3ca*^{H1047R} mutation leads to severe defects in glucose homeostasis. In a normal setting, the insulin-dependent activation of the PI3K pathway occurs after a meal, when the increase in blood glucose induces the pancreatic release of insulin. Once released, insulin stimulates metabolically active tissues, such as, muscle and adipose tissue, to take up more glucose and promote

glycolysis and glycogenesis in liver and muscle, until the levels of blood glucose are brought back to physiological levels (Roder et al., 2016). Therefore, pathologic perturbation of the PI3K pathway has been associated with insulin resistance (Winnay et al., 2016), obesity and diabetes (Huang et al., 2018; Winnay et al., 2016), and tumour growth, especially of tumours that switch to a heavily glucose-dependent metabolism to meet their increased metabolic demand (Lien et al., 2016).

Interestingly, the levels of insulin in the plasma of mutant mice were exceptionally low, compared to control mice, and basal levels (prior stimulation with glucose) and the administration of glucose did not result in increased levels of insulin in the plasma (Kinross et al., 2015). It was thought that one possibility for the reason behind the unexpected lack of insulin release, post glucose stimulation, could lie in the design of the experiment. The most common methods for the administration of glucose in rodents are by oral gavage and by single i.p. injection. Both routes of administration are widely accepted as appropriate methods for administering glucose. However, the i.p injection is subject to error as a result of injection into intestines or stomach rather than the intraperitoneal space (Arioli and Rossi, 1970), which affects the rate at which the glucose appears in the circulation and the plasma insulin response. The absorption of glucose from the gut leads to the incretin-mediated release of gastrointestinal hormones, primarily the Glucagon-like peptide 1 (GLP1), which in turn potentiates glucose-induced insulin release (Pais et al., 2016). The effect of incretin following oral delivery of glucose results in a more elevated insulin secretory response which consequently lowers blood glucose levels relative to i.p. glucose injection, where the incretin effect is absent.

To investigate if the delivery of glucose by i.p. injection was masking the release of insulin in mice used in the previous study, plasma samples from a cohort of *Pik3ca*^{H1047R} and control mice that had received glucose by oral gavage were collected and thus, the plasma insulin levels were measured by ELISA. Surprisingly, the results confirmed what had previously been observed. In addition to initial low insulin levels in the plasma of mutant mice, the insulin levels did not increase after stimulation with glucose. This excluded the hypothesis that hypoglycaemia was due to the scavenger effect of insulin on glucose.

Similarly, the GTT analysis performed after administration of glucose by oral gavage showed similar results of to that previously observed when glucose was administered by i.p. injection (Kinross et al., 2012). *Pik3ca*^{H1047R} mice cleared the glucose at the same rate as control mice and did not show defects in tolerating glucose. This data indicates that both i.p. injection and oral gavage are valid methods for the glucose administration for assessing plasma insulin and/or GTT analysis and that hypoglycaemia and hypoinsulinemia were not mutually dependent events.

The hypothesis that mutations in the PI3K pathway lead to inhibition of insulin release from the pancreatic islets was tested in this chapter by using the PI3K inhibitor BYL-719. Administration of the inhibitor restored insulin release in *Pik3ca*^{H1047R} mice, suggesting that the drug can remove the inhibitory effect of PI3K, therefore allowing the islets to respond to glucose stimulation by releasing insulin. To assess whether the inhibitory effect of PI3K was directly affecting the pancreas, as opposed to be the indirect consequence of the effect of BYL-719 on other metabolic tissues, pancreatic islets were isolated from *Pik3ca*^{H1047R} and matched control mice and their ability to release glucose was investigated *in vitro*. Stimulation with a high concentration of glucose induced insulin release in control mice, whilst it had no effect on insulin secreted by the pancreatic islets isolated from *Pik3ca*^{H1047R} mutant mice. This data further supports the hypothesis that the expression of a mutation in the PI3K pathway inhibits pancreatic release of insulin. A similar trend towards a reduction in insulin release in the presence of 20mM glucose was also observed in *Pik3ca*^{H1047R}*Pten*^{-/-} double mutant mice. However, the difference between the insulin released by the pancreas of control and *Pik3ca*^{H1047R}*Pten*^{-/-} mice was not significant. This data was surprising since double mutant mice showed the same defects of insulin release as single mutant mice and might be due to an incomplete activation of the mutations with tamoxifen administration. This hypothesis is yet to be tested.

Despite no increase in insulin release in *Pik3ca*^{H1047R} mice, an increase in glucose uptake in the lungs, kidney and spleen of mutant mice was observed, compared to control mice when using the ¹⁸F-FDG tracers. The fact that the amount of glucose taken up by the tissues of mutant mice increased even in the presence of low levels of plasma insulin, is possibly due to two factors: first, since the PI3K pathway is ubiquitously expressed in the body it is possible that the tissues, have switched to aerobic glycolysis and therefore need to uptake more

glucose to generate energy in the form of ATP. This is typical of cancer cells or highly proliferative cells, which need more intermediates to generate new macromolecule. Since *Pik3ca*^{H1047R} mice present organomegaly, it is likely that the tissue need more glucose to sustain their increased energetic demand. Second, the induced hyperactivation of the PI3K mutation, mimics the insulin pathway so that, even in absence of insulin in the plasma, the body activates the normal response to the insulin/PI3K activation.

As described above, one of the first responses to increased insulin is induction of glucose uptake by the tissues in order to re-establish the physiological levels of glucose in the circulation. Despite an increase in ¹⁸F-FDG uptake in some tissues, no significant changes were observed in the ¹⁴C-2DG tracer uptake between control and mutant mice. Mutant mice, indeed, had a slight increase in 2-DG uptake, compared to control mice but it was not statistically significant. A limitation of the 2DG uptake assay is that only 30mg of the tissues were used to quantify the tracer uptake (¹⁴C), as opposed to the ¹⁸F-FDG uptake, where the radioactivity is counted in the whole tissue (¹⁸F). Since the distribution of these tracers is not homogenous within the tissue, using only part of the tissue instead of its whole might not give a real representation of glucose uptake. Another difference between the two techniques is that, whilst ¹⁸F-FDG is administrated intravenously in the tail of the mice, ¹⁴C-2DG is administrated by i.p. injection. The two routes of administration might result in differences in glucose uptake in the tissues. However, Koon-Pong Wong and colleagues proved that the i.p. is a valid alternative route, providing pharmacokinetic data equivalent to data from tail-vein injection for small-animal ¹⁸F-FDG PET (Wong et al., 2011). Therefore the hypothesis that the differences observed between the two methods were attributable to the route of administration of the tracers was excluded.

Discrepancies between the ¹⁸F-FDG bio-distributions in individual tissues and the PET scan done on the whole body were also observed. This might be due to the low intrinsic resolution of the PET which does not allow the characterisation of the glucose distribution within the tissues. New imaging techniques have been coupled with ¹⁸F-FDG to improve the imaging of intracellular distribution of glucose, including the fluorescence diffuse optical tomography technology (Garofalakis et al., 2013). However, high resolution distributions of glucose in an animal model are still missing.

In this chapter the levels of lactate have been measured in the plasma of control and mutant mice to support the hypothesis that mutations in the PI3K pathway lead to increased aerobic glycolysis. Otto Warburg, indeed, showed that tumour cells uptake high amounts of glucose producing large volumes of lactate even in the presence of oxygen (Warburg, 1956).

It was observed that mutations in the PI3K pathway do not alter the levels of plasma lactate. However, under physiological conditions, lactate is taken up by the heart, brain and skeletal muscle. The uptake of lactate by the tissues determines its clearance from the blood (Gladden, 2004; Philp et al., 2005). According to this, two potential mechanisms could have determined the lack of difference in lactate levels between control and mutant mice: 1) control and mutant mice produced the same amount of lactate; 2) the increase in lactate production in control or mutant mice was accompanied by increased uptake of lactate by the tissues. However, it was not possible to discriminate between these two events. The use of radio-labelled nutrients could help to better investigate the shuttling of lactate *in vivo*. Many studies, indeed, have used radio-labelled tracer to investigate lactate metabolism *in vivo* and *in vitro* (Bok et al., 2019; Faubert et al., 2017; Park et al., 2016).

The liver plays a major role in maintaining glucose homeostasis, as it is the main organ for glucose storage, in the form of glycogen, as well as a major source of endogenous glucose production. In conditions of low nutrients, insulin levels are decreased and glucagon is secreted from pancreatic α cells to promote HGP (Sharabi et al., 2015). HGP is achieved by glycogen breakdown (glycogenolysis) as well as by *de novo* glucose synthesis from available precursors (gluconeogenesis). In this chapter it was shown that expression of the *Pik3ca*^{H1047R} mutation in the liver inhibits gluconeogenesis in the liver, by inhibiting the two glycolytic *G6pc* and *Pck1* in the mutant mice. This is in agreement with the well-known role of the insulin-PI3K axis in stopping gluconeogenesis by regulation of the glycolytic enzymes and is described more in detail in the section 1.4.6 of this thesis. It is also shown that the *Pik3ca*^{H1047R} mutation inhibits HGP. Consistent with this finding, Liu and colleagues found that Irisin, a newly identified myokine, induces the browning of white adipose tissue, inhibits hepatic gluconeogenesis and increases glycogen synthesis via the PI3K/AKT pathway in type 2 diabetic mice and hepatocytes (Liu et al., 2015). It is also well established that insulin resistance interferes with hepatic glucose production (Santoleri and Titchenell, 2019).

Other than the liver, the kidneys' contribute to maintaining glucose homeostasis by releasing glucose into the circulation, taking up glucose from the circulation to satisfy their energy needs, and through reabsorption of glucose at the level of the proximal tubule. Renal release of glucose into the circulation is the result of glycogenolysis and gluconeogenesis, respectively involving the breaking down and formation of glucose-6-phosphate from precursors, such as lactate (Triplitt, 2012). Although, renal gluconeogenesis has not been investigated in this thesis, it might affect the level of blood glucose in mice harbouring systemic activation of the PI3K pathway. The kidneys normally absorb as much glucose as possible from plasma through glucose transporter proteins that are present in cell membranes within the proximal tubules. If the capacity of these transporters is exceeded, glucose appears in the urine (Triplitt, 2012). The ability of the kidneys to filter glucose from the proximal tubes was tested by measuring the levels of glucose in the urine of the mice. Preliminary data didn't show any difference between the level of glucose in the urine of control and mutant mice. However, the data available is not sufficient to exclude the role of kidneys in the hypoglycaemia observed in the *Pik3ca*^{H1047R} mouse model. Therefore, the data has not been shown in this thesis.

The PI3K pathway can be enhanced by activating mutations in the *PIK3CA* gene or loss of its negative regulator, *PTEN*. Inactivating mutations in the *PTEN* gene are often found in cancer and are responsible for metabolic reprogramming (Chen et al., 2018a; Chen et al., 2018b; Morani et al., 2014; Patel et al., 1996). PTEN is a dual phosphatase with both protein and lipid phosphatase activities, that reduces the level of PIP₃ to PIP₂, therefore acting as a tumour suppressor by returning the PI3K/AKT pathway back to basal levels and suppressing insulin sensitivity. It has also been shown that the deletion of the *Pten* gene leads to enhanced insulin and metabolic sensitivity in adipose tissue (Kurlawalla-Martinez et al., 2005), liver (Stiles et al., 2004; Stiles et al., 2006) and pancreatic β -cells (Stiles et al., 2006).

In this chapter we have demonstrated that the loss of *Pten* induces a similar metabolic response to *Pik3ca*^{H1047R} *UbCre*⁺ mice, resulting in reduced survival, increased body weight and tissue size, a drop in blood glucose and hypoinsulinemia, suggesting that the alteration of the PI3K/AKT pathway is responsible for the observed phenotype despite where in the PI3K pathway the mutation occurs. However, since mice harbouring the *Pten*^{-/-} mutation manifested the phenotype later in time compared to mice harbouring the *Pik3ca*^{H1047R}

mutation, this suggests that overexpressing an activator of the PI3K/AKT pathway is more efficient than blocking its negative regulator.

It was demonstrated that the two mutations, *Pten*^{-/-} and *Pik3ca*^{H1047R} have synergistic effect on systemic metabolism leading to severe hypoglycaemia and hypoinsulinemia and early death. However, *Pik3ca*^{H1047R}*Pten*^{-/-}*UbCre*⁺ mice did not show organomegaly and increased body weight, most likely due to the early lethality of double mutant mice and thus insufficient time for increased organ and/or body size to manifest. The synergistic effect of the two mutations is in agreement with the fact that mutations in the *Pik3ca* gene, do not induce spontaneous tumorigenesis when endogenously activated, but rather cooperate with mutations in *Pten* (Kinross et al., 2012; Pearson et al., 2018; Van Keymeulen et al., 2015) to enhance the initiation and/or progression of tumorigenesis.

The metabolic effects that were observed in mice harbouring the *Pten*^{-/-} and/or *Pik3ca*^{H1047R} mutations are opposite to what is described in the literature, where the role of mutations in the PI3K/AKT pathway have been well described as responsible for insulin insensitivity and type 2 diabetes (Stiles et al., 2004; Wijesekara et al., 2005). This might be due to the fact that mutations in the PI3K pathway are expressed ubiquitously in our mouse models as opposed to mouse models present in the literature, where mutations in the PI3K pathway are induced only in selective tissues. How the tissues influence each other's metabolism and how the ubiquitous expression of mutations in the PI3K pathway affect this inter-communication will be explored in the following chapter. Another difference in the mouse model presented in this thesis is that the *Pik3ca* mutation has been induced in the *Pik3ca* endogenous gene as opposed to inducing a transgenic overexpression of *Pik3ca*. It has been shown, indeed, that mice expressing mutations at endogenous levels have different responses compared to when overexpressed. For instance, whilst endogenous expression of *Pik3ca*^{H1047R} mutation did not induce tumorigenesis in mice, unless occurring together with a mutation in a second gene, when the *Pik3ca*^{H1047R} mutation was driven by exogenous promoters, the mice developed tumours (section 1.8.1).

3.3.1 Overall findings

Overall, this chapter showed that hyperactivation of the PI3K pathway leads to reduced survival and metabolic alterations of mice, independently on whether the mutations occur in the *Pik3ca* or *Pten* gene. Both the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations induces organomegaly in mice. However, mice harbouring both the mutations do not manifest organomegaly due to their early death.

This chapter also showed that mutations in the PI3K pathway lead to hypoglycaemia associated with defects in hepatic gluconeogenesis in *Pik3ca*^{H1047R}, *Pten*^{-/-} mice and double mutant mice. Furthermore, hepatic glucose production was inhibited in *Pik3ca*^{H1047R} mice, but this has not yet been tested in *Pten*^{-/-} and *Pik3ca*^{H1047R} *Pten*^{-/-} mice. Therefore, further analysis need to be done to assess the role of *Pten* loss and its combination with the *Pik3ca*^{H1047R} mutation in HPG.

Finally, this chapter has shown that ubiquitous expression of the *Pik3ca*^{H1047R} mutation in mice directly inhibits pancreatic islets to release insulin, therefore driving hypoinsulinemia in mice.

CHAPTER 4

INVESTIGATING THE EFFECTS OF THE UBIQUITOUS EXPRESSION OF THE *Pik3ca*^{H1047R} MUTATION ON SYSTEMIC METABOLISM

4.1 INTRODUCTION

In the previous chapter it was demonstrated that the expression of the *Pik3ca*^{H1047R} mutation in every organ of the mouse caused abnormal growth of a large number of organs and a decrease in blood glucose and plasma insulin to below physiological levels. Since the metabolic alterations were not limited to individual tissues but affected the entire organism, the studies were extended to the systemic metabolism of the mice to determine what metabolic adaptation the body had undergone to sustain its growth. This chapter presents the *in vivo* analysis of the body composition and the energetic metabolism of the mice, combined with *in vivo* heavy water (²H₂O) labelling and *ex vivo* analysis of lipid metabolism in mice harbouring the *Pik3ca*^{H1047R} mutation.

4.1.1 Characterise the energetic metabolism of mice harbouring the *Pik3ca*^{H1047R} mutation.

Mammals derive energy from nutrients through the oxidation of food substrates to carbon dioxide (CO₂), water (H₂O) and heat in the presence of oxygen (O₂). The energy generated can be measured by direct and indirect calorimetry. Direct calorimetry measures the energy as the heat exchanged by the animal and the environment (Kenny et al., 2017), whereas indirect calorimetry measures the respiratory gases, O₂ and CO₂, used during metabolic processes (Mtaweh et al., 2018). Indirect calorimetry presents a couple of advantages compared to the direct calorimetry (Speakman, 2013). First, whilst it is possible to measure O₂ and CO₂ gases with great accuracy, measuring small changes in heat is technically difficult. Second, direct calorimetry generates errors in the calculation of the energy expenditure (EE) because it does not account for the amount of heat that is stored in the body during the measurement period. Indirect calorimetry does not have this limitation since no significant levels of O₂ and CO₂ are stored in the body (Speakman, 2013).

The ratio of O₂ consumption to CO₂ production, gives the respiratory quotient (RQ), which is used to determine which substrate is oxidised to produce energy. Since RQ can only be calculated at cellular levels it is generally interchanged with the RER, which is calculated by using the air expelled through the lungs (Speakman, 2013). RER is an indicator of the type of fuel that is being metabolized: if the proportion between the volume of CO₂ (VCO₂) generated to the volume of O₂ (VO₂) consumed through respiration is equal or just above 1, carbohydrates are the main source of energy. When the VCO₂/VO₂ ratio is about 0.7, fat is the main source of energy, whilst a RER between 0.7 and 1 indicates that both carbohydrates and fat are used to generate energy (Haugen et al., 2007).

The energy expenditure is usually carried out using indirect calorimetry. Until 1949 the EE was calculated using the following formula: total EE (kJ/min) = kilojoules per litre O₂ consumed × VO₂ (Speakman, 2013). Since this formula did not account for the nitrogen produced via the urine to calculate protein oxidation, Weir, in 1949, proposed a new method to accurately calculate EE using RER, which also adjusts for estimated protein metabolism, based on dietary composition (Weir, 1949). The method was empirically validated in 2019 by Kaiyala and colleagues (Kaiyala et al., 2019), who defined it as a valid alternative method to the standard EE calculator. However, the Weir equation has further been simplified to exclude the urinal nitrogen. Therefore, the most recent Weir formula [metabolic rate (kcal per day) = 1.44 (3.94 VO₂ + 1.11 VCO₂)] only accounts for the VO₂ consumption in millilitres per minute and VCO₂ production in millilitres per minute (Mehta et al., 2015). In most of the recent studies the EE is calculated using this simplified version of the Weir equation.

Daily total energy expenditure is the net amount of energy utilized to support core physiological functions and locomotion. It accounts for three components: basal metabolic rate, the thermic effect of food, and physical activity (von Loeffelholz and Birkenfeld, 2000). Physical activity can be categorized into non-exercise activity thermogenesis and exercise-related activity thermogenesis (Psota and Chen, 2013). Many factors affect EE, including age, body composition, ambient and body temperature, the status of thyroid hormones and the activity of the sympathetic nervous system (von Loeffelholz and Birkenfeld, 2000). Metabolic diseases, such as chronic renal disease and cirrhosis, also affect EE (Psota and Chen, 2013). Sex is also an important factor to consider when study metabolism. The estrous

cycle, indeed, has been shown to influence EE and RER of mice (Parker et al., 2001). To remove the sex component, male and female mice were analysed separately in this work.

The EE of the mice was measured using the Promethion Metabolic System (<https://www.sablesys.com/products/promethion-line/>); a comprehensive system that registers metabolic and behavioural activities of the mice. It calculates the EE on the basis of the VO_2 consumed and the VCO_2 produced, using the most recent version of the Weir equation. The Promethion Metabolic System also allows the measurement of movement, body mass, feeding, and drinking, which, used in combination with the EE, gives a more complete understanding of the metabolism of the mice.

4.1.2 Identifying the cause of abnormal tissue growth observed in mice harbouring the *Pik3ca*^{H1047R} mutation.

Abnormal growth can arise from adaptive changes in cells in response to various types of stimuli and adverse environmental changes. These adaptations include hypertrophy (increased tissue size via enlargement of cells, caused by an increase in organelles and structural proteins), hyperplasia (increase in cell number) and dysplasia (disordered growth of cells).

In this chapter the causes of abnormal tissue growth, in response to the expression of the *Pik3ca*^{H1047R} mutation were investigated. For this purpose, conscious mice were administered heavy-deuterated water ($^2\text{H}_2\text{O}$), so called because the hydrogen is substituted by its isotope deuterium (^2H) and because its density is 10% higher than H_2O . The introduction of low concentrations of $^2\text{H}_2\text{O}$ into biological systems results in the incorporation of ^2H into C-H bonds of the macromolecules (Hellerstein, 2003; McCabe and Previs, 2004). The analysis of the ^2H incorporation into lipids, proteins, DNA, RNA and other macromolecules provides a highly sensitive measure of their rate of synthesis.

Here, a new method, described in 2016 by Foletta and colleagues (Foletta et al., 2016), was used. The method allows the simultaneous extraction of nucleic acid, protein, lipid and polar metabolite fractions from a single sample, in combination with the gas chromatography-mass

spectroscopy (GC-MS). Therefore, the rate at which the brain, liver, spleen and intestine of mutant and control mice synthesise DNA and proteins was analysed.

4.1.3 Characterise the lipid metabolism of mice harbouring the *Pik3ca*^{H1047R} mutation.

In this chapter *ex vivo* analysis was performed to characterise the lipid metabolism of the mice by calculating lipolysis in the adipose tissue and the FFA oxidation in skeletal muscle and liver.

During food intake, excess of dietary non-esterified FFAs are esterified to triglycerides (TAGs), which are then stored in the cytosolic lipid droplets of adipocytes. Upon increased energetic demand, such as in a condition of glucose restriction, TAG stores are mobilized following their hydrolytic cleavage and the resulting FFAs and glycerol are delivered via the circulation to peripheral tissues, particularly heart, liver and skeletal muscle. In these tissues FFAs are used for β -oxidation and ATP production (Lass et al., 2011).

The hydrolysis of TAGs to FFAs and glycerol is called lipolysis and depends upon specific hydrolases. In particular the adipose triglyceride lipase and HSL (Chapter 1, Section 1.6.5) have been found to be responsible for more than 90% of the lipolytic capacity in murine adipose tissue (Schweiger et al., 2006). In a condition of elevated energetic demand, the switch towards a higher rate of lipolysis in the adipose tissue is a fundamental metabolic adaptation. Alterations in lipolysis are frequently associated with obesity and insulin resistance, as well as reduced responsiveness to the stimulation of lipolysis (Large et al., 1999; Reynisdottir et al., 1995).

The oxidation of FFAs occurs inside the mitochondria, where they are broken down into Acetyl-CoA units. In order to enter the mitochondria FFAs need to be activated (Houten and Wanders, 2010). First, FFAs react with Acetyl-CoA forming Acyl-CoA units in a process that produces adenosine monophosphate from ATP. This reaction is catalysed by the enzyme acyl-carnitine transferase I. Next carnitine binds Acyl-CoA, inside the cytoplasm, forming acyl-carnitine, which allows the transport of FFAs inside the mitochondria. The transport is catalysed by the enzyme acyl-carnitine transferase II. Once inside the inner mitochondrial membrane, acyl-carnitine is converted back to long-chain Acyl-CoA to enter the FFA β -

oxidation pathway. Each cycle of β -oxidation produces one Acetyl-CoA, which enters the mitochondrial tricarboxylic acid (TCA) cycle. The nicotinamide adenine dinucleotide (NADH) and the flavin adenine dinucleotide (FADH₂) produced by both FFA β -oxidation and the TCA cycle are used by the electron transport chain to produce ATP (Houten and Wanders, 2010).

4.1.4 The browning of white fat

Mammalian adipose tissue is categorized into white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the primary site of energy storage and secretes some endocrine factors like leptin for regulating satiety, whilst BAT stores lower levels of fat and can be used to oxidise fatty acids to maintain body temperature. Whilst brown adipocytes contain many mitochondria and many lipid droplets, white adipocytes have few mitochondria and a single large lipid droplet. Importantly, brown adipocytes express the mitochondrial uncoupling protein 1 (UCP1), which uncouples oxidative respiration from ATP synthesis (Wang and Seale, 2016).

A third type of fat, consisting of beige adipocytes, has been found to derive from precursors of white adipocytes. This phenomenon is generally referred as browning of WAT (Wang and Seale, 2016). Beige adipocytes are found in various WAT deposits, especially in the subcutaneous inguinal WAT. Beige adipocytes are similar to the brown adipocytes in BAT, having many mitochondria, many lipid droplets and expression of UCP1, but have a specific gene expression pattern and, therefore, are distinct cell types (Wu et al., 2012). Beige fat cells, indeed, exhibit distinct gene expression compared to either WAT or BAT, combining expression pattern of genes characteristic to WAT (adiponectin, adipocyte protein 2 gene (*aP2*), adipsin) and BAT (*Ucp1*, *Cidea*, *Pgc-1 α*) and genes specific for beige adipocytes, such as a developmental transcription factor T-box1 (*Tbx1*), a fatty acid transporter solute carrier family 27 member 1 (*Slc27a1*), and transmembrane protein 26 (*TMEM26*) as well as molecules of immune and inflammatory response pathways, such as *CD40* and *CD137* (Kaisanlahti and Glumoff, 2019). Beige fat cells develop in response to cold and certain other stimuli such as diet and β -adrenergic agonist treatment. A more detailed list of browning

agents is reported in a recent review from Kaisanlahti and Glumoff (Kaisanlahti and Glumoff, 2019).

The hypothalamic arcuate nucleus (arc) plays an important role in WAT browning. Within the arc are two neuronal populations characterized by the expression of specific neuropeptides: the POMC and CART neurons population and the AgRP and NPY population (Varela and Horvath, 2012). POMC and CART neuropeptides decrease food intake and body weight. By contrast, the AgRP and NPY neuropeptides increase food intake. During a state of energy surplus, AgRP levels are diminished and POMC levels are elevated, therefore stimulating WAT browning. On the contrary, stimulation of AgRP neurons suppresses WAT browning (Varela and Horvath, 2012). Insulin has been previously shown to stimulate WAT browning by activating POMC neurons. Therefore, whether mutations in the PI3K pathway are responsible for WAT browning in mice has been investigated.

4.1.5 Aims

This chapter aims to:

- characterise the effects of mutations in the *Pik3ca* gene on the systemic metabolism of mice.
- characterise the metabolic reprogramming that tissues harbouring the *Pik3ca*^{H1047R} mutation undergo to sustain their growth.

4.2 RESULTS

4.2.1 Ubiquitous expression of the *Pik3ca*^{H1047R} mutation leads to severe drop of body fat and slight increase of body fluids.

As described in chapter 3 (Section 3.2.3-Figure 3.3), *Pik3ca*^{H1047R} mice had increased body weight, compared to control mice. To verify if the increase in body weight was associated with accumulation of adipose tissue and/or fluids in the body, the body composition of each mouse was determined. This was achieved using the Bruker LF50H Minispec, which uses Time Domain Nuclear Magnetic Resonance (TD-NMR) to measure fat mass, lean mass and free fluids in conscious mice. As the body composition varies throughout the day, TD-NMR was performed at a similar time of the day for each cohort, in order to improve consistency between the data obtained from each individual cohort. Before each scan, the body weight of the mouse was recorded and used to express the proportion of fat mass, lean mass and free fluids, as percentage of the total body weight.

Control and mutant mice were treated with tamoxifen and scanned 6 days after drug administration, which is when the body weight of mutant mice was already abnormally increased, compared to control mice.

The body composition of control and mutant mice is shown as the total grams of fat, lean mass and fluids in male and female mice (Figures 1A and B) and as the percentage of fat mass (Figure 1C), lean mass (Figure 1D) and body fluids (Figure 1E), relative to the body weight of the mice.

The total and percentage body fat drastically dropped in both male and female mutant mice, compared to control mice (Figures 4.1 A, B and C), with females showing about 90% reduction of the total body fat and males losing about 40% body fat. The lean mass of male mutant and control mice remained unchanged (Figures 4.1 A and D), whilst female mutant mice had a higher percentage of lean mass than control mice (Figures 4.1B and D). Interestingly, body fluids were higher in mutant mice, compared to control mice (Figure 4.1 A, B and E), and this difference was slightly accentuated in female mice (~25% higher than control mice), compared to male mice (~10% higher than control mice). However, since the NMR only calculates the total amount of body fluids, it was not possible to define which type of fluid (blood, water, urine) and in which proportion, had contributed to this increase.

Overall, both male and female mutant mice had lower body fat and higher body fluids, compared to control mice, whilst the lean mass was higher only in female mutant mice, compared to control mice.

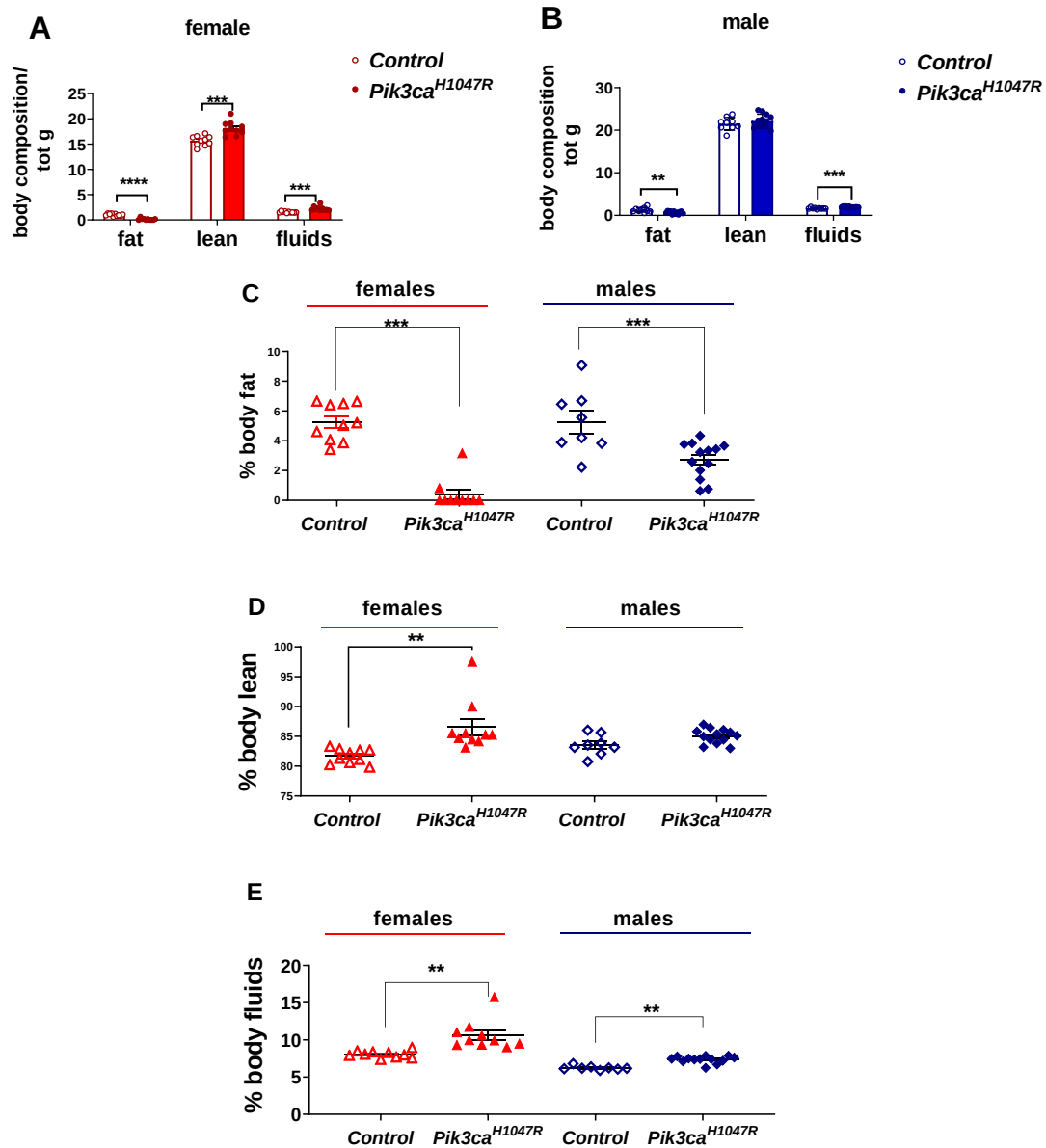


Figure 4. 1: Ubiquitous expression of the *Pik3ca*^{H1047R} mutation reduces body fat and increases body fluids in mice.

Control and *Pik3ca*^{H1047R} mice were administered tamoxifen (200mg/kg) by oral gavage on two consecutive days. Six days post tamoxifen treatment mice were scanned using the Bruker LF50H Minispec, which uses TD-NMR. The graphs display the total grams of fat, lean and fluids of female (A) and male (B) *Pik3ca*^{H1047R} and control mice. The graphs display the percentage of (C) body fat mass, (D) lean mass and (E) body fluids relative to the total body weight (g) of *Pik3ca*^{H1047R} and control mice. Female mice are represented in red, whilst male mice are represented in blue. N female mice = 10 control, 10 *Pik3ca*^{H1047R}. N male mice = 8 control, 13 *Pik3ca*^{H1047R}. Error bars represent mean $\pm$ SEM. ** = p.value $\leq$ 0.01, *** = p.value $\leq$ 0.001.

4.2.2. Ubiquitous expression of the *Pik3ca*^{H1047R} mutation increases hepatic cell proliferation and neuronal protein synthesis in mice.

The previous section demonstrates that the increase in body weight observed in mice *Pik3ca*^{H1047R} was not associated with adiposity. Instead, the size of several tissues had increased in mutant mice, compared to control mice, 7 days post tamoxifen administration (Chapter 3-Section 3.2.3).

Whether the tissue growth was due to increased cell proliferation or increased cell size was investigated by using the ²H₂O metabolic tracer. As explained in the introduction to this chapter, the administration of ²H₂O into the body water pool, *in vivo*, results in the incorporation of deuterium (²H) into C-H bonds of deoxyribose and amino acids. Subsequent analysis of ²H labelling of these constituents in DNA and proteins provides a highly sensitive measure of the rates of synthesis/turnover of these macromolecules. To assess if the tissues became hyperplastic, the fraction of ²H incorporated into the DNA-derived deoxyribose of the tissues was measured. Higher enrichment of ²H into DNA indicates higher proliferation rate of the tissues. For instance, an increment of 50% ²H enrichment means that 50% of the DNA strands are old and 50% of the DNA has been newly synthesised. This indicates that, on average, all cells have undergone 1 cell division event.

To verify if the tissue growth was occurring by hypertrophy, the incorporation of ²H protein-derived alanine was analysed. Tissues that did not have increased *de novo* synthesis of DNA, but had higher *de novo* synthesis of proteins, were considered hypertrophic. Small intestine, liver, spleen and brain were analysed since the size of these tissues was significantly increased (Chapter 3-Section 3.2.3-Figure 3.4). Mice were administered a dose of ²H₂O at the moment of gavage of tamoxifen and kept on 5% ²H₂O in drinking water for 7 days, before being sacrificed. The tissues were harvested and the ²H enrichment in DNA and proteins was analysed by GC-MS.

Figure 4.2A shows that the ²H₂O enrichment in the plasma of control and mutant mice was constant over time, indicating that the system had reached a steady state at the time that the tissues were harvested. The DNA of the liver and spleen of *Pik3ca*^{H1047R} mice had significantly higher incorporation of ²H, corresponding to 60% increased “*de novo*” DNA synthesis in the liver and 25% newly synthesised DNA in the spleen of mutant mice,

compared to control mice (Figure 4.2B). However, since the DNA was extracted from the entire organ, and no sorting of specific cell types by flow cytometry was performed, it was not possible to ascertain which cell type had been dividing. Cells of the small intestine were rapidly proliferating in both control and mutant mice (~ 40% $^2\text{H}_2\text{O}$ enrichment) but the synthesis of new DNA occurred at the same rate between the two groups (Figure 4.2 B). On the contrary, the brain of both control and mutant mice did not show any incorporation of ^2H , indicating that no new DNA synthesis was present in the brain of both control and mutant mice. Therefore, in 7 days the cells of the brain did not undergo division, irrespective of the mouse genotype (Figure 4.2 B).

Despite the lack of cell division, new proteins had been synthesised in the brain of both control and mutant mice (Figures 4.2 C-E). The ^2H enrichment in the brain proteins of *Pik3ca*^{H1047R} mice was 10% higher than control mice. The protein content is shown as pool size (the protein content of the tissue per mg tissue; Figure 4.2 C) fraction newly synthesized (the portion of the total protein pool that is new; Figure 4.2 D) and the absolute amount of protein synthesized (the product of fraction synthesized and protein pool size; Figure 4.2 E).

As the protein turnover of liver, spleen and intestine happens faster than cellular division (DNA synthesis), and DNA synthesis was already high in these tissues, 7 days post tamoxifen administration, the protein turnover in these biomass components was not analysed. Indeed, the ^2H labelling of the proteins of these tissues was likely to have reached 100% labelling after 7 days, therefore not allowing a meaningful comparison between the two groups.

Overall, the increased DNA synthesis observed in the liver and spleen of mutant mice indicates an increased rate of proliferation, and the higher rate of protein synthesis in the brain of mutant mice in the absence of any new DNA synthesis, indicates increased growth or hypertrophy, compared to control mice. It was not possible to define if intestine was growing by hypertrophy or hyperplasia using this method.

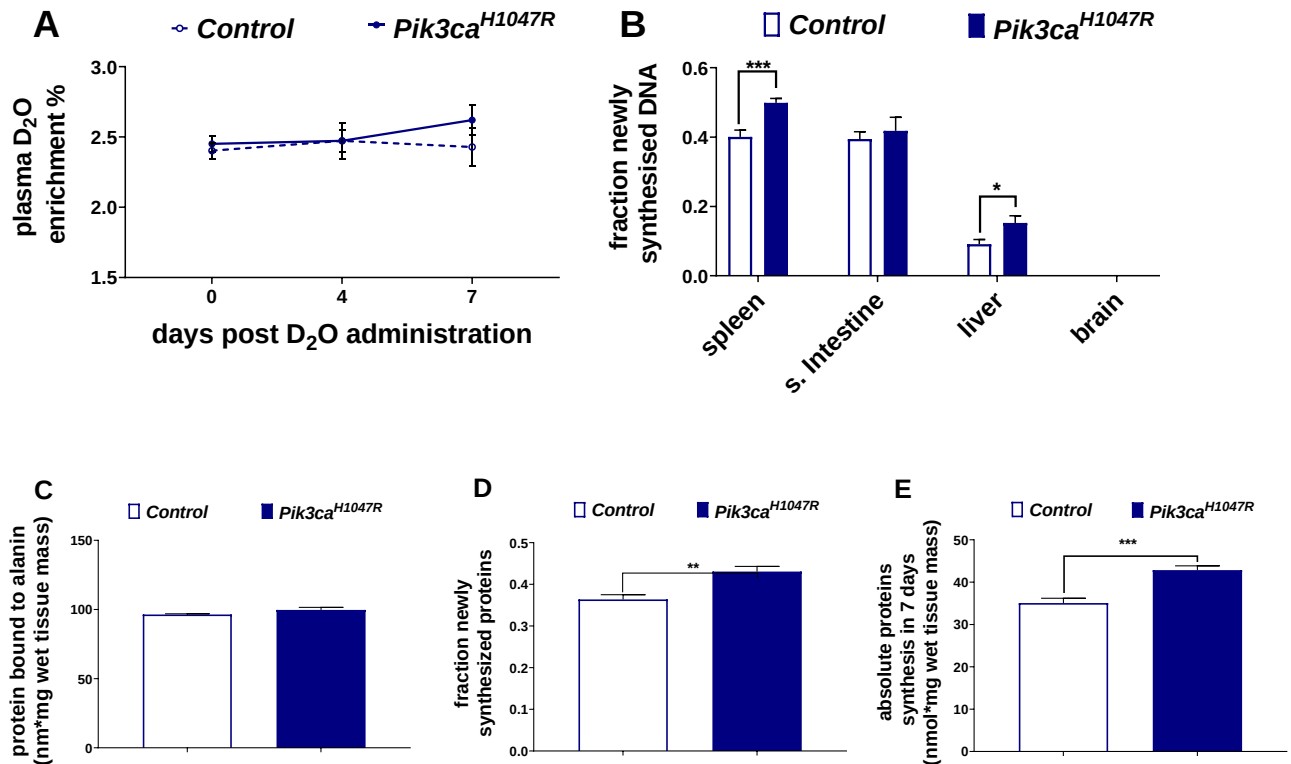


Figure 4. 2: Ubiquitous expression of the *Pik3ca^{H1047R}* mutation increases hepatic cells proliferation and neuronal protein synthesis in mice.

Pik3ca^{H1047R} and control mice were administered tamoxifen (200mg/kg), via oral gavage, on two consecutive days. At the time of the first administration of tamoxifen, deuterated water (²H₂O), (containing 0.9% w/v NaCl), was also delivered by i.p. injection (20 µl/g of body weight). Mice had access to 5% (v/v) ²H-labeled-drinking water during the whole duration of the test. Seven days post ²H₂O injection the mice were sacrificed and the brain, liver, spleen and small intestine (s.intestine) were extracted. Plasma samples were collected through eye bleeding (~30µl whole blood = ~10-15µl plasma) at day 1, 4 and 7 post ²H₂O injection. Graphs display the **(A)** ²H enrichment in the plasma of *Pik3ca^{H1047R}* and control mice, **(B)** ²H incorporation in the DNA of spleen, small intestine, liver and brain of *Pik3ca^{H1047R}* and control mice expressed as fractional units. Graphs display the brain protein pool size expressed as **(C)** protein content of the tissue per mg wet tissue, **(D)** fraction of newly synthesized proteins, and as **(E)** the absolute amount of protein synthesized expressed as the product of fraction synthesized and protein pool size. *Pik3ca^{H1047R}* mice are represented by a blue solid line or solid bars, whilst control mice are represented by a blue dashed line or empty bars. ²H incorporation in DNA and proteins of the tissues were obtained by GC-MS. N mice= 9 control, 9 *Pik3ca^{H1047R}*. Error bars represent mean ± SEM. ** = p.value ≤ 0.01.

4.2.3 Effect of the ubiquitous expression of the *Pik3ca*^{H1047R} mutation on food consumption and ambulatory locomotion of mice.

It has been shown in chapter 3 (Section 3.2.4-Figure 3.5) that mice harbouring the *Pik3ca*^{H1047R} mutation have a severe reduction in blood glucose levels. Furthermore, *Pik3ca*^{H1047R} mice have a significant loss of total body fat (Section 4.2.1). Since hypoglycaemia and loss of body fat can be associated with fasting, food intake was measured to verify if a reduction in nutrient uptake was responsible for the drop in body fat and/or carbohydrates observed.

For the purposes of this study, mice were enclosed in the Promethion metabolic cages, and the movement and food intake were monitored in real time for each mouse individually. The ambulatory locomotion (1 cm/second within the x, y, z beam-break matrix) is indicated as “pedestrian metres”; (<https://www.sablesys.com/products/promethion-line/>) of each mouse. This was recorded to assess if changes in the EE of the mice was associated with the intensity of the movements. Mice were kept in single cages over a period of 60 hours. Since the first 12 hours were used to allow the acclimation of each mouse to the new cage, the data collected during this period of time were not taken into account. The ambulatory locomotion of each mouse inside the cage and the food taken up by each mouse in the 24 hours following the acclimation time are shown in figures 4.3 A-H. In the last 24 hours of the study, a wheel was added to each cage to investigate the effect of exercise on the metabolism of the mice. The data collected during this time period were analysed separately and are discussed below in sections 4.2.6 and 4.2.7.

Since gender plays an important role in body metabolism (Burke et al., 2016; Dionne et al., 1999; Mauvais-Jarvis, 2015; Wu and O'Sullivan, 2011), the data related to the energetic metabolism of female and male mice are presented separately. The data are shown as the single measurements obtained every 30 minutes over a period of 24 hours as well as the average of the measurements obtained during daytime and night-time, (recorded every 5 minutes over a period of 24 hours). Figures 4.3A-F show that both control and mutant mice consumed higher amounts of food (g) at night than during the day. Whilst no difference in food intake was observed between female control and mutant mice, male mutant mice had

significantly lower food intake than control mice. However this difference was observed only at night-time.

Both control and mutant mice were more active at night, than during the day (Figures 4.3 G-L). Female *Pik3ca*^{H1047R} mice had reduced ambulatory locomotion at night compared to control mice. The reduction of movement was also observed in male mutant mice during the night time, compared to control mice, but the difference was not statistically significant.

Overall, female mutant mice were more active than control mice at night, a time at which they consumed the same amount of food as control mice. Male mice, instead, were slightly less active than control mice during the night, a time at which they consumed significantly less food than control mice. No differences between control and mutant mice were observed during the day.

The body mass of the mice was also monitored using the Promethion metabolic cages, which allow for body weight measurements in real time, without additional handling of the mice, which can induce stress. Figure 4.4 shows that, whilst the body weight of mutant female mice was significantly higher than control mice, the body weight of *Pik3ca*^{H1047R} male mice was not different from the body weight of control mice, which is in contrast to what was observed in our previous studies (Chapter 3-Section 3.2.3-Figure 3.3). The body mass of all the mice remained relatively constant during the 24h analysis, with little increase in the feeding hours.

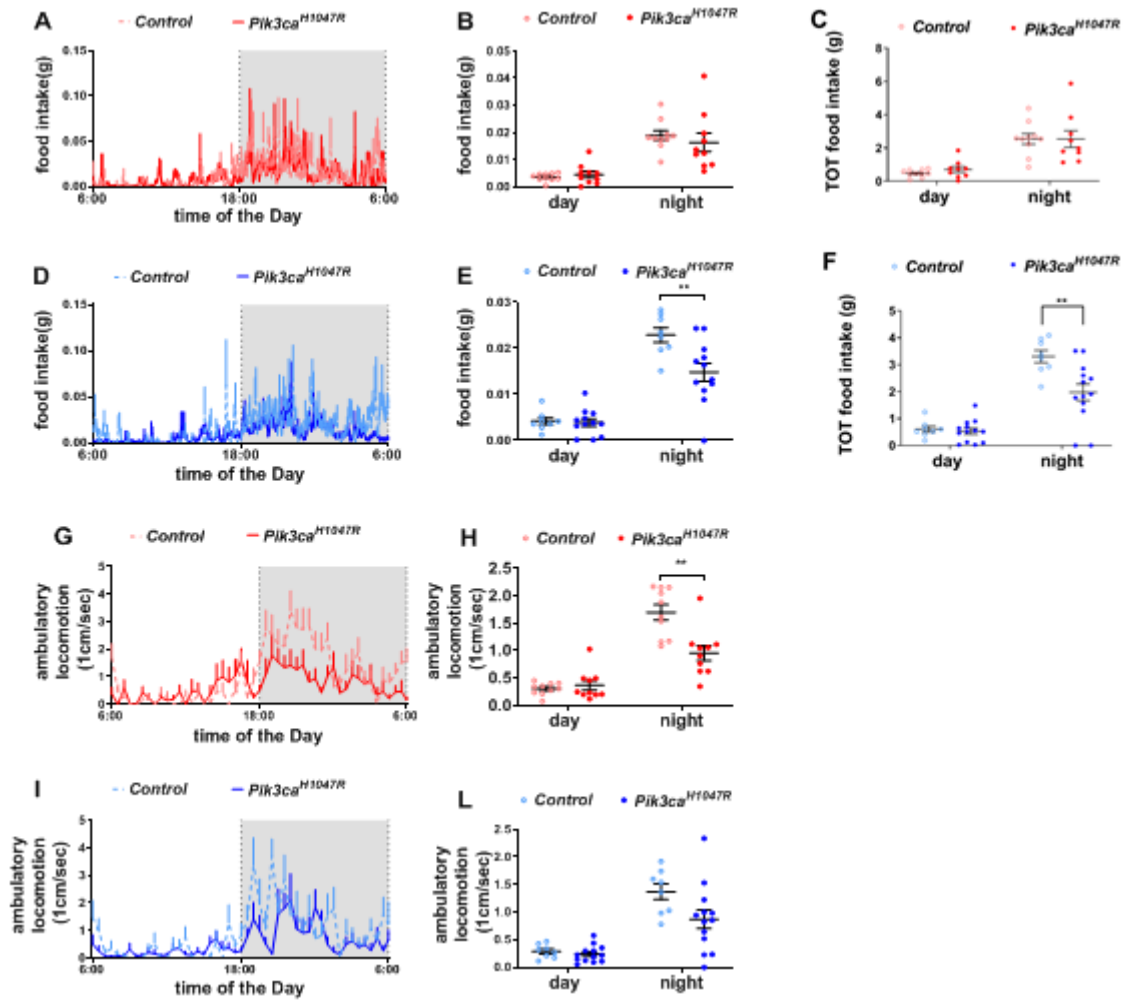


Figure 4. 3: Food intake and ambulatory locomotion of *Pik3ca*^{H1047R} and control mice over a period of 24h in the Promethion Metabolic cages.

Mice were administered tamoxifen (200mg/kg) on two consecutive days. 6 days post tamoxifen administration mice were moved to the Promethion metabolic cages and acclimatised for 12 hours. **(A -C)** graphs show food intake of *Pik3ca*^{H1047R} and control female mice every 30 minutes over a period of 24 hours, as well as the average and total food intake recorded at night and during the day. **(D-F)** graphs show food intake of *Pik3ca*^{H1047R} and control male mice every 30 minutes over a period of 24 hours, as well as the average and total food intake recorded at night and during the day. **(G-H)** Graphs show ambulatory locomotion of *Pik3ca*^{H1047R} and control female mice every 30 minutes over a period of 24 hours, as well as the average ambulatory activity, recorded at night and during the day. **(I-L)** Graphs show ambulatory locomotion of *Pik3ca*^{H1047R} and control male mice every 30 minutes over a period of 24 hours, as well as the average ambulatory activity, recorded at night and during the day. Female mice are represented in red, whilst male mice are represented in blue. Control mice are represented by dashed lines or empty dots, whilst mutant mice are represented by solid lines and solid bars. N male mice = 8 control, 13 *Pik3ca*^{H1047R}. N female mice = 10 control, 10 *Pik3ca*^{H1047R}. The grey area on the graph represents night time. Error bars represent mean ± SEM. ** = p.value ≤ 0.01.

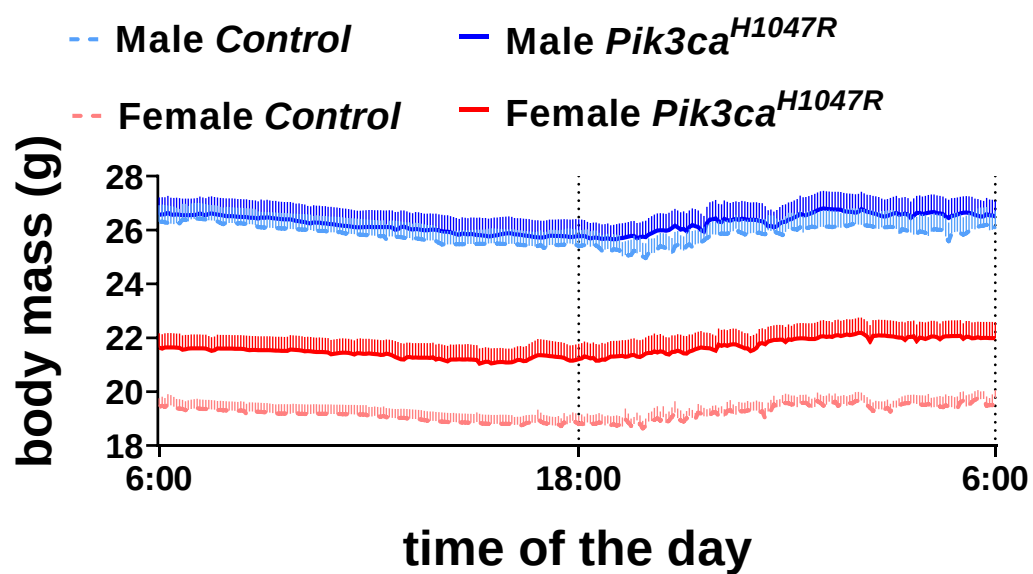


Figure 4. 4: Body mass of *Pik3ca*^{H1047R} and control mice over a period of 24h in the Promethion Metabolic cages.

Mice were administered tamoxifen (200mg/kg) on two consecutive days. 6 days post tamoxifen administration mice were moved to the Promethion metabolic cages and acclimatised for 12hours. The body mass of male and female *Pik3ca*^{H1047R} and control mice was monitored every five minutes over a period of 24 hours. Female mice are represented in red, whilst male mice are represented in blue. Control mice are represented by dashed lines, whilst mutant mice are represented by solid lines. The area between the dotted lines at time 18:00 and 6:00 indicate night time, whilst the area between 6:00 to 18:00 represents day time. N male mice = 8 control, 13 *Pik3ca*^{H1047R}. N female mice = 10 control, 10 *Pik3ca*^{H1047R}. Error bars represent mean $\pm$ SEM. ** = p.value $\leq$ 0.01.

4.2.4 Effect of the ubiquitous expression of the *Pik3ca*^{H1047R} mutation on the RER of the mice.

It has been shown in the previous chapter (Chapter 3-Section 3.2.3-Figure 3.4) that *Pik3ca*^{H1047R} mice had increased organ size despite the low levels of blood carbohydrates and low body fat. Since both the main sources of body energy (sugar and fat) were almost exhausted in these mice, how these two nutrients contribute to tissue growth was investigated. Therefore, the RER of the mice was calculated over a period of 24 hours, using the Promethion metabolic cages.

As described in the introduction to this chapter, RER is commonly used to determine which nutrients are metabolised to produce energy. A RER = 1 indicates that carbohydrates are being predominantly used, whereas a RER = 0.7 suggests lipid oxidation. The measurements collected over the 24 hours period after the acclimation of the mice, and before the addition of a wheel to each cage, are shown in Figures 4.5 and 4.6. The data are presented as the single measurements obtained every 5 minutes over a period of 24 hours as well as the average of the measurements obtained during daytime and night-time.

Figure 4.5 shows the respiratory rate of female mice. RER of both control and mutant female mice was close to 0.7 during day-time, indicating that fat was the predominant source of energy used during the day, independent of the genotype. A mix of fat and carbohydrates ($0.7 < \text{RER} < 1$) were instead used at night to generate energy by both control and mutant female mice (Figures 4.5 E and F). However, in the time period between 4:00 to 6:00 a.m., mutant mice had lower RER, compared to control mice. A reduction of the RER of mutant mice was also visible from 06:00-10:00 p.m. However, the RER was still between values of 0.7 and 1, suggesting that the mice were using both fat and carbohydrates to make energy at that specific time of the day. In agreement with the RER, both VO_2 and VCO_2 of female mice were higher at night than during the day. VO_2 and VCO_2 also remained similar between control and mutant mice during the whole duration of the study, except between 4:00 to 6:00 a.m., when both VO_2 and VCO_2 of mutant mice were lower than control mice.

Figure 4.6 shows the respiratory rate of male mice. VCO_2 and VO_2 were higher at night than during the day for both genotypes (Figure 4.6 A-D). However, *Pik3ca*^{H1047R} mice had generally lower VCO_2 and VO_2 than control mice, which was statistically significant at 6:00

a.m.-12:00 a.m. (start of the study) and at 2:00-6:00 a.m. (end of the study). According to the VCO_2 consumed and the O_2 produced, the total RER of both control and mutant male mice was higher at night, with fat being the preferential source of energy and lower during the day, when both fat and carbohydrates were used to generate energy (Figures 4.6 E and F). Although mutant and control male mice had a similar trend throughout the majority of the day, mutant mice had a drop in RER in the morning, from 6:00 a.m. to 9:30 a.m. (start of the study) and from 4:30 a.m. to 6:00 a.m. (end of the study).

Overall, all the mice used in this study had RER close to 0.7 during the day with fat being the preferential source of nutrients. The RER of all mice rose during the night, when both fat and carbohydrates were consumed. However, both male and female mutant mice had lower RER than control mice, during the early time of the day. The fact that the drop in RER of mice harbouring the *Pik3ca*^{H1047R} mutation overlapped with reduction in food uptake (Figure 4.3 A and D), suggests that during fasting mutant mice obtain most of the body energy from fat. The impact of oncogenic *Pik3ca*^{H1047R} on food intake and ambulatory activity of female vs male mice is recapitulated in table 3.

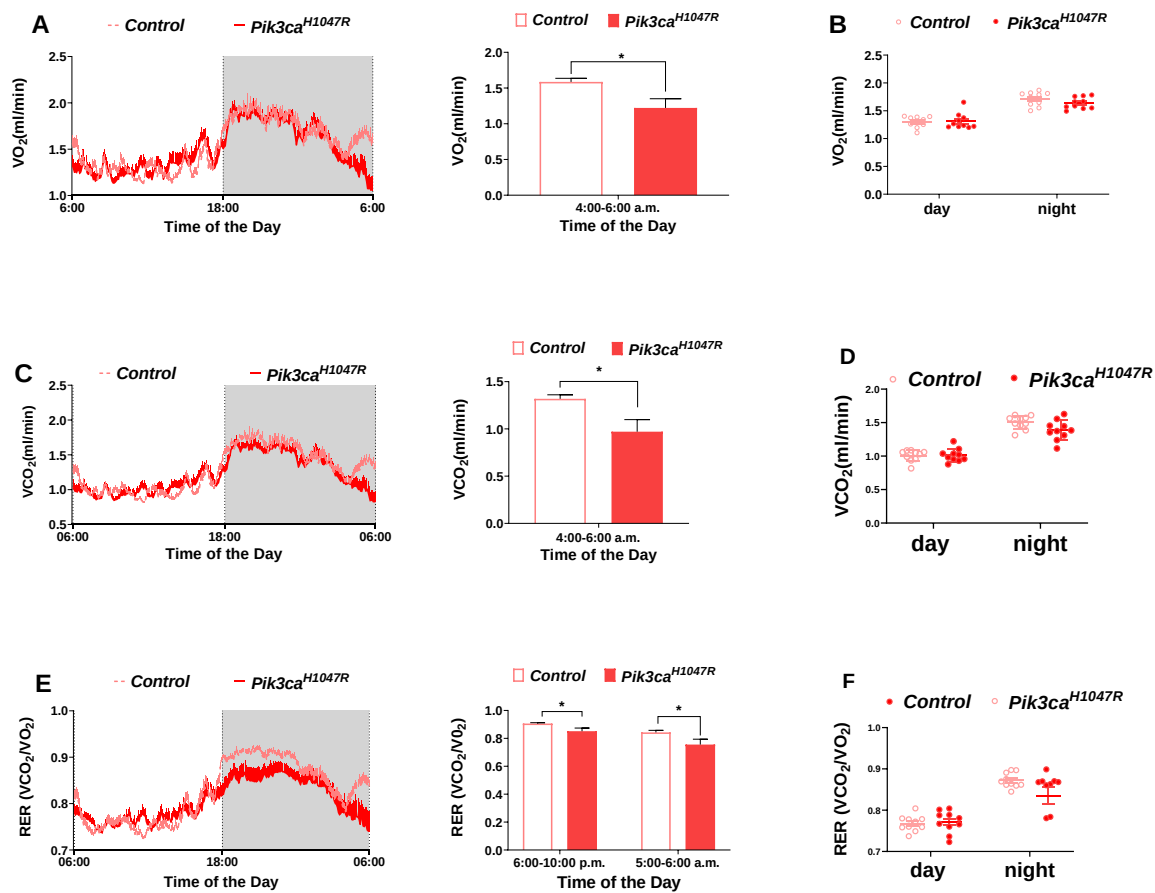


Figure 4. 5: Ubiquitous expression of the *Pik3ca*^{H1047R} mutation alters the respiratory exchange rate (RER) of female mice during fasting.

Female mice were administered tamoxifen (200mg/kg) on two consecutive days. 6 days post tamoxifen administration mice were moved to the Promethion metabolic cages and acclimatised for 12 hours. The graphs display volume of O_2 (VO_2) used by *Pik3ca*^{H1047R} and control mice every 5 minutes over a period of 24hours (**A**), as well as the average O_2 used at night and during the day (**B**) and expressed in ml/min. The graphs display volume of CO_2 (VCO_2) produced by *Pik3ca*^{H1047R} and control mice every 5 minutes over a period of 24h (**C**) as well as the average of CO_2 generated at night and during the day (**D**) and expressed in ml/min. The graphs display the RER (VCO_2/VO_2) of *Pik3ca*^{H1047R} and control mice every 5 minutes over a period of 24hours (**E**) as well as the average RER during the day and at night (**F**). To each category (RER, VCO_2 and VO_2) a histogram shows the time of the day when statistically significant differences between mutant and control mice were observed. The area between the dotted lines at time 18:00 and 6:00 indicate night time, whilst the area between 6:00 to 18:00 represents day time. N mice= 10 control, 10 *Pik3ca*^{H1047R}. Control mice are represented by dashed lines, empty bars or empty dots, whilst mutant mice are represented by solid lines, solid bars and solid dots. Error bars represent mean $\pm$ SEM. * = p.value $\leq$ 0.05.

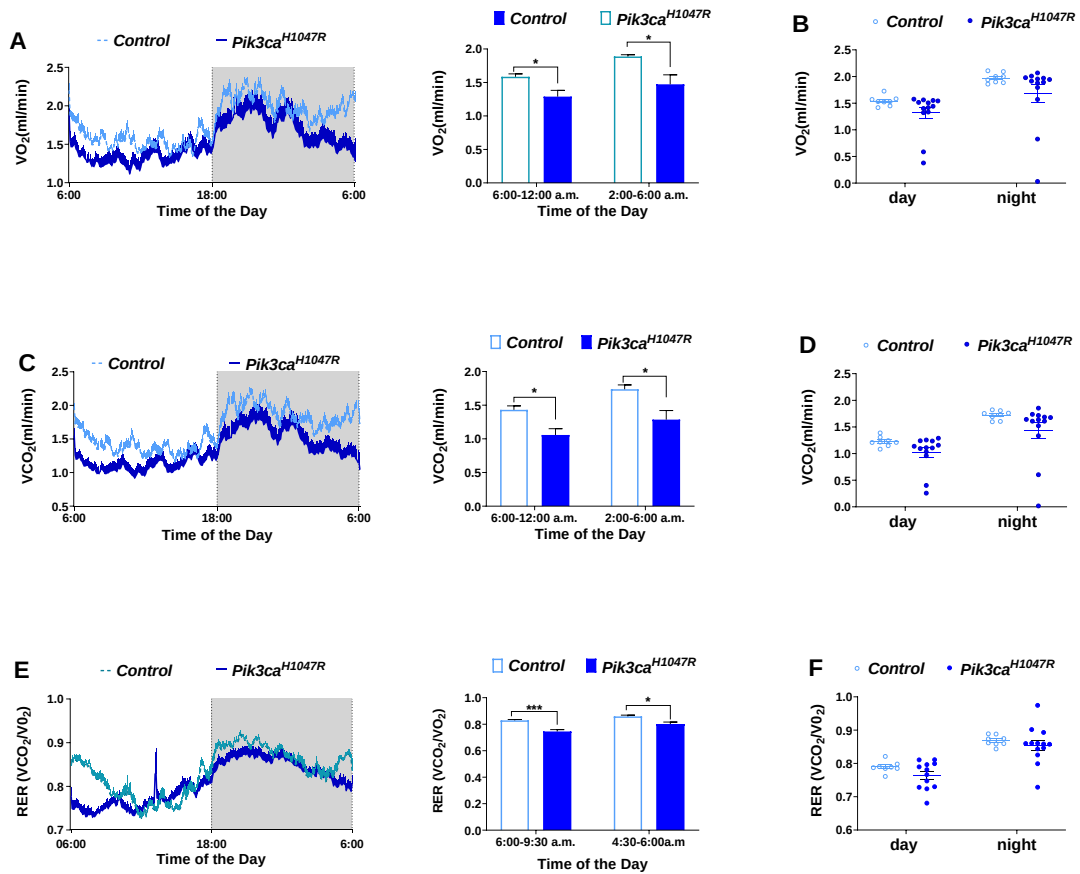


Figure 4.6: Ubiquitous expression of the $Pik3ca^{H1047R}$ mutation alters the respiratory exchange rate (RER) of male mice during fasting.

Male mice were administered tamoxifen (200mg/kg) on two consecutive days. 6 days post tamoxifen administration mice were moved to the Promethion metabolic cages and acclimatised for 12 hours. The graphs display volume of O_2 (VO_2) used by $Pik3ca^{H1047R}$ and control mice every 5 minutes over a period of 24hours (A), as well as the average O_2 used at night and during the day (B) and expressed in ml/min. The graphs display volume of CO_2 (VCO_2) produced by $Pik3ca^{H1047R}$ and control mice every 5 minutes over a period of 24h (C) as well as the average of CO_2 generated at night and during the day (D) and expressed in ml/min. The graphs display the RER (VCO_2/VO_2) of $Pik3ca^{H1047R}$ and control mice every 5 minutes over a period of 24hours (E) as well as the average RER during the day and at night (F). To each category (RER, VCO_2 and VO_2) a histogram shows the time of the day when statistically significant differences between mutant and control mice were observed. The area between the dotted lines at time 18:00 and 6:00 indicate night time, whilst the area between 6:00 to 18:00 represents day time. N mice= 8 control, 13 $Pik3ca^{H1047R}$. Control mice are represented by dashed lines, empty bars or empty dots, whilst mutant mice are represented by solid lines, solid bars and solid dots.. Error bars represent mean $\pm$ SEM. * = p .value ≤ 0.05 . *** = p .value ≤ 0.001 .

4.2.5 Effect of the ubiquitous expression of the *Pik3ca*^{H1047R} mutation on the energy expenditure (EE) of the mice.

VCO₂ and VO₂ are used by the Promethion system to calculate the EE of the mice, based on the Weir equation. EE is a measure of the amount of energy stored for daily use by the mice and is expressed as Kcal/h. EE is strictly related to fat-free mass and body weight (Garby et al., 1988; Ravussin et al., 1988). However, since it is not always possible to obtain the body composition of the mice, the EE is generally adjusted only to the body weight of the animals. In this chapter the measure of EE obtained from the Promethion metabolic cages has been normalised only to the body weight of the mice.

The data are shown as single measurements obtained every 5 minutes over a period of 24 hours as well as the average of the measurements obtained during daytime and night-time. Figures 4.7 A-D show that both control and mutant mice had higher EE during the day than at night, independent of the sex. Female *Pik3ca*^{H1047R} mice had significantly lower EE, compared to control mice, both during the day and at night (Figures 4.7 A and B). Male *Pik3ca*^{H1047R} mice used less energy than control mice during day-time, a time at which both ambulatory activity and food intake were unchanged with respect to control mice (Figures 4.3 E-H). However, a trend towards a decreased EE, with respect to control mice, was also observed at night and possibly associated to lower food intake and ambulatory activity. The impact of oncogenic *Pik3ca*^{H1047R} on RER and EE of female vs male mice is recapitulated in table 4.

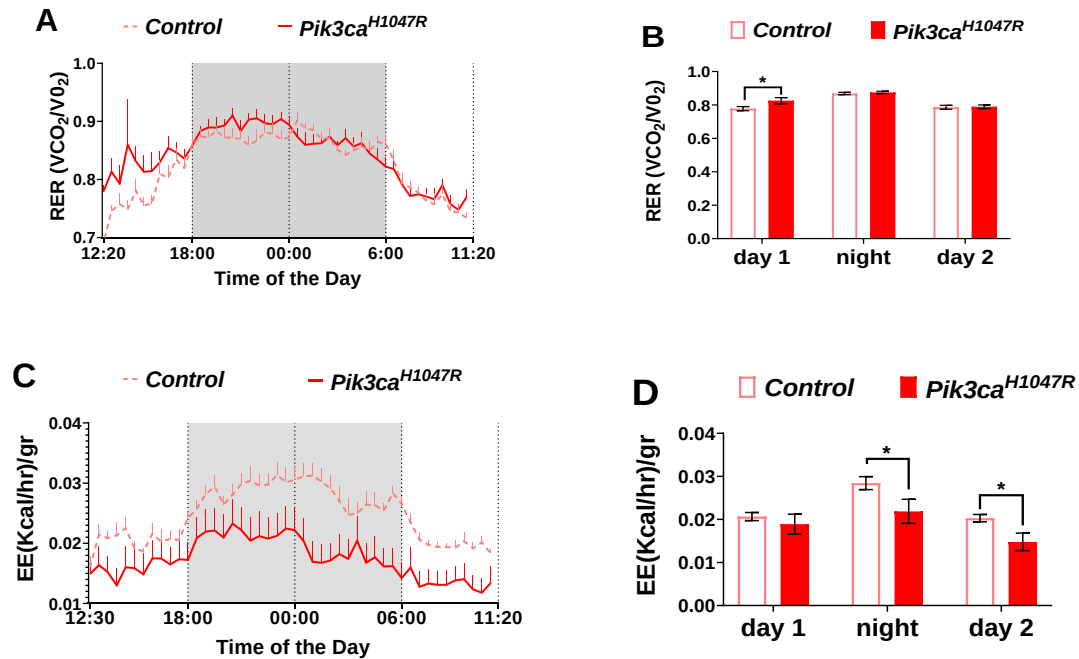


Figure 4. 7: Ubiquitous expression of the *Pik3ca*^{H1047R} mutation alters the energy expenditure (EE) of female mice.

Mice were administered tamoxifen (200mg/kg) on two consecutive days. 6 days post tamoxifen administration mice were moved to the Promethion metabolic cages and acclimatised for 12hours. Graphs display the EE of female (A-B) and male (C-D) mice as calories used every 5 minutes over a period of 24hours, as well as the average of the measurements recorded at night and during the day and expressed as kcal/h/lean mass (g). Female mice are represented in red, whilst male mice are represented in blue. The area between the dotted lines at time 18:00 and 6:00 indicate night time, whilst the area between 6:00 to 18:00 represents day time. N female mice = 10 control, 10 *Pik3ca*^{H1047R}; N male mice = 8 control, 13 *Pik3ca*^{H1047R}. Control mice are represented by dashed lines or empty dots, whilst mutant mice are represented by solid lines and solid dots. Error bars represent mean $\pm$ SEM. * = p.value $\leq$ 0.05, ** = p.value $\leq$ 0.01.

4.2.6. Voluntary exercise, ambulatory locomotion and food intake of mice ubiquitously expressing the *Pik3ca*^{H1047R} mutation.

Physical activity is defined as every movement carried out by the skeletal muscles that require energy. Therefore, the ambulatory locomotion of the mice can be defined as physical activity. It is distinct from voluntary exercise, which is structured, repetitive and intentional movement. Exercise impacts the cardiorespiratory system, strength, endurance, flexibility and body composition. In order to measure the amount of exercise for each mouse, a running wheel was added to each cage for a period of 24 hours. The food intake of the mice was also measured during the same period of time.

Figures 4.8 A and B show that both female control and mutant mice used the wheel more at night-time than during day-time. However, female *Pik3ca*^{H1047R} mice exercised less than control mice during the night. Furthermore, mutant female mice had significantly lower ambulatory activity than control mice (Figures 4.8 C and D) and similar food intake to control mice, apart from a slight increase in food consumption in the early hours of the study (Figures 4.8 E and F). As opposed to female mice, the rate of exercise of mutant male mice was similar to control mice and both the genotypes exercised more at night than during the day (Figure 4.9 A and B). However, mutant male mice had significantly lower ambulatory activity and food intake than control mice (Figures 4.9 C-F).

Overall, both male and female *Pik3ca*^{H1047R} mice moved and/or ate less than control mice during the 24 hours of the study undertaken in the presence of the wheel. Mutant female mice, but not male mice showed reduced exercise on the wheel, compared to control mice. The impact of oncogenic *Pik3ca*^{H1047R} on food intake and ambulatory activity of female vs male mice during exercise is recapitulated in table 3.

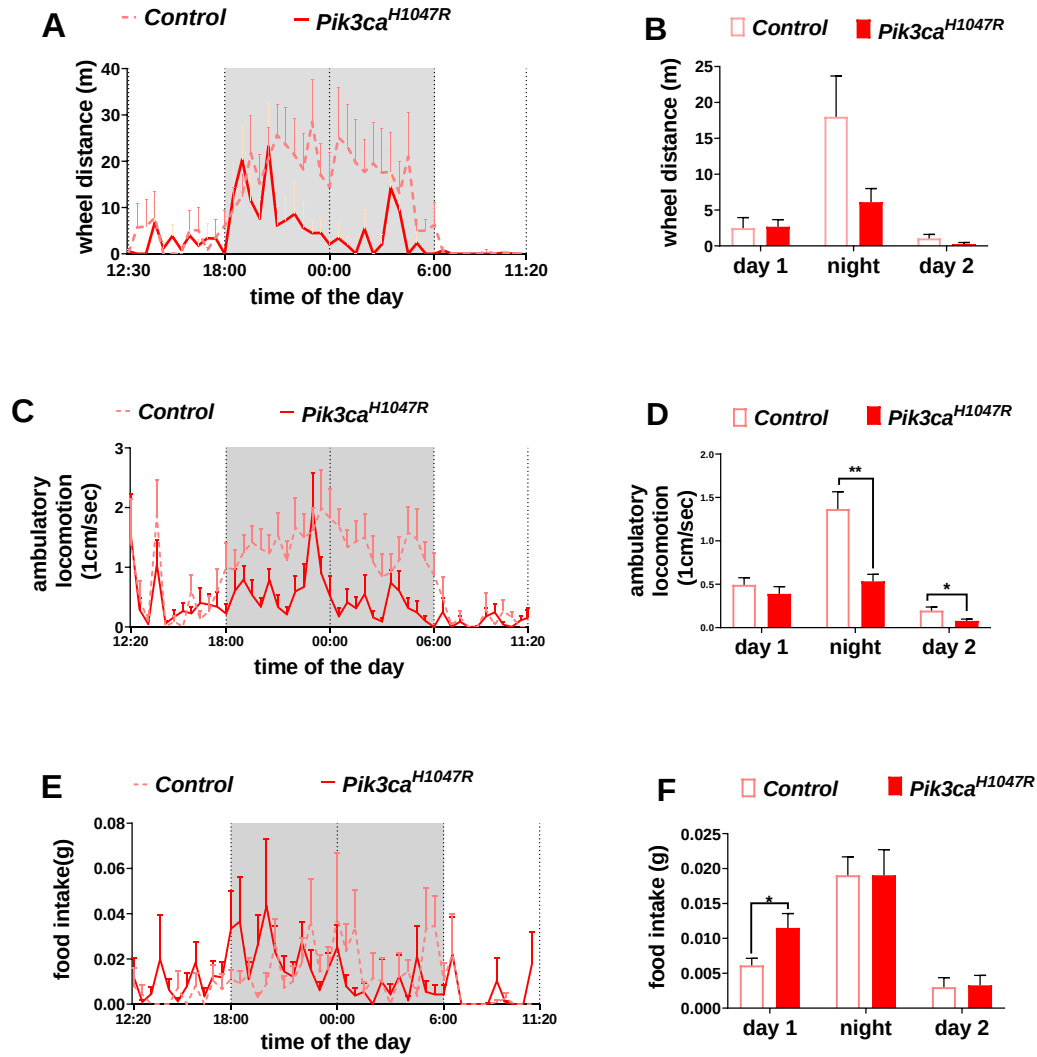


Figure 4. 8: Voluntary activity, food intake and pedestrian metres of *Pik3ca*^{H1047R}*UbCre*⁺ and control female mice over a period of 24h in the Promethion Metabolic cages.

Female mice were administered tamoxifen (200mg/kg) on two consecutive days. 6 days post tamoxifen administration mice were moved to the Promethion metabolic cages and acclimatised for 12 hours. After 24 hours a running wheel was added to each cage. Graphs display wheel metres of *Pik3ca*^{H1047R} and control mice (**A**) recorded every 30 minutes over a period of 24 hours, as well as (**B**) the average of the wheel metres recorded at night and during the day. Graphs display ambulatory locomotion of *Pik3ca*^{H1047R} and control mice (**C**) every 30 minutes over a period of 24 hours as well as (**D**) the average ambulatory locomotion recorded at night time and during the day time and expressed in cm/sec in the presence of the wheel. Graphs display food intake of *Pik3ca*^{H1047R} and control mice (**E**) recorded every 30 minutes over a period of 24 hours as well as (**F**) the average food intake recorded at night and during the day and expressed as g, in the presence of the wheel. Control mice are represented by dashed lines or empty bars, whilst mutant mice are represented by solid lines and solid bars. N mice = 10 control, 10 *Pik3ca*^{H1047R}. The grey area on the graph represents night time Error bars represent mean ± SEM. * = p.value ≤ 0.05, ** = p.value ≤ 0.01

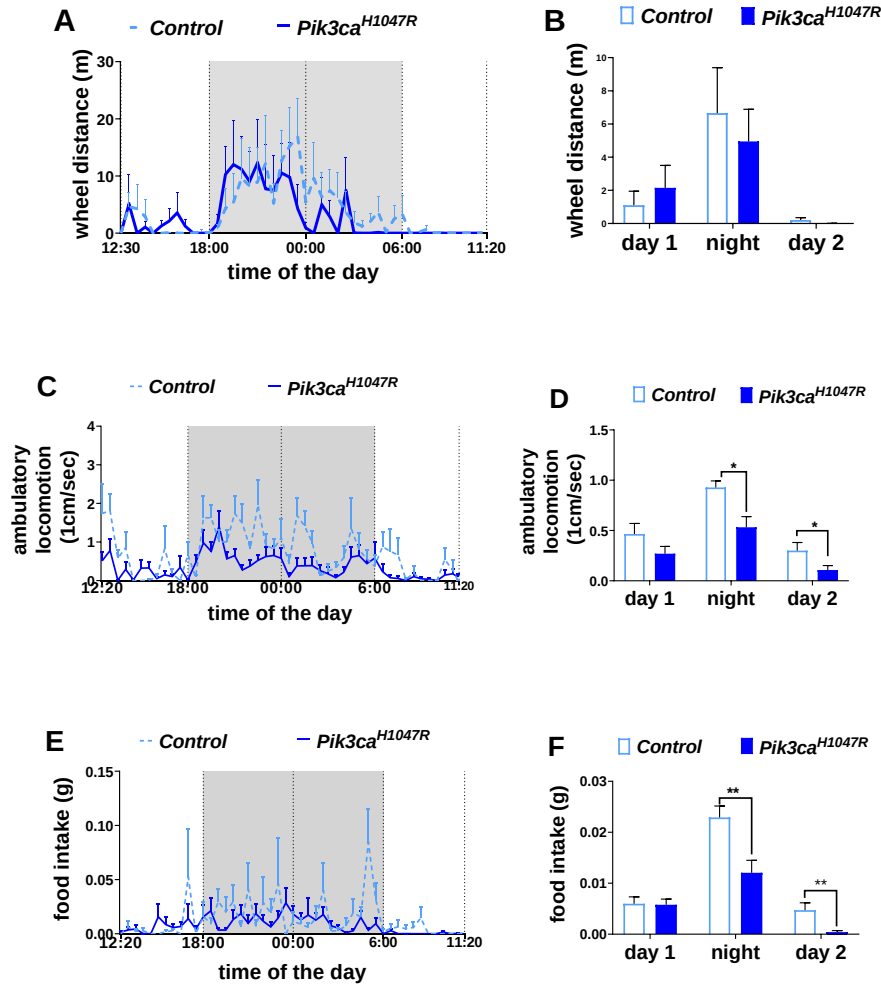


Figure 4. 9: Voluntary activity, food intake and ambulatory locomotion of *Pik3ca*^{H1047R}UbCre⁺ and control male mice over a period of 24h in the Promethion Metabolic cages.

Male mice were administered tamoxifen (200mg/kg) on two consecutive days. 6 days post tamoxifen administration mice were moved to the Promethion metabolic cages and acclimatised for 12 hours. After 24 hours a running wheel was added to each cage. Graphs display wheel metres of *Pik3ca*^{H1047R} and control mice (**A**) recorded every 30 minutes over a period of 24 hours, as well as (**B**) the average of the wheel metres recorded at night and during the day. Graphs show ambulatory locomotion of *Pik3ca*^{H1047R} and control mice (**C**) every 30 minutes over a period of 24 hours as well as (**D**) the average ambulatory locomotion recorded at night time and during the day time and expressed in cm/sec in the presence of the wheel. Graphs show food intake of *Pik3ca*^{H1047R} and control mice (**E**) recorded every 30 minutes over a period of 24 hours as well as (**F**) the average food intake recorded at night and during the day and expressed as g, in presence of the wheel. Control mice are represented by dashed lines or empty bars, whilst mutant mice are represented by solid lines and solid bars. N mice = 8 control, 13 *Pik3ca*^{H1047R}. The grey area on the graph represents night time. Error bars represent mean ± SEM. * = p.value ≤ 0.05, ** = p.value ≤ 0.01.

4.2.7 RER of mice ubiquitously expressing the *Pik3ca*^{H1047R} mutation during volunteer exercise.

Several lines of evidence suggest that RER and EE vary with exercise (Garland et al., 2011; O'Neal et al., 2017; Paes et al., 2016; Ramos-Jimenez et al., 2008). The relationship between the voluntary exercise of mice harbouring the *Pik3ca*^{H1047R} mutation and their energetic metabolism was investigated by adding the running wheel to each cage. In the 24 hours that followed the addition of the wheel to the cages, the levels of VCO₂ and VO₂ of the mice were monitored and used to calculate the RER and EE of the mice during exercise.

Figures 4.10A and B show that both control and mutant female mice used a mix of fat and carbohydrates at night time, indicated by a RER between 0.8 and 1, whilst the RER dropped during daylight hours, at indication that most of the energy used during the day was derived from lipid oxidation. *Pik3ca*^{H1047R} female mice had similar RER as control mice throughout the day, apart from a slight increase during the first hours of the study. Female mutant mice had lower EE than control mice particularly at night (Figures 4.10 C and D), when their movements in the cages were also reduced (Figures 4.9 C and D). The RER of male mutant mice was similar to control mice throughout the 24 hours (Figures 4.11 A and B). However, the EE of *Pik3ca*^{H1047R} male mice was significantly lower than control mice, particularly after midnight (Figures 4.11 C and D), when the food intake and physical activity were also lower than control mice (Figures 4.9 C-F).

Overall, both male and female mutant mice had similar RER to control mice, but significantly lower EE than control mice, especially during the resting hours (low movements) and during fasting (low food intake). The impact of oncogenic *Pik3ca*^{H1047R} on RER and EE of female vs male mice during exercise is recapitulated in table 4.

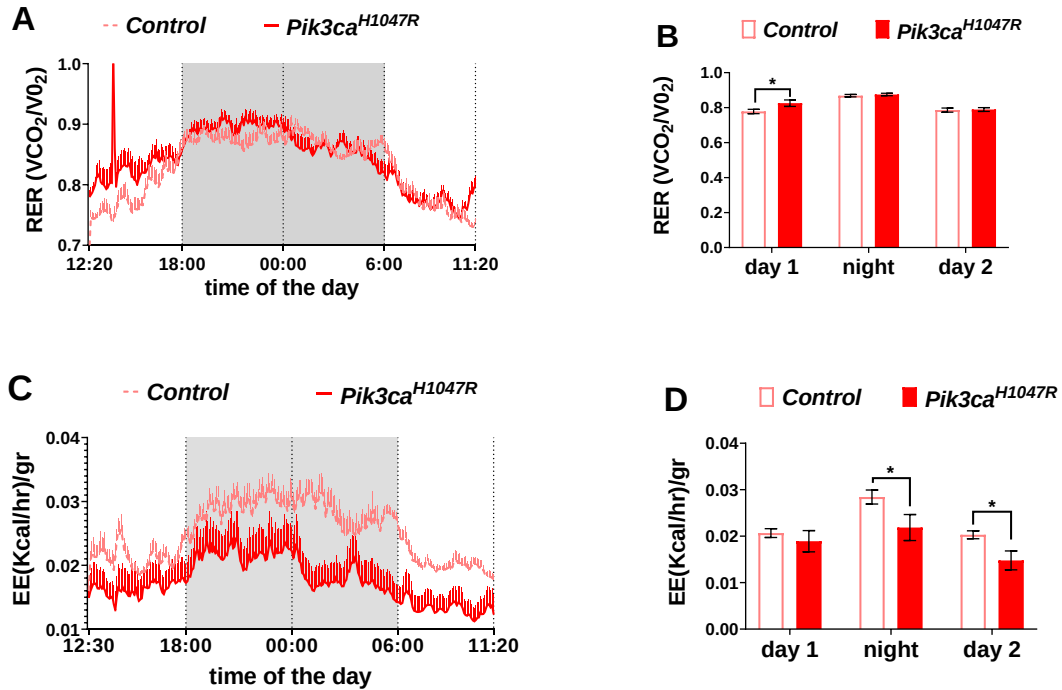


Figure 4. 10: RER and EE of *Pik3ca*^{H1047R} and control female mice over a period of 24h in the Promethion Metabolic cages.

Female mice were administrated tamoxifen (200mg/kg) on two consecutive days. 6 days post tamoxifen administration mice were moved to the Promethion metabolic cages. After 36 hours a running wheel was added to each cage. The graphs display the RER of *Pik3ca*^{H1047R} and control mice (**A**) every 30 minutes over a period of 24 hours, as well as (**B**) the average of the measurements recorded at night and during the day, in presence of the wheel and indicated as the VCO₂/VO₂ ratio. The graphs show the energy expenditure EE of *Pik3ca*^{H1047R} and control mice (**C**) every 30 minutes over a period of 24 hours, as well as (**D**) the average of the measurements recorded at night and during the day and expressed as Kcal/h, in presence of the wheel. Control mice are represented by dashed lines or empty bars, whilst *Pik3ca*^{H1047R} are represented by solid lines and solid bars. N mice= 10 control, 10 *Pik3ca*^{H1047R}. Error bars represent mean ± SEM. * = p.value ≤ 0.05.

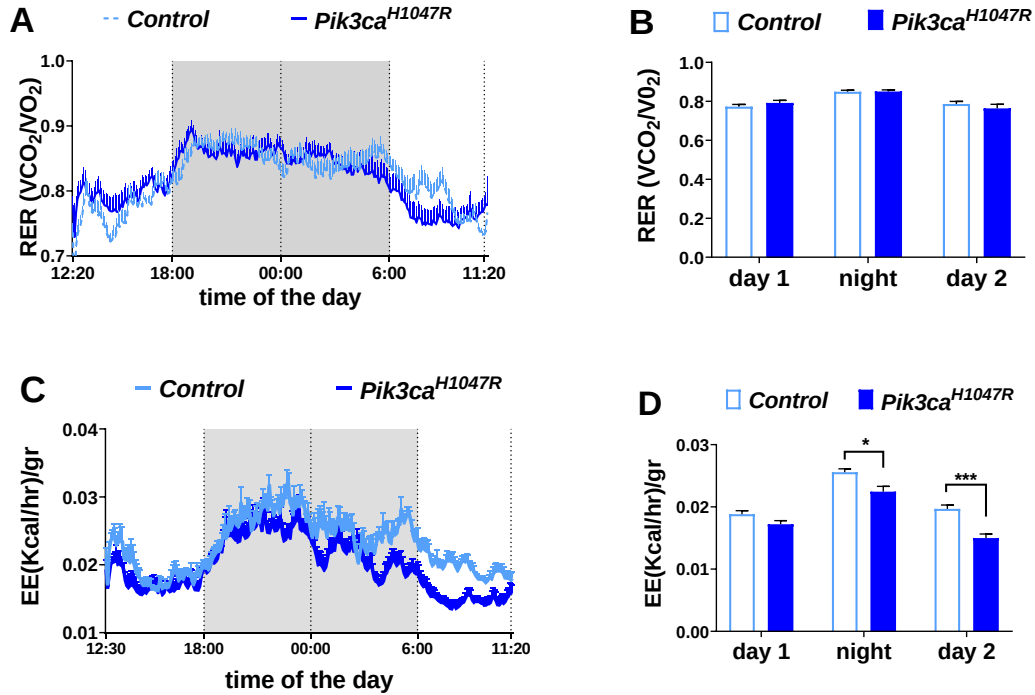


Figure 4. 11: RER and EE of *Pik3ca*^{H1047R} and control male mice over a period of 24h in the Promethion Metabolic cages.

Male mice were administered tamoxifen (200mg/kg) on two consecutive days. 6 days post tamoxifen administration mice were moved to the Promethion metabolic cages. After 36 hours a running wheel was added to each cage. The graphs display the RER of *Pik3ca*^{H1047R} and control mice (A) every 30 minutes over a period of 24 hours, as well as (B) the average of the measurements recorded at night and during the day, in presence of the wheel and indicated as the VCO₂/VO₂ ratio. The graphs show the energy expenditure EE of *Pik3ca*^{H1047R} and control mice (C) every 30 minutes over a period of 24 hours, as well as (D) the average of the measurements recorded at night and during the day and expressed as Kcal/h, in presence of the wheel. Control mice are represented by dashed lines or empty bars, whilst *Pik3ca*^{H1047R} are represented by solid lines and solid bars. N mice= 8 control, 13 *Pik3ca*^{H1047R}. Error bars represent mean $\pm$ SEM. * = p.value $\leq$ 0.05.

Table 3: Impact of oncogenic *Pik3ca*^{H1047R} on resting metabolism in male vs female mice

Mice	Male		Female	
Time	Day	Night	Day	Night
Food intake	ns	↓	ns	ns
Ambulatory locomotion	ns	ns	ns	↓
VO ₂	ns	ns	ns	ns
VCO ₂	ns	ns	ns	ns
RER	ns	ns	ns	ns
EE	ns	ns	ns	ns

Table showing the metabolic changes observed in *Pik3ca*^{H1047R} mice, compared to control mice. Ns = non-significant changes as compared to control mice. ↓ = decrease as compared to control mice.

Table 4: Impact of oncogenic *Pik3ca*^{H1047R} on metabolism in male vs female mice during exercise

Mice	Male			Female		
Time	Day 1	Night	Day 2	Day	Night	Day 2
Wheel distance	ns	ns	ns	ns	ns	ns
Food intake	ns	↓	↓	↓	ns	ns
Ambulatory locomotion	ns	↓	↓	ns	↓	↓
RER	ns	ns	ns	↓	ns	ns
EE	ns	↓	↓	ns	↓	↓

Table showing the metabolic changes observed in *Pik3ca*^{H1047R} mice, compared to control mice. Ns = non-significant changes as compared to control mice. ↓ = decrease as compared to control mice.

4.2.8 Ubiquitous expression of the *Pik3ca*^{H1047R} mutation increases lipolysis in the adipose tissue, but does not alter FFA oxidation in the skeletal muscle and liver of the mice.

It has been demonstrated that mice harbouring the *Pik3ca*^{H1047R} mutation have general loss of total adipose tissue (Section 4.2.1), compared to control mice and use more fat than carbohydrates during fasting (Figures 4.5-4.6, 4.10-4.11). To support these data the rate of lipolysis was assessed in control and mutant mice.

Lipolysis is the hydrolysis of triglycerides into glycerol and FFAs. The glycerol and FFAs are then released into the bloodstream or culture media. Lipolysis was assessed *ex vivo*, in adipose tissue, at the basal state, and in the presence of lipolytic agonists, isoproterenol (pan- β -adrenergic agonist), or forskolin (activator of adenylate cyclase and cAMP production independent of β -adrenergic activation). Glycerol released by the adipose tissue into the media was measured using a colorimetric assay based on the use of isoproterenol. The activation of the β -adrenergic receptors by isoproterenol results in the production of cAMP from ATP, which in turn activates the hydrolysis of triglycerides by HSL. Lipolysis is then determined by measuring a colorimetric product with absorbance at 570nm (A_{570}) proportional to the amount of glycerol present.

Figure 4.12 shows that isoproterenol and forskolin stimulated lipolysis of both control and mutant mice, compared to the basal state. Interestingly, whilst no difference between the genotypes was observed after addition of the lipolytic agonists, at the basal state (no stimulation) mutant mice had increased lipolysis, compared to control mice. Due to the limited availability of male mice, lipolysis was studied only in female mice.

The FFAs generated through lipolysis are transported into the blood stream and reach surrounding tissues such as the muscle where they get oxidised in the mitochondria to produce energy. Therefore, it was investigated if the FFAs produced by lipolysis resulted in increased FFA oxidation in the skeletal muscle and in the liver of control and mutant mice, by studying the incorporation of ¹⁴C labelled oleate into acid-soluble metabolites and lipids, using thin layer chromatography.

Figures 4.13 A-H show that the FFA uptake in the skeletal muscle as well as in the liver of mutant mice remained unchanged with respect to control mice. The rate of FFA oxidation as well as the storage of FFAs into lipids (cholesterol, triglycerides, diglycerides, ceramide, phospholipids) was similar between mutant mice and control mice.

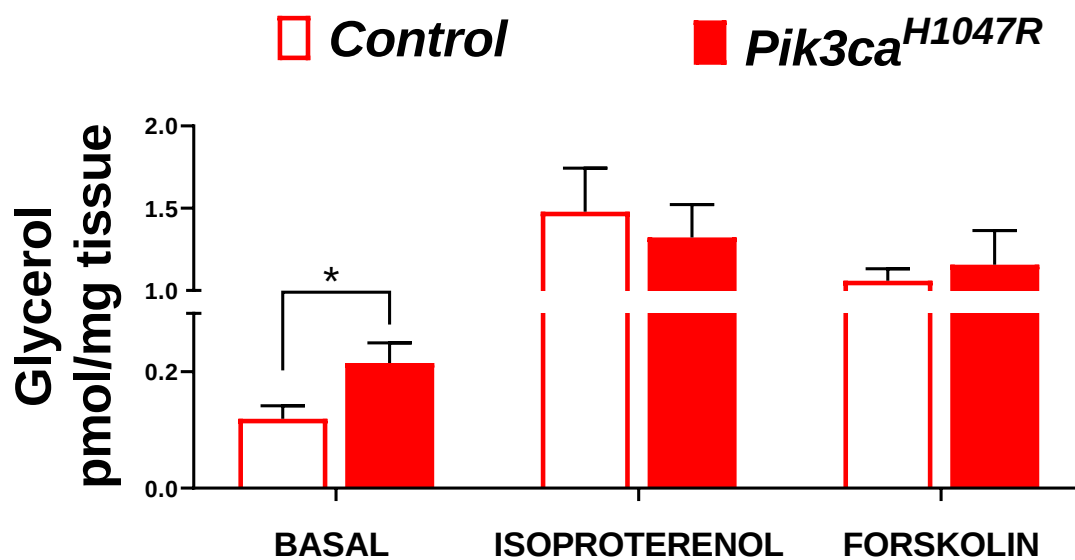
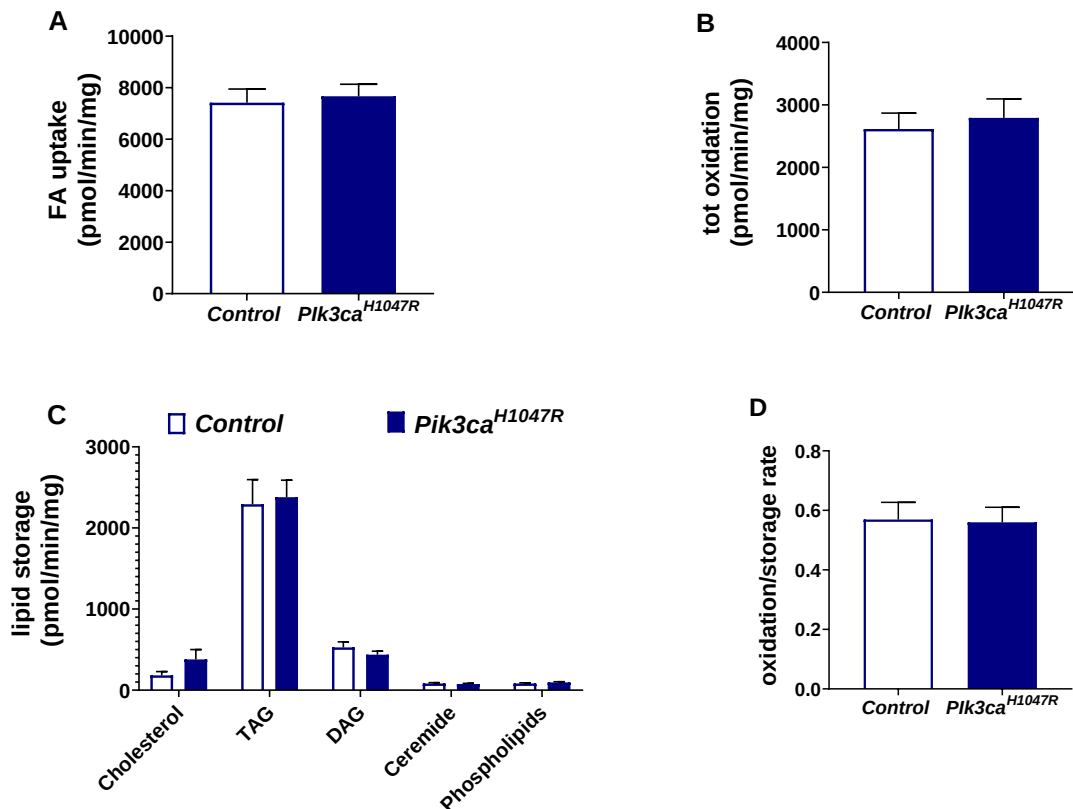


Figure 4. 12: Ubiquitous expression of the *Pik3ca*^{H1047R} mutation increases lipolysis in the adipose tissue of the mice.

Female *Pik3ca*^{H1047R} and control mice were administered tamoxifen (200mg/kg) on two consecutive days. 6 days post tamoxifen administration mice were moved to the Promethion metabolic cages and sacrificed 60 hours after. The graph shows the lipolysis in the adipose tissue, obtained *ex vivo*, by measuring a colorimetric product with absorbance at 570 nm (A_{570}) proportional to the amount of glycerol present into the media, in the basal state, and in the presence of isoproterenol or forskolin. Control mice are represented by empty bars, whilst *Pik3ca*^{H1047R} mice are represented by solid bars. Error bars represent mean $\pm$ SEM. * = p.value $\leq$ 0.05.

SKELETAL MUSCLE



LIVER

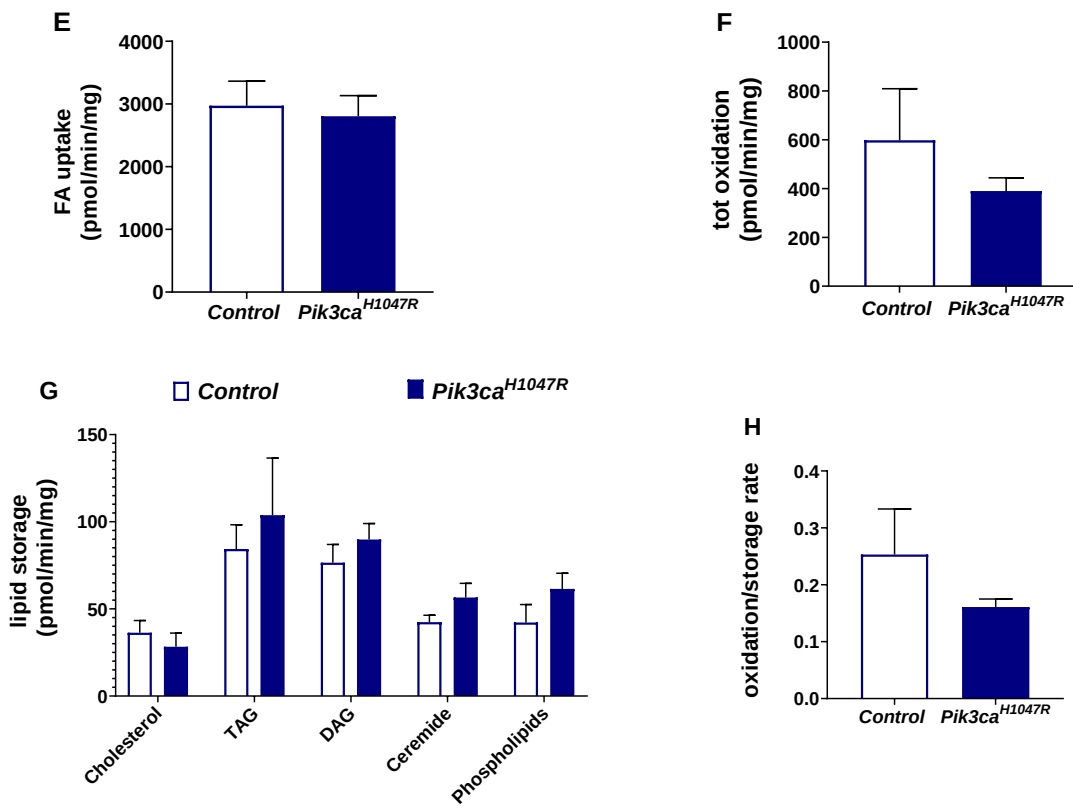


Figure 4. 13: Ubiquitous expression of the *Pik3ca*^{H1047R} mutation does not alter FFAs oxidation in the skeletal muscle and liver of mice.

Pik3ca^{H1047R} and control female mice were administered tamoxifen (200mg/kg) on two consecutive days. 8 days post tamoxifen administration mice were sacrificed and liver and skeletal muscle harvested. Graphs display (A) FFA uptake, (B) FFA oxidation (C) FFA storage into lipids and (D) fatty acids storage to oxidation ratio in the skeletal muscle of *Pik3ca*^{H1047R} and control mice. Graphs display (E) FFA uptake, (F) FFA oxidation (G) FFA storage into lipids and (H) FA storage to oxidation ratio in the liver of *Pik3ca*^{H1047R} and control mice. N mice used for the analysis of FFAs oxidation in the muscle= 18 control, 18 *Pik3ca*^{H1047R}. N mice used for the analysis of FFAs oxidation in the liver = 6 control, 6 *Pik3ca*^{H1047R}. Control mice are represented by empty dots and empty bars, whilst *Pik3ca*^{H1047R} mice are represented by solid dots and solid bars. CHO = cholesterol, TAG = triacylglycerides, DAG = diacylglycerides, CE = ceramide, PL = phospholipids. Error bars represent means $\pm$ SEM. * = p.value $\leq$ 0.05.

4.2.9 Ubiquitous expression of the *Pik3ca*^{H1047R} mutation reduces plasma ketone bodies.

In conditions of low glucose the liver generates ketone bodies which are then used by peripheral tissues as an energy source. The main ketone bodies are acetoacetate, 3- β -hydroxybutyrate (3- β -HB) and acetone. Ketogenesis is the process by which FFAs are transformed into acetoacetate and 3- β -HB. Ketone bodies form from acetyl CoA, derived from FA oxidation, which condense with oxaloacetate, produced by glycolysis.

To further assess whether FA oxidation occurred in the liver of mutant mice, the formation of ketones was investigated. For this purpose the levels of 3- β -HB were measured in the plasma of *Pik3ca*^{H1047R} and matched control mice. Figure 4.14 shows that mutant mice had decreased levels of plasma ketones, compared to control mice, 4 days post tamoxifen administration and the levels remained low until the end of the study (day 7). This data is in agreement with the lack of FA oxidation observed above.

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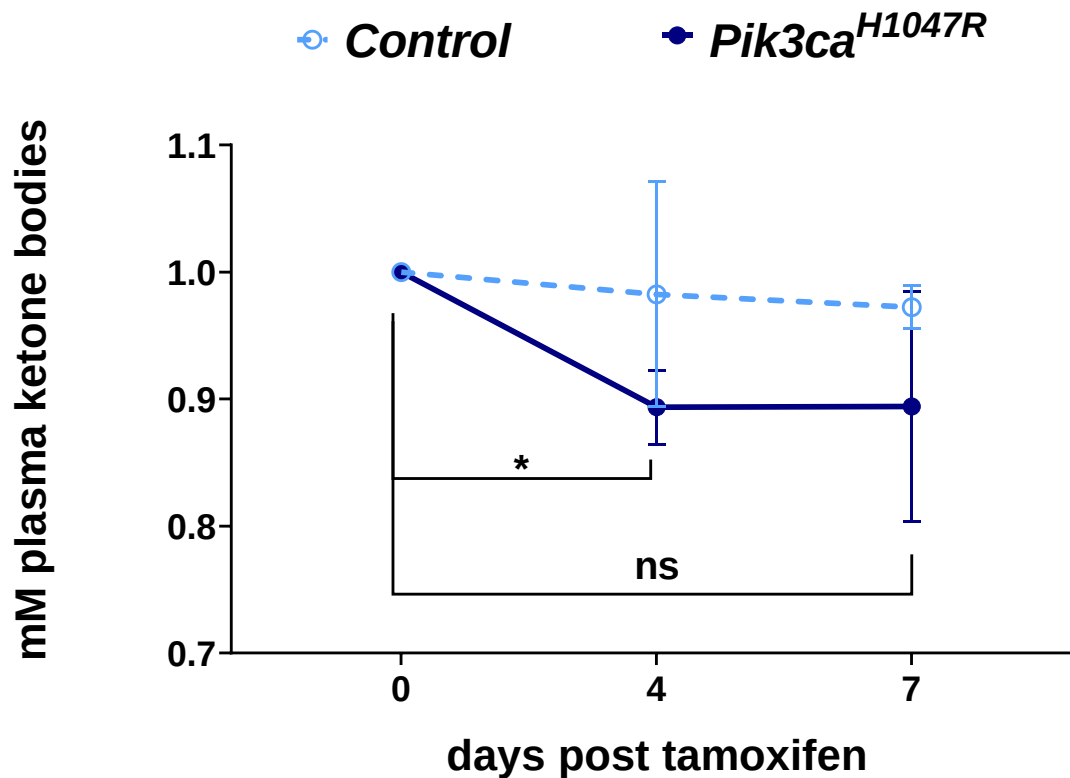


Figure 4. 14: Ubiquitous expression of the *Pik3ca*^{H1047R} mutation leads to decreased levels of plasma ketones.

Pik3ca^{H1047R} and control mice were administered tamoxifen (200mg/kg) on two consecutive days. Plasma samples were collected 0, 4 and 7 days post tamoxifen administration. Plasma ketones were measured by using a colorimetric assay (Abcam) which utilizes 3-β-HB dehydrogenase to generate a product which reacts with a colorimetric probe with an absorbance band at 450 nm. Control mice are represented by a dashed line, whilst *Pik3ca*^{H1047R} mice are represented by a solid line. Error bars represent means ± SEM. N = 5 control, 6 *Pik3ca*^{H1047R} * = p.value ≤ 0.05. ns= non-significant (p.value > 0.05).

4.2.10 Ubiquitous expression of the *Pik3ca*^{H1047R} mutation leads to WAT browning.

It has been shown above that a mutation in the PI3K pathway result in loss of body fat and increased lipolysis. Furthermore, in previous studies conducted on *Pik3ca*^{H1047R} mice, increased vascularisation at the level of the inguinal fat pad was observed in mutant mice,

compared to control mice (Kinross et al., 2015). In association with increased vascularisation, inguinal fat pad in *Pik3ca*^{H1047R} mice had a distinct gross morphology (Fig 4.15 A) and histological morphology (Fig. 4.15 B), characterised by the presence of small adipocytes clusters with a multilocular lipid droplet morphology, typical of BAT, suggesting the generation of beige adipocytes within the WAT of mutant mice. In agreement with this, the mRNA expression of genes specific for the beige adipocytes (*Tmem26* and *Cd137*) was increased in the BAT and WAT of mutant mice, compared to control mice (Figures 4.15 C and D).

Previous studies have established that the PI3K pathway regulates WAT browning by activating the appetite-suppressing proopiomelanocortin (POMC) and suppressing the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP)-neuropeptide expressing neurons in the arcuate nucleus (ARC) of the hypothalamus (Dodd et al., 2015). Thus, we examined whether *Pik3ca*^{H1047R} mice alters the mRNA expression of *Pomc*, *Npy* and/or *Agrp*. No change in *Pomc*, *Agrp*, and *Npy* neuropeptides mRNA expression was observed in *Pik3ca*^{H1047R} mice compared to control mice (Figures 4.15 G).

Interestingly, despite the generation of beige adipocytes within the WAT of mutant mice, no expression of the *Ucp1* mRNA (Figures 4.15 E) and protein (Figures 4.15 F) was detected. Whilst *Ucp1* mRNA expression seemed to be lower in the BAT of mutant mice, compared to control mice (Figure 4.15E), immunohistochemistry analysis showed high expression of Ucp1 in the BAT of both control and mutant mice (Figure 4.15F).

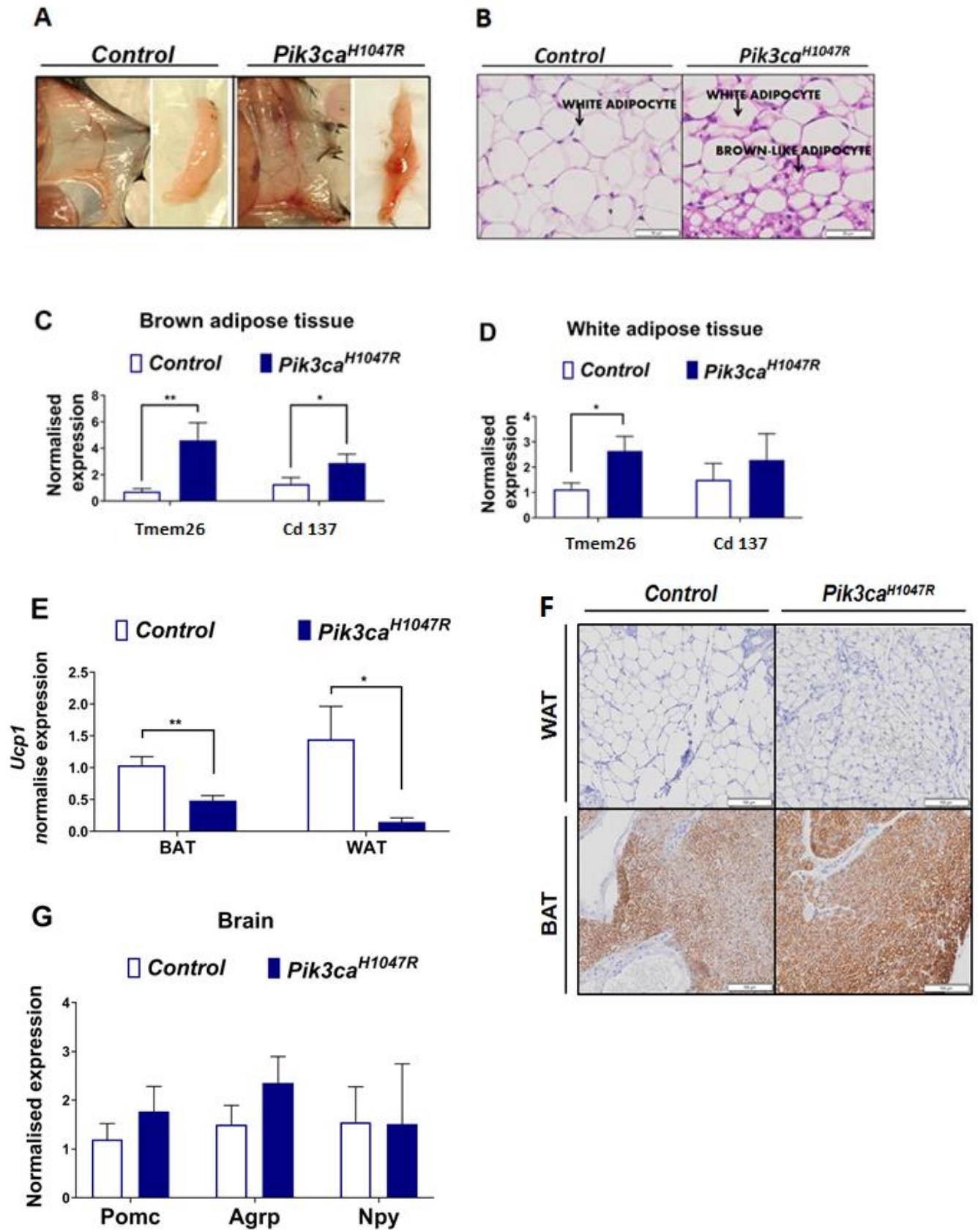


Figure 4. 15: Ubiquitous expression of the *Pik3ca*^{H1047R} mutation stimulates WAT browning but does not induce Ucp1 expression in WAT.

Mice were administered tamoxifen (200mg/kg) on two consecutive days. 7 days post tamoxifen administration mice were sacrificed and the hypothalamus, BAT and WAT were isolated. **A)** Gross morphology and **(B)** Histological [hematoxylin and eosin (H&E) staining] morphology of the inguinal fat pad of control and *Pik3ca*^{H1047R} mice. Graphs show mRNA expression of *Tmem26* and *Cd137* in **(C)** the BAT and **(D)** the WAT of control and *Pik3ca*^{H1047R} mice. **E)** Graph shows mRNA expression of *Ucp1* in the BAT and the WAT of control and *Pik3ca*^{H1047R} mice. **F)** Immunohistochemistry showing the levels of the Ucp1protein (brown) in the BAT and the WAT of control and *Pik3ca*^{H1047R} mice. Cell nuclei and eosin were counterstained staining with hematoxylin and eosin. **G)** Graph shows mRNA expression of *Npy*, *Agrp* and *Pomc* neurotransmitters in the brain of control and *Pik3ca*^{H1047R} mice. Error bars represent means $\pm$ SEM. N = 3-7 control, 3-7 *Pik3ca*^{H1047R} * = p.value $\leq$ 0.05, ** = p.value $\leq$ 0.01.

4.3 DISCUSSION

This chapter shows that mutations in the PI3K pathway alter the systemic metabolism of mice, resulting in increased lipolysis and altered energetic metabolism of the adipose tissue.

In this chapter the energetic metabolism of mice ubiquitously expressing the *Pik3ca*^{H1047R} mutation has been characterised. The body composition of *Pik3ca*^{H1047R} mutant mice and matched control mice was measured using TD-NMR. Another method, the dual energy X ray absorptiometry (DXA) is also used to study the body composition of animals (Sjogren et al., 2001). The two methods have been shown to provide comparable levels of reproducibility in measurements of body composition of mice (Halldorsdottir et al., 2009). However, DXA also provides information on the exact area of fat accumulation, which is not possible to obtain using NMR. Since this study did not aim to characterise the exact localisation of the fat in the body, NMR was chosen over DXA, because it allows faster measurements and does not require the animal to be anesthetized.

NMR showed that a mutation in the PI3K pathway results in severe loss of total body fat in mutant mice, compared to control mice. Although both female and male mutant mice had reduction of adipose tissue, female mice had a more drastic loss of total body fat, compared to male mice. These data support the well-established influence of sex on body composition (Bredella, 2017; Petruzzelli and Wagner, 2016). For instance, female mice, but not male mice, were found to respond to caloric restriction by increasing their lipolytic activity and lipid-oxidation. Furthermore, estrogen receptor alpha (ER α)-deficient female mice, but not male mice, have reduced adipose tissue lipolysis (Benz et al., 2012).

The rate of lipolysis in the adipose tissue of *Pik3ca*^{H1047R} mice, compared to control mice was also investigated in this chapter. Lipolysis is the process used to obtain energy from fat by hydrolysis of TAGs into FFAs and glycerol during times of energy deprivation. The storage of energy in the form of TAGs, and the ability to rapidly mobilize these reserves as FFAs to fuel the energetic demand, represents a highly adaptive metabolic response, typical of cancer cells (Duncan et al., 2007).

The first step of lipolysis is regulated by lipolytic hormones, such as norepinephrine, epinephrine, and adrenocorticotrophic hormone, which activate adenylate cyclase. Adenylate cyclase, in turn, stimulates the synthesis of cAMP, leading to activation of cAMP-dependent

protein kinase and subsequent phosphorylation and activation of HSL. Subsequent translocation of HSL from the cytosol to the surface of lipid storage droplets activates lipolysis (Brasaemle et al., 2000; Egan et al., 1992).

Lipolysis occurs in all tissues of the body where the resulting FFAs are used endogenously for energy production or biosynthetic pathways. The WAT is an exception as it releases FFAs and glycerol to supply nutrients to non-adipose tissues. For this study two lipolytic agents, isoproterenol and forskolin were used to stimulate the translocation of HSL to the membrane of the adipocytes (Morimoto et al., 2001).

Lipolysis in absence of these two agents was also measured to define the basal (non-stimulated) lipolysis in the adipose tissue. In the presence of both forskolin and isoproterenol the rate of adipose tissue lipolysis was the same in control and mutant mice. However, in basal conditions, the rate of lipolysis in the adipose tissue of mutant mice was higher than control mice.

Due to the well-established role of the PI3K pathway in lipid biosynthesis and lipolysis it was particularly surprising to observe an increased rate of lipolysis in mice harbouring the *Pik3ca*^{H1047R} mutation. It is well known, indeed, that insulin and AKT, suppress lipolysis and FFA release from adipose tissue through inhibition of the transcription factor FOXO1 (Chakrabarti and Kandror, 2009; Gross et al., 2009).

Lipolysis is essential in non-adipose tissues for instant provision of FFAs, particularly in highly oxidative tissues such as liver and skeletal muscles (Schweiger et al., 2014). Increased lipolysis is associated with alterations in FFA uptake from the surrounding microenvironment, *de novo* lipogenesis, storage as lipid droplets and β -oxidation.

The data presented in this chapter show that, despite an accentuated lipolysis in the adipose tissue of *Pik3ca*^{H1047R} mice, compared to control mice, the FFA uptake in the skeletal muscle, and liver which are the principal tissue deputed to FFA β -oxidation, remained unchanged between mutant and control mice. Additionally, the rate of FFA oxidation and the storage of FFAs into lipids was the same in control and mutant mice in both the organs. One possible explanation for the inconsistency between increased lipolysis and unchanged FFA oxidation is that, whilst lipolysis has been investigated in mice that were kept in metabolic cages for 60 hours prior to extraction of the adipose tissue, the FFA oxidation was studied in tissues of

mice that were not kept in the metabolic cages. During the last 24 hours spent inside the cages, indeed, the mice had the opportunity to exercise on the wheel and this exercise might have changed their lipid metabolism in favour of increased lipolysis and possibly increased lipid oxidation. A study conducted in 352 healthy male adults has shown that high-intensity exercise favours a lesser body fat deposition which might be related to an increase in post-exercise energy metabolism mediated by β -adrenergic stimulation (Yoshioka et al., 2001).

A simple way to test the efficiency of the FFA uptake test is to analyse the levels of FFAs inside the blood of mutant mice, versus control mice. Since an increased uptake of FFAs was not observed either in the liver or in the muscle of *Pik3ca*^{H1047R} mice, the levels of FFAs in the blood stream of mice harbouring the mutation should be higher than control mice: the FFAs generated through lipolysis, indeed, are released into the blood stream before being taken up by the tissues. Furthermore, the expression of FA transporters could help to validate the uptake of FFAs by the tissues. Indeed, it would not be surprising if mutations in the PI3K pathway altered the expression of FAs transporters. For instance, it was recently observed that the deletion of CD36 in highly aggressive *Pten*^{-/-} prostate cancers results in reduced FFA uptake and slowed cancer progression (Watt et al., 2019). Furthermore, to test the effect of the *Pik3ca*^{H1047R} on lipolysis, the activity of the nervous system could be tested. As discussed in the introduction to this chapter (Section 4.1.4), WAT browning is under the control of specific neurone populations in the arc of the hypothalamus. During a state of energy surplus, POMC/CART neurones stimulate WAT browning, whilst AgRP/NPY neurons are suppressed. Both neuropeptides AgRP/NPY and POMC are important to maintain normal lipid and glucose homeostasis in peripheral tissues, such as the liver, muscle and the pancreas (Varela and Horvath, 2012). In general, sympathetic neurons transmit their signal by releasing noradrenalin, which subsequently binds to adrenergic receptors, located at the postsynaptic membrane on the target organ (Fluck, 1972). Stimulation of β -adrenergic receptors in WAT triggers adenyl cyclase to release cAMP. The release of cAMP activates PKA, which in turn phosphorylates adipose tissue TG lipase (ATGL), HSL, and perilipins, ultimately resulting in lipolysis of stored TG (Bartness and Song, 2007). Whilst the role of the nervous system has been partially investigated in this thesis, as discussed below, the levels of PKA, ATGL and HSL are currently under investigation in our laboratory.

In this chapter it was also observed that *Pik3ca*^{H1047R} mice have reduced levels of ketone bodies, compared to control mice. Ketone bodies are the product of the condensation of acetyl CoA, derived from FA oxidation with oxaloacetate, produced by glycolysis. This reduction might be associated with the lack of FA oxidation, which does not provide sufficient acetyl CoA, or to the lack of gluconeogenesis in the liver (Chapter 3, Section 3.2.9). Indeed ketogenesis requires efficient mitochondrial β -oxidation of fatty acids (Grabacka et al., 2016). Oxaloacetate is derived from pyruvate during glycolysis. Therefore, it is essential to have a level of glycolysis that provides sufficient oxaloacetate to condense with acetyl CoA. If glucose levels become too low, then oxaloacetate is preferentially utilized in the process of gluconeogenesis, instead of condensing with acetyl CoA (Laffel, 1999). In the presence of oxaloacetate, instead, acetyl CoA is mainly used in the TCA cycle. Another possibility is that, since the insulin-PI3K pathway is active in the liver of mutant mice, the liver does not sense the lack of nutrients, therefore inhibiting ketogenesis. In circumstances where insulin levels are relatively high, indeed, the synthesis of ketones is inhibited. In situations of relative insulin deficiency, such as starvation or type I diabetes, FFA oxidation is increased, driving the production of alternative energy-producing substrates, ketone bodies, for use by the brain, as well as peripheral tissues. High levels of ketones, indeed, have been associated to higher risk of diabetes (Kanikarla-Marie and Jain, 2016).

Due to its role in lipid metabolism, PI3K has a central role in the development of hepatic lipogenesis (Yecies et al., 2011) and obesity (Huang et al., 2018). In agreement with this, the expression of a dominant negative human PI3K mutation in mice leads to reduced weight gain and adipose accumulation (Solheim et al., 2018). In addition, accumulation of lipid droplets is typical of cancers with mutations in the PI3K pathway, (Koundouros and Poulogiannis, 2020) such as breast cancer (Kinlaw et al., 2006; Orešić, 2012), gastrointestinal tumours (Li et al., 2017), colon cancer (Fazolini et al., 2015) hepatocellular carcinoma (Guri et al., 2017) and prostate cancer (Audet-Walsh et al., 2018; Yue et al., 2014).

In this chapter the lipid metabolism of mice harbouring the *Pik3ca*^{H1047R} mutation and control mice was further investigated by indirect calorimetry, which measures the rate of endogenous FFAs oxidation *in vivo* from the exchange of oxygen (O₂) and carbon dioxide (CO₂). The ratio between the volume of O₂ consumed and the volume of CO₂ generated is indicated as respiratory RER (Parker et al., 2001). It was observed that the volume of O₂ consumed and

CO₂ generated by the mice as well as their RER was higher at night time than during daytime, independently of the sex and the genotype. All the mice used in this study consumed more fat than carbohydrates during the day (RER~0.7) to generate energy, whilst they metabolised mostly carbohydrates at night (RER~0.9). No overall change in the RER of mutant mice, compared to control mice was observed, with exception of specific times of the day at which the drop in RER overlapped with reduced food intake, at indication that mutant mice used more fat during fasting, than control mice. When carbohydrates were not supplied through the diet, mutant mice were not able to use their hepatic reserves of glucose, and therefore switch towards lipid metabolism. In normal conditions, glucose taken up during a meal is stored in the form of glycogen in the liver and muscle and it is ready to be used to generate energy when needed.

During short-term fasting periods, the liver produces and releases glucose mainly through glycogenolysis, which is the breakdown of glycogen to glucose (Zhang et al., 2018). In the fed state, the PI3K/AKT signalling pathway reduces hepatic glucose production (HGP) and glycogenolysis (Huang et al., 2018). The activation of the PI3K/AKT signalling pathway in all the organs of the mouse by tamoxifen administration might be sensed by the tissues as a constant fed state, even in the absence of nutrients, therefore inhibiting glucose release from the liver. It was, indeed, shown in chapter 3 (Section 3.2.12) that mutations in the PI3K pathway inhibit gluconeogenesis and HGP in mice. Therefore, mutant mice have to burn more lipids, when there is a lack of food, because they are not able to utilise their hepatic reserves of glucose.

In this chapter it is also shown that both control and mutant mice have higher EE during the day than at night, independently of the sex. The increase in EE is associated with higher activity and food intake during the night. Both female and male mutant mice had reduced energy expenditure (EE), compared to control mice associated with lower ambulatory activity. This data was in disagreement with the previously described role of insulin in increasing EE (Klaman et al., 2000; Zong et al., 2011). Dodd and colleagues found that insulin can increase energy expenditure by inducing the browning of WAT (Dodd et al., 2015). This discrepancy might be due to the fact that the mouse model used in this study carries a whole body/systemic mutation, whilst the mouse model used by Dodd and colleagues harbours a localised manipulation of insulin signalling in the hypothalamus.

In this study it was shown that mice harbouring the *Pik3ca*^{H1047R} mutation had *Pik3ca*^{H1047R} mice had a distinct gross morphology and histological morphology, characterised by the presence of small adipocytes clusters with multilocular lipid droplet morphology, typical of BAT. Furthermore, mutant mice had higher expression of the genes specific for beige adipose tissues, *Tmem26* and *Cd137* in BAT and WAT, suggesting that WAT and BAT had increased beige adipocytes, compared to control mice. However, the mutation had not caused an increased expression of *Ucp1* (Section 4.1.4), both at the mRNA and protein level. This might be due to the fact that *Ucp1* is lost after the induction of the stimuli in beige fat. For instance, Rosenwald and colleagues showed that cold-induced beige adipocytes lose the expression of *Ucp1*, but can persist in the tissue after the cold stimulus is removed (Rosenwald et al., 2013). However, the study also showed that after the stimuli beige adipocytes change both their morphology and their gene expression profile to that of a white adipocyte, which does not explain the high expression levels of *Tmem26* and *Cd137* observed in *Pik3ca*^{H1047R} mice. We therefore, speculated that the *Pik3ca*^{H1047R} mutation stimulates the generation of beige adipocytes within the WAT, but these are *Ucp1* negative, therefore limiting the consumption of energy. This is in agreement with the lower EE observed in mutant mice, compared to control mice.

Physical activity is the second main factor influencing EE. It is distinct from exercise which is a repetitive and volunteer movement aimed to improve body fitness. The role of exercise on energy expenditure has been largely explored. Exercise may influence resting energy expenditure in 3 ways: (a) a prolonged increase in post-exercise metabolic rate; (b) a chronic increase in resting metabolic rate; and (c) a possible increase in energy expenditure during non-exercising time (Poehlman et al., 1991). Timothy J. O’Neal and colleagues observed that the effect of voluntary exercise - wheel running - for 3 to 7 days increased daily energy expenditure of rodents. However, total daily energy expenditure remained stable after the first week of wheel access, despite further increases in wheel use, due to the existence of compensatory mechanisms that restore basal energy expenditure levels after the first week.

Here the relationship between voluntary exercise – wheel running – and total daily energy expenditure of mice carrying the *Pik3ca*^{H1047R} mutation was investigated for the first time. It was observed that female but not male mutant mice exercised for a shorter time than control mice. Furthermore, both male and female mutant mice had reduced ambulatory activity,

compared to control mice. This suggests that, whilst male mutant mice continued to keep active by using the wheel as opposed to moving around the cage, female mice harbouring the *Pik3ca*^{H1047R} mutation, lowered both their movements and their rate of exercise during the study. Whilst female mice consumed the same amount of food as control mice for the whole duration of the study, with the exclusion of the first few hours following the addition of the wheel to the cages, mutant male mice had significantly lower food intake during the night and in the second day-time post addition of the wheel.

Finally, during time spent in the metabolic cages, the body mass of the mice was monitored and recorded. In disagreement with what was observed in the previous chapter (Chapter 3-Section 3.2.3- Figure 3.3), the body weight of mutant male mice remained unchanged with respect to control mice. This might be because the body weight is monitored each time the mice sit in the weight module (cabin) inside the cage. The body weight monitor has two limitations. First, body weight is recorded only when the animal interacts with the device (mouse inside the cabin), therefore changes in body weight are not recorded when the mouse does not access the cabin inside the cage. Second, if the mouse brings food inside the cabin, the weight of the food is added to the body weight of the mouse, therefore causing errors in the measurements of body weight.

Aside from the studies on systemic metabolism, this chapter has presented studies on single tissues labelled with ²H₂O (heavy water). In chapter 3 it has been shown that mice harbouring the *Pik3ca*^{H1047R} mutation have increased body weight (Chapter 3-Section 3.2.3- Figure 3.3) and increased organs size (Chapter 3-Section 3.2.3- Figure 3.4).

In this thesis it has been demonstrated that the increased body weight is not associated with adiposity, making it clear that the increase in organ size is the only cause responsible for increased body weight in *Pik3ca*^{H1047R} mice. ²H₂O has been used to identify the cause of the growth of the tissues. During ²H₂O administration, dividing cells incorporate deuterium into the covalent C–H bonds of the deoxyribose of the DNA. Non-dividing cells do not incorporate deuterium into deoxyribose. Newly divided cells, therefore can be identified based on the deuterium isotopic enrichment in ²H in DNA. Mice were treated with ²H₂O for 7 days post administration of tamoxifen and the incorporation of ²H into the deoxyribose of DNA and alanine of the proteins, was studied by GC-MS.

It has been found that the *Pik3ca*^{H1047R} mutation leads to hyperplasia in liver and spleen of mutant mice. Interestingly, no increased proliferation was observed in the intestine of mutant mice compared to control mice. This result is likely due to the time point at which the analysis was performed. Compared to other tissues, the intestine has a high turnover rate (Creamer et al., 1961). Therefore, a possible reason why a difference in the proliferation rate between control and mutant mice was not observed is that the intestine had reached 100% ²H₂O labelling in both control and mutant mice, 7 days post tamoxifen treatment.

Cell cycle analysis and bromodeoxyuridine labelling could be used in the future to test this hypothesis, by looking at the proliferation rate of intestine 7 days post tamoxifen administration. Possibly performing the ²H₂O test at earlier time points could have shown a difference in the rate at which the tissues of control and mutant mice replicate. For instance, in a study on the DNA synthesis rates of colon epithelial cells, animals were given ²H₂O for 3 days only, instead of 7 as in this study, before being sacrificed (Kim et al., 2005).

The enrichment of ²H in the DNA of the brain was, instead, equal to zero, both in control and mutant mice, suggesting that the cells of the brain did not undergo any cell division in 7 days. This is in agreement with the slow rate at which the brain replicates, suggesting that the experiment needs to be performed at a later time point. However, it has been shown that 7 days post tamoxifen administration the size of the brain of mutant mice was already increased compared to control mice (Chapter 3-Section 3.2.4-Figure3.4). This increase was not associated with hyperplasia since the cells of the brain were not dividing. However, it was found the ²H enrichment in brain proteins of mutant mice was higher than in control mice, suggesting that the tissue was growing by hypertrophy.

This data is in agreement with a mouse model of human *Pik3ca*-related brain overgrowth, generated by Roy and colleagues (Roy et al., 2015), which demonstrates increased neuronal cell size. Future analysis across a time course would allow more detailed characterisation of these changes over time in response to the *Pik3ca*^{H1047R} mutation in mice.

4.3.1 Overall findings:

In this chapter it has been shown that mice harbouring the *Pik3ca*^{H1047R} mutation have severe loss of total body fat, compared to control mice. Accordingly, *Pik3ca*^{H1047R} mice have higher rate of lipolysis in the adipose tissue, compared to control mice. Furthermore, RER analysis showed that mutant mice use more fat than carbohydrates during fasting, probably due to their inability to release glucose from the liver, as described in chapter 3.

However, despite the increased lipolysis, the rate of FFA uptake and oxidation in the skeletal muscle of *Pik3ca*^{H1047R} mice remained unchanged with respect to control mice. Even more surprisingly, the FFA uptake in the liver of mutant mice was reduced compared to control mice. Further analyses are required to validate this result.

It has also been shown that mice harbouring the *Pik3ca*^{H1047R} mutation have lower EE than control mice.

The *Pik3ca*^{H1047R} mutation is associated with increased WAT browning which is independent from the levels of neurotransmitters. The beige adipocytes are Ucp1 negative, in support of the lower EE observed in mutant mice, compared to control mice.

Additionally, in this chapter, the mechanisms responsible for the tissue growth occurring in mutant mice has been characterised, showing that the *Pik3ca*^{H1047R} mutation leads to hyperplastic growth of some of the tissues, including spleen and liver, and hypertrophic growth of the brain. Further analyses are required to confirm these data. In particular further analysis on the proliferation rate of the brain need to be performed at a later time point, post activation of the mutations with tamoxifen, *in vivo*. This would allow assessing the possibility that the lack of hyperplasia observed in the brain of mutant mice was due to the slow division rate of neuronal cells.

CHAPTER 5

IDENTIFYING METABOLIC ALTERATIONS RESULTING FROM MUTATIONS IN THE PI3K PATHWAY THROUGH TRANSCRIPTOMICS AND PROTEOMICS

5.1 INTRODUCTION

In the previous chapters it has been shown that mice harbouring mutations in the PI3K pathway have aberrant systemic metabolism, associated with hypoglycaemia and hypoinsulinemia, loss of body fat, increased lipolysis and organomegaly. However, the mechanism by which the tissues support their growth and the metabolic pathways used to sustain the energetic demand remained unclear.

As described in the introduction to this thesis (Chapter 1, Section 1.4), PI3K–AKT signalling regulates cellular metabolism, directly by phosphorylating metabolic enzymes and nutrient transporters, or indirectly by activating downstream effectors that play a major role in cellular metabolic reprogramming, including mTORC1, GSK3 and FOXO transcription factors. The activation of mTORC1, in particular, drives the increased protein synthesis occurring during growth. Different mechanisms through which mTORC1 regulates protein synthesis have been described and summarised in Hoxhaj review (Hoxhaj and Manning, 2020). mTORC1 induces protein synthesis by favouring the release of 4E-BP from eIF4E at the 5' cap of mRNAs, by enhancing ribosomal DNA transcription through both RNA polymerase I and III or by inducing the dimerization of eIF4B, with the RNA helicase eIF4A, thereby enhancing the unreel of 5' untranslated regions with complex secondary structures.

Due to the effect of the PI3K-AKT pathway on both transcription and translation, the metabolic reprogramming resulting from mutations in the PI3K pathway has been investigated in this chapter at both the transcriptional and translational levels, by using transcriptomic and proteomic techniques. In order to track the major number of changes occurring in the transcriptome and proteome in response to PI3K activation, *Pik3ca*^{H1047R}*Pten*^{-/-} mice were used for this study. Indeed, co-occurrence of the mutations, determines a higher activation of the PI3K pathway, compared to the when a single mutation is present (Kinross et al., 2012).

Three mouse tissues harbouring *Pik3ca*^{H1047R}*Pten*^{-/-} mutation were analysed in this study: the brain, the intestine and the liver. The brain and the intestine were investigated for two reasons: 1) mutations in the PI3K pathway determine an increase in their growth (Chapter 3-Section 3.2.3); 2) 3D culture of neuronal spheroids and intestinal organoids were available in our laboratory, therefore enabling the use of *in vitro* systems for the validation of the data resulting from the transcriptomic analysis.

Studies on the transcriptome of the liver were performed to add additional information to what was already learned about liver metabolism in this thesis (Chapter 3-Section 3.2.9-3.2.11 and Chapter 4-Section 4.2.9). Furthermore, the same three tissues were used for *in vivo* ²H₂O labelling studies (Chapter 4-Section 4.2.2). Therefore, transcriptomic data can be combined with the previous studies to have a more complete understanding of the effect of PI3K mutations on the metabolism of these three tissues.

5.1.1 High-dimensional biology

The advances in genomic technology obtained during the past 20 years have made it possible to access the information contained within the human genome and investigate the genetic variants associated with diseases. However, diseases are not always associated with genetic mutations, but can also be determined by post-transcriptional (Corbett, 2018; Kim and Kyung Lee, 2012) or post translational regulation (Gajjala et al., 2015; Li et al., 2010; Pascovici et al., 2018; Ramesh et al., 2020).

High-dimensional biology (HDB) also referred to as "omics" sciences combines the studies of the genes (genomics), mRNAs (transcriptomics), proteins (proteomics) and metabolites (metabolomics) to have a greater understanding of normal physiological processes and also to investigate changes occurring in disease (Romero et al., 2007).

5.1.2 Analysis of the transcriptome

The analysis of the transcriptome can capture all the mRNA variants generated from alternative splicing, RNA editing or alternative transcription initiation and termination sites.

Since 1976, when Fiers and colleagues (Fiers et al., 1976) sequenced the RNA of the bacteriophage MS2 for the first time, many techniques have been developed to study the transcriptome. The most modern techniques are based on the use of reverse complementary DNA (cDNA) sequencing in combination with RT-PCR, which allows the sequencing of amplified cDNA

5.1.2.1 RNA-seq

RNA-seq is one of the most recently developed techniques, which is used to examine the quantity and sequences of RNA in a sample using next generation sequencing (NGS). The technique has been heavily influenced by the development of Illumina technologies (San Diego, CA) which is able to sequence thousands or millions of DNA molecules (Lowe et al., 2017). Gene set enrichment analysis, based on the functional annotation (gene ontology) of the differentially expressed genes, is commonly used for interpreting gene expression data. In this study the ensemble of gene set enrichment analyses (EGSEA) has been used. EGSEA combines the results from twelve algorithms to arrive at a consensus gene set ranking to identify biological themes and pathways perturbed in an experiment (Alhamdoosh et al., 2017). It provides access to a diverse range of gene signature collections, that includes more than 25,000 gene sets for human and mouse (Alhamdoosh et al., 2017).

5.1.3 Analysis of the proteome

Proteomics includes the study of all the proteins of a cell. It supplements genomic and transcriptomics to characterise the identity of proteins of an organism, and to define the structure and function of a particular protein (Aslam et al., 2017). One important consideration, when studying the proteome, is its huge complexity associated with the large number of possible three-dimensional conformations that primary sequence polypeptides can fold into, and the several chemical modifications (phosphorylation, glycosylation, lipidation) that a protein can undergo to turn into its final functional product (Manzoni et al., 2018). Proteins extracted in the initial step for protein expression analysis are separated by gel electrophoresis or liquid chromatography and then visualised through mass spectroscopy

(MS) (Chuthapisith et al., 2007). MS allows the study of protein–protein interactions, the mapping of numerous organelles, and the generation of quantitative protein profiles from diverse species based on their molecular weight (Aslam et al., 2017). The main advantage of label-free MS is that it requires minimal manipulation of the sample, without the need to modify peptides/proteins with stable isotope-containing compounds or to add heavy isotope-labelled internal standards to the sample (Xie et al., 2011).

5.1.4 Three-dimensional *in vitro* cell culture models

Whilst mouse models were useful to study the metabolism at a systemic level and to investigate how several organs harbouring mutations in the PI3K pathway affected each other's metabolism, they presented some limitations. Studies performed on the entire organ did not enable the characterisation of specific cell type metabolism. For instance, the use of drugs that target metabolism can be lethal for the mice. The use of 3D cultures enables the investigation of the metabolic reprogramming resulting from specific mutations, in real time and without affecting the animals.

In this study the metabolism of 3D cultures of neurospheres and intestinal organoids was studied by using the Seahorse Extracellular flux (XF) Analyzer which studies key cellular functions such as mitochondrial respiration and glycolysis in live cells by measuring their oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The assay is based on the use of drugs that target mitochondrial respiration and glycolysis, which would be lethal if administrated *in vivo*.

5.1.5 Aim

- characterise the effect of mutations in the PI3K pathway on the transcription and translation of genes involved in cellular metabolism.

5.2 RESULTS

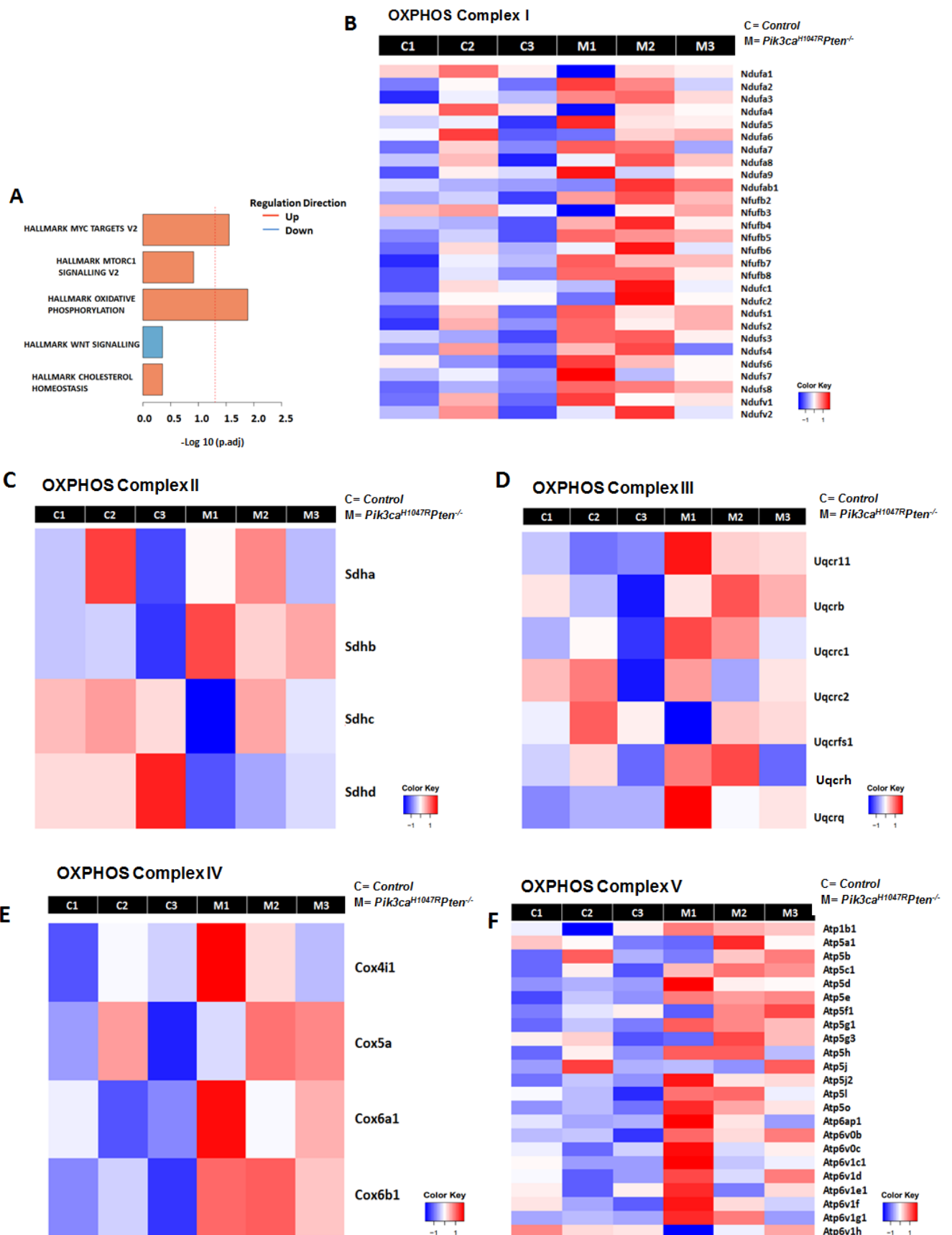
5.2.1 3'RNA-seq of the brain of mice harbouring the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations.

In order to understand how mutations in the PI3K pathway affect brain metabolism, RNA-seq was performed in the brain of *Pik3ca*^{H1047R}*Pten*^{-/-} and matched control mice. Mice were treated with tamoxifen (200mg/kg) on two consecutive days to induce the expression of the *Pik3ca*^{H1047R} mutation and also knockout *Pten*. One day after the second dose of tamoxifen, mice were sacrificed and the brain harvested. RNA was extracted and 3'-RNA-seq was performed by the molecular genomics core (Peter MacCallum Cancer Centre). RNA-seq reads were aligned using the hierarchical indexing for spliced alignment of transcripts (HISAT) (Kim et al., 2015), and gene expression quantified using high-throughput sequencing (HTS) software (Anders et al., 2015). Normalised expression was measured in count-per-million (CPM) in log₂ scale, with library size adjustment using Trimmed Mean of M-values method (Robinson and Oshlack, 2010), using edgeR (Robinson et al., 2010) R package.

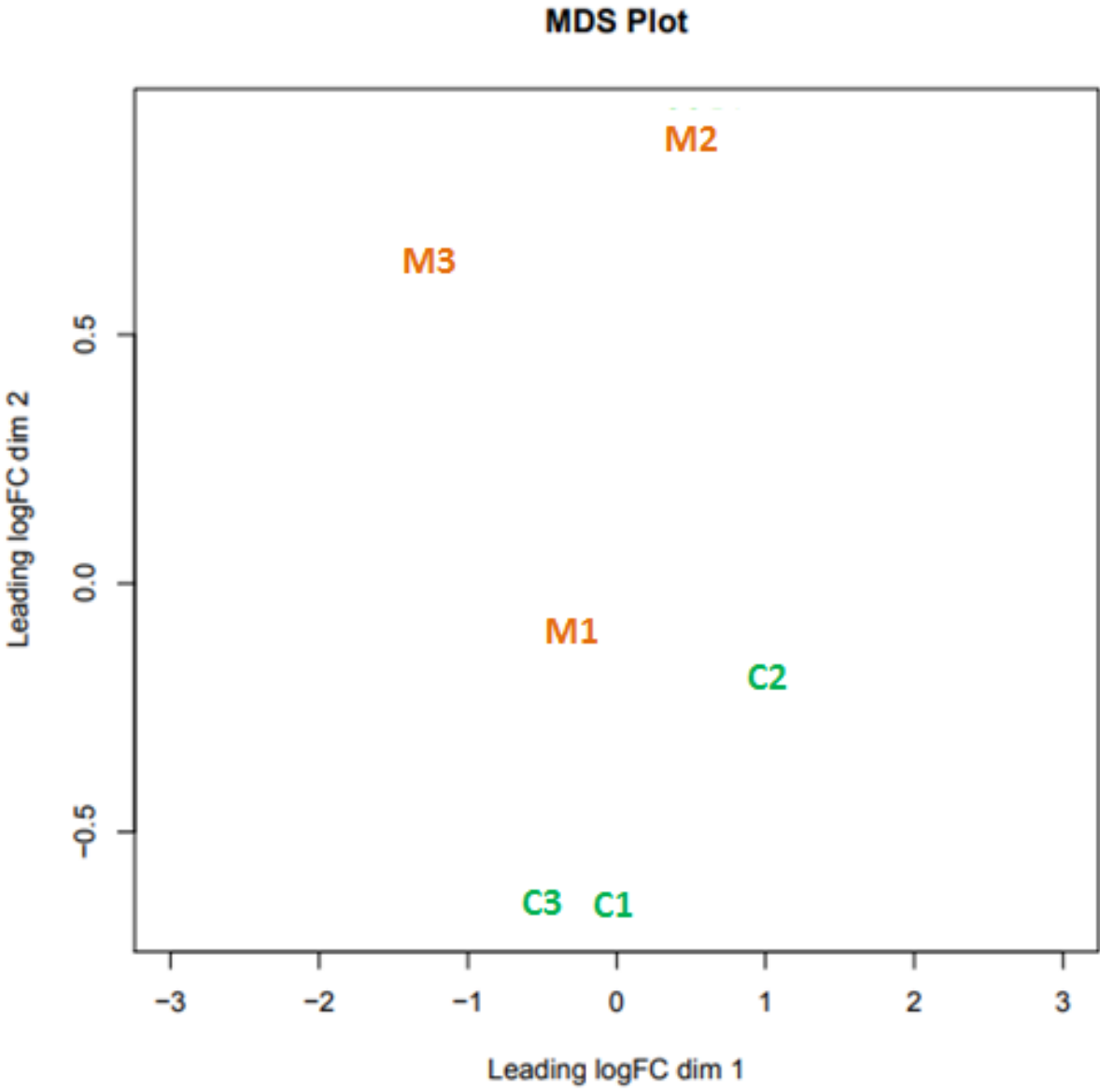
Figure 5.1A shows the gene sets enriched in the brain of mutant mice as compared to control mice. Data are displayed as a summary bar chart where the colour of the bars is based on the direction of regulation of the gene sets: red for up-regulated, blue for down-regulated. The mTORC and MYC pathways, downstream from PI3K, as well as cholesterol metabolism and oxidative phosphorylation (OXPHOS) pathways were upregulated in the brain of *Pik3ca*^{H1047R}*Pten*^{-/-} mutant mice, whilst the Wnt/ β -catenin signalling pathway was downregulated. Interestingly, the OXPHOS pathway was the most enriched pathway in the brain of mutant mice, compared to control mice. In particular, genes that encode components of OXPHOS complexes I, III, IV and V were upregulated in the mutant mice (Figures 5.1 B, D-F). The effect of the *Pik3ca*^{H1047R}*Pten*^{-/-} mutation on complex II was less pronounced with some of the genes being upregulated (*Sdhb*), some downregulated (*Sdhd*) and some unchanged (*Sdha* and *Sdhc*) (Figure 5.1 C). Multiresolution Correlation Analysis (MCA) showed that the distribution of the samples was scattered (Figure 5.1G), at indication that variability was present between the samples.

Multiple protein-protein interaction analysis performed on genes that were upregulated in mutant, compared to control mice showed that genes belonging to OXPHOS (red) were

upregulated in *Pik3ca*^{H1047R}*Pten*^{-/-} mice, with respect to control mice (Figure 5.1 H-I). Surprisingly, as opposed to what is shown by 3'RNA-seq data, no difference in the mRNA expression of genes involved in OXPHOS was observed between mutant and control mice when using RT-PCR (Figure 5.2 A). Seven of the top differentially expressed genes were used for the RT-PCR and their expression levels were normalised to mouse β -actin. Similarly, no change in the levels of representative proteins for the five OXPHOS complexes of the mitochondria was observed in the brain of *Pik3ca*^{H1047R}*Pten*^{-/-} mice, compared to control mice (Figure 5.2 B).



G



OXPHOS

Other pathways

H

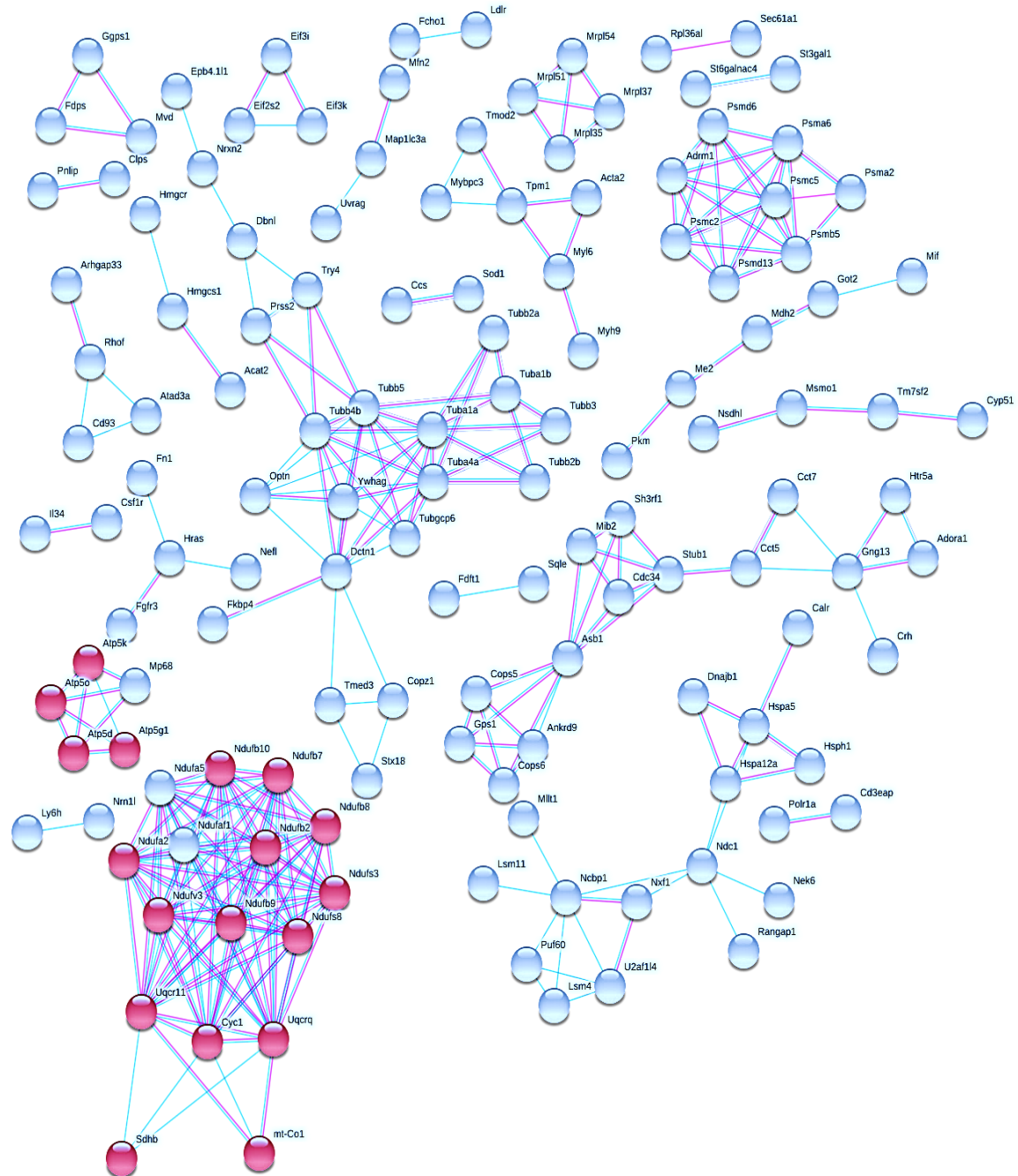


Figure 5. 1: 3'RNA-seq performed on the brain of mice harbouring the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations showed increased expression of genes belonging to the OXPHOS pathway.

Pik3ca^{H1047R}*Pten*^{-/-} and control mice were administered tamoxifen (200mg/kg), via oral gavage, on two consecutive days. One day post the second dose of tamoxifen mice were sacrificed and the brain harvested. The RNA was extracted and the sequencing was performed by the Molecular Genomics core (Peter MacCallum Cancer Centre). **(A)** The bar plot displays the $-\log_{10}$ (p. adj) values for the top 5 enriched gene sets obtained using EGSEA. The orange bars represent up-regulation, whilst the blue bar represents down-regulation. Heatmaps of the normalized read counts of genes belonging to the OXPHOS **(B)** complex I, **(C)** complex II, **(D)** complex III, **(E)** complex IV and **(F)** complex V in control vs mutant mice. C= control, M= *Pik3ca*^{H1047R}*Pten*^{-/-}. The threshold was set to p-value ≤ 0.05 , and $\log_2$ fold change ≥ 0.5 . Colors range from blue for low expression to red for high expression. Some of the genes belonging to complex III are missing due to an error in loading the complete gene sets on Galaxy. **G)** Multiresolution Correlation Analysis (MCA) showing the samples distribution. **H)** Protein-protein interaction network obtained using the String protein interaction network platform (p-value ≤ 0.05). **I)** Magnification of the OXPHOS complex genes are highlighted in red. N= 3 Control, 3 *Pik3ca*^{H1047R}*Pten*^{-/-}.

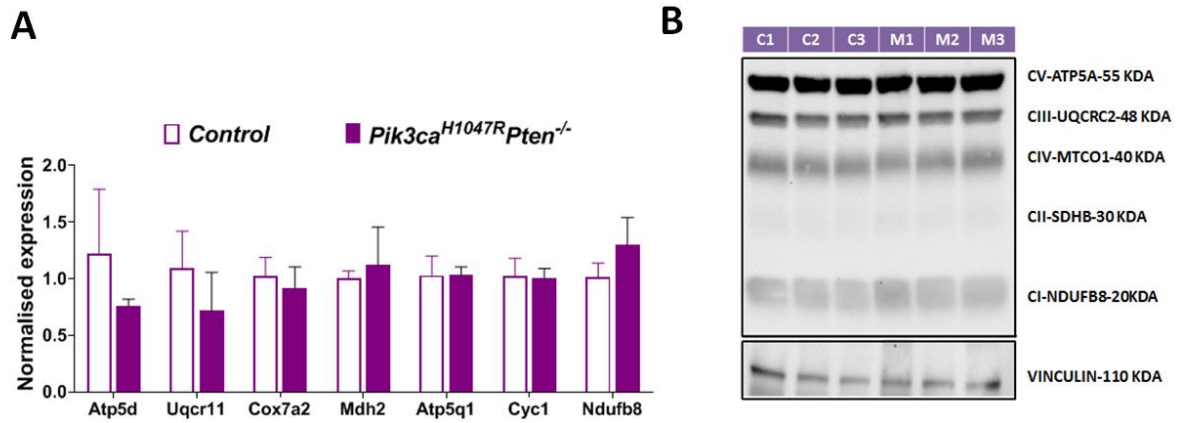


Figure 5. 2: Analysis of the OXPHOS pathway expression in the brain of mice ubiquitously expressing the *Pik3ca^{H1047R}Pten^{-/-}* double mutation.

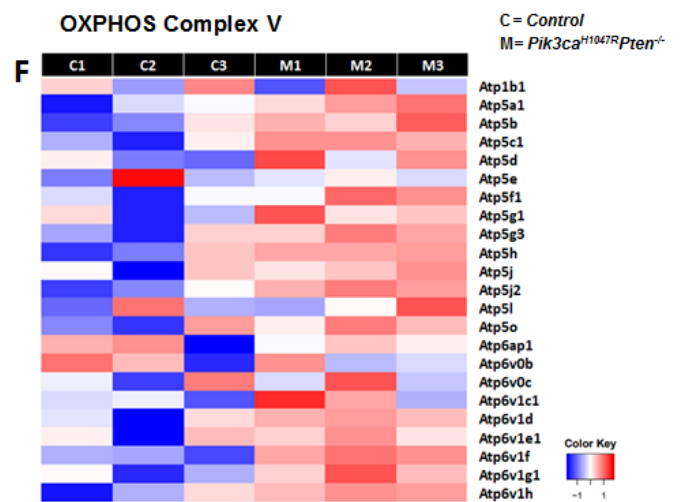
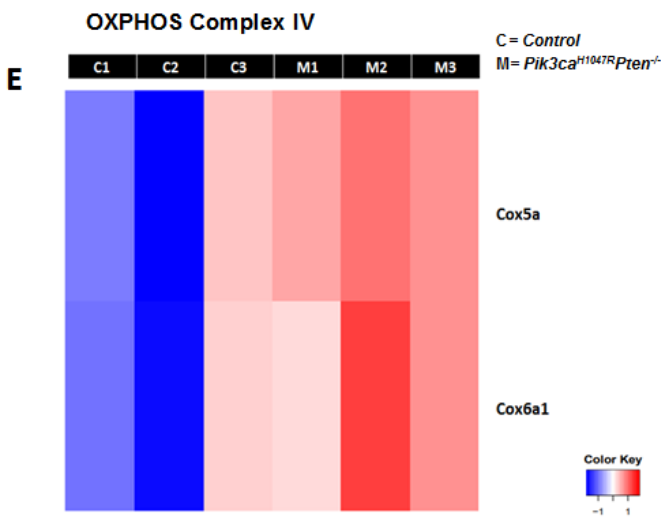
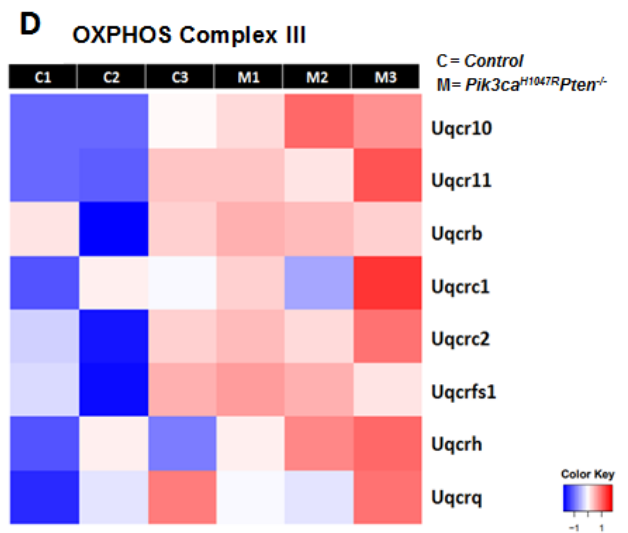
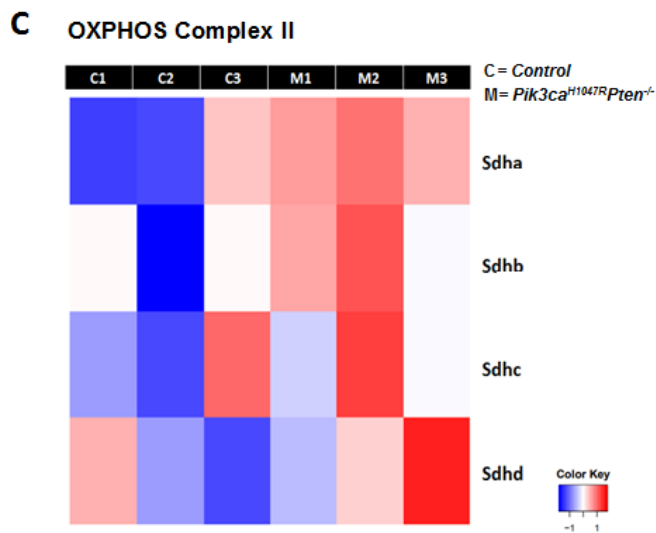
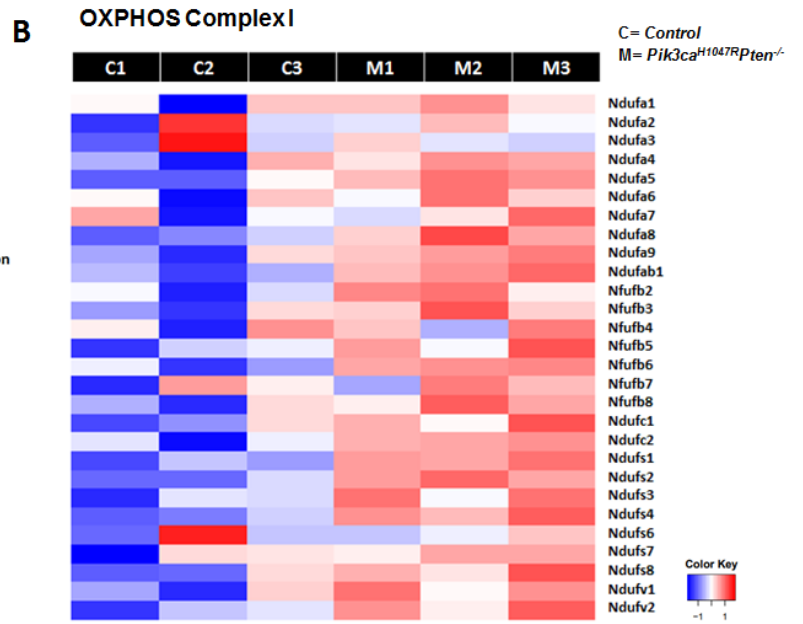
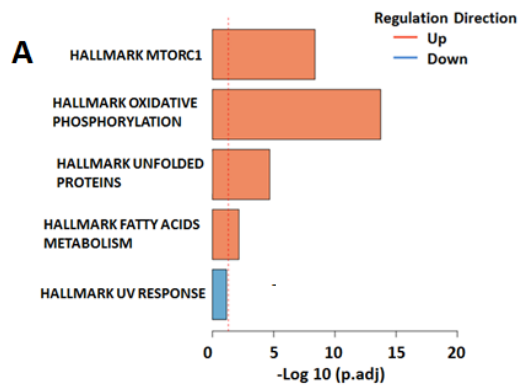
Pik3ca^{H1047R}Pten^{-/-} and control mice were administered tamoxifen (200mg/kg), via oral gavage, on two consecutive days. One days later mice were sacrificed and the brain harvested. Proteins and RNA were harvested and the cDNA synthesised. **(A)** The graph displays the mRNA levels of representative genes belonging to OXPHOS pathway, quantified via q-RT-PCR. The expression levels are normalised to mouse β -actin. Gene expression in control mice is represented by empty bars, whilst gene expression in mutant mice is represented by solid bars. **(B)** Western blot of representative proteins from the five OXPHOS complexes (CI-CV). Vinculin was used as loading control. N = 3 Control, 3 *Pik3ca^{H1047R}Pten^{-/-}*. Error bars represent the mean $\pm$ SEM. *Atp5d* = ATP synthase subunit delta; *Atp5q1* = ATP synthase subunit gamma; *Uqcrl1* = ubiquinol-cytochrome-c reductase; *Mdh2* = malate dehydrogenase 2; *Cyc1* = cytochrome bc1 complex; *Ndufb8* = NADH:Ubiquinone Oxidoreductase Subunit B8; ATP5A = ATP synthase subunit alpha; UQCRC2 = ubiquinol-cytochrome-c reductase core protein 2; MTCO1 = Cytochrome c oxidase subunit I; SDHB = succinate dehydrogenase complex iron sulfur subunit B.

5.2.2 3'RNA-seq of the intestine of mice harbouring the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations.

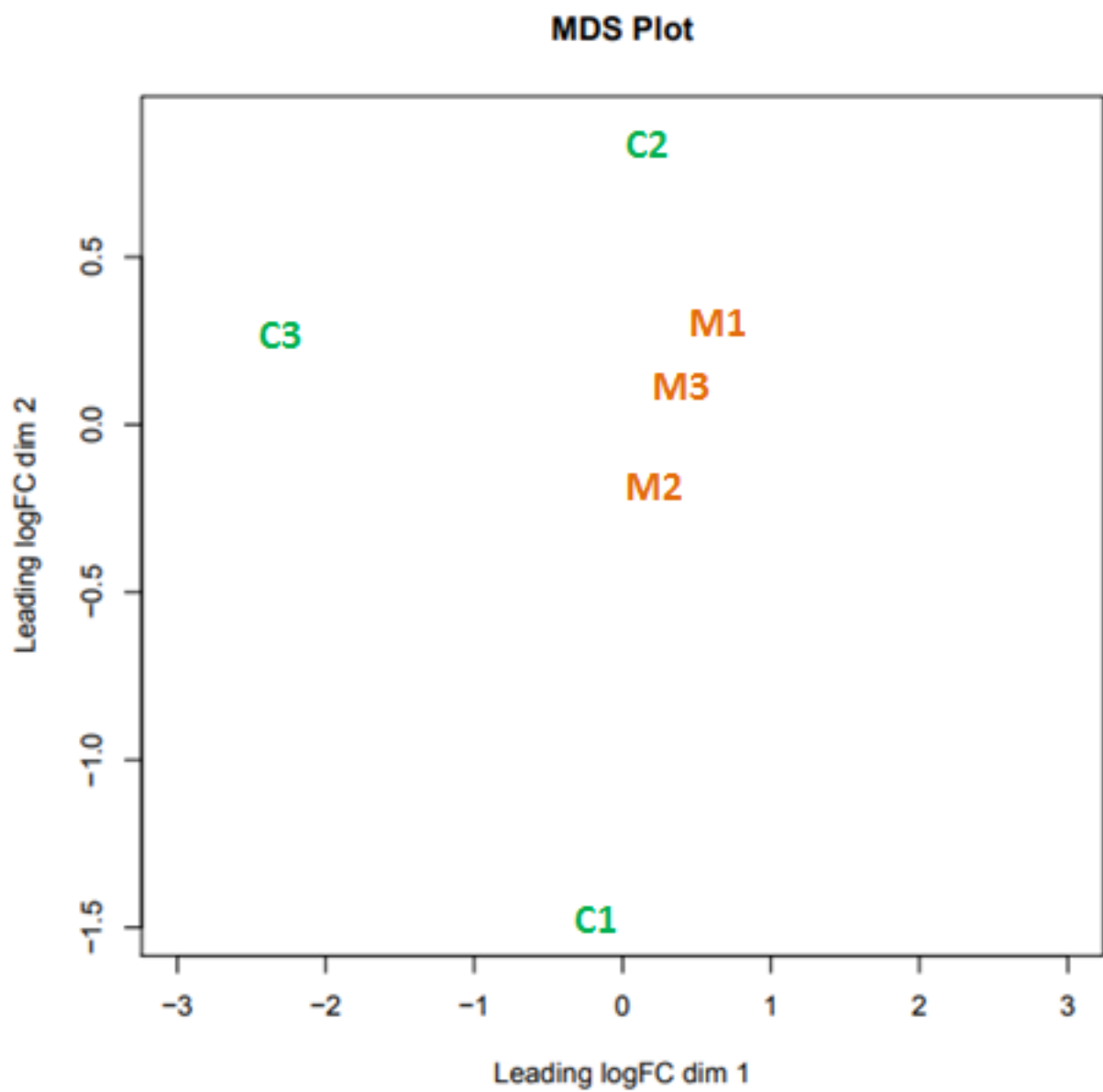
In order to analyse changes in the intestinal transcriptome resulting from mutations in the PI3K pathway, 3'RNA-seq was performed on the intestines of *Pik3ca*^{H1047R}*Pten*^{-/-} and matched control mice.

Figure 5.3A shows that the mTORC and MYC pathways, downstream from PI3K, as well as the unfolded proteins metabolism, fatty acids metabolism and OXPHOS pathways were upregulated in the intestine of *Pik3ca*^{H1047R}*Pten*^{-/-} mutant mice, compared to control mice, whilst genes implicated in the response to UV were downregulated. Similar to what was observed in the brain (Figure 5.1 A), the most enriched gene set was OXPHOS. All five OXPHOS complexes were upregulated in the intestines of mutant, compared to control mice (Figures 5.3 B-F). Multiresolution Correlation Analysis (MCA) showed that whilst mutant samples were clustered, control samples were scattered (Figure 5.3G), at indication that variability was present between the controls.

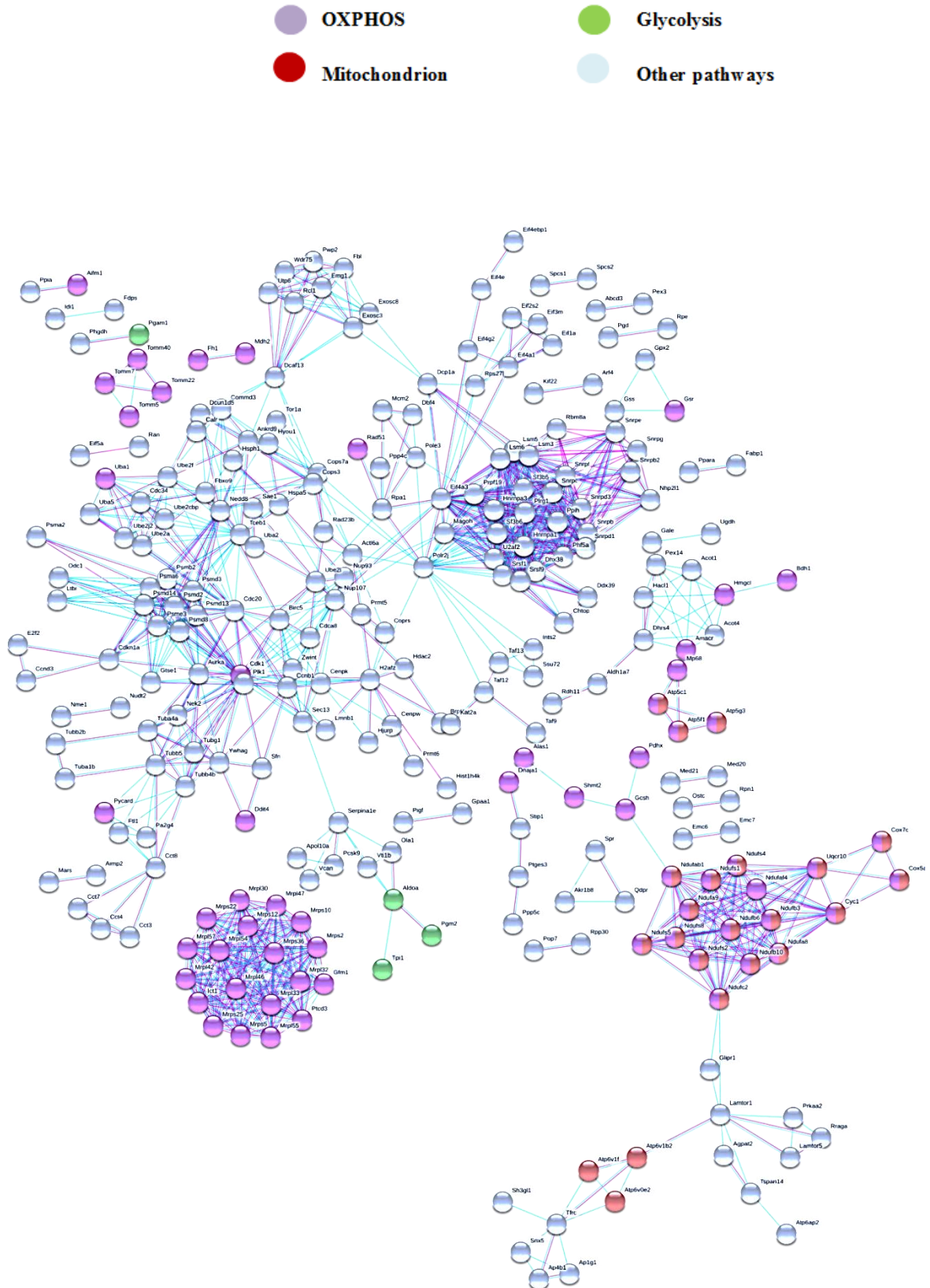
Multiple protein-protein interaction analysis showed increased expression of genes belonging to several metabolic pathways: OXPHOS (red), components of the mitochondrion (purple), genes belonging to glycolysis and gluconeogenesis (green) (Figure 5.3H-I). However, when the expression of representative genes involved in OXPHOS were analysed using q-RT-PCR, no changes were observed between the intestine of control and mutant mice (Figure 5.3 L). Seven of the top differentially expressed genes were used for the RT-PCR and their expression levels was normalised to mouse β -actin.



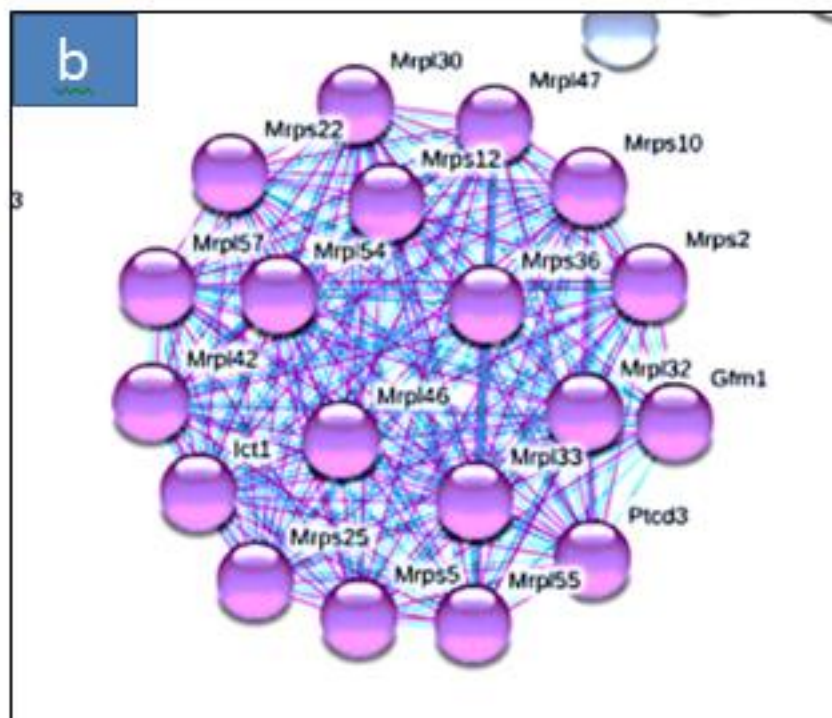
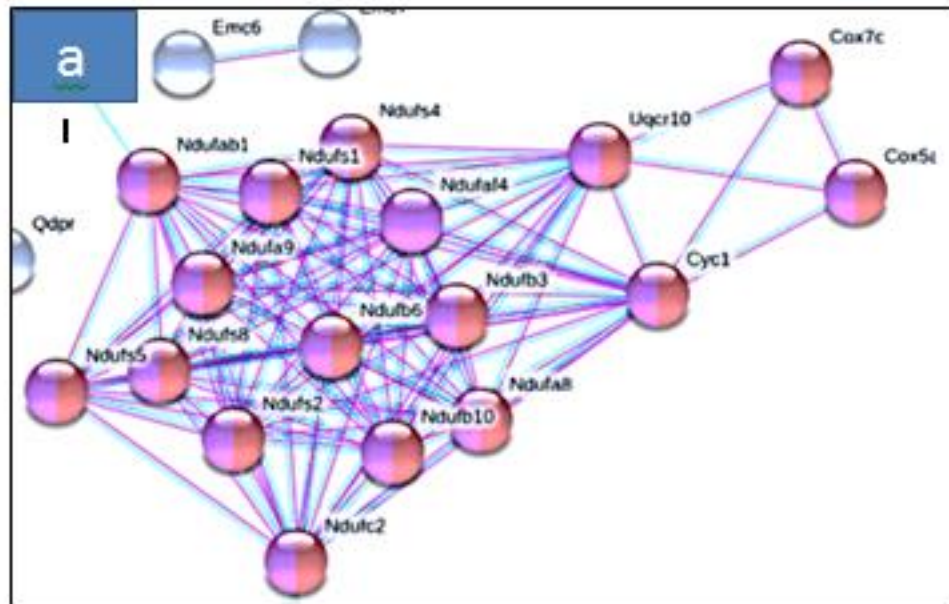
G



H



OXPHOS
Mitochondrion



L

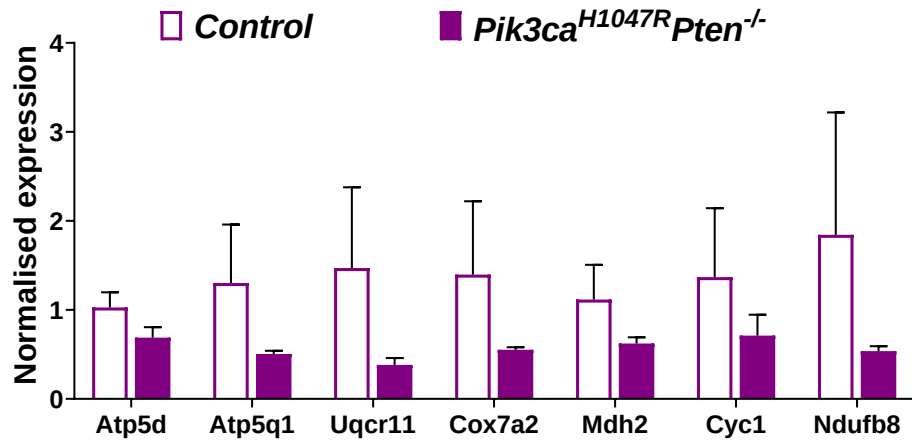


Figure 5. 3: intestine of mice ubiquitously expressing the *Pik3ca^{H1047R}Pten^{-/-}* double mutation.

Pik3ca^{H1047R}Pten^{-/-} and control mice were administered tamoxifen (200mg/kg), via oral gavage, on two consecutive days. Two days post tamoxifen administration mice were sacrificed and the intestine harvested. The RNA was extracted and the sequencing was performed by the Molecular Genomics core (Peter MacCallum Cancer centre). **(A)** The plot bar displays the $-\log_{10}$ (p. adj) values for the top 5 enriched gene sets obtained using EGSEA. The red bars represent up-regulation, whilst the blue bars represent down-regulation. Heatmaps of the normalized read counts of genes belonging to the **(B)** complex I, **(C)** complex II, **(D)** complex III, **(E)** complex IV and **(F)** complex V of the OXPHOS pathway in control vs mutant mice. C= control, M= *Pik3ca^{H1047R}Pten^{-/-}*. The threshold was set on p-value ≤ 0.05 , and $\log_2$ fold change ≥ 0.5 . Colors range from blue for low expression to red for high expression. Some of the genes belonging to the complex III and IV are missing due to an error in loading the complete gene sets on Galaxy. **(G)** Multiresolution Correlation Analysis (MCA) showing samples distribution. **(H)** Protein-protein interaction network obtained using the String protein interaction network platform (p-value ≤ 0.05). N= 3 Control, 3 *Pik3ca^{H1047R}Pten^{-/-}*. Red spheres represent the mitochondrion, purple spheres represent genes belonging to OXPHOS, green spheres represent genes belonging to glycolysis, whilst the rest of the spheres represent genes belonging to other pathways. **(I)** Magnification of figure G showing a) genes belonging to the **L** OXPHOS complex and b) genes belonging to the mitochondrion complex.) The graph display the mRNA levels of representative genes belonging to the the OXPHOS pathway, quantified via q-RT-PCR. The expression levels are normalised on to mouse β -actin. Gene expression in control mice is represented by empty bars, whilst gene expression in mutant mice is represented by solid bars. Error bars represent the mean $\pm$ SEM. *Atp5d* = ATP synthase subunit delta; *Atp5q1* = ATP synthase subunit gamma; *Uqcr11* = ubiquinol-cytochrome-c reductase; *Mdh2* = malate dehydrogenase 2; *Cyc1* = cytochrome bc1 complex; *Ndufb8* = NADH:Ubiquinone Oxidoreductase Subunit B8.

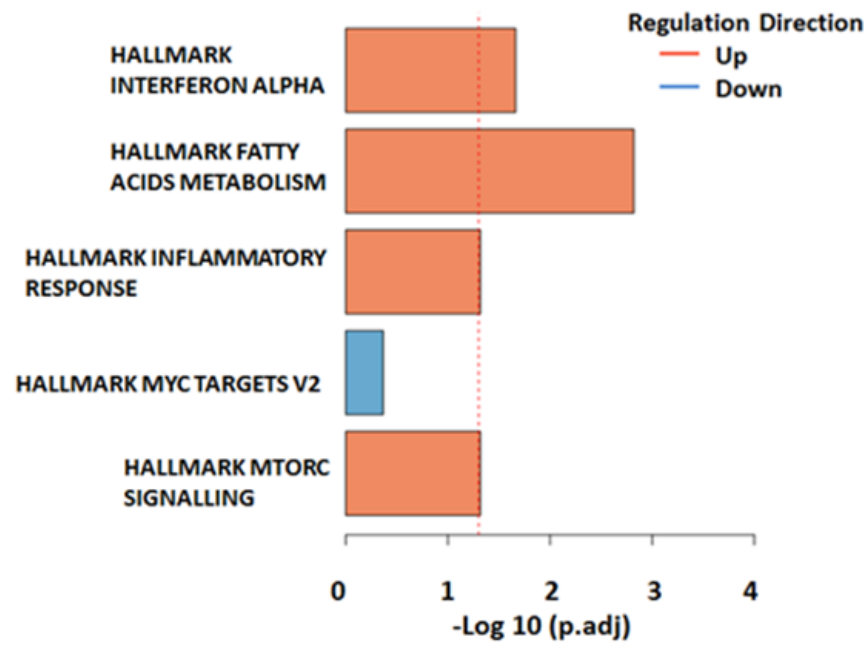
5.2.3 3'RNA-seq of tissues harbouring the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations revealed increased fatty acids metabolism in the liver of mice.

In order to analyse any changes in the liver transcriptome resulting from mutations in the PI3K pathway, 3'RNA-seq was performed on the liver of *Pik3ca*^{H1047R}*Pten*^{-/-} and matched control mice.

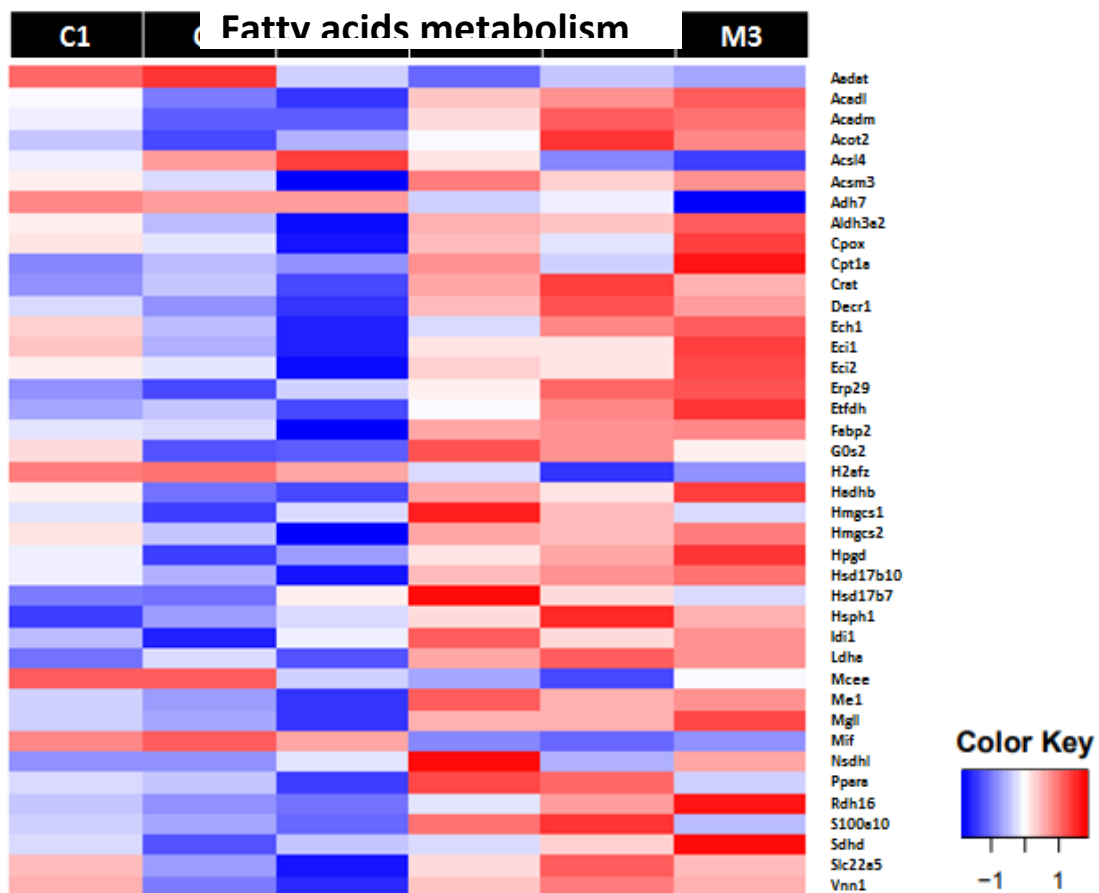
Figure 5.4A shows that the mTORC pathway, interferon alpha pathway, inflammatory response and fatty acid metabolism pathways were upregulated in the liver of *Pik3ca*^{H1047R}*Pten*^{-/-} mutant mice, compared to control mice, the cMYC pathway was downregulated. The most enriched pathway was fatty acid metabolism as shown by the heatmap (Figures 5.4 B). Multiresolution Correlation Analysis (MCA) showed that both control and mutant samples had a clustered distribution, with the exception of one of the samples per group (Figure 5.4C).

Multiple protein-protein interaction analysis of differentially expressed genes (p-value ≤ 0.05) performed using String (<https://string-db.org/>) showed upregulation of the lipid (blue) and fatty acid metabolism (green) in mutant mice, compared to control mice. The disconnected nodes are not shown (Figure 5.4 D-E). However, when the mRNA expression of genes involved in fatty acid metabolism was analysed, no changes were observed between the liver of control and mutant mice (Figure 5.4 F). Four of the top differentially expressed genes were used for the RT-PCR and their expression levels were normalised to mouse β-actin. Since the lipid and fatty acid metabolism of mice harbouring mutations in the PI3K pathway has been investigated in chapter 4 (Section 4.2.9), no further analysis of the liver of mice has been performed.

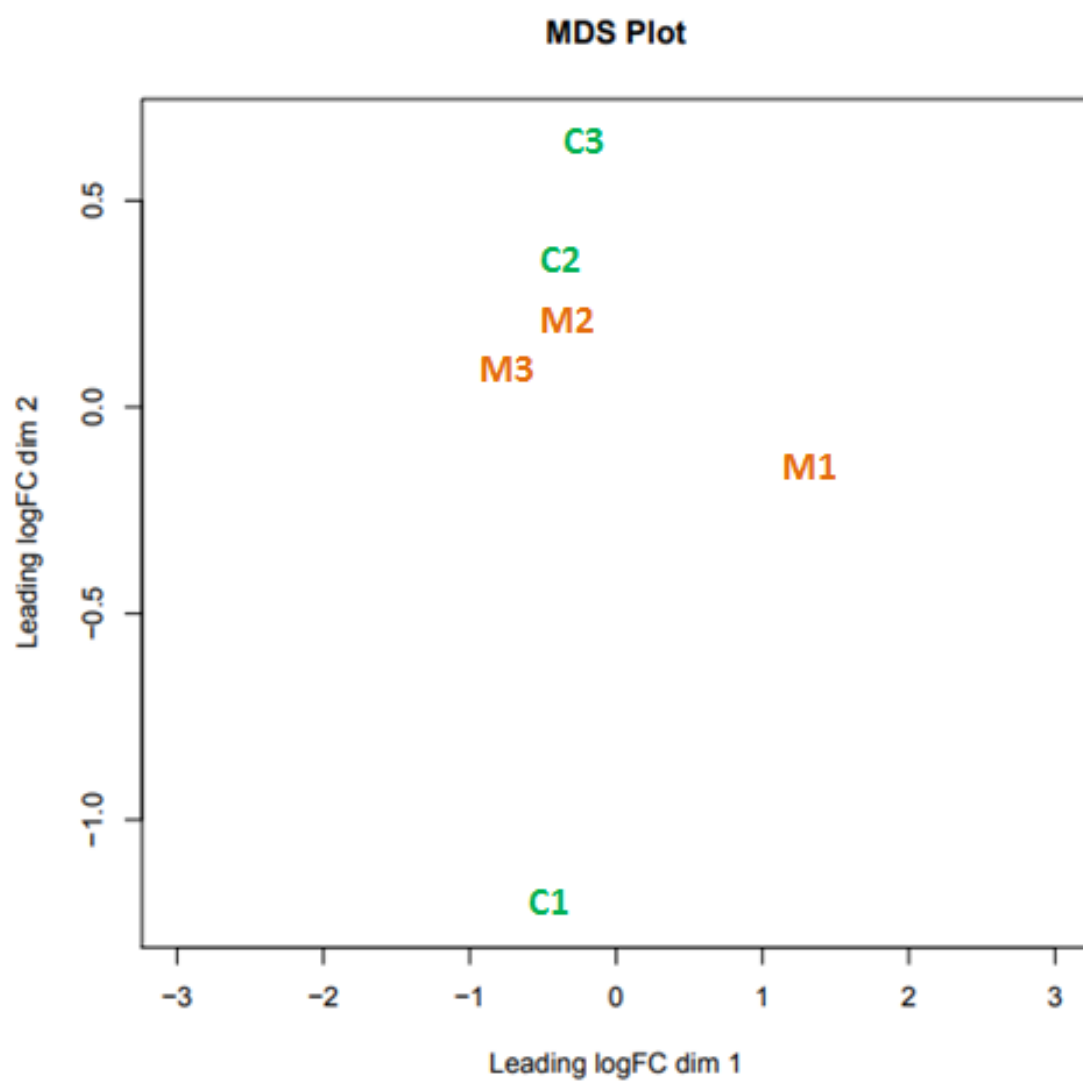
A



B



C



F

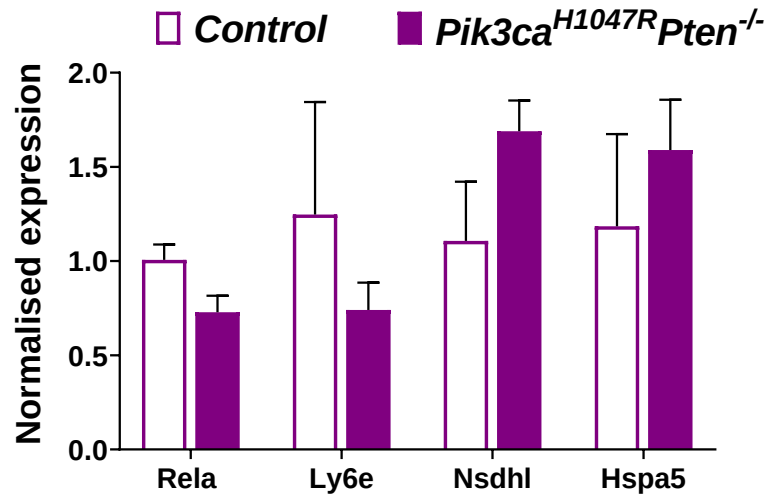


Figure 5. 4: Analysis of the fatty acid metabolism expression in the liver of mice ubiquitously expressing the *Pik3ca*^{H1047R}*Pten*^{-/-} double mutation.

Pik3ca^{H1047R}*Pten*^{-/-} and control mice were administered tamoxifen (200mg/kg), via oral gavage, on two consecutive days. One day later mice were sacrificed and the liver harvested. The RNA was extracted and the sequencing was performed by the Molecular Genomics core (Peter MacCallum Cancer centre). **A)** The plot bar displays the $-\log_{10}$ (p. adj) values for the top 5 enriched gene sets obtained using EGSEA. The red bars represent up-regulation, whilst the blue bars represent down-regulation. **B)** Heatmaps of the normalized read counts of genes belonging to the fatty acids metabolism in control vs mutant mice. The threshold was set on p-value ≤ 0.05 , and log2 fold change ≥ 0.5 . Colors range from blue for low expression to red for high expression. **C)** Multiresolution Correlation Analysis (MCA) showing the samples distribution. **D)** Protein-protein interaction network obtained using the String protein interaction network platform (p-value ≤ 0.05). Blue spheres represent genes belonging to the lipid metabolism pathway whilst green spheres represent genes belonging to the FA metabolism pathway. The rest of the spheres represent genes belonging to other pathways. **E)** Magnification of figure C showing genes belonging to the lipid metabolism and fatty acids metabolism **F)** The graph displays the mRNA levels of genes belonging to the fatty acids metabolism, quantified via q-RT-PCR. The expression levels are normalised on mouse β -actin. Gene expression in control mice is represented by empty bars, whilst gene expression in mutant mice is represented by solid bars. N= 3 Control, 3 *Pik3ca*^{H1047R}*Pten*^{-/-}. Error bars represent the mean $\pm$ SEM. Ly6e = lymphocyte antigen 6 family member E; Nsdhl = NAD(P)d steroid dehydrogenase-like; Hspa5 = heat shock protein family A (Hsp70) Member 5.

5.2.4: 3'RNA-seq of tissues of mice harbouring the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations shows that the PI3K pathway has tissue-specific effect on metabolism.

Overall the RNA-seq performed on mice carrying the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations showed that mutations in the PI3K pathway have tissue specific effect on metabolism (Figure 5.5). The fact that all tissues presented upregulated mTORC pathway validates the efficiency of the mutations as mTORC is activated downstream to PI3K. Both the intestine and brain of mutant mice showed upregulated OXPHOS, compared to control mice, whilst such pathway was not altered in the liver of the mice. The intestine and the liver of mutant mice shared alteration of the FA metabolism, compared to control mice. FA metabolism was, instead, unchanged in the brain of control and mutant mice. Tissue-specific effects of the mutations were observed in each tissue with hyperactivation of the cholesterol metabolism and Wnt pathway in the brain, proteins folding and UV response in the intestine and the inflammatory response in the liver.

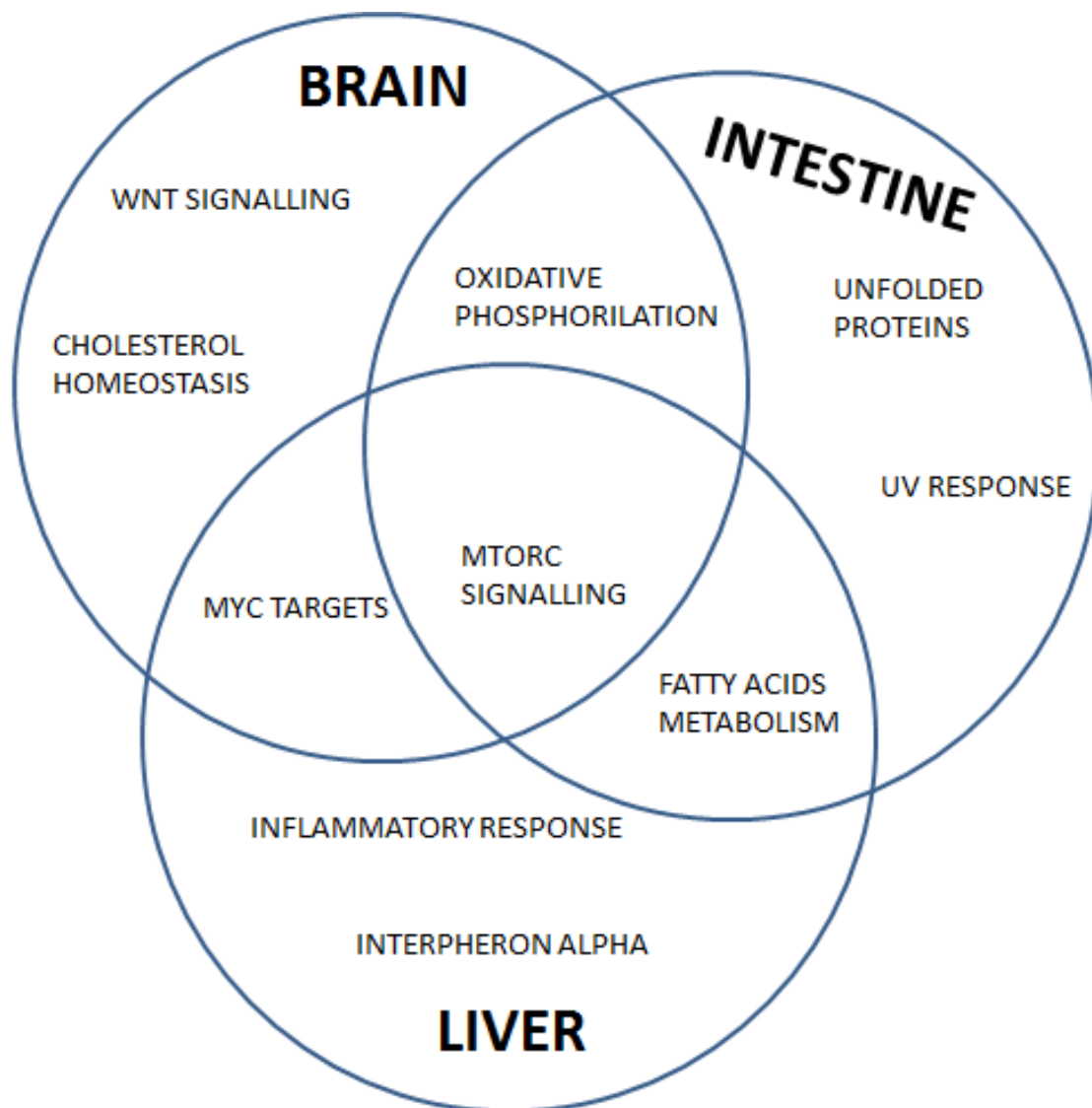


Figure 5. 5 Venn diagram summarizing the pathways that were significantly enriched in the brain, intestine and liver of *Pik3cd*^{H1047R}*Pten*^{-/-} mice. The data obtained from the gene ontology analysis were organised in a Venn diagram showing the pathways that were enriched in individual tissues or in more than one tissue

5.2.4 Proteomic analysis of brain and intestine harbouring the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations.

To further integrate data obtained from the RNA-seq, proteomic analysis was performed in the brain and the intestine of *Pik3ca*^{H1047R}*Pten*^{-/-} and matched control mice. For this purpose, mice were treated with tamoxifen, on two consecutive days and sacrificed the following day. The tissues were then harvested and protein extracted. Proteins were separated by liquid chromatography and then visualised through mass spectroscopy, based on their molecular weight (The Bio21 Institute of Molecular Science and Biotechnology, University of Melbourne). Changes in protein levels are plotted comparing the mutant vs. control mice, in both intestine and brain (Figure 5.6 A and B). The set threshold was p-value ≤ 0.05 and log₂ fold change ≥ 0.5 .

No overlap was observed between the significant differentially expressed genes (found by RNA-seq) and the protein profile resulting from the proteomic analysis in the brain. However, two key regulators for normal brain function: the Sodium-Dependent Glutamate/Aspartate Transporter 1 (SLC1A3 transporter) and the monoamine oxidase A (MAOA) were found to be downregulated in the brain of mutant mice, compared to control mice (Figure 5.6A).

Proteomic analysis performed in the intestine (Figure 5.6 B), instead, revealed that Isovaleryl-CoA dehydrogenase (IVD), a mitochondrial matrix enzyme that catalyzes the third step in leucine catabolism was downregulated in the intestine of mutant mice, compared to control mice. The Acylpyruvase FAHD1 and the ATP-binding cassette sub-family D member 3 (ABCD3) were more highly expressed in *Pik3ca*^{H1047R}*Pten*^{-/-} mice compared to controls. FAHD1 is an enzyme able to hydrolyze acetylpyruvate and fumarylpyruvate *in vitro* (Manjasetty et al., 2004; Pircher et al., 2011). It was previously identified as oxaloacetate decarboxylase (ODx) (Pircher et al., 2015), and as such acts as a regulatory enzyme in the TCA cycle. ABCD3 is one of three ABC transporters present in the peroxisomal membrane which catalyses ATP-dependent transport of substrates for metabolic pathways localized in peroxisomes. Peroxisomes play a major role in human cellular lipid metabolism, including fatty acid β -oxidation. FFAs can enter peroxisomes through passive diffusion or by means of ABC transporters.

Two proteins belonging to OXPHOS (NDUFS 5 and 6) were slightly upregulated in the brain of mutant mice, whilst only NDUFS 5 was found to be more expressed in the intestine of mutant mice, compared to control mice.

Aside from changes in mitochondrial proteins, increased expression of the glycolytic enzyme phosphoglycerate mutase 1 (PGAM1), which catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate, was observed in the intestine of mutant mice, compared to control mice (Figure 5.3B). However differential protein levels were under the set cut off. Interestingly the gene coding for the PGAM protein was also upregulated at the transcriptional levels in the intestine of mutant, compared to control mice, as shown in the RNA-seq analysis (Figure 5.3 G).

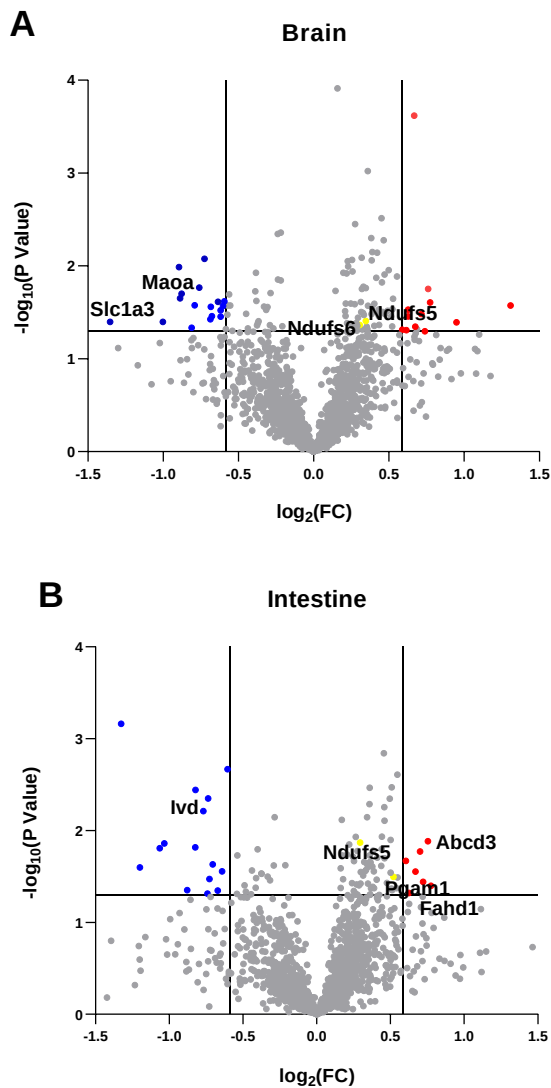


Figure 5.6: Proteomic analysis of brain and intestine of mice ubiquitously expressing the *Pik3ca*^{H1047R}*Pten*^{-/-} double mutation.

Pik3ca^{H1047R}*Pten*^{-/-} and control mice were administered tamoxifen (200mg/kg), via oral gavage, on two consecutive days. The following day mice were sacrificed and the intestine and brain harvested. Proteins were extracted and analysed through LC-MS (The Bio21 Institute of Molecular Science and Biotechnology, University of Melbourne). The volcano plots display the log₂ fold change (x-axis) and log₁₀ p-value (y-axis) of **(A)** neuronal proteins and **(B)** intestinal proteins. The cut offs are set at 0.05 for p-value and 1.5 fold change. The red dots indicate upregulation, whilst the blue dots indicate downregulation. The yellow dots indicate proteins that were more expressed in the mutant, compared to control mice but had a fold change < 1.5. N= 4 Control, 4 *Pik3ca*^{H1047R}*Pten*^{-/-}. MaoA = monoamine oxidase A; Slc1a3 = excitatory amino acid transporter 1; Ndufs = NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit; Ivd = Isovaleryl-CoA dehydrogenase; Abcd3= ATP Binding Cassette Subfamily D Member 3; Fahd1= Fumarylacetoacetate Hydrolase Domain Containing 1; Pgalm1= Phosphoglycerate Mutase 1.

5.2.5 Functional characterisation of the effects of the *Pik3ca*^{H1047R}*Pten*^{-/-} mutation in mouse intestinal organoids and neurospheres.

As described in the introduction to this chapter, animal work is often supported by *in vitro* studies using 3D culture, which can overcome problems such as the toxicity of drugs that target metabolism in animals. Therefore, 3D culture systems of neurospheres and intestinal organoids were used to study the metabolic effects of mutations in the PI3K pathway on epithelial cells and neurones by using drugs that target the mitochondrial and glycolytic function of the cells. Neurospheres were obtained from the Department of Microbiology and Immunology (DMI, University of Melbourne, Parkville, Victoria 3010, AU), where they were generated using neural stem/progenitor cells (NSPCs) (Daniel et al., 2018).

Figure 5.7 A shows representative images of the neurospheres 1 and 5 days post passaging. Both control and mutant neurospheres maintained the sphere-like morphology.

Mouse intestinal organoids were generated from the crypts of the small intestine of the mice. Mice were treated with tamoxifen (200 mg/kg on two consecutive days) *in vivo*. The following day mice were sacrificed and the small intestine was removed and used to generate organoids. Figure 5.7 B shows representative images of the organoids 1 and 6 days post crypt isolation. Mature organoids (after 6 days of culturing), highly resemble the *in vivo* intestinal epithelium.

Control and *Pik3ca*^{H1047R}*Pten*^{-/-} intestinal organoids and neurospheres were used for *in vitro* analysis of mitochondrial activity using the Seahorse XF technology. The Agilent Seahorse XF Cell Mito Stress Test assay measures key parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of the cells in real time, through the serial addition of drugs that target mitochondrial activity. The drugs used in this assay are oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), rotenone and antimycin A (Figure 5.8 A). Oligomycin inhibits ATP synthase (complex V), therefore decreasing the electron flow through the ETC and the OCR. FCCP uncouples respiration and disrupts the mitochondrial membrane potential, resulting in the release of the inhibition of flow through the ETC. As a consequence the OCR reaches the maximum. The FCCP-stimulated OCR is used to calculate spare respiratory capacity, which reflects of the ability of the cell to respond to stress. It is defined as the difference between maximal respiration (the

maximal oxygen consumption rate attained by adding the uncoupler FCCP) and basal respiration (energetic demand of the cell before addition of the drugs). Rotenone and antimycin A block the complex I and III respectively, and therefore completely shut down the mitochondrial respiration. This enables the amount of respiration driven by processes outside the mitochondria to be calculated (Figure 5.8A).

Figure 5.8 shows the mitochondrial respiration in neurospheres and intestinal organoids. In both systems, the mitochondrial respiration under basal conditions was similar between control and mutant mice. The addition of oligomycin determined a decrease in mitochondrial respiration which was similar between the two genotypes. Similarly, no difference in the OCR of control and mutant mice was observed after addition of FCCP and rotenone and antimycin A (Figure 5.8 B-E). Overall, no changes in the mitochondrial activity were observed between control and mutant mice in the two *in vitro* systems used in this study, suggesting that the observed changes in the transcriptome do not affect mitochondrial function.

Since the mitochondrial function of mutant mice did not appear to be altered, with respect to control mice, a second metabolic assay, the XF glycolytic rate assay, which measures the glycolytic function of the cells as opposed to the mitochondrial respiration, was performed. The seahorse XF glycolytic rate assay measures the glycolytic rate of the cells based on their proton efflux rate (PER). The proton efflux from live cells comprises both glycolytic and mitochondrial-derived acidification. In order to calculate the mitochondrial-associated acidification, the mitochondrial function is inhibited during the assay by rotenone and antimycin A. The second injection is 2-deoxy-D-glucose (2-DG), a glucose analog which inhibits glycolysis through competitive binding of glucose hexokinase. Subtraction of mitochondrial acidification to the total proton efflux rate results in the glycolytic PER (Figure 5.9 A).

Figures 5.8 B and C show that intestinal organoids harbouring mutations in the PI3K pathway had a similar glycolytic rate as the control organoids, both at basal conditions and after administration of the drugs, suggesting that the *Pik3ca*^{H1047R}*Pten*^{-/-} double mutation did not alter the glycolytic activity of intestinal cells.

As opposed to intestinal organoids, the glycolytic function of neurospheres harbouring the *Pik3ca*^{H1047R}*Pten*^{-/-} double mutation was higher than control mice, under baseline conditions (Figures 5.9 D and E). However, mutant neurospheres responded to the addition of the drugs in a similar manner as control neurospheres, suggesting that, despite the mutations having caused an increase in the aerobic glycolysis in the brain of mice, it did not influence the ability of the cells to adapt to a metabolic stress.

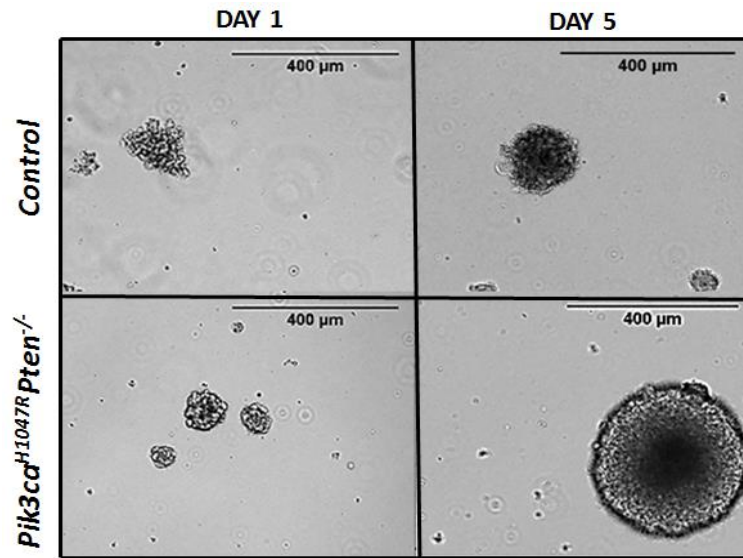
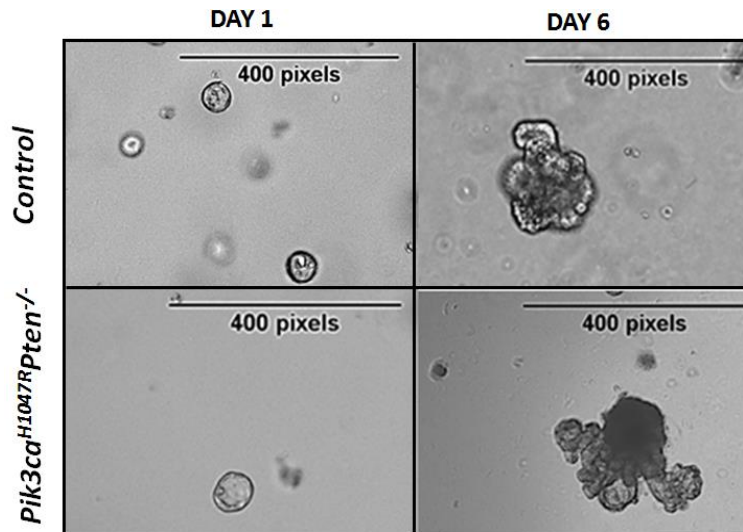
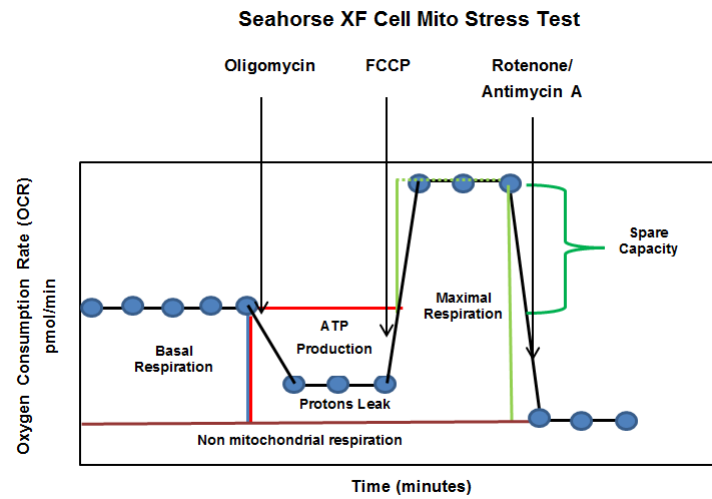
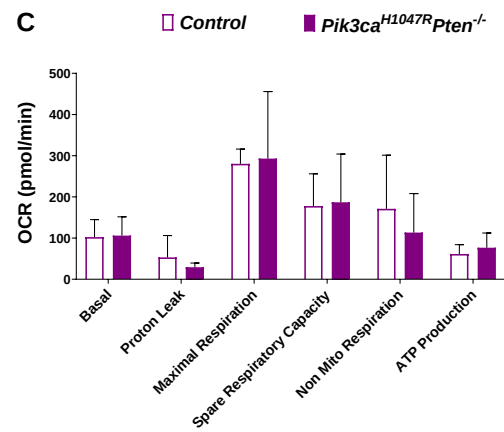
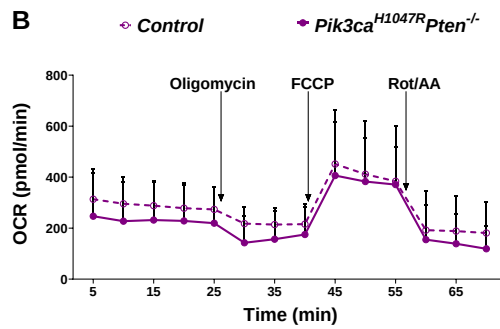
A**B**

Figure 5. 7: Phase contrast images of a developing mouse intestinal neurospheres and organoid in culture. Figures display representative image of (A) control and *Pik3ca^{H1047R}Pten^{-/-}* mouse neurospheres 1 and 5 days post passaging and (B) control and *Pik3ca^{H1047R}Pten^{-/-}* mouse intestinal organoids 1 and 6 days post crypt isolation. Neurospheres were generated from the stem cells present in the lateral ventricles of the brain and grown in ultra-low attachment 6 well plates. Intestinal organoids were generated from the crypts of the small intestine and grown in 48 well plates on the scaffold Matrigel™. The images were taken using the Evos cell imaging systems microscope (Thermo Fisher scientific).



INTESTINAL ORGANOIDs



NEUROSPHERES

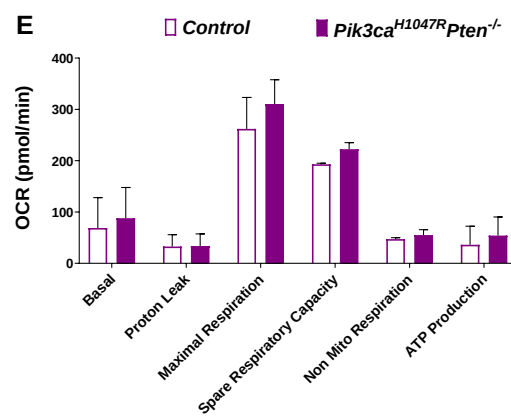
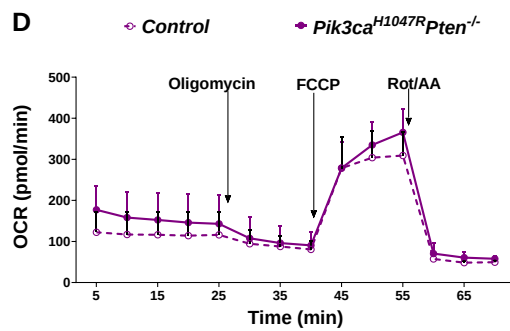
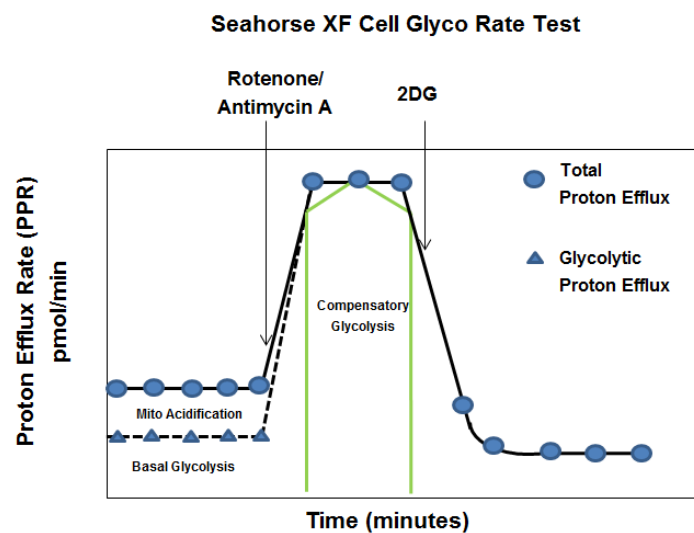
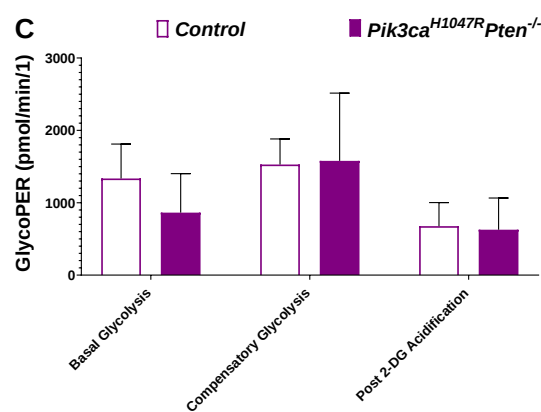
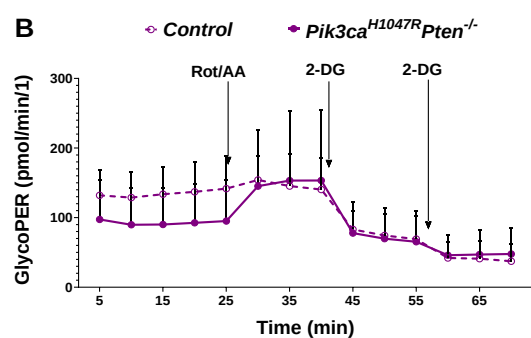


Figure 5. 8: Agilent Seahorse XF Cell Mito Stress Test assay performed on intestinal organoids and neurospheres generated from mice ubiquitously expressing the *Pik3ca*^{H1047R} and *Pten*^{-/-} mutations.

The mitochondrial function of *Pik3ca*^{H1047R}*Pten*^{-/-} and control mouse neurospheres and intestinal organoids was measured using the Agilent Seahorse XF Cell Mito Stress Test assay. **A)** Schematic representation of the seahorse XF cell mito stress test. Oxygen consumption rate (OCR) was measured continuously following treatment with the mitochondrial inhibitors oligomycin (20 µM), FCCP (20 µM), rotenone and antimycin A (Rot/AA) (5 µM). Graphs display **(B)** the mitochondrial respiration profile of intestinal organoids before and after addition of the mitochondrial modulators and **(C)** the basal OCR, proton leak, maximal respiration, spare respiratory capacity, non mitochondrial respiration and ATP production of intestinal organoids, calculated from the OCR profiles. Graphs display **(D)** the mitochondrial respiration profile of neurospheres before and after addition of the mitochondrial modulators and **(E)** the basal OCR, proton leak, maximal respiration, spare respiratory capacity, non mitochondrial respiration and ATP production of neurospheres, calculated from the OCR profiles. Data were normalised on the protein content of organoids and neurospheres. Error bars represent the mean ± SEM of n=2 independent experiments.



INTESTINAL ORGANOIDS



NEUROSPHERES

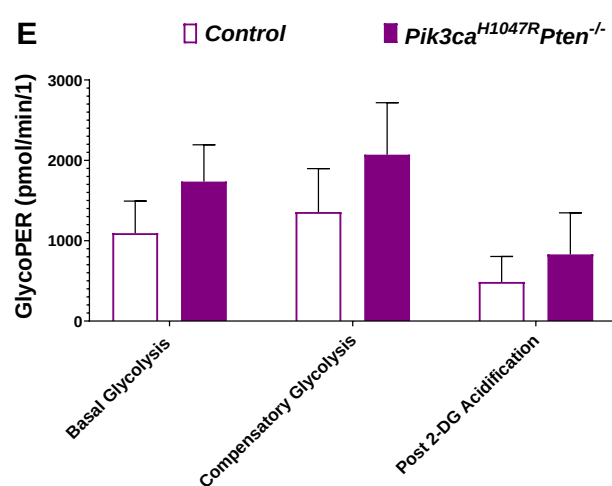
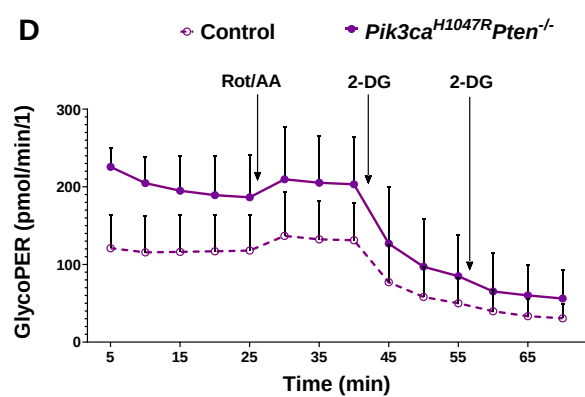


Figure 5. 9: Agilent Seahorse XF Glyco Rate Test assay performed on intestinal organoids and neurospheres generated from mice ubiquitously expressing the *Pik3caH1047R* and *Pten*^{-/-} mutations.

The glycolytic function of *Pik3ca*^{H1047R}*Pten*^{-/-} and control mouse intestinal organoids and neurospheres was assessed using the Agilent seahorse XF glycolytic rate assay. **A)** Schematic representation of the Seahorse Glyco rate test. The glycolytic PER (glyco-PER) was measured continuously following treatment with rotenone/antimycin A (Rot/AA) (5 μ M) and 2 deoxyglucose (2DG) (200 μ M). Graphs display **(B)** the glyco-PER of intestinal organoids before and after addition of the mitochondrial modulators and 2DG and the **(C)** basal glycolysis, compensatory glycolysis and post 2-DG acidification of intestinal organoids. Graphs display **(D)** the glyco-PER of neurospheres before and after addition of the mitochondrial modulators and 2DG and **(E)** the basal glycolysis, compensatory glycolysis and post 2-DG acidification of neurospheres. Error bars represent the mean $\pm$ SEM of n=2 independent experiments.

5.3 DISCUSSION

This chapter has shown that oncogenic activation of the PI3K pathway alters metabolism by regulating the expression of specific genes and proteins, in a tissue-specific fashion.

In this chapter the effects of mutations in the PI3K pathway on the transcription and translation of metabolic enzymes has been investigated. The study of the transcriptome was performed using RNA-seq technology. RNA-seq was chosen over other techniques because it can detect transcripts from organisms with previously undetermined genomic sequences (Yan, 2013), it has a typical dynamic range of 5 orders of magnitude, therefore requiring a much lower input of RNA (nanogram quantity) compared to microarrays (microgram quantity) (Lowe et al., 2017), and it has a very low background noise compared to hybridization-based technology (Yan, 2013).

One limitation of this study was the low sample size. Since the tissues extracted from only 3 control and 3 mutant mice were sequenced and since there was high variability between the three samples, it was not possible to observe significant differences between control and mutant mice, when comparing gene expression using a threshold of adjusted p-value ≤ 0.05 . Although the adjusted p-value is preferably used when comparing multiple test as it adjusts for repeated test, it was replaced by the nominal p-value (≤ 0.05) in this study in order to define significant changes in the gene expression between control and mutant mice.

RNA-seq analysis showed that intestine, brain and liver of mutant mice had upregulated expression of the mTORC pathway, compared to control mice. Since mTORC acts downstream of PI3K, this data validates the efficiency of the sequencing. The cMYC pathway was also upregulated in the intestine and brain of mutant mice, compared to control mice. This is in agreement with the well established role of MYC as a target of PI3K (Zhu et al., 2008). Interestingly, the *Pik3ca*^{H1047R}*Pten*^{-/-} double mutation caused downregulation of the MYC pathway in the liver of mutant mice, with respect to control mice. However, only genes belonging to the V2 subgroup (58 genes) of MYC targets generated using the hallmark genes collection, were downregulated, whilst no changes in the subgroup V1, which includes 200 genes belonging to the MYC pathway, were observed.

Interestingly, within the five top enriched gene sets, alteration of metabolic pathways were observed in the three organs and confirmed by the protein-protein interaction analysis. The effect of the *Pik3ca*^{H1047R}*Pten*^{-/-} double mutation was different depending on the tissue analysed. Whilst brain and intestine of mutant mice had upregulation of the oxidative phosphorylation pathway, compared to control mice, the liver of mutant mice had increased fatty acid metabolism, with respect to control mice. Cholesterol metabolism was also altered in the brain of mutant mice, whilst fatty acid metabolism was increased in the intestine of mutant mice, with respect to control mice. No increase in the aerobic glycolysis was observed in any of the tissues, in disagreement with the well established role of the PI3K pathway to support glycolysis. However, since mutant mice had severe hypoglycaemia it is plausible that in absence of glucose, the tissues switched to alternative pathways to support their growth. The availability of nutrients determined the tissue-dependent metabolism switch. The liver, which generally has high levels of lipids, responds to increase energetic request by increasing lipid metabolism. Since lipolysis was increased in the adipose tissue of mutant mice (chapter 4, Section 4.2.9), we could speculate that the FA produced through lipolysis were oxidised in the liver to generate energy. However the data shown in chapter 4 indicate that FA oxidation was not increased in the liver of mutant mice as opposed to control mice.

These data underline the organ-dependent effect of mutations in the PI3K pathway on metabolism, consistent with what has been described in the literature. For instance, the PI3K/AKT signalling pathway is known to have different effects on the metabolism of skeletal muscle, adipose tissue, liver, brain and pancreas, and its imbalance in these tissues leads to the development of obesity and type 2 diabetes mellitus (Huang et al., 2018). *In vivo* and *in vitro* evidence suggested that PIK3R3 promotes fatty acid β -oxidation in the liver by inducing PPAR α expression and its target genes (Yang et al., 2018). The effect of PI3K is also cell-dependent. In mouse embryo fibroblasts, for instance, PTEN elevation shifts cellular energy metabolism toward mitochondrial respiration, whilst prostate *Pten*-null epithelial cancer cells are more vulnerable to inhibition by complex I inhibitors (Naguib et al., 2018).

Mutations of the PI3K pathway also have different outcome on the metabolism of cancer cells. Activation of the PI3K pathway is known to induce the Warburg effect in many cancers, including glioblastoma (Cheng et al., 2009) and gastrointestinal cancers (Sawayama, 2014). In colorectal cancer, instead, PI3K sustains the TCA cycle by upregulating glutamate

pyruvate transaminase 2, which converts glutamate to α KG (Hao et al., 2016). Genome-scale vulnerability screens revealed the dependency of tumours harbouring the *PIK3CA* mutation on the mitochondrial TCA cycle enzyme oxoglutarate dehydrogenase (Ilic et al., 2017a). Previous transcriptomic studies have observed increased oxidative phosphorylation following activation of the MAPK pathway in the brain (Baek et al., 2017). Furthermore melanoma brain metastasis with significant overexpression of multiple activation-specific markers in the PI3K–AKT pathway had significantly enriched oxidative phosphorylation (Fischer et al., 2019). Studies performed on breast cancer cell lines, instead, revealed that mutations in the PI3K pathway effect several metabolic pathways, including a decrease in glycerophosphocholine levels, increases in glutaminolysis, de novo fatty acid synthesis and pyruvate entry into the TCA cycle (Lau et al., 2017).

To follow up the data generated through RNA-seq, mRNA expression levels of metabolic genes were investigated through q-RT-PCR. Several studies have compared RNA-seq results to q-RT-PCR data, and have found excellent correlation between these methods (Asmann et al., 2009; Griffith et al., 2010; Shi and He, 2014; Wu et al., 2014a). However, in this study a discrepancy was observed between the results obtained through RNA-seq and q-RT-PCR, whereby differential expression of the selected genes analysed was not confirmed between control and mutant mice. This discrepancy might be associated to the efficacy of the primers used for the q-RT-PCR, which depends on what region of the cDNA they amplified and on their concentration.

Another way to validate gene expression obtained through RNA-seq is to study the non-transcriptional responses, for instance by looking at protein expression levels. Therefore, a western blotting was performed on protein samples extracted from the brain of control and mutant mice, using antibodies that target OXPHOS complex I to V of the mitochondria. No changes in the protein levels of any of the five OXPHOS complexes was found between the two genotypes. However, since only five representative proteins of the OXPHOS complexes (one for each complex) were used for the western blot, it was not possible to exclude that other proteins belonging to the complexes might have been differentially expressed in mutant mice, as opposed to control mice.

To expand on the transcriptional analysis, proteomic studies were performed on the intestine and brain of mutant and control mice using liquid chromatography associated to MS. One of the primary observations was the protein PTEN, which is lost in *Pik3ca*^{H1047R}*Pten*^{-/-} double mutant mice, but it was not detected in control mice either. This might be associated with the use of trypsin to digest the proteins before performing MS. Trypsin cleaves proteins into peptides with an average size of 700-1500 daltons, which is in the ideal range for MS (Laskay et al., 2013). Trypsin is highly specific, cutting at the carboxyl side of arginine and lysine residues. This high specificity makes it harder to detect small peptides like PTEN (403aa), where the probability to find close arginine and lysine residues is lower than in big peptides. The use of trypsin in proteomics may impose other limits in the ability to detect the full proteome. Tightly-folded proteins can resist trypsin digestion. Post-translational modifications also affect the trypsin activity. For instance, glycans often limit trypsin access to cleavage sites, and acetylation makes lysine and arginine residues resistant to trypsin digestion (Saveliev et al., 2013).

Another observation resulting from the proteomic studies was that only a few proteins detected by MS overlapped with gene expression identified using RNAseq, including the phosphoglycerate mutase1 PGAM 1, which was upregulated in the intestine of mutant mice, compared to control mice and the two OXPHOS proteins, NDUF5-6 which were overexpressed in both brain and intestine of mutant mice, compared to control mice. PGAM1 catalyzes the interconversion of 3-phosphoglycerate and 2-phosphoglycerate during glycolysis. It has been reported to be upregulated in many human cancers (Hitosugi et al., 2012; Jiang et al., 2014). Overexpression of PGAM1 has been shown to be stimulated by mTOR through HIF1 α -mediated transcriptional activation, therefore mediating the Warburg effect in non-small cell lung cancer (Sun et al., 2018). Activation of PGAM1 leads to an increase in 2-phosphoglycerate which increases the glycolytic flow and energy production through the TCA cycle in the mitochondria (Chaneton and Gottlieb, 2012). Furthermore, PGAM1 modulates two other biosynthetic pathways derived from glycolysis: the oxidative branch of the pentose phosphate pathway (PPP) and the serine biosynthesis pathway (Hitosugi et al., 2012). PGAM1 is highly expressed both in brain and intestine (The Human Protein Atlas) and ubiquitously expressed in mouse tissues (The National Center for Biotechnology Information). However it was upregulated only in the intestine of mutant mice, compared to control mice, further underlying the context-specific effect of the *PIK3CA*

mutation on cell metabolism. Further studies need to be performed to investigate the functional consequences of increased expression of PGAM1 in mutant mice, compared to control mice.

Multiple mitochondrial proteins were found to be differentially expressed in the intestine and brain of mutant mice, compared to control mice. This finding was surprising since the whole cell lysate was used for the proteomic analysis, as opposed to isolated mitochondrial protein extracts. Identifying the mitochondrial proteome, in fact, is technically challenging given their low abundance and high dynamic range of proteins. Therefore, using mitochondrial extracts increases the chance to detect mitochondrial proteins by MS (Calvo and Mootha, 2010). Proteins belonging to OXPHOS complex I, NDUF6 and/or NDUF5 were more expressed in the brain and in the intestine of mutant mice, compared to control mice. However their differential expression levels were under the established threshold, suggesting that only small changes in the protein levels of OXPHOS complex I occurred in response to mutations in the PI3K pathway. This is in agreement with what was observed in the RNA-seq dataset, where only small transcriptional changes between control and mutant mice were observed.

Two mitochondrial proteins, MAOA and SLC1A3 were downregulated in the brain of mutant mice, compared to control mice. MAOA is a mitochondrial enzyme which catalyzes the oxidative deamination of amines, such as dopamine, norepinephrine, and serotonin. Loss of MAOA in the brain is associated with development of antisocial behaviours (Godar et al., 2016) and severe developmental delay, intermittent hypotonia and stereotypical hand movements (Whibley et al., 2010). Animal models with total or partial loss-of-function of *MaoA* have been shown to recapitulate numerous psychological and neurofunctional phenotypes observed in humans (Godar et al., 2016). On the contrary, increase expression of *MaoA* contributes to high-grade aggressive prostate cancer by inducing epithelial-to-mesenchymal transition (EMT), stabilization of the transcription factor Hif1 α , and elevation of ROS (Wu et al., 2014b). Using double knockout *Pten/MaoA* mice, Liao and colleagues showed that loss of *MaoA* leads to a decrease in prostate cancer (Liao et al., 2018). Conversely, there is no current evidence that loss of *MaoA* could induce tumours.

The glutamate-aspartate transporter SLC1A3 was also less expressed in the brain of mutant mice, compared to control mice. Solute carriers (SLCs) are a major superfamily of membrane transporters with 439 members classified into 65 families, which mediate the exchange of substances, such as ions, nutrients, signaling molecules and drugs across biological membranes (Hu et al., 2020). A total of 287 SLC genes have been identified in the brain and are fundamental for the maintenance of its internal homeostasis and metabolism (Hu et al., 2020). Mutations in SLCs are responsible for major depression, attention deficit and hyperactivity disorder, epilepsy, movement disorders, psychosis, and other brain disorders. Mutations in the *SLC1A3* transporter have also been associated with ataxia and vertigo (Iwama et al., 2018). Whilst loss of SLC1A3 is associated with neuronal diseases, high expression levels of SLC1A3 have also been observed in some tumor types. Sun and colleagues found that SLC1A3 contributes to L-asparaginase resistance in solid tumors by refilling the cells with aspartate and glutamate, therefore sustaining tumour cell proliferation (Sun et al., 2019). Furthermore, it was observed that p53 promotes the expression of SLC1A3, therefore enabling the utilization of aspartate as fuel for the TCA cycle in the absence of extracellular glutamine. This phenomenon determines tumour adaptation to glutamine starvation (Tajan et al., 2018). A breast epithelial cell line with an oncogenic *PIK3CA* mutation was found to have SLC1A3 increased at both the mRNA and protein expression level.

Overall, the RNA-seq revealed that mutations in the PI3K pathway alter the function of neuronal genes associated with the physiological functionality of the brain, inducing events of epilepsy, seizures and ataxia. This is in agreement with previous studies that have shown that mice homozygous for *Pten* deletion developed seizures and ataxia and had an enlarged brain (Backman et al., 2001).

Proteomic analysis on proteins extracted from the intestine showed upregulation of the two mitochondrial proteins ABCD3 and FAHD1. ABCD3 is a member of the superfamily of ABC transporters associated with the peroxisomal import of fatty acids and/or fatty acyl-CoAs in the organelle (Kawaguchi and Morita, 2016). It has been shown to act as an independent prognostic factor for colorectal cancer. However, its expression was reduced in tumour compared to normal colon (Zhang et al., 2020). There is no current evidence that PI3K modulates ABCD3 expression or activity. Although, its upregulation would be in

agreement with increased fatty acids metabolism observed in the intestine of mutant mice through the use of RNA-seq, more studies need to be performed to define the functional effect of increased ABCD3 in the intestine of mutant mice.

FAHD1 is a member of the fumarylacetoacetate hydrolase (FAH) superfamily of metabolic enzymes. It has recently been identified as an eukaryotic oxaloacetate decarboxylases (ODx)(Pircher et al., 2015), the enzyme that catalyzes the irreversible decarboxylation of oxaloacetate to pyruvate and CO₂. There is currently no evidence that PI3K modulates FAHD1. However, it has been shown that FAHD1 depletion induces a reduction in mitochondrial ATP-coupled respiration in human endothelial cells (Petit et al., 2017), and, therefore, that FAHD1 is essential for mitochondrial function in endothelial cells. In agreement with this it could be speculated that the increased expression of FAHD1 in the intestine of mutant mice is the result of increase mitochondrial activity. This would be in agreement with increased OXPHOS in the intestine of *Pik3ca*^{H1047R}*Pten*^{-/-} mice. However, further studies need to be performed to confirm this hypothesis.

In this chapter, 3D cell culture has also been used to investigate the effect of the *Pik3ca*^{H1047R}*Pten*^{-/-} double mutation on the metabolism of intestinal epithelial cells and neurones, taking advantage of the fact that organoid culture can be performed in the microplates which are compatible with standard high-throughput assays. The metabolic reprogramming of both neurospheres and intestinal organoids were then studied by using the Agilent Seahorse XF technology following the protocol published in 2015 by Fan and colleagues (Fan et al., 2015). The Seahorse analyzer has largely been used to investigate differences between normal and cancerous cell lines, effects of microenvironmental stress and the ability of drugs to alter the metabolic phenotypes of a 2D cell culture monolayer (Baenke et al., 2016; Bailey et al., 2014; O'Neill et al., 2019; Simoes et al., 2015; Wen et al., 2018). However less studies have been performed using 3D cultures.

Until recently, only few works dealt with assessment of mitochondrial function of the stem cells in intestinal organoids (Bas and Augenlicht, 2014) (Fan et al., 2015) and neurospheres (Khacho et al., 2016; Vlashi et al., 2011; Wang et al., 2010).

In this study metabolic studies performed on neurospheres and intestinal organoids showed that mutations in the PI3K pathway do not alter the mitochondrial function of epithelial cells

or neurones. However, the *Pik3ca*^{H1047R}*Pten*^{-/-} double mutation resulted in an increase in the glycolytic function of neurospheres (but not intestinal organoids) under baseline conditions, suggesting that a mutation in the PI3K pathway results in a switch from OXPHOS to aerobic glycolysis in neurospheres (but not intestinal organoids). This is in agreement with a study performed by Ilic and colleagues who showed that the PI3K mutation alters the glycolytic function but not the mitochondrial phenotype of tumour cells (Ilic et al., 2017a). Similarly *PIK3CA* E542K and E545K mutations were shown to promote glucose metabolism and proliferation in cervical cancer cells (Jiang et al., 2018a).

Seahorse studies were in disagreement with RNA-seq data, which showed increased OXPHOS in both the intestine and the brain of mice harbouring the *Pik3ca*^{H1047R}*Pten*^{-/-} double mutation compared to control mice. This discrepancy might be due to the fact that when growing in culture the cells are isolated from the rest of the body, whilst in the context where the PI3K-insulin pathway is activated in every organs, *in vivo*, the tissues might mutually influence each others metabolism. Furthermore, since the mutations were activated *in vivo*, during the growth in culture, the cells might have adapted and reprogrammed their metabolism. Therefore, future studies should be repeated by activating the mutations directly *in vitro* or even during the Seahorse assay. Alternatively, specific regions of the brain and intestine could be studied *ex vivo* using the Seahorse technology, soon after extraction, to maintain the signals derived from other tissues, *in vivo*, using newly designed tissue restraints to adapt the Agilent XFe96 metabolic analyzer for whole tissue analysis (Neville et al., 2018).

In this chapter, 3D cell culture has also been used to investigate the metabolism of spheroids generated from neural stem/progenitor cells and organoids generated from intestinal crypts. Mice were treated with tamoxifen to activate the *Pik3ca*^{H1047R}*Pten*^{-/-} mutations before isolating the tissues and generating intestinal organoids/neurospheres. The metabolic reprogramming of both neurospheres and organoids were then studied by using the Agilent Seahorse XF technology following the protocol published in 2015 by Yang-Yi Fan and colleagues (Fan et al., 2015). Seahorse XF Analyzers measure OCR and extracellular acidification rate (ECAR) of live cells in real time through the use of mitochondria and glycolytic modulators. It was observed that mutations in the PIK3 pathway do not alter the mitochondrial metabolism of either neurospheres or organoids. The glycolytic metabolism

was also unchanged in mutant organoids/neurospheres, compared to control mice, despite a slight increase in the glycolytic function of intestinal organoids. This is inconsistent with a study performed on cancer cell lines, where, following inhibition of PI3K–AKT–mTOR signaling and decreased glycolysis the tumours were able to adapt by increasing apoptosis, which supplies substrates for mitochondrial respiration and redox homeostasis (Lue et al., 2017).

The functional analysis was in disagreement with what was observed by RNA-seq. This discrepancy might be due to the fact that when growing in culture the cells are isolated from the rest of the body, whilst in the context where the PI3K-insulin pathway is activated in every organs, *in vivo*, the tissues might mutually influence each other metabolism. Furthermore, since the mutations were activated *in vivo*, during the growth in culture, the cells might have adapted and reprogrammed their metabolism. Therefore, future studies should be repeated by activating the mutations directly *in vitro* or right during the seahorse assay. Alternatively, the whole brain and intestine could be studied *ex vivo* using the seahorse technology, soon after extraction, to maintain the signals derived from other tissues, *in vivo*, using newly designed tissue restraints to adapt the Agilent XFe96 metabolic analyzer for whole tissue analysis (Neville et al., 2018).

Previous studies performed using the seahorse technology showed controversial results. Ilic and colleagues showed that the PI3K mutation alters the glycolytic function but not the mitochondrial phenotype of tumour cells (Ilic et al., 2017b). Similarly *PIK3CA* E542K and E545K mutations were shown to promote glucose metabolism and proliferation in cervical cancer cells (Jiang et al., 2018a). Similarly, studies on mouse embryo fibroblasts (MEFs) showed that *Pten* elevation shifts cellular energy metabolism toward mitochondrial respiration. On the contrary prostate *Pten*-null cancer cells were proven to be more vulnerable to inhibition to complex I inhibitors, suggesting a dependence on the mitochondrial respiration. These studies were performed in 2D cell lines, which have the main disadvantage to grow on a monolayer, therefore receiving homogenous amounts of nutrients and growth factors from their growth medium. This characteristic of 2D cell lines makes the system different from the *in vivo* setting and might give bias results when studying metabolism. For

instance the comparison of the metabolic profile of 2D vs. 3D models demonstrated differences in response to mitochondrial stress test drugs (Russell et al., 2017).

In contrast, 3D organoids have been found to mimic the metabolism of *in vivo* models. Lagies and colleagues (Lagies et al., 2020) showed that spheroids differentiate and start to produce metabolites typical for their tissue of origin (Lagies et al., 2020) and can, therefore, replace animal testing, as opposed to 2D cultures.

5.3.1 Overall findings

In this chapter it has been shown that mutations in the PI3K pathway lead to transcriptional induction of genes involved in metabolic pathways. The metabolic alterations observed in this study were tissue specific, with increased oxidative phosphorylation in the brain and intestine and increased fatty acids metabolism in the liver of mutant mice, compared to control mice.

Functional analyses were not able to support the transcriptomic data. However, proteomic analysis revealed changes in mitochondrial proteins in the brain and the intestine of mutant mice, compared to control mice. Interestingly, the effect of mutations in the PI3K pathway on protein expression was organ-specific, with none of the proteins found both in the brain and in the intestine, with exception of NDUF 5. This further highlights the context-specific effect of PI3K on cellular metabolism.

This chapter has also shown that mutations in the PI3K pathway induce upregulation of the glycolytic enzyme PGAM1 at the transcriptional and translational level, underlying the role of the PI3K pathway in glycolysis.

In this study the entire protein lysate extracted from the brain and the intestine of mice was used to look at changes in protein expression caused by the activation of the PI3K pathway. Since the PI3K pathway is well known to act by phosphorylating its downstream targets, further analysis of phosphoproteomics need to be performed in the future.

The effect of mutations in the PI3K pathway on the metabolism of tissues investigated in this chapter might be specific to our mouse model, due to the fact that not only the PI3K mutations are present inside individual tissues, but are expressed at systemic level. Since

every tissue influences each other metabolism, the metabolic changes observed in this mouse model might be different from the metabolic reprogramming occurring in mice harbouring the PI3K mutation in only one tissue or in cancer. For instance, the brain and the liver might have had increased OXPHOS due to the low level of blood glucose (Chapter 3, Section 3.2.4), which is the substrate for glycolysis, therefore increasing an alternative metabolic pathway to sustain their growth. Likewise, the intestine might have increased expression of the gene PGAM in order to redirect the pyruvate inside the TCA cycle or PPP, to sustain the energetic demand of the tissue, in lack of glucose. On the other hand, the increased FA oxidation and lipid metabolism in the brain might be a consequence of the effect of PI3K in inducing lipolysis in the adipose tissue, as shown in chapter 4 (Section 4.2.9).

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

The PI3K signalling pathway is one of the most important intracellular pathways, which regulates a wide range of cellular functions including metabolism (Yang et al., 2019). Under physiological conditions the effect of PI3K/AKT/mTOR signalling pathway on systemic metabolism is initiated by insulin, which is released by the pancreas in response to elevation of blood glucose. Once released, insulin acts on metabolic peripheral tissues, where it activates the PI3K/AKT/mTOR pathway, therefore restoring the physiological levels of blood glucose (Huang et al., 2018).

Alterations in the PI3K/AKT/mTOR pathway occur in a wide range of human cancers (Yang et al., 2019) and metabolic diseases including diabetes, obesity (Huang et al., 2018) and in benign overgrowth syndromes, collectively known as PIK3CA-related overgrowth spectrum (PROS) (Madsen et al., 2018). Due to the well characterised role of the PI3K pathway in metabolism, current studies focus on finding therapeutic strategies to target metabolism in cancer cells harbouring mutations in the PI3K pathway.

Through an inducible “exon switch” approach, our laboratory has previously generated mice ubiquitously expressing a mutation in *Pik3ca*, the gene coding for the subunit p110 α of PI3K. By using this mouse model (*UbCre^{ER}Pik3ca^{H1047R}*) has been shown that mutations in the PI3K pathway lead to dramatic increase in body weight that is not associated with adiposity. Additionally, expression of the mutation in *Pik3ca* leads to severe defects in glucose homeostasis resulting in insulin independent hypoglycaemia.

This study aimed to characterise the cause responsible for the alteration in glucose homeostasis observed in mutant mice, as well as to understand whether these effects are specific for the *Pik3ca^{H1047R}* mutation or can be induced by mutations occurring in other genes belonging to the PI3K pathway. In particular if the loss of *Pten*, the negative regulator of PI3K, induced the same phenotype as the *Pik3ca^{H1047R}* mutation, or synergises with the *Pik3ca^{H1047R}* mutation, was investigated. Sustained activation of the PI3K signalling was obtained by inducing a conditional expression of the *Pik3ca^{H1047R}* mutation and/or loss of the negative regulator *Pten* in every organ of the mouse.

As anticipated, two of the effects of the mutations immediately characterised were: reduced survival rate (Chapter 3; Section 3.2.2) and severe hypoglycaemia (Chapter 3; Section 3.2.4). Furthermore, mutant mice showed reduced levels of plasma insulin, compared to control mice (Chapter 3; Section 3.2.5). Whilst hypoglycaemia could potentially explain the low level of basal plasma insulin observed in mutant mice, compared to control mice, the fact that the plasma insulin did not rise after stimulation with glucose suggested a defect in pancreatic insulin production or release. Since previous studies had shown that the number of pancreatic islets was unchanged in mutant mice, with respect to control mice (Kinross et al., 2015), the hypothesis of a defect in pancreatic insulin production was excluded. Therefore, whether mutations in the PI3K pathway prevented the pancreas from releasing insulin was further examined. The treatment of *Pik3ca*^{H1047R} mutant mice with an inhibitor of the PI3K pathway, BYL-719, restored glucose-mediated insulin release. In agreement with this, *in vitro* glucose stimulation of pancreatic islets isolated from mutant mice did not increase insulin release, in contrast to the increased insulin release by the pancreatic islets isolated from control mice (Chapter 3; Section 3.2.4).

Since hyperinsulinemia could not be ascribed as the reason for hypoglycaemia, glucose uptake and hepatic gluconeogenesis were investigated (Chapter 3; Section 3.2.7-3.2.10). Increased ¹⁸F-FDG uptake was observed in various tissues of mice harbouring the *Pik3ca*^{H1047R} or the *Pten*^{-/-} mutations, compared to control mice. However, no changes in glucose uptake in the tissues of double mutant mice were observed. Furthermore, no significant difference in the ¹⁴C-2-DG bio-distribution was present between the tissues of control and mutant mice. Therefore, increased glucose uptake alone could not account for hypoglycaemia.

However, this thesis has demonstrated that hyperactivation of the PI3K pathway inhibits gluconeogenesis as shown by the lack of pyruvate metabolism to glucose, a reduced expression of the two enzymes responsible for gluconeogenesis, *Pck1* and *G6pc*, and by the inhibition of hepatic glucose production. Although the inhibition of hepatic gluconeogenesis is known as a physiological response to the activation of the PI3K pathway, in response to elevated levels of blood glucose, it is generally a transitory event which only lasts until the normal levels of blood glucose are restored (Roder et al., 2016). However, in the mouse model used in this study, the PI3K pathway was constitutively active in every organ of the

mouse leading to a sustained and prolonged inhibition of gluconeogenesis, which, together with increased glucose uptake from some of the tissues, induced severe hypoglycaemia.

Another visible effect of the activation of the PI3K pathway in mice was a remarkable enlargement of several organs and increased body weight (Chapter 3; Section 3.2.3), which could not be ascribed to adiposity. On the contrary, hyperactivation of the pathway caused an outstanding loss of body fat, compared to control mice (Chapter 4; Section 4.2.1) and increased lipolysis in the adipose tissue (Chapter 4; Section 4.3.8). These data were in disagreement with the well-established role of PI3K in inhibiting lipolysis (Choi et al., 2010). The fact that the PI3K pathway is activated in every organ of the mouse, as opposed to specific tissues, might provide a plausible explanation of why such a discrepancy was observed. Since our mouse model lacked the glucose necessary to generate energy due to severe hypoglycaemia, it is plausible that adipose tissue lipolysis was stimulated in order to supply an alternative energetic source. This data suggest that the effect of the PI3K pathway in individual organs is influenced by the systemic metabolism and by the total body energetic demand.

The decreased adiposity observed in mutant mice could not be ascribed to food intake, which was unchanged between control and mutant mice, or ambulatory activity, which was lower in mutant mice, compared to control mice. However, adiposity could be associated with nutrient utilisation. Indeed, mutant mice showed lower RER, compared to control mice, suggesting a higher consumption of fat than carbohydrates which could explain the reduced adiposity observed in these mice (Chapter 4; Section 4.2.3 and 4.2.4).

The *Pik3ca*^{H1047R} mouse model showed enhanced angiogenesis at the level of the inguinal fat pad, which appeared darker than control mice. A dark colour of the WAT is a common aspect of the WAT browning, a phenomenon which consists in the generation of beige adipocytes (also called brown-like adipocytes) within the WAT (Wang and Seale, 2016). Insulin-PI3K signalling has been shown to modulate this phenomenon by activating a population of POMC/CART neurones and inactivating AGRP/NCY neurones (Dodd et al., 2015). This thesis showed that PI3K activation determines the expression of beige adipocytes in WAT, as shown by the histological presence of beige adipocytes interspaced between white adipocytes, and by the expression of genes specific for beige adipocytes (*Tmem26* and

Cd137). However, the expression levels of *Ucp-1*, which uncouples oxidative respiration from ATP synthesis, were not increased in the WAT of mutant mice, compared to control mice. Furthermore, no significant changes in the expression of *Pomc*, *Agrf* and *Npy* neurotransmitters were observed between control and mutant mice, suggesting that while hyperactivation of the PI3K pathway stimulates the generation of beige adipocytes, they are inactive (Chapter 4; Section 4.2.10). Although it was not possible to ascribe the reason for the inactivation of brown adipocytes, this inactivation could explain the low energy expenditure observed in mutant mice as opposed to control mice.

Although mice harbouring the *Pik3ca*^{H1047R} mutation had increased lipolysis no changes in FA uptake, lipid storage or oxidation were observed between the liver and skeletal muscle of mutant mice and control mice, suggesting that the fatty acids generated through the hydrolysis of triglycerides within intracellular lipid droplets were not used by the liver or muscle to generate energy (Chapter 4; Section 4.2.8). This might imply that FAs accumulate into the blood stream of the mice. Future studies need to be performed in the future to assess this hypothesis.

In summary, the first part of this study demonstrates that the activation of the PI3K pathway in every organ of the mice inhibits glucose-dependent release of insulin whilst inducing insulin independent-glucose uptake in peripheral tissues, inhibition of hepatic gluconeogenesis and hypoglycaemia. Due to low levels of glucose, mutant mice activate lipolysis in the adipose tissue, which provides an alternative source of energy to the mice. Finally this thesis shows that the activation of the PI3K pathway stimulates the generation of beige adipocytes inside the WAT, which are *Ucp-1* negative and therefore inactive, keeping the energy expenditure at low levels (Figure 6.1). The fact that both fat and carbohydrates were exhausted in mutant mice and that the beige adipocytes were inactive (therefore unable to generate energy as heat), might be a plausible reason for the short survival rate of the mice.

Since the PI3K pathway and systemic metabolism mutually influence each other, changes in the metabolism of individual tissues might result either from the direct effect of the PI3K pathway activation in specific tissues or from the indirect effect the PI3K pathway activation on systemic metabolism. In some cases it was possible to discriminate between these two possibilities. *Ex-vivo* studies, indeed, show that PI3K activation has a direct effect in the

pancreas, where it inhibits insulin release (Chapter 3; Section 6), and in the liver, where it inhibits gluconeogenesis (Chapter 3; Section 3.2.11). However, since previous mouse models harbouring tissue specific mutations in the PI3K pathway did not present increased lipolysis, we can speculate that the activation of lipolysis is dependent on the activation of PI3K and the consequential hypoglycaemia observed.

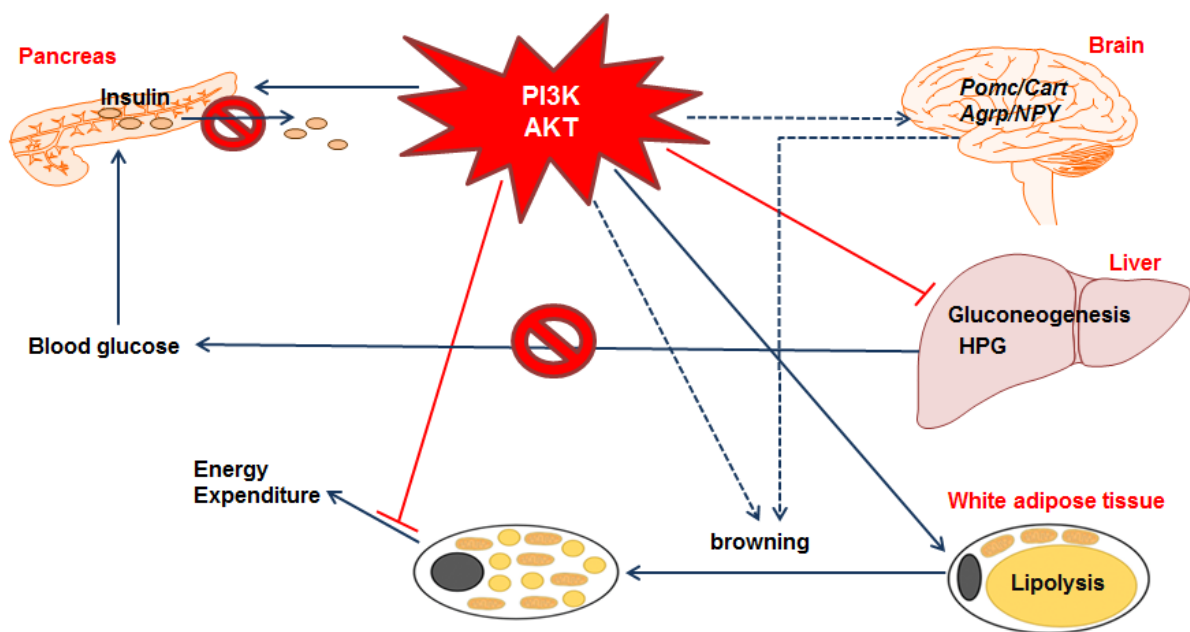


Figure 6. 1: Metabolic regulation in mice ubiquitously expressing *Pik3ca*^{H1047R}.

Schematic summarising the effects of the constitutive activation of the PI3K signalling on systemic metabolism. Activation of the PI3K pathway in the pancreas of mice inhibits glucose-stimulated release of insulin. Activation of the PI3K pathway in the liver of mice inhibits gluconeogenesis and hepatic glucose production (HGP), therefore leading to hypoglycaemia. Activation of the PI3K pathway in the adipose tissue of mice stimulates lipolysis. Activation of the PI3K pathway stimulates white adipose tissue browning, but does not lead to increased energy expenditure. Broken lines indicate pathway that have not been fully characterised in this thesis.

Understanding the molecular mechanism through which mutations in the PI3K pathway affect systemic metabolism might offer a therapeutic approach for the treatment of patients carrying mutations in the PI3K pathway, in combination with PI3K inhibitors. Thus, this study explored the molecular mechanisms through which the activation of the PI3K pathway affects organ metabolism by analysing the expression and activity of enzymes involved in metabolism. For this purpose transcriptional and translational analysis were performed in the brain, liver and intestine of *Pik3ca*^{H1047R}*Pten*^{-/-} and matched control mice, through RNA-seq and proteomics (Chapter 5; Section 5.2.1-5.2.4).

Using RNA-seq analysis this study has shown that the role of PI3K on metabolism is tissue dependent, with OXPHOS upregulated in the brain and intestine of mutant mice, compared to control mice, and FA oxidation upregulated in the liver of mutant mice, compared to control mice. These results highlighted the context-specific effect of PI3K, where each tissue reprograms their metabolism, according with their access to nutrients. The liver, which generally has high levels of lipids, responds to increase energetic request by increasing lipid metabolism, whilst the brain and intestine fuel the TCA cycle by upregulating OXPHOS. However, since functional metabolic analysis of OCR and ECAR in intestinal organoids and brain neurospheres could not confirm RNA-seq data, further work needs to be performed to understand the significance of OXPHOS in the tissues of mutant mice. Metabolism of individual tissues, *in vivo*, is influenced by systemic metabolism. As such, it is possible that organoids, grown in culture, may have lost the influence of systemic metabolic signals, which may explain the discrepancy between the analyses. As such, metabolic assays could be performed *ex-vivo* by directly measuring the OCR and ECAR in the individual tissues, rather than in tissue specific organoid cultures.

Proteomic analysis performed in the brain and intestine of mutant and control mice showed increased expression of some of the mitochondrial proteins in consequence of the mutations in the PI3K pathway, including NDUF 5 and NDUF 6, as well as the overexpression of the glycolytic enzyme PGAM1. Interestingly, whilst the OXPHOS proteins, NDUF 5 and 6 were expressed in the intestine and brain of mutant mice, with respect to control mice, the expression of PGAM1 was limited to the intestine, further indicating that the PI3K pathway has a tissue specific effect on metabolic reprogramming. These data suggest that targeting metabolism in patients carrying mutations in the PI3K pathway may be beneficial as a therapeutic strategy towards cancers or diseases that carry mutations in the PI3K pathway. However, due to the variety of effect that PI3K signalling can have on metabolism and its dependence upon the location in which mutations of the PI3K pathway occur, it is necessary to design more personalised therapies. Targeting the metabolic effect caused by mutations in the PI3K pathway would allow selective targeting of PI3K mutant tissue, removing the wider systemic consequences of a PI3K inhibitor. For instance, targeting the mitochondrial activity as well as the expression of PGAM1 is currently possible thanks to the presence of inhibitors on the market (Huang et al., 2019; Sharif et al., 2019; Wen et al., 2013). The use of these inhibitors has been reported to be efficient for the treatment of human cancers (Bernal et al.,

1982; Capozzi et al., 2012; Griffin et al., 2011; Huang et al., 2019). Therefore, although further investigations are necessary, this study opens the way for future investigations aimed at targeting the oxidative phosphorylation and the pentose phosphate pathway in patients harbouring mutations in the PI3K pathway.

6.1 CONCLUSIONS

Research in the past two decades has defined how aberrant activation of the PI3K signalling pathway drives tumorigenesis and metabolic diseases. Future research aims at refining novel therapeutic strategies. The failure of PI3K inhibitors is associated, at least in part, to the off-target effects on systemic metabolism. Ubiquitous expression of the PI3K signalling pathway has opposite effects to what observed is in mouse models with tissue-specific activation of the pathway including severe hypoglycaemia, hypoinsulinemia and increased lipolysis in the adipose tissue. The effects of the mutations are tissue specific and depend upon the availability of nutrients. These data expand our knowledge on the tissue specific effects of the activation of the PI3K pathway, taking into account the systemic alterations that such activation generates. This knowledge may be utilised in future studies to lead to improved patients treatment.

6.2 FUTURE STUDIES

Although the initial studies presented in this thesis were conducted on 3 mouse models- *Pik3ca*^{H1047R}, *Pten*^{-/-} and *Pik3ca*^{H1047R}*Pten*^{-/-} (Chapter 3), part of the studies presented in this thesis were performed exclusively using the *Pik3ca*^{H1047R} mouse model or the *Pik3ca*^{H1047R}*Pten*^{-/-} mouse model. For instance, analysis of the systemic metabolism, as well as analysis of the lipids metabolism (Chapter 4), were performed only on *Pik3ca*^{H1047R} mice. Genetic and functional analysis, indeed, were performed using the *Pik3ca*^{H1047R}*Pten*^{-/-} mouse model (Chapter 5). Although similar effects on glucose homeostasis were observed between mice harbouring the *Pik3ca*^{H1047R}, *Pten*^{-/-} or *Pik3ca*^{H1047R}*Pten*^{-/-} mutation (Chapter 3), further metabolic studies could be conducted using all the 3 mouse models, in order to compare the

effect that the single mutations or the combination of the two mutations on systemic metabolism, gene expression and protein function.

This thesis has shown that the *Pik3ca*^{H1047R} mutation stimulates WAT browning in mice. However, it was not possible to ascertain whether PI3K activation stimulates WAT browning directly in adipose tissue or if its effect is dependent upon the signal sent by the brain. To validate these hypotheses further studies will be performed by inhibiting the sympathetic neuronal transmission from the brain to peripheral tissues (Dodd et al., 2015).

The mouse models used for this study harbour mutations in the PI3K pathway in every organ. Due to the role of PI3K on systemic metabolism it was not possible to ascertain whether the effects observed in individual tissues were directly caused by the activation of the PI3K mutation in the tissue or were the reflexion of the systemic changes caused by the ubiquitous expression of the PI3K activation. To discriminate between these two hypothesis future studies will be performed by inducing the conditional expression of mutations in the PI3K pathway in specific organs of the mice (brain, liver, pancreas, muscle) and investigate the metabolism of the tissue expressing the mutation, as well as the effect of the mutation on systemic metabolism.

3'RNA-seq and proteomic analysis have highlighted the importance of the PI3K pathway in metabolism. Importantly, the mutations affect metabolism in a tissue-dependent manner. One of the genes resulting from both the 3'RNA-seq and the proteomics analysis was the glycolytic enzyme PGAM. PGAM1 overexpression is found in several human cancers where it stimulates is tumour growth, survival, and invasion. (Hare et al., 2014). Since overexpression of PGAM1 has been shown to be stimulated by mTOR through HIF1 α -mediated transcriptional activation, therefore mediating the Warburg effect (Sun et al., 2018), further analysis will be conducted to investigate PGAM1 as therapeutic target in combination with PI3K inhibitors for the treatment of patients harbouring mutations in the PI3K pathway. For this purpose PGAM inhibitors currently available on the market (Huang et al., 2019; Sharif et al., 2019; Wen et al., 2013) could be used for in vitro studies, by using cancer cell lines harbouring mutations in the PI3K pathway or by using a mouse model available in our laboratory, which harbour the *Pik3ca*^{H1047R} mutation and loss of the gene APC, therefore developing intestinal carcinoma (Hare et al., 2014).

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