



Development of in vitro models to study effects of compounds that modulate cellular functions in patients with mitochondrial disease

Tiffany Ann Lodge

Green Templeton College

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Abstract

Development of *in vitro* models to study effects of compounds that modulate cellular functions in patients with mitochondrial disease.

Background: Adverse drug reactions caused by off-target activity can limit the use or withdrawal of otherwise effective drugs. Mitochondria have been recognised as potential target for inadvertent drug activity thus causing toxicity. It is difficult to detect drug induced mitochondrial toxicity in pre-clinical animal models and in human trials. Drug trials are carried out in healthy humans and it is not until a large population has been exposed to the drug that adverse reactions leading to mitochondrial toxicity come to light. The development of pre-clinical *in vitro* assays to detect drug induced mitochondrial toxicity can help reduce adverse drug reactions and drug development costs. In this project, we used different *in vitro* cell models (with or without mitochondrial disease) to study the effect of the anticonvulsant drug, sodium valproate (VPA) on cellular functions such as mitochondrial respiration, under both normal conditions (glucose) and energetic stress (galactose). To investigate changes to the morphology of the mitochondrial network, we developed a novel high-throughput imaging method. **Results:** The results presented in this thesis indicate that sodium valproate treatment did not have the same effect on cellular activity and mitochondrial network morphology in all the *in vitro* models used. VPA treatment inhibited mitochondrial respiration in the cell model without mitochondrial disease. However, in the models harbouring mitochondrial disease the effect was variable. Treatment under normal and energetic stress condition caused increased cell death and a reduction in cell number in all the cell models. We also evaluated a novel high-throughput imaging method and found that VPA treatment affects mitochondrial network morphology with fragmentation observed in two of cell models. It was found that not all cell models are suitable for this type of high-throughput imaging. **Conclusion:** Our results suggest that the effect of VPA under normal and energetic stress condition varies according to the cell model used. The induction of energetic stress by galactose is possibly too acute for certain cell models leading to complications in data interpretation. Depending upon the model used, reducing the glucose concentration could potentially be sufficient to detect drug induced mitochondrial toxicity.

Scientific research is an equal measure of frustration and fun

Anon

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Abbreviations

3-MA	3-Methyladenine
AB	Alamar Blue
ADP	Adenosine diphosphate
ADR	Adverse drug reaction
ADS	Autism spectrum disorder
AHS	Alpers-Huttenlocher syndrome
ALF	Acute liver failure
ANOVA	One-way analysis of variance
AD	Alzheimer's disease
ATP	Adenosine triphosphate
BODIPY	4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene
CCCP	Carbonyl cyanide m-chlorophenyl hydrazine
CCD	Charge-coupled device
CoA	Coenzyme A
CSF	Cerebrospinal fluid
D-loop	Displacement-loop
DMSO	Dimethyl sulfoxide
Drp1	Dynamin-related protein 1
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
E&P	E64d and Pepastatin A
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
FADH ₂	Flavin adenine dinucleotide, reduced
FBS	Foetal bovine serum
FCCP	Trifluoromethoxyphenylhydrazine
FITC	Fluorescein isothiocyanate
GABA	Gamma-aminobutyric acid
HBSS	Hank's balanced salt solution
HDAC	Histone deacetylase 1

HSP	Heavy strand promoter
HUVEC	Human umbilical vein endothelial cells
IMM	Inner mitochondrial membrane
KSS	Kearns-Sayre syndrome
LC3	Microtubule-associated protein light chain 3
LOHN	Leber's hereditary optic neuropathy
LSP	Light strand promoter
MEF	Mouse embryonic fibroblasts
MELAS	Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes
MERRF	Myoclonic epilepsy with ragged-red fibres
Mfn1 and 2	Mitofusin1 and 2
mtDNA	Mitochondrial DNA
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)
NAD ⁺	Nicotinamide adenine dinucleotide, oxidised
NADH	Nicotinamide adenine dinucleotide, reduced
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
nDNA	Nuclear DNA
OCR	Oxygen consumption rate
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PD	Parkinson's disease
PEO	Progressive external ophthalmoplegia
PFA	Paraformaldehyde
PGC-1 α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI	Propidium iodide
PINK1	PTEN-induced putative kinase 1

Plasticity	The ability of cells to adjust their mitochondrial response to changes in glucose concentration (high to low) or to energetic stress (galactose)
POLG	Polymerase gamma
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
RT	Room temperature
TCA cycle	Tricarboxylic acid cycle
TOM20	Translocase of outer membrane 20
TRF	Time resolved fluorescence
UCP1	Uncoupling protein 1
VDAC	Voltage dependant anion channels
VPA	Sodium valproate

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Chapter 1

Introduction

1 General Introduction

1.1 Mitochondria

The mitochondria are double membrane organelles found in nearly all eukaryotic cells, mature erythrocytes being the exception. Although, best known for being ‘the power house’ of the cell, producing adenosine triphosphate (ATP), mitochondria have many other cellular functions. A typical human cell can contain hundreds of mitochondria, depending on the cell’s energy requirement (Lee *et al.*, 2009). Additionally, the mitochondrion has been shown to play important roles in apoptosis, Ca^{2+} homeostasis and intracellular signalling. Furthermore, important metabolic pathways such as the tricarboxylic acid cycle (TCA cycle) and beta-oxidation takes place in the mitochondrial matrix. The organelle contains several copies of its own genome, which unlike the nuclear genome which is inherited from both parents is maternally inherited.

1.1.1 Mitochondrial structure

Mitochondria are bounded by a double membrane, figure 1.1. The outer mitochondrial membrane (OMM) has a fairly simple topology and composition, low cardiolipin content and low protein and phospholipid ratio. Key essential structures housed on the OMM include voltage dependant anion channels (VDAC) and translocases of the outer membrane (TOM). VDACs make the OMM permeable to ions and small molecules up to 5kDa such as adenosine diphosphate (ADP) and ATP (Colombini, 2016). Furthermore, VDACs situated on dysfunctional mitochondria in mouse embryonic fibroblasts can recruit Parkin, thereby initiating the PINK/Parkin mitophagy pathway (Sun *et al.*, 2012). Mitophagy is mitochondrial directed autophagy and will be

discussed later. Nuclear encoded proteins targeted to the mitochondria are imported via the TOM import complex. TOM complexes consist of seven different subunits; TOM40 forms the protein conducting channel and associates with the subunits TOM22, TOM20 and TOM70 are receptors. TOM6 and TOM7 are smaller proteins and are involved in the stability of the TOM complex. There is a further TOM protein, TOM5 which participates in transfer of precursor proteins to the translocation pore (Becker *et al.*, 2011). The inner mitochondrial membrane (IMM) has a complex topology as the membrane folds into 'compartments' called cristae, figure 1.1. The IMM is rich in cardiolipin and has a high ratio of protein to phospholipids. Only lipophilic molecules can be transported freely across the IMM (Walther and Rapaport 2009). The composition of the IMM makes it impermeable to H^+ ions which are vital for oxidative phosphorylation (OXPHOS). Furthermore, the complexes of the electron transport chain (ETC) are embedded in the IMM (Lemasters *et al.*, 2012; Navarro and Boveris, 2007). As mentioned above the mitochondrial matrix accommodates several important metabolic pathways including the TCA cycle and is rich in metabolites such as nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide, reduced ($NADH$), ADP and ATP (Logan, 2006).

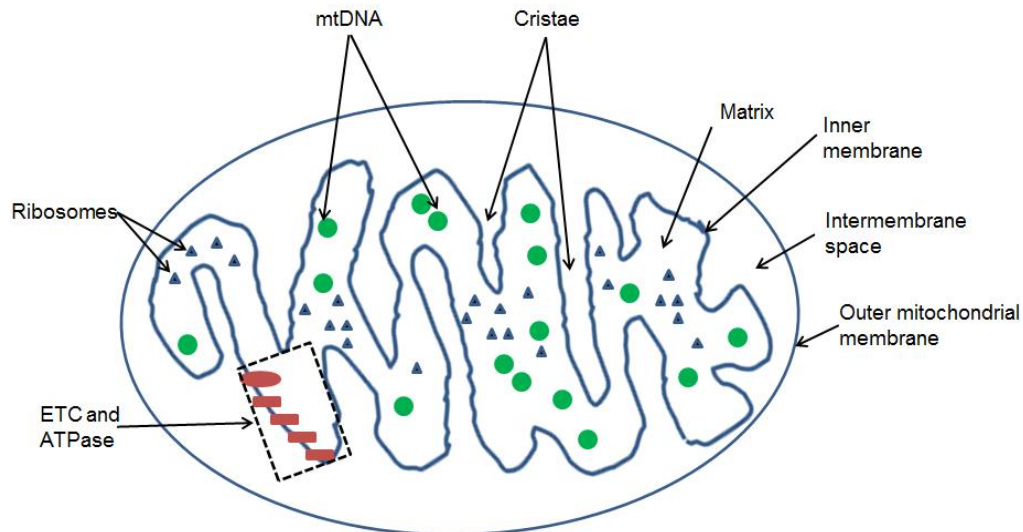


Figure 1.1. Illustration of the structure of the mitochondrion. Green circles denotes mtDNA nucleoids, blue triangles ribosomes and black dashed box the electron transport chain (ETC) and ATPase (adapted from Logan, 2006).

The mitochondria are unique organelles containing double stranded 16.5kb circular DNA molecule known as the mitochondrial DNA (mtDNA), having 37 genes, figure 1.1. MtDNA is located in the mitochondrial matrix and encodes for 13 proteins of the ETC, 22 transfer RNAs and two ribosomal RNAs (Falkenberg *et al.*, 2007). The complexes of the ETC requires 83 proteins and the mtDNA only encodes for 13 of these, the remaining are nuclear encoded and imported into the mitochondria for assembly, see table 1.1 (Wallace and Chalkia, 2013). Each mitochondrion will contain multiple copies of mtDNA, packed into structures called nucleoids (Bogenhagen, 2012; Spelbrink, 2010).

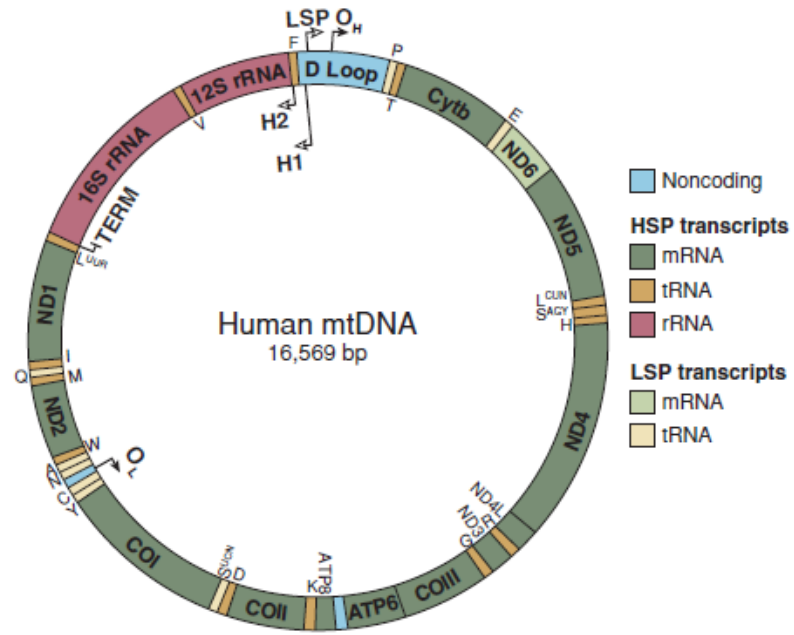


Figure 1.2. Map of human mtDNA. Abbreviations, clockwise: Cytb (cytochrome b); ND6 (NADH dehydrogenase subunit 6); ND5 (NADH dehydrogenase subunit 5); ND4 (NADH4, NADH dehydrogenase subunit 4); COIII (cytochrome c oxidase subunit III); COII (cytochrome c oxidase subunit II); COI (cytochrome c oxidase subunit I); ND2 (NADH dehydrogenase subunit 2); ND1 (NADH dehydrogenase subunit 1); HSP (heavy strand promoter); LSP (light strand promoter). Adapted from Falkenberg *et al.*, 2007.

Table 1.1. Genetic origin of the subunits required for a fully functional electron transport chain.

Complex	Number of subunits	nDNA encoded	mtDNA encoded
I	41	34	7(ND1-6)
II	4	4	0
III	11	10	1 (cytochrome b)
IV	13	10	3 (cytochrome oxidase I,II and III)
V	14	12	2 (ATPase 6 and 7)

Abbreviation; ND1-6 ; NADH dehydrogenase subunits 1-6. nDNA; nuclear DNA (Wallace and Chalkia, 2013)

1.2 mtDNA replication

Cells contain hundreds of mitochondria each with several copies of mtDNA located in the mitochondrial matrix (Stumpf and Copeland, 2011, Copeland, 2008). Unlike nuclear DNA (nDNA) which is wound around histones, mtDNA is arranged into nucleoids (Kukat *et al.*, 2011). Nucleoids are substructures that contain mtDNA, transcription factor A mitochondrial (TFAM) and single-stranded DNA-binding protein (mtSSB) which are central in mtDNA biogenesis (Bogenhagen *et al.*, 2014; Kukat *et al.*, 2011). MtDNA consists of two strands, the cytosine rich light strand and the guanine rich heavy strand. There are no introns present in the mitochondrial genome with each strand having its own promoter region for transcription initiation, the light strand promoter (LSP) and the heavy strand promoter (HSP), figure 1.2. MtDNA has a single non-coding region and this is found the displacement-loop (D-loop). This region contains the origin of replication of the heavy strand (O_H) and both the LSP and HSP. Two modes of mtDNA replication have been proposed, the asynchronous strand displacement model and strand-coupled bidirectional replication model. In the

asynchronous strand displacement model, mtDNA synthesis is primed by transcription through O_H within the D-loop and the mtDNA is asymmetrically replicated (Brown *et al.*, 2005). Whereas in the second model, strand-coupled bi-replication, initiation of mtDNA replication occurs in a zone near the O_H and is followed by the progression of two replication forks around the circular mtDNA (Bowmaker *et al.*, 2003; Yakusawa *et al.*, 2005).

mtDNA replication machinery

MtDNA is maintained and replicated by the mitochondrial DNA replication machinery, figure 1.3. The only known mitochondrial polymerase is the polymerase gamma (PolG). PolG is made up of two nuclear encoded subunits imported into the organelle from the cytosol. PolG A also known as PolG1 or PolG and has a molecular mass of 140kDa. The smaller subunit PolG B (PolG2) has a molecular mass of 55kDa. PolG A, the catalytic subunit has 3'-5' exonuclease and 5'-3' deoxyribose phosphate lyase activity. The accessory subunit PolG B promotes processivity during mtDNA synthesis. Also part of the mtDNA replication machinery is the helicase TWINKLE (PEO1) which unwinds the mtDNA template in a 5'-3' direction. The mitochondrial stranded binding protein (mtSSB) provides stability of the unwound DNA and stimulates DNA synthesis (Falkenberg *et al.*, 2007; Stewart *et al.*, 2011), figure 1.3.

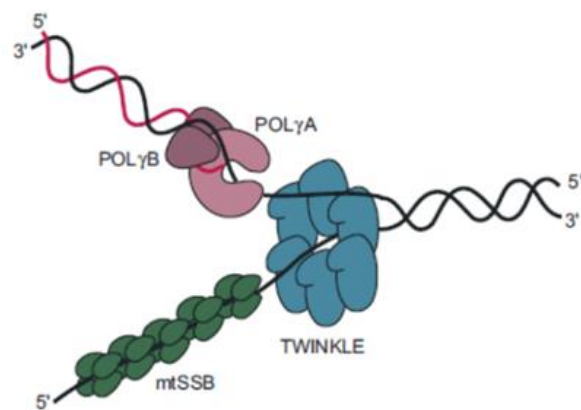


Figure 1.3. The mtDNA replication machinery. Consisting of polymerase γ (POLG) for DNA synthesis. The TWINKLE helicase which unwinds the DNA template and the single mitochondrial stranded DNA-binding protein (mtSSB). Adapted from Falkenberg *et al.*, 2007.

1.3 Mitochondrial disease

The mitochondrion has a central role in maintaining cellular functions by being the major source of ATP production together with its crucial role in apoptotic cell death. Defects in mitochondrial function can result in a range of diseases that can be single organ orientated such as Leber's hereditary optic neuropathy (LHON) or multi organ for example myoclonic epilepsy with ragged-re fibres (MERRF). The severely affected organs are those with a high energy demand such as the brain, heart, liver and skeletal muscle however, any organ or tissue in the body can be affect (Davis and Williams, 2012; DiMauro *et al.*, 2013; Osellame *et al.*, 2012). Mitochondrial disorders can present at any age. Table 1.2 provides a brief overview of common mitochondrial disorders and related genetic mutations. In a healthy individual, the mtDNA will be identical (homoplasmic) however, when a sequence variation occurs as with patients with certain mitochondrial diseases, the cell will contain both wild-type (normal) and mutated mtDNA this is termed heteroplasmy. Not all mtDNA variants are pathogenic and therefore disease causing as this will depend on mutation site within the mitochondrial genome but also the proportion of wild-type to mutant mtDNA within the cells in the affected organ or organs (Lightowlers *et al.*, 1997; Fernadez-Silva *et al.*, 2003; Zeviani and Di Donaro, 2004). The mutation rate in mtDNA is higher than for nDNA as the mismatch repair system is much less efficient. MtDNA also lack protective histones, leaving it vulnerable to the mutagenic effects of reactive oxygen species (ROS) which can be found at high levels in mitochondria. The location of the mutation within the mtDNA genome can influence the resulting pathogenic phenotype (Tucker *et al.*, 2010). Mutations found in genes which alter an amino acid will affect the function or expression of a single protein whereas mutations in tRNAs or rRNAs can have wider ramifications as it can have an impact on the translations of all 13 mtDNA encoded proteins.

Table 1.2: Mitochondrial disorders and associated mutations. A brief overview of some of the common mitochondrial diseases and related genetic mutations.

Disorder	nDNA/mtDNA mutation	Approx. age of onset	Effect on mitochondrial function
Leigh Syndrome	nDNA mutations, in cytochrome c oxidase in e.g. <i>SURF1</i> , <i>GFM1</i> , <i>NDUFS4</i>	Infancy~1	ETC Complex assembly and translation factors
LHON (Leber hereditary optic neuropathy)	mtDNA point mutations, m.3460G>A, 1178G>A, 14487T>C	Young adult (15-30)	Complex 1 dysfunction
MELAS (myopathy, encephalopathy, lactic acidosis, stroke like episode)	mtDNA point mutations, m.3243A>G, mtDNA COX genes	Childhood but also <40 years	ETC assembly and stability. ATP deficiency
MERRF (myoclonus, epilepsy, ragged red fibres)	mtDNA point mutations (tRNA), m.8344A>G most common, <i>MT-TK</i> gene	Childhood (5-15)	Impaired protein synthesis due to <i>mttRNA^{Lys}</i> gene
Mitochondrial DNA depletion syndromes, Alpers-Huttenlocher	nDNA mutations in genes, <i>DGK</i> , <i>TK2</i> , <i>TWINKLE(PEO1)</i> , <i>POLG</i>	Congenital or infantile	Reduction in mtDNA copy number

Adapted from Chinnery, 2010; Schapira, 2006; Naviaux and Nguyen, 2004

Mitochondrial disease in humans comprises of a broad range of metabolic disorders resulting in a wide range of disease phenotypes from myopathies to visual and hearing impairment, organ failure and respiratory disorders (Chinnery, 2010; DiMauro and Schon, 2003; DiMauro, 2004, Taylor and Turnbull, 2005). Furthermore, impaired and abnormal mitochondrial function has been implicated in neurodegenerative diseases such Parkinson's disease, Alzheimer's disease, Huntington's disease and autism spectrum disorder and in the normal ageing process (Gui *et al*, 2015; Mizuno *et al*, 1995; Piaceri *et al.*, 2012; Tang *et al.*, 2013).

1.3.1.1 Mitochondrial disease linked to mtDNA deletions, duplication and depletion

Deletions

Deletions are defects of the mitochondrial genome from which several genes or parts of genes have been lost. The fragments lost range from two to ten kilobases. MtDNA deletions have been found to be tissue specific or present in wide range of tissue. In Kearns-Sayre syndrome (KSS), mtDNA deletions are found in several tissues such as muscle, retinal pigment epithelial cells and cardiomyocytes whereas, in progressive external ophthalmoplegia (PEO), mtDNA deletions are restricted to skeletal muscle tissue (DiMauro and Hirano, 2011; Kabunga *et al.*, 2015). KSS is a multi-systemic disorder with an onset before the age of 20. PEO is characterised by drooping of the eyelids, paralysis of the extrocular muscles (ophthalmoplegia) and variable proximal limb weakness. PEO may present as a single clinical phenotype or as part of other mitochondrial disease such as KSS (DiMauro and Hirano, 2011). Mitochondrial DNA deletions are rarely inherited and can arise by *de novo* mtDNA deletions during foetal development. MtDNA deletions can occur in the oocyte during germline developments or in the embryo during embryogenesis. For example at the blastocyst stage, mtDNA containing deletions can enter the three germ layers and cause KSS, or segregate to muscle causing PEO (DiMauro and Schon, 2003).

Duplication

A complete or partial duplication of the mitochondrial genome can occur resulting in mitochondrial dysfunction (DiMauro, 2004; Poulton *et al.*, 1989; Tengan *et al.*, 2002). Studies have found high incidences of duplication in patients with KSS. Although mtDNA duplications do not lead to loss of mtDNA genes it may interfere with mitochondrial function via abnormal (excessive) transcription of proteins in the

duplicated regions (Poulton *et al.*, 1995; Odoardi *et al.*, 2002). Duplications alone have not been shown to cause disturbed ETC activity (Odoardi *et al.*, 2003).

Depletion

MtDNA depletion is due to defects in the mtDNA maintenance machinery caused by mutations in the nuclear genes that encodes for proteins involved in mitochondrial nucleotide synthesis *thymidine kinase2* (*TK2*) and *deoxyguanosine kinase* (*DGOUK*) or mtDNA replication (*PolG* and *PEO1*). All the proteins involved in mtDNA replication and maintenance are encoded by the nuclear genome. Mutations in these genes can compromise the mitochondrial replication and maintenance machinery, resulting in depletion of the mtDNA content, leading to impaired ATP production due to insufficient synthesis of electron transport chain components in the affected organs. MtDNA depletion syndromes are autosomal recessive disorders that have a broad genetic and clinical spectrum (El-Hattab and Scaglia, 2013). *TK2* and *DGOUK* are genes encoding for two of the 11 proteins known to be involved in the replication and maintenance of the mtDNA. *TK2* encodes for thymidine kinase2 (*TK2*) and *DGOUK* encodes for deoxyguanosine kinase (*dGK*). As mtDNA is replicated independently of the nuclear genome there is a need for a constant supply of deoxyribonucleoside triphosphates (*dNTPs*), *TK2* and *dGK* are the key proteins involved in the maintenance of the *dNTP* pool (El-Hattab and Scaglia, 2013). Key proteins involved in mtDNA replication are polymerase gamma (*PolG*) encoded by *PolG* and *TWINKLE* encoded by *PEO1*. Furthermore, depletion of mtDNA due to *PolG* mutations has been found in PD patients (Gui *et al.*, 2015).

1.3.1.2 Mitochondrial disease linked to mtDNA point-mutations

MtDNA point mutations follow maternal inheritance pattern and both genders can be affected. Commonly, point mutations affecting tRNA genes may affect protein synthesis but not OXPHOS as such, whereas, mutations in genes encoding proteins for the ETC subunits will affect OXPHOS function. Over 250 mtDNA point mutations have been described as pathogenic (Russell and Turnbull, 2014; Taylor and Turnbull, 2005). One of the most common pathogenic point mutation found is the m.3243A>G transition of the tRNA^{Leu} gene, with a carrier rate of 7.8 per 100,000, adult population with mitochondrial disease (Gorman *et al.*, 2015). The resulting clinical outcomes are mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MEALS). Other clinical outcomes linked to m.3243A>G mutation are impaired hearing and diabetes (El-Hattab *et al.*, 2015).

1.3.2 Polymerase gamma (PolG) related mitochondrial disease

As previously mentioned, the nuclear encoded PolG proteins are essential proteins involved in the replication of mitochondrial genome. Heterozygous mutations in the smaller PolG2 subunit have been associated with mitochondrial diseases such as PEO (Young *et al.*, 2013) PolG1 (PolG) is the larger subunit with mutations in its linker region influencing its association with the smaller subunit PolG2 resulting in mtDNA depletion and/or mtDNA deletions. From herein PolG1 will be referred to as PolG.

The diseases associated with PolG mutations are many and diverse. Over 180 pathogenic mutations have been attributed to mutations of the PolG gene, figure 1.4 (Saneto *et al.*, 2013; Cohen and Naviaux, 2010). Disease onset and severity varies in PolG-related conditions and can evolve over time with an overlapping spectrum of

symptoms. The A467T mutation in PolG, a substitution of alanine with threonine, occurs in 36% of all PolG patients. In the general population the frequency of this mutation is estimated to be 0.2% to 1%. Patients who are homozygous for the A467T mutation, typically presents with symptoms in late to early teens (Stumpf *et al.*, 2013). However most patients are heterozygous and may have the A467T mutations with another PolG disease causing mutation on the other allele i.e. the W748S, substitution of tryptophan with serine. PolG A467T mutations cause a loss of interaction between the two PolG subunits, reducing polymerase activity causing stalling of mtDNA synthesis (Chan *et al.*, 2005). The W748S mutation has also been associated with parkinsonism (Remes *et al.*, 2008). PolG mutations have been found in several mitochondrial diseases including Alpers-Huttlencocher syndrome, progressive external ophthalmoplegia (PEO) and ataxia neuropathy spectrum (Stumpf *et al.*, 2013).

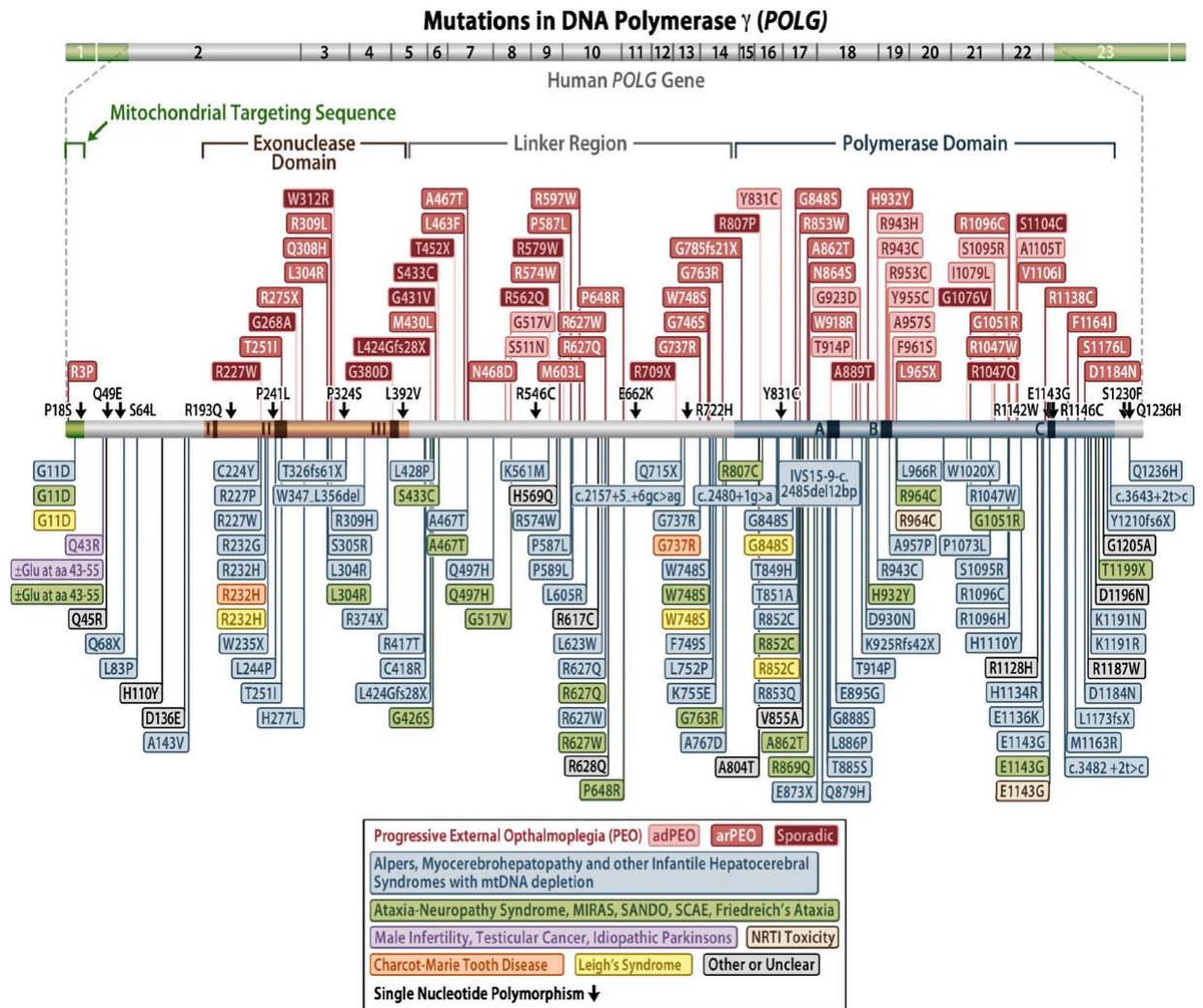


Figure 1.4. Pathogenic mutations in the *PoIG1* gene. The *PoIG1* gene comprises of three domains, polymerase, linker and exonuclease. Pathogenic mutations can be found in all three, (Cohen and Naviaux, 2010).

Alpers-Huttenlocher syndrome

Alpers-Huttenlocher syndrome (AHS or Alpers) was initially identified in 1931 when Bernard Alpers first published findings describing a severe epileptic syndrome with rapid onset of dementia and cortical blindness in young children. In 1976, Peter Huttenlocher described the hepatic features of the syndrome. However, it was not until 2004 (review by Cohen and Naviaux, 2010) that the genetic background was published attributing mutations in the *PolG* gene as the genetic cause (Cohen and Naviaux, 2010).

AHS is the most severe of the *PolG* related mitochondrial diseases and is generally fatal. However, the age of onset, rate of neurological degradation, hepatic failure and the age of death can vary (Stumpf *et al.*, 2013; Cohen and Naviaux, 2010). A key clinical feature of AHS is intractable seizures and this is one of the initial symptoms observed in children, followed by developmental delay. As the disease progresses, cortical visual loss leading to blindness is common. Hepatic dysfunction leading to rapid liver failure can also occur but the timing is highly variable. AHS associated liver disease includes microvesicular steatosis, bile ductular proliferation and disorganisation of normal lobular structure (Saneto *et al.*, 2013). Furthermore, certain *PolG* mutations, those attributed to AHS, result in patients becoming prone to liver failure brought on by sodium valproate treatment (Nanau and Neuman, 2013).

1.4 Diagnosis and treatment options for patients with mitochondrial disease.

Diagnosing mitochondrial disease is challenging due to the broad spectrum of clinical presentations and age of onset. The diagnosis of mitochondrial disease involves several diagnostic systems, a general clinical evaluation (myopathy, ataxia and failure to thrive and lactic acidosis in neonates), electroencephalography (when seizures are present) and blood tests followed by more invasive investigation such as tissue biopsy. Furthermore, a detailed medical family history is generally required due to the genetic origin of mitochondrial diseases (Haas *et al.*, 2008; McFarland *et al.*, 2010). Laboratory investigations for mitochondrial disease include metabolic analysis of blood, cerebrospinal fluid (CSF) and urine. Increased lactate and pyruvate in the blood or CSF can be signs of increased anaerobic metabolism and an indication of mitochondrial dysfunction (Haas *et al.*, 2007). To establish the presence of mitochondrial disease tissue biopsies are required. As skeletal muscle is often affected in mitochondrial disease, muscle biopsies are obtained for light and electron microscopy histological analysis to assess structure and organisation of the muscle fibres. If a mosaic or global pattern of cytochrome c oxidase deficient muscle fibres and red ragged fibres are observed, then mitochondrial disease may be present. However, red ragged fibres may not be present in paediatric patients as these fibres accumulate over time (Milone and Wong, 2013). Electron microscope can be used to investigate mitochondrial morphology. Furthermore, muscle and skin biopsies can be used to measure ETC activity and coenzyme Q quantification thus evaluating mitochondrial function (Haas *et al.*, 2008; McFarland *et al.*, 2010; Cohen and Naviaux, 2010). The most recent tool used to diagnose mitochondrial disease is next generation sequencing (NGS) to investigate levels of mtDNA mutations and mutations in nuclear genes. Over the years the cost of NGS has been dramatically reduced making it a valuable addition

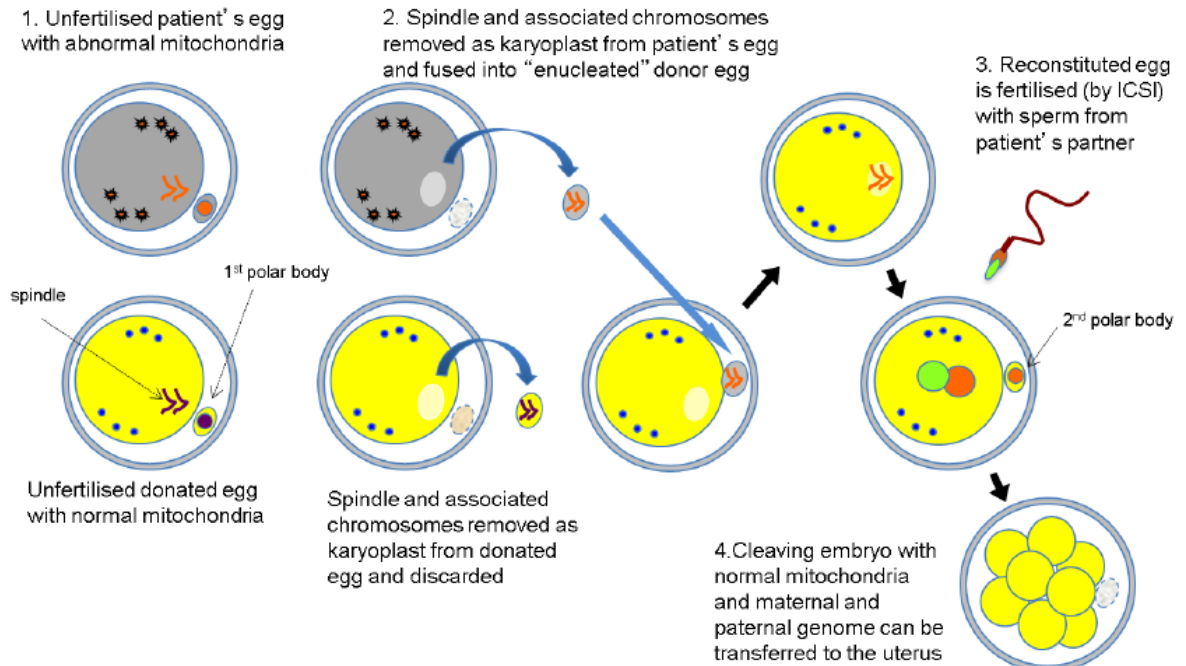
to the diagnosis process as it provides the information regarding the genetic mutation/s present (Wong, 2013).

Mitochondrial disease is not curable, with pharmacological treatment options for patients limited and largely supportive (McFarland *et al.*, 2010). Vitamin supplementation (thiamine (B₁₂) has been used as treatment for Leigh syndrome (Distelmaier *et al.*, 2014; Fassone *et al.*, 2013). Coenzyme Q₁₀ and its analogue idebenone and riboflavin have had some success in patients with complex I deficiency (McFarland *et al.*, 2010). Riboflavin is a precursor for FAD and has been used in patients with ETC complex I deficiency (Bernsen *et al.*, 1993). Other treatments used are dietary and exercised based. In a small clinical study, a diet that was low in carbohydrates but high in fat (ketogenic diet) was shown to enhance mitochondrial function by increasing fatty acid oxidation and reduce glycolysis (Gano *et al.*, 2014). Patients with mitochondrial associated myopathies have responded well to aerobic and resistance exercise as this improved mitochondrial respiration and tissue perfusion in the short-term. However, exercise intolerance is common amongst patients with mitochondrial disease (Mancuso *et al.*, 2012).

Recent advances in *in vitro* fertilisation (IVF) techniques have allowed treatments to be developed which could potentially prevent mitochondrial disease transmission. There are two strategies available, maternal spindle transfer (oocyte repair) and pronuclear technique (zygote repair), figure 1.5. Both approaches require a healthy donor egg. Proof of concept has been established for both techniques (Craven *et al.*, 2010; Amato *et al.*, 2014). These preventative approaches were deemed safe to be tried in humans and approved by authorities in the UK in 2015. To date there has been no publications reporting on successful implementation in humans of either of these techniques. Mitochondrial gene replacement raises a number of ethical issues, such as modification of the germlines thereby creating 'designer babies'. Furthermore, we do

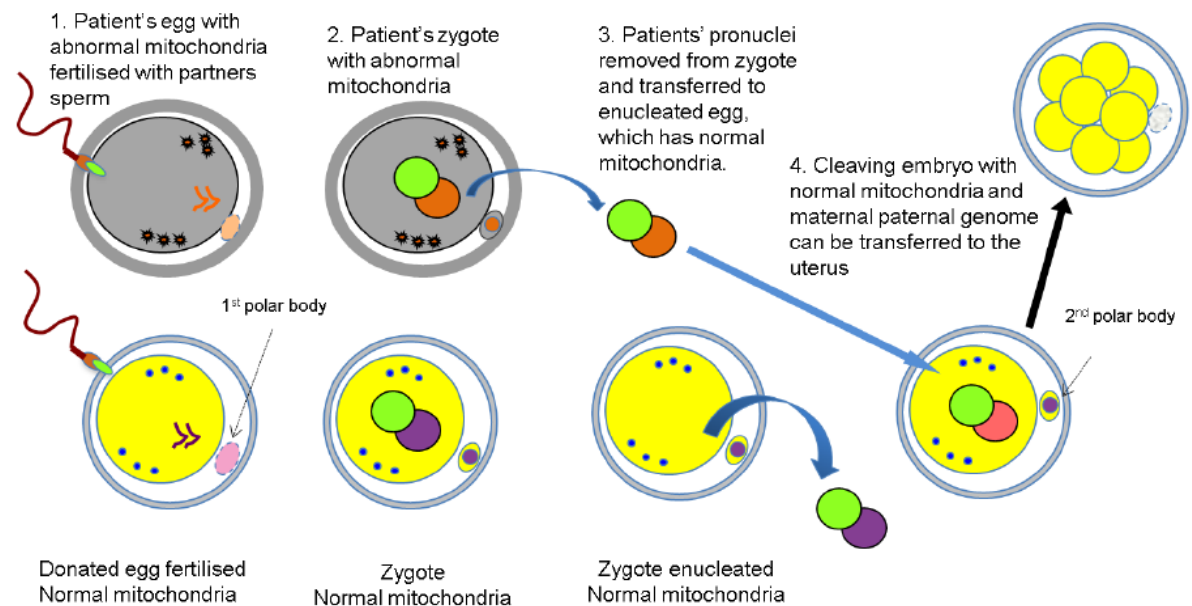
not fully understand the mtDNA and nDNA interactions in early embryo development
nor do we know what long term ramifications the treatment may have on the child.

A



The 1st polar body is a diploid product of the first meiotic division, which occurs at ovulation. The 2nd polar body is a haploid product of the second meiotic division, which occurs on fertilisation (or after activation). The polar bodies fail to persist and do not contribute to the resulting embryo.

B



The 1st polar body is a diploid product of the first meiotic division, which occurs at ovulation. The 2nd polar body is a haploid product of the second meiotic division, which occurs on fertilisation (or after activation). The polar bodies fail to persist and do not contribute to the resulting embryo.

Figure 1.5. MtDNA replacement strategies for the prevention of maternally inherited mitochondrial diseases. A) maternal spindle transfer technique. B) Pronuclear transfer technique. (Brendenoord and Braude, 2010; Human Fertilisation and Embryology Authority, 2014)

1.5 Drug induced mitochondrial dysfunction

The aim of this project was to develop new investigative techniques to further elucidate the effect of drugs and chemical compounds on mitochondrial physiology. Although, pharmaceutical companies can screen new compounds for mitochondrial toxicity using *in vitro* assays, it is sometimes difficult to predict which compounds will lead to specific organ associated toxicities in patients.

1.5.1 Uncouplers

Uncoupling of the mitochondria renders the inner mitochondrial membrane permeable to protons and allows for the oxidation of NADH and the reduction of O₂ without the synthesis of ATP. Commonly used chemical uncouplers are Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) these protonophores depolarise plasma and mitochondrial membranes. There are also naturally occurring uncouplers such as the thermogenin found in brown adipose tissue. The mitochondrial inner membrane protein thermogenin also known as uncoupling protein 1 (UCP1) is a proton transporter which allows for mitochondrial protons to re-enter into the mitochondrial matrix by-passing ATP synthase. Without the synthesis of ATP, mitochondrial driven oxidation releases energy as heat. The oxidation of dietary energy substrates (carbohydrates, lipids and proteins) eventually results in the production of NADH and FADH₂ needed to drive OXPHOS to maintain adequate ATP levels (Palou *et al.*, 1998; Jastroch *et al.*, 2010). The uncoupling of mitochondria and subsequent proton leak contributes to whole body energy expenditure, making the organelle a target for antiobesity drugs (Thrush *et al.*, 2013).

1.5.2 Inhibitors

Antimycin A and oligomycin A are examples of inhibitors of mitochondrial function.

Antimycin A inhibits complex III by binding to cytochrome c reductase inhibiting the oxidation of ubiquinol thus blocking the electron transfer between cytochrome b and c (Ma *et al.*, 2011). The inhibition of the electron transfer causes the proton gradient to collapse and loss of mitochondrial membrane potential. Inhibition of complex III also leads to increased production of reactive oxygen species (ROS). Antimycin A has been shown to suppress proliferation in human pulmonary adenocarcinoma Calu-6 cells by inducing complex III mediated oxidative stress and G1 phase cell cycle arrest.

Antimycin A was found to reduce key proteins involved in the progression of G1 phase (Han and Park, 2009). Another inhibitor of mitochondrial function is oligomycin A which inhibits the ATP synthase by binding the F_0 subdomain (proton channel) inhibiting the proton translocator and blocking the flow of proton (Jonckheere *et al.*, 2012). This causes the collapse of the proton gradient and results in the disruption of the mitochondrial membrane potential (Brand and Nicholls, 2011; Jastroch *et al.*, 2010).

1.6 Sodium valproate

Sodium valproate was first synthesised by Beverly Burton in 1881-2 as an organic solvent of valeric acid and for many years used as an organic solvent for testing epileptic compounds. Its anticonvulsive properties were discovered by chance in 1963. Meunire and Carraz were investigating the anticonvulsive properties of khellin using valproic acid as the solvent (Lopez-Munoz *et al.*, 2012). They found that it was the solvent, valproic acid, and not the compound tested in the animal model (rodent) which had the anticonvulsive effect (Meunier *et al.*, 1963 in Lopez-Munoz *et al.*, 2012). Sodium valproate (VPA) was first prescribed as a treatment for epilepsy in 1967, France and U.S Food and Drug Administration (FDA) approved use in 1983 (Terbach *et al.*, 2011).

1.6.1 Mechanism of action

Sodium valproate (VPA) also known as valproic acid, 2-n-propylpentanoic acid, is a widely prescribed anticonvulsant for different types of epilepsy (Tomson *et al.*, 2016). It is also prescribed for other neurological disorders including bipolar disorders and migraines (Pourahmad *et al.*, 2012; Silva, 2008).

VPA is an eight carbon branch chain fatty acid, figure 1.6, with several metabolic pathways being involved in the biotransformation of the parent drug in the liver. VPA is converted into over 50 different metabolites by several pathways including β -oxidation and cytochrome P450 (CYP2A6, CYP2B6, CYP2C9), microvesicular ω -oxidation and glucuronidation. Many of the metabolites are highly toxic (Aries *et al.*, 2010; Kiang *et al.*, 2006; Luis *et al.*, 2007; Silva *et al.*, 2008). VPA is first metabolised by cytochrome P450 (CYP2C2) to 4-ene-VPA and then undergoes further metabolism by β -oxidation to form 2,4-diene-VPA. VPA can be directly metabolised to 2-ene-VPA by β -oxidation.

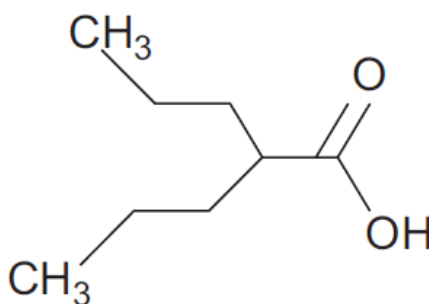


Figure 1.6. The chemical structure of VPA, a branch chain fatty acid.

The half-life of VPA is 11 to 20 hours and the drug is nearly completely metabolised with only trace amounts detected in patients' urine. The therapeutic range is between

300-700 μ mol/l (40 to 80 μ g/ml) however, this is difficult to establish due to differences in individual rates of drug metabolism (Callaghan *et al.*, 1985; Johannessen, 1980).

1.6.2 VPA treatment in disease

In its role as an anti-seizure agent, VPA alters the activity of the neurotransmitter gamma-aminobutyric acid (GABA) by increasing its synthesis and release and decreasing its turnover. This activity is specific to GABA α receptors in the substantia nigra region and therefore thought to be involved in the control of seizure generation (Bowden, 2009; Loscher, 1999; Owens and Nemeroff, 2003). In the last 30 years, VPA has not only been found effective in the treatment of epilepsy but also in the treatment of migraine. It was found that VPA can influence the balance of GABA and glutamate levels in the brain thereby modulating the biochemical occurrence of aura and/or affect the nociception via modulation of GABA and glutamate mediated neurotransmission in migraines with aura. Furthermore, it was also shown that VPA blocks neurogenic inflammation via GABA receptor mediated inhibition (Cutrer *et al.*, 1996). Human healthy controls treated with VPA, showed decreased cerebral glucose metabolism due to inhibition of the TCA cycle as VPA inhibits α -ketoglutarate, a rate limiting enzyme of the cycle. This has also been observed in mice (Gillard *et al.*, 1996; Johannessen *et al.*, 2002; Leiderman *et al.*, 1991).

A more recent study has found that VPA has histone deacetylase 1 (HDAC) inhibitory actions (Santoro *et al.*, 2013). This is significant because a steady level of core histone acetylation is dynamically regulated by a balance between histone acetyl transferases and HDACs. Acetylation relaxes the chromatin structure thus allowing access to transcription factors but deacetylation represses the access to transcription. In some cancerous cells it has been found that histone deacetylation is increased and prevents

the transcription of cell cycle inhibitors resulting aberrant cell proliferation and tumour formation. Because of VPA's inhibition of deacetylation this makes VPA a potential anti-cancer drug. VPA has shown to inhibit growth of prostate cancer xenografts decreasing cell proliferation in PCa cells (Sidana *et al.*, 2012). However, using VPA as an anti-tumour agent may have unwanted side effects. A recent publication by Santoro *et al* (2013) reported that HDAC inhibitors such as VPA can exert different effects on different tumour stages. In tumours arising from prostate cancer VPA appears to prevent tumour growth by inhibiting cell proliferation however, in transgenic pre-leukaemic mice VPA administration increased the onset of leukaemia compared to the control group. This suggests that HDAC inhibitors such as VPA could selectively block intrinsic anti-tumour functions of HDACs depending on the stage of tumorigenesis and the cancer cells targeted. Much of the current research into VPA's anti-cancer properties has been in mouse models and *in vitro* cell models. There have been several small clinical trials in patients with advanced stages of malignant solid tumours and in the treatment of myeloid leukaemia, reviewed by Duenas-Gonzalez *et al.*, (2008). These patients were subject to multiple other cancer treatment prior to VPA administration. It is therefore difficult to deduce the true effect of the VPA therapy on tumour progression and patient's survival time furthermore there were no control subjects to compare with. However, clinical samples from patients treated with VPA and hydralazine (vasodilator) for breast and cervical tumours were subjected to global gene expression micro array analysis to identify any upregulated genes. Interestingly, ribosome and OXPHOS genes emerged as the two pathways with the most up regulated genes. Ribosome proteins are essential in protein synthesis (Duenas-Gonzalez *et al.*, 2008). Interestingly, upregulation of OXPHOS genes could indicate a switch in metabolism from anaerobic glycolysis to OXPHOS. The true benefit of VPA as an anti-cancer drug needs to be investigated further.

1.6.3 VPA toxicity

VPA is associated with a range of adverse side effects and carries a 'black box' warning by the U.S FDA on VPA related products such as Depakote, Depakene and Stavzor (www.fda.gov/drug/drugSafety). These adverse side effects include alopecia, drowsiness, hepatotoxicity and tremors. Weight gain and insulin resistance has also been observed in children prescribed VPA. It has also been found to be a teratogen (Masuccio *et al.*, 2010; Phiel *et al.*, 2001). Furthermore, it has been discovered that children born to mothers who had taken VPA during pregnancy may have some developmental delay and behaviour problems (Deshmukh *et al.*, 2016; Tomson *et al.*, 2016; Veiby *et al.*, 2013). VPA and its metabolites such as 4-ene VPA has been shown to cross the placenta in rodent models and in humans, levels of whole drug and metabolites has been detected in cord blood of mothers who had taken VPA during pregnancy (Nau and Loscher, 1984; Kondo, *et al.*, 1987; Semczuk-Sikora, *et al.*, 2010).

Patients on multiple drug treatments combined with VPA are more prone to hepatotoxicity and non-alcoholic fatty liver disease (NAFLD) than those on VPA as a single therapy. NAFLD is the accumulation of triglycerides within hepatocytes that exceeds 5% of the liver weight (Kneeman *et al.*, 2012). VPA is potentially hepatotoxic even at therapeutic doses as a dose-dependent increase in liver enzymes and a decrease in fibrinogen can occur (Koenig, 2006; Silva *et al.*, 2008). This is reversible and a full recovery can be made upon cessation of the drug. In rare cases irreversible idiosyncratic fatal acute liver failure (ALF) can also happen. To prevent ALF from developing, early intravenous administration of L-carnitine has been used to increase chances of survival in these patients (Koenig, 2006; Lheureux *et al.*, 2009; Schmid *et al.*, 2013). Furthermore, VPA therapy has been found to cause weight gain and insulin resistance (Leuf *et al.*, 2009; Verrotti *et al.*, 2011). Although other AEDs such as carbamazepine and lamotrigine have been found to cause weight gain, VPA has by far

the highest incidents of weight gain and NAFLD (Leuf *et al* 2009). This may in part be related to inhibition of β -oxidation by VPA, causing an accumulation of triglycerides (Huo *et al* 2014; Zhang *et al* 2014). Furthermore, serum levels of palmitate (16:0) and linoleic acid (18:2) was found to be lower in patients treated with VPA who had abnormal liver function compared to treated patients with normal liver function. These findings are consistent with impaired lipid secretion, metabolism and abnormal liver function. Inhibition of lipid secretion can result in the build-up of triglycerides, leading to impaired liver function (Huo *et al.*, 2014). VPA sequestration with coenzyme A (CoA) causes valproyl –CoA to accumulate in the cytosol of hepatocytes on entering the mitochondria a disturbance of β -oxidation can occur. Toxic VPA metabolites can prevent fatty acid oxidation (FAO) by direct inhibition of β -oxidation or indirectly by depleting the intramitochondrial CoA pool (Begrache *et al.*, 2011; Pessayre *et al.*, 1999). As a branch chain fatty acid VPA can be oxidised to several products competing with other lipids for enzymes involved in the fatty acid oxidation pathway (Silva *et al.*, 2008). This can impact on lipid metabolism which in turn causes microvesicular steatosis and impaired liver function (Willebrords *et al.*, 2015). For patients with the mitochondrial disease Alpers-Huttenlocher (POLG), VPA administration can cause acute liver failure, leading to death (Silva *et al.*, 2008).

After 50 years of research, VPA treatment is beneficial in a range of diseases but its mode of action is still not fully understood. The development of a model to study VPA's effect on cellular functions is therefore needed.

1.7 Adverse drug reactions resulting in liver damage

Adverse drug reactions are the unwanted side effects of a drug beyond its anticipated therapeutic benefit. Adverse drug reactions (ADR) may occur following a single dose or after prolonged administration or as a result of the combination of two or more drugs. There are predisposing risk factors for ADRs to occur such as age, gender and genetic variation. Elderly patients are at higher risk of ADR as they often take multiple drug treatments to treat different pathologies and toxicity profiles were generally developed in healthy young animals and human controls. Thus increasing drug-drug interaction coupled with age related impaired drug metabolism may lead to ADR. Children and neonates are also at high risk of ADR due to immature renal and hepatic function (Scott and Thompson, 2014). The risk of ADR is greater for women than men and is likely due to pharmacokinetic difference in hepatic clearance and female hormonal influences (Scott and Thompson, 2014). Genetic factors can predispose patients to ADR and subsequent drug induced liver injury. The efficacy of drug biotransformation is subjected to genetic variation linked to variants of the cytochrome P450 (CYP) enzymes. One such example is the CYP2C9 variant CYP2C9*2 which has been found to cause decreased biotransformation of VPA to the metabolite 4-ene-VPA resulting in higher plasma concentrations of the parent drug. It is not yet clear if this has a beneficial or detrimental effect on the patient as 4-ene-VPA is understood to be involved in VPA induced hepatotoxicity (Kian *et al.*, 2006; Tan *et al.*, 2010).

1.8 Energy metabolism

During cellular energy production through glycolysis, digested food is broken down into sugars such as glucose from starch and glycogen, fructose from fruit and the hydrolysis of refined sugars, and galactose from the hydrolysis of lactose (Dashty, 2013). Glucose and galactose are imported into the cells and can be converted to glycolytic intermediates. In this project, the focus was on how glucose and galactose affect the cellular function when exposed to various chemical compounds. An *in vitro* model, where cells are cultivated in a glucose rich environment will derive ATP from glycolysis therefore potentially masking mitochondrial dysfunction. However, by replacing glucose with galactose as the carbohydrate source should drive the cells to generate ATP via mitochondrial dependant respiration thus, unmasking mitochondrial function (Gohil *et al.*, 2010; Marroquin *et al.*, 2007). Can underlying mitochondrial dysfunction caused by drug treatment such as VPA be elucidated by switching the cells fuel source?

1.8.1 Glycolysis

Glycolysis takes place in the cytosol when sugars enter the cell via glucose transporters (GLUT). The type of GLUT found varies between tissues, GLUT1 and GLUT3 are found in most mammalian tissue and involved in basal glucose uptake. GLUT2 is located in liver cells and pancreatic β cells where it removes excess glucose from the blood and has a role in insulin regulation. GLUT4 found in muscle and fat cells with GLUT5 expressed in the small intestine where it has a role as the primary fructose transporter (Mueckler and Thorens, 2013; Voet and Voet, 2004). GLUT1 is highly expressed in HepG2 cell line and is a major contributor of glucose uptake (Takanaga *et al.*, 2008). Galactose also enters the cell via glucose transporters, GLUT1 and GLUT2, however, the affinity is far weaker (Mueckler and Thorens, 2013; Scheepers *et al.*, 2004). Once galactose has entered the cell, it is converted to glucose-6-phosphate, figure 1.7 A.

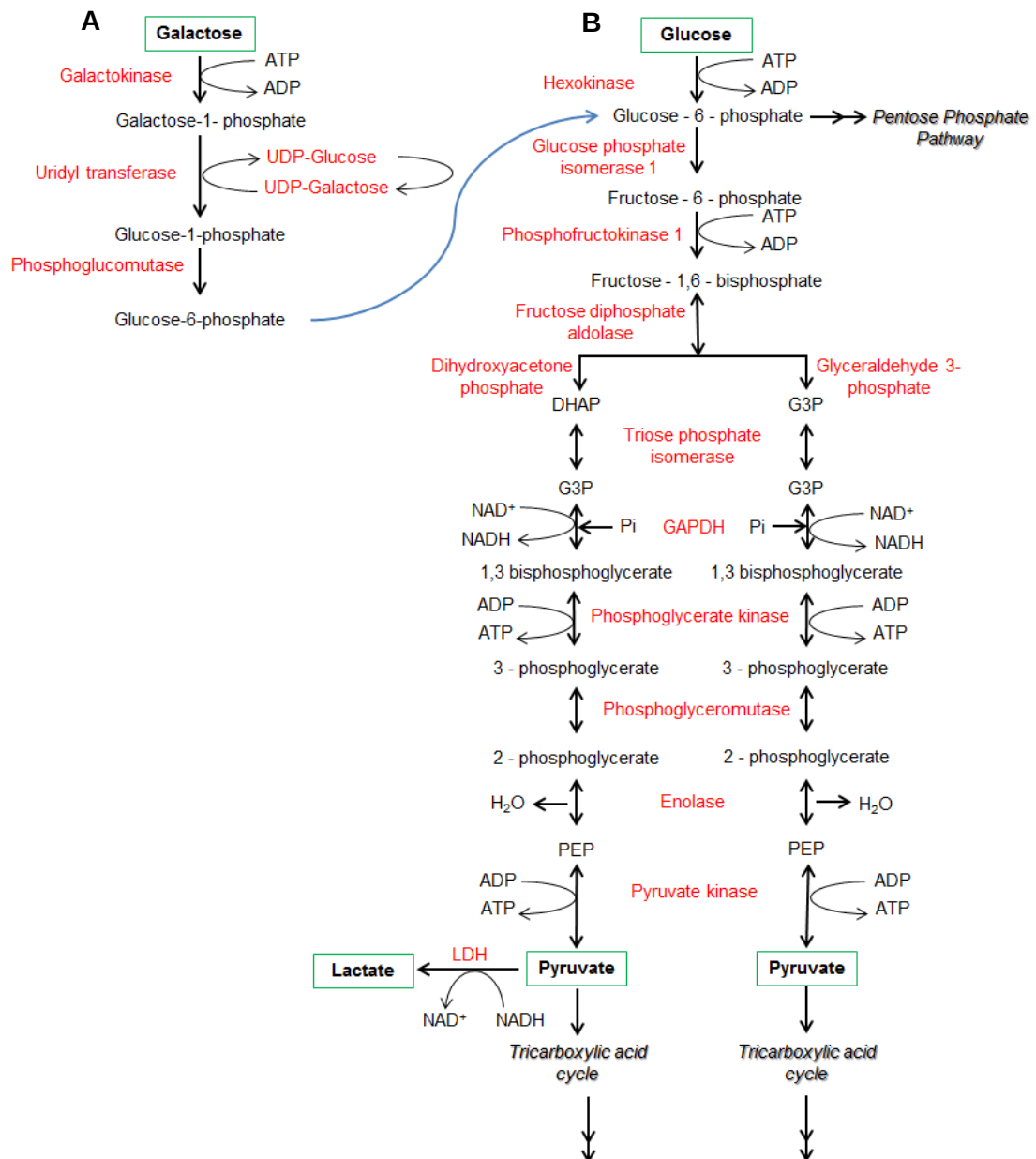


Figure 1.7. Galactose and glucose metabolism. The conversion of galactose to glucose-6-phosphate (A) and glycolysis (B). Breakdown of glucose-6-phosphate via the glycolytic pathway results in pyruvate. Pyruvate can either enter the tricarboxylic acid cycle to produce more ATP and intermediates for the electron transport chain or be converted to lactate by lactate dehydrogenase (LDH). ADP, adenosine diphosphate. ATP, adenosine triphosphate. NAD⁺, nicotinamide adenine dinucleotide. NADH, Nicotinamide adenine dinucleotide reduced. GAPDH, glyceraldehyde phosphate dehydrogenase. DHAP, dihydroxyacetone phosphate. G3P, glyceraldehyde-3-phosphate. Pi, inorganic phosphate. PEP, phosphoenolpyruvate. Adapted from Lunt and Vander Heiden, 2011.

In many cell types glucose is metabolised primarily via glycolysis to generate pyruvate and lactate. The key regulatory enzymes for the pathway are hexokinase, phosphofructokinase and pyruvate kinase. The pathway is adjusted in response to conditions inside and outside of the cell and does not require oxygen (Lunt and Vander Heiden, 2011). The conversion of pyruvate to lactate by lactate dehydrogenase recycles NAD^+ . Depending on the cellular metabolic status, pyruvate can be transported into the mitochondrion for use in the tricarboxylic acid cycle (TCA) and ATP production through the electron transport chain (ETC). Furthermore, glycolysis and the pentose phosphate pathway (PPP) are metabolically linked by the common intermediate glucose-6-phosphate. The PPP is important in the production of precursors for nucleotide biosynthesis by conversion of glucose-6-phosphate to ribulose 5-phosphate. It also generates NADPH (nicotinamide adenine dinucleotide phosphate) which is required for the biosynthesis of nucleotides and lipids (Lunt and Vander Heiden, 2011). The act of glycolysis itself only yields 2 mol ATP per 1mol glucose compared to 36mol ATP per 1mol glucose through oxidative phosphorylation (OXPHOS). Although, glycolysis is not an efficient way of producing ATP, the cellular reliance on glycolysis could be more to do with generation of anabolic precursors for other essential pathways than just energy production (Lunt and Vander Heiden, 2011). The end product of glycolysis is pyruvate, which can be converted to lactate via lactate dehydrogenase or acetyl-Coenzyme A (acetyl-CoA) in the mitochondria via pyruvate dehydrogenase complex (PDH) for entry into the TCA cycle. OXPHOS will be discussed later in this chapter.

1.8.2 The TCA cycle

The TCA cycle is a metabolic pathway located in the mitochondrial matrix, figure 1.8. This cyclical pathway consists of sequential steps, generating NADH and FADH₂ which in turn feeds electrons to the ETC. Furthermore, the TCA cycle produces biosynthetic precursors including citrate needed for lipid biosynthesis and α -ketoglutarate used in the generation of nonessential amino acids; asparagine, aspartate and proline (DeBerardinis *et al.*, 2008).

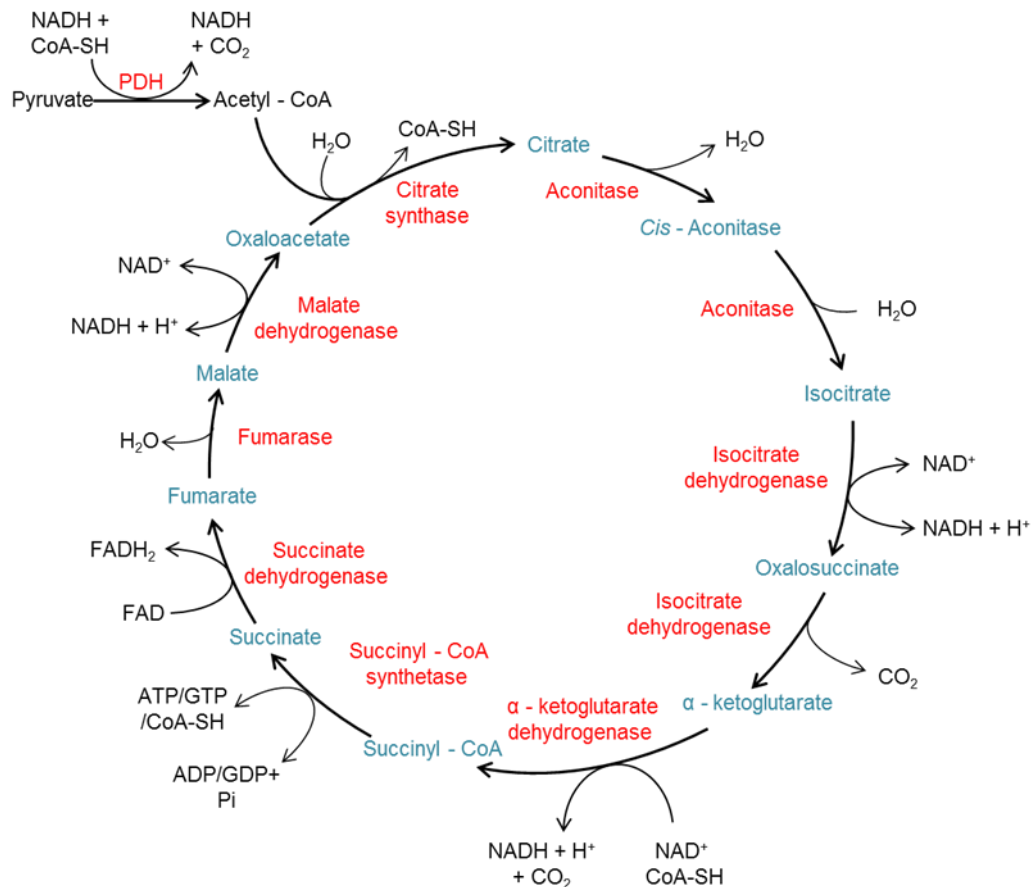


Figure 1.8. The Tricarboxylic Acid (TCA) Cycle. The enzymes required for the TCA cycle are located in the mitochondrial matrix. Forward cycling, solid arrows. Reverse cycling, dashed arrows. Adapted from Lunt and Vander Heiden, 2011.

1.9 Mitochondrial energy production – From glycolysis to OXPHOS

As discussed earlier, glycolysis is the initial pathway that converts glucose to pyruvate which takes place in the cytosol. Glucose metabolism through glycolysis produces a small amount of ATP and does not require oxygen. The pyruvate produced by glycolysis is transported to the mitochondria, for conversions to ATP via the TCA cycle, figure 1.8 and oxidative phosphorylation is carried out, figure 1.9.

1.9.1 Oxidative phosphorylation (OXPHOS)

Oxidative phosphorylation (OXPHOS) is carried out by means of electron flow between complex I, III and IV or from complex II, III and IV, in the inner mitochondrial membrane (IMM), figure 1.8. These complexes are multi-subunit enzymes and are major parts of the ETC. Protons are pumped through the inner mitochondrial membrane by complexes I, III and IV, creating a proton gradient. When protons move back into the mitochondrial matrix via complex V, ATP is synthesised from ADP and phosphate, figure 1.8. The other components of the ETC are ubiquinone, also known as coenzyme Q, between complex I or II and III, and cytochrome c between complex III and IV. Furthermore, oxidation of NADH to NAD^+ at complex I and FADH_2 to FAD at complex II, the resulting electrons are passed through the chain to the terminal electron acceptor oxygen at complex IV.

The complexes

Complex I (NADH dehydrogenase) is a large complex comprising of 46-48 subunits of which only seven are encoded by mtDNA. NADH which was reduced by the TCA cycle is oxidised by complex I and two electrons are donated and transferred through

complex I to the redox carrier ubiquinone, which then transfers the electrons to complex III. Transportation of electrons causes conformational changes to complex I allowing for four H^+ ions to pass through the IMM to the inter membrane space (Al-Ghoulem, 2011; Navarro and Boveris, 2007; Osellame *et al.*, 2012).

Complex II (succinate dehydrogenase) is composed of four subunits and is a component of the TCA cycle and an interregional part of OXPHOS. Complex II reduces flavin adenine dinucleotide (FAD) to flavin adenine dinucleotide reduced ($FADH_2$) and the donated electrons are transferred to ubiquinone (coenzyme Q, CoQ). Coenzyme Q diffuses freely through the IMM and acts as a redox carrier. Once coenzyme Q is reduced by either complex I or complex II it shuttles the electrons to complex III (Al-Ghoulem, 2011; Navarro and Boveris, 2007; Osellame *et al.*, 2012).

Complex III (ubiquinol cytochrome c oxidoreductase) is a complex of 11 subunits of which one is encoded by mtDNA, cytochrome b. It facilitates the electron transfer from reduced coenzyme Q to cytochrome c. The redox reaction enables complex III to transfer four H^+ ions to the inter membrane space. The reduced cytochrome c transfers the electrons to complex IV (Al-Ghoulem, 2011; Navarro and Boveris, 2007; Osellame *et al.*, 2012).

Complex IV consists of 13 subunits of which three are encoded by mtDNA. Complex IV accepts electrons upon oxidation of cytochrome c. The electrons are then passed to oxygen (O_2), which is reduced to water (H_2O) and four H^+ ions are transferred to the inter membrane space. Complex IV is the final redox complex in the ETC and consumes O_2 (Al-Ghoulem, 2011; Navarro and Boveris, 2007; Osellame *et al.*, 2012).

The activities of complexes I, III and IV create an H^+ ion gradient over the IMM where the mitochondrial matrix has a negative charge relative to the intermembrane space consequently creating an electric voltage, the mitochondrial membrane potential. The H^+ ion gradient provides the energy for the synthesis of ATP, the final step in

OXPHOS, by the influx of H^+ ion through ATP synthase. Complex V (ATP synthase) is the complex responsible for ATP production. It comprises of 17 subunits (mammalian cells) and is formed by two subdomains. Subdomain F_0 is embedded in the IMM and is a transmembrane channel for H^+ ions influx to the mitochondrial matrix. Subdomain F_1 is a membrane protein on the matrix side and forms an asymmetrical stalk linked to the stationary subdomain F_0 . The H^+ ion flow causes the F_1 subdomain to rotate which in turn rotates the stalk linked to the stationary F_0 subdomain. This results in the binding of ADP and phosphate to form ATP (Al-Ghoulem, 2011; Brand and Nicholls, 2011; Jastroch *et al.*, 2010; Navarro and Boveris, 2007; Osellame *et al.*, 2012)

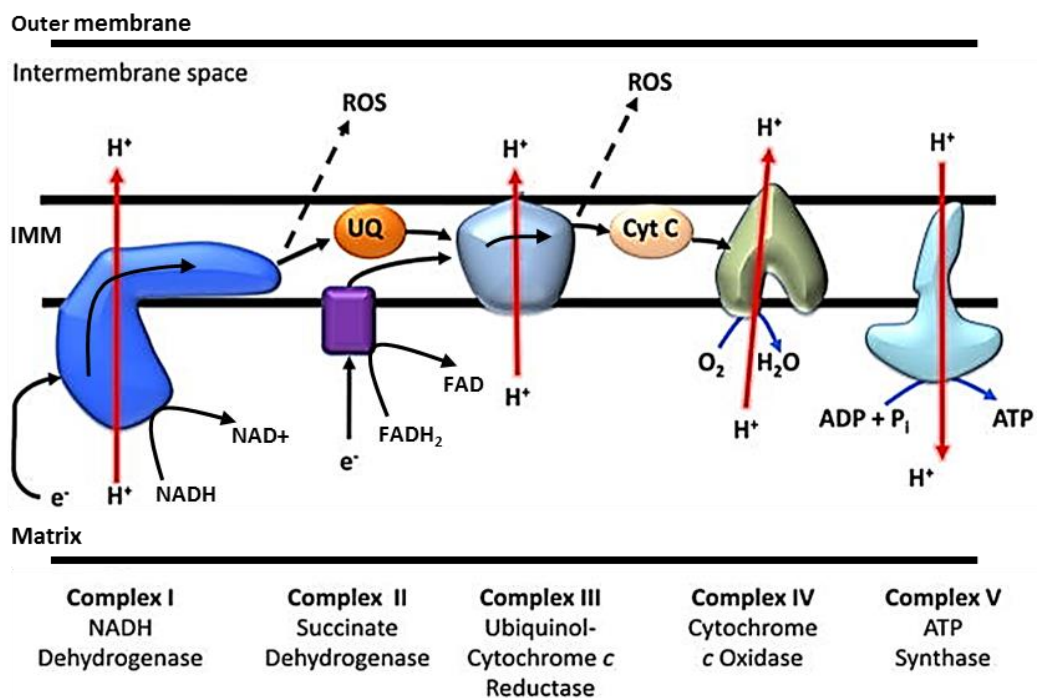


Figure 1.9. The electron transport chain. Electrons (e^-) flow in black arrows and hydrogen ions (H^+) red arrows. UQ, ubiquinone; Cyt C, cytochrome c; ROS, reactive oxygen species; IMM, inner mitochondrial membrane. (Al-Ghouleh, 2011).

The structural conformations of each complex are known, depicted in the cartoon figure 1.9, shows complex I as L shaped. The cartoon depicts a schematic organisation of complexes in the ETC and the movement of electrons and protons between the IMM and the mitochondrial matrix (Baradaran *et al.*, 2013). The arrangements of these complexes have been subject to debate and several models have been proposed. There are three models suggested; fluid model; where the complexes are spaced apart, solid state where the complexes are tightly associated and the plasticity model which is a mix of tightly packed complexes and spaced apart (Acin-Perez and Enriquez, 2014; Chaban, *et al.*, 2014). Furthermore, the fluidity of the mitochondrial membrane may also influence the efficiency and configuration of the complexes (Kuter *et al.*, 2016)

1.10 Mitochondrial network – the importance of a dynamic network during stress and survival.

Unlike early electron micrographs which showed the mitochondria to exist as single lozenge shaped organelles we now believe mitochondria to be dynamic networks which undergo fusion and fission (review by Shirihai *et al.*, 2015).

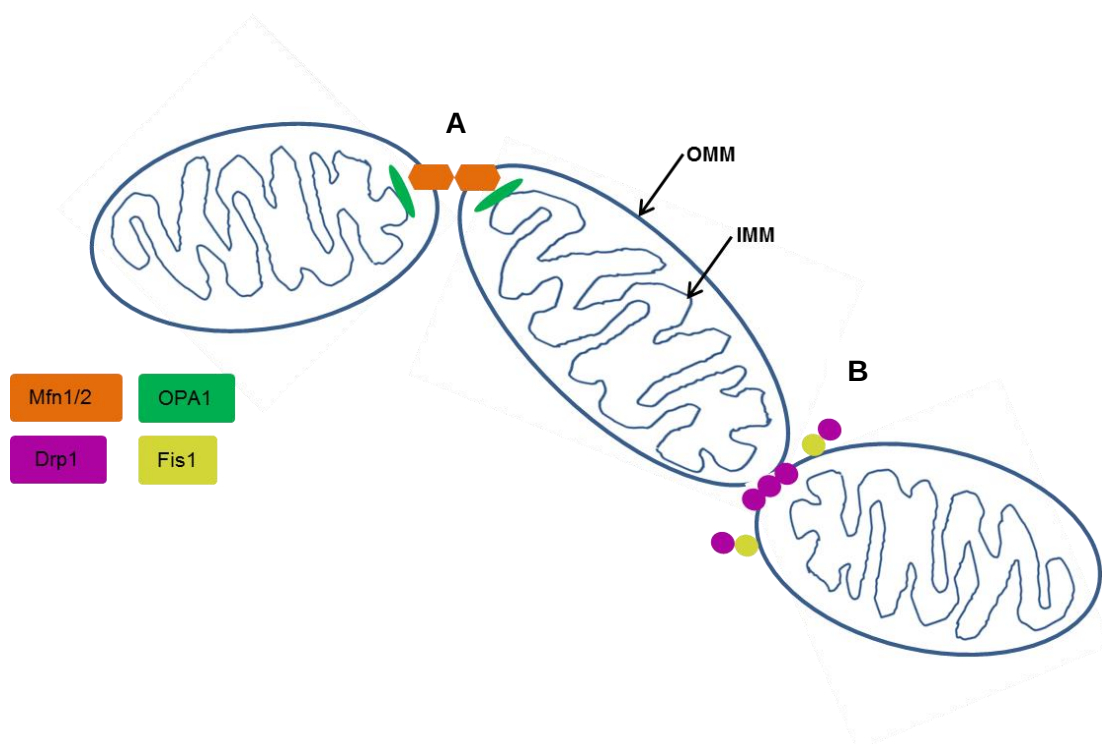


Figure 1.10. Mitochondrial fusion and fission in mammalian cells. (A) Fusion- mitofusins 1 and 2 are located on the OMM and facilitates the tethering and fusion of the OMM in conjunction with OPA1 which facilitates the fusion of the inner membrane.(B) Fission- Drp1 is recruited from the cytosol to Fis1

When two mitochondria come together the first step of the fusion process is the tethering of two OMMs via their carboxyl terminal domains of mitofusins, Mfn1 or 2. Once the OMM has fused OPA1 promotes the fusion of the IMM, figure 1.10. During fission events, the cytosolic protein Drp1 (dynamin-like protein1) binds to Fis1 and

drives the constriction and severs both the OMM and the IMM (Twig and Shirihai, 2011). These fusion and fission events are thought to be a protective response to cellular stress and occur under stressful condition such as nutrient starvation, the mitochondrial network becomes fragmented with larger individual mitochondria as fission is inhibited as a protective measure against excessive autophagosomal degradation of the organelle (Rambold *et al.*, 2011). An opposing response is increased fusion (Gomes *et al.*, 2011) here the networked can become hyperfused. Stress induced mitochondrial hyperfusion (SIMH), was observed by Tondera and colleagues (2009) when a mild toxin, actinomycin D was administered *in vitro* to MEFs. The mitochondrial network morphology can shift from fragmented to fused as a protective mechanism. It is also seemingly essential to cellular health as mutations in the fusion proteins can cause disease. In humans, point mutations in Mfn2 leads to the neurodegenerative disease, Charcot-Marie-Tooth type 2a. Mutations in OPA1 cause dominant optic atrophy (Alexander, 2000; Zuchner 2004 in Tondera *et al.*, 2009). Damaged fragments of mitochondria then becomes a target for the autophagy and/or mitophagy pathways

1.11 Autophagy and mitophagy

Mitochondrial fusion and fission events can occur as a response to intra and extracellular events including insults increasing oxidative stress or reducing mitochondrial membrane potential, figure 1.11.

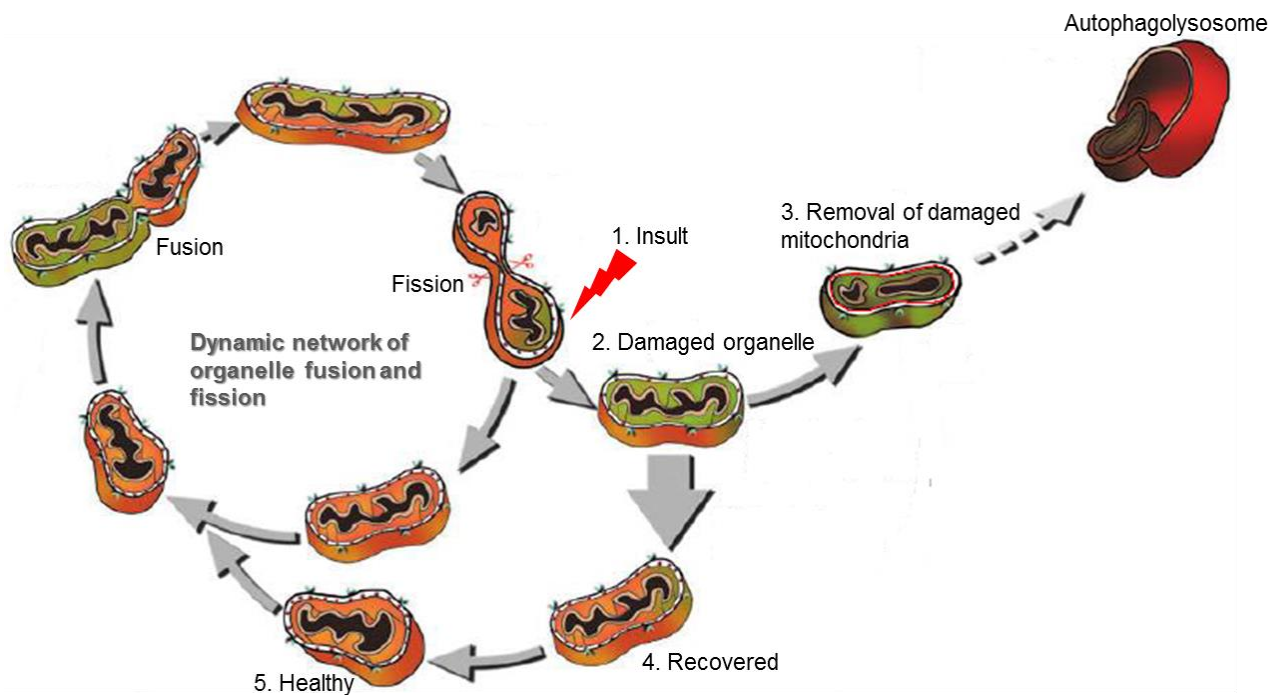


Figure 1.11. The dynamics of the mitochondrial network. The mitochondrion undergoes fusion and fission in response to intracellular and extracellular stimuli. When an insult to parts of the network occurs, the damaged part (2) undergoes fission and if unable to recover will be removed by way of mitophagy (3) or re-joins the network by fusion (4 and 5). Adapted from Twig and Shirihai, 2011.

The selective degradation of the mitochondria by a process known as mitophagy takes place when damage accumulates to the point where recovery of mitochondrial membrane potential is not possible (Kim *et al.*, 2007; Nerendra *et al.*, 2008). Reduced mitochondrial membrane potential triggers the recruitment of a cytosolic ubiquitin ligase Parkin by PINK1 (PTEN-induced putative kinase 1) to the mitochondrial membrane. PINK1 undergoes voltage dependant proteolysis in polarised mitochondria but when the membrane becomes depolarised PINK1 accumulates on the mitochondrial outer membrane where it phosphorylates Mfn2 thus promoting the recruitment of Parkin and ubiquitin. Furthermore, polyubiquitinated of outer mitochondrial membrane proteins binds p62 linked to autophagosomal LC3 thereby marking the mitochondria for mitophagy. PINK1/Parkin induced mitophagy is the

pathway that is the most studied with mutations in *Park2* gene being associated with early onset Parkinson's disease (Narendra *et al.*, 2008). The molecular mechanism leading to mitochondrial selective autophagy (mitophagy), occurs in sequential steps and the key events and components are; the tethering and fusion of OMM is mediated by Mfn1 and 2 (mitofusins) and fusion of the IMM OPA1 (optic atrophy1), fission is mediated by Drp1 (dynamin-related protein 1), figures 1.10 and 1.11. Mitochondrial membrane depolarisation occurs which interrupts normal proteolytic processing of PINK1 this causes accumulation of the protein on the outer mitochondrial membrane (OMM) and phosphorylates Mfn2, promoting recruitments of Parkin and ubiquitin (Youle and Narendra, 2011).

1.11.1 Autophagy and mitophagy, the detection of cellular quality control pathways

The mitochondrion forms dynamic networks that undergo fusion and fission, as described in the above section. The cellular mechanism for maintaining this network is mitochondrial directed autophagy, which was described in 1957 however the term mitophagy was coined by Lemasters in 2005. Mitophagy enables the removal and recycling of impaired and damaged mitochondrion thereby maintaining an optimal functional mitochondrial network preventing the release of pro-apoptotic proteins. Mitophagy is not just for mitochondrial quality control but also for the complete removal of all mitochondria from the cell as in the case of mature erythrocytes which lack mitochondria. During the maturation of reticulocytes, mitochondria are completely removed by the time mature erythrocytes emerge from the development pathway (Mortensen *et al.*, 2009). There are multiple proteins involved in the targeting and labelling of spent organelles for the removal by autophagy or mitophagy and some of

these are used as markers in the study of mitophagy. However, detecting and interpretation autophagy is fraught with difficulties and inconsistencies, a comprehensive review by Klionsky *et al.*, was published in 2012.

The microtubule-associated protein light chain 3 better known as LC3 is widely used to monitor autophagy. There are several variants present in mammals, LC3A - C, where LC3B has a known role and is commonly used for assessing autophagy. Recently, it was found that LC3A is also involved in mitochondrial uncoupled (CCCP) induced autophagy but it is unclear what role LC3C has in autophagy (Bai *et al.*, 2012). Here and in subsequent chapters LC3B will be referred to as LC3.

LC3 is present on isolation membranes where it participates in the closure of the autophagosome (Fujita *et al.*, 2008). LC3 is recycled and is modified by cleavage of the carboxyl terminal region by Atg4 to form the cytosolic form LC3I. LC3I is converted to a membrane bound form by Atg7 and conjugated with phosphatidylethanolamine, forming LC3II (Tanida *et al.*, 2005). Experimentally autophagy is monitored by detecting the conversion of LC3I (cytosolic) to LC3II (membrane-bound), using Western immunoblotting method. As LC3I has a molecular mass of between 16-18kD and LC3II 14-16kD, the two forms can be distinguished using SDS page electrophoresis. The LC3II band indicates the level of autophagosomes present. However, the accuracy of this method has been criticised over the years as not being robust due to LC3II itself is degraded by autophagosomes (Klionsky *et al.*, 2012; Mizushima *et al.*, 2010). Furthermore, an increase in LC3II signal can be interpreted several ways, an upregulation of autophagosome formation or a block in autophagosome degradation pathway causing an accumulation of autophagosomes. It is not possible to distinguish between the two different events using Western immunoblotting as both upregulation of autophagosome formation and blockage of autophagosome degradation would results in an increase of the LC3II signal (Mizushima *et al.*, 2010). This in turn means that it would be difficult to establish a

pharmacological compound's effect on autophagy as inducer or inhibitor due the relationship between LC3I and LC3II is not easy to decipher using Western immunoblot. Not only are the basal levels of LC3I and LC3II cell specific but can also change during passages in cell culture (Mizushima *et al.*, 2010). Additionally, the conversion of LC3I to LC3II is also linked to the cell metabolism; both starvation and mitochondria uncoupling by CCCP are known to increase autophagy/mitophagy, with increased LC3II signal detected by immunoblotting. However, this may differ between cell lines (Mizushima *et al.*, 2010). As in SH-SY5Y cells only a slight increase in LC3II and a reduction in LC3I was detected after starvation but a stronger increase in LC3II was reported after CCCP treatment (Klionsky *et al.*, 2010).

1.11.2 Autophagy and mitophagy modulators

The dynamics of autophagy/ mitophagy has been previously described section 1.11. In summary, membrane structures referred to as isolation membranes or phagophores form near the target i.e. damaged mitochondria. The target is then fully engulfed in the membrane structures forming the auto/mitophagosome. Newly formed auto/mitophagosome then fuse with an endosome or pre-lysosome and become a mature auto/mitophagosome which will degrade the cargo (damaged mitochondria) via proteolysis. The steps in the pathway can be either inhibited or induced by drug treatments, figure 1.12, these compounds will be discussed below. Drug treatments affecting the autophagy/mitophagy pathway are of fundamental importance in studying mitophagy/autophagy and will be discussed in detail in later sections of this thesis.

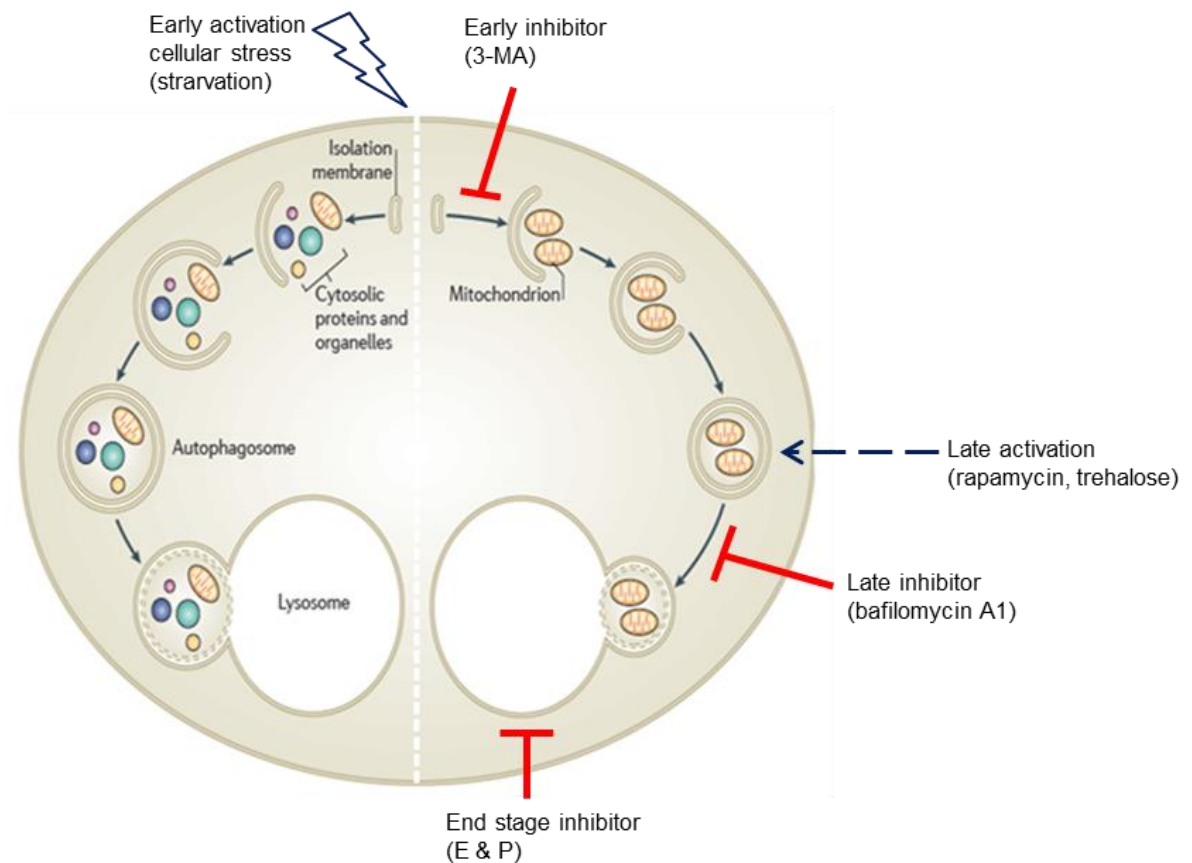


Figure 1.12. Pharmacological targets of the autophagy pathway. Early activation of autophagy can be induced by cellular stress such as starvation. A later activation can be achieved by treating with rapamycin or trehalose. 3-MA (3-methyladenine) is deemed early inhibitor of the pathway as it prevents the formation of the phagosome. E64D & pepstatin A (E&P) are end stage inhibitors as they prevent the degradation of the autophagolysosome (Adapted from Youle and Nerendra, 2011).

Bafilomycin A1

Treatment with bafilomycin A1 (bafA1) inhibits vacuolar-type H(+) ATPases that are found in lysosomes and endosomes. BafA1 is a specific inhibitor of these ATPases. Furthermore, BafA1 has been found to prevent the fusion of autophagosomes and lysosomes in rat hepatoma cell line (Yamamoto *et al.*, 1998).

Carbonyl cyanide m-chlorophenyl hydrazine (CCCP)

Treatment with CCCP uncouples OXPHOS and causes reduced mitochondrial membrane potential, disrupting the mitochondrial network. CCCP is the classic and a commonly used agent to induce mitophagy via the PINK1/Parkin pathway. Following depolarisation, PINK1 on the outer mitochondrial membrane recruits PARK2 which in turn will ubiquitinate several proteins and this will mark the mitochondria for degradation (Narendra *et al.*, 2008). Additionally, high concentrations of CCCP (10 μ M) have been found to change intra cellular pH resulting in increased auto/mitophagy (Berezhnov *et al.*, 2016)

Colchicine

At low concentrations, colchicine inhibits the formation of microtubules however at higher concentrations promotes microtubule depolymerisation (Ren *et al.*, 2014, Larocque *et al.*, 2014).

E64D and Pepstatin A

E64D is a cysteine protease inhibitor that blocks the activity of lysosomal hydrolases and is combined with pepstatin which is an aspartyl protease inhibitor. These compounds inhibit the degradation of mitochondrial elements within the lysosomal lumen (Sato *et al.*, 2007).

Rapamycin

Rapamycin inhibits mTOR and thereby induce autophagy. mTOR is ubiquitously expressed and regulates many cellular processes such as growth, proliferation and protein synthesis (Tanemura *et al.*, 2012; Dai, *et al.*, 2014).

Trehalose

Trehalose induces autophagy which is independent of the mTOR pathway (Sarkar, *et al.* 2006). Furthermore, it was found *in vitro* to improve the clearance of mutant huntingtin and α -synuclein aggregates. Both these proteins are disease causing elements in Huntington and Parkinson, respectively.

3-methyladenine (3-MA)

3-MA blocks the early stage of autophagy by inhibiting class III PI3K (phosphatidylinositol -3 kinase signalling pathway, which will prevent the formation of the phagosome. However, it can also inhibit class I PI3K which are shown to activate the formation of phagosome thus in long term experiments promote autophagy. The PI3K pathway is involved in many biological processes such as cell cycle progression, cell survival, including the formation of the phagosome (Castino *et al.*, 2010; Hou *et al.*, 2012).

1,10 Phenanthroline

Phenanthroline has been found to induce mitochondrial fragmentation and dysfunction by membrane depolarisation and network disturbances by inhibiting the Drp1 thereby blocking mitochondrial fission (Park *et al.*, 2012). Drp1 being a GTPase protein that controls mitochondrial fission.

Sodium valproate (VPA)

There is currently very little literature investigating the impact of VPA on autophagy/mitophagy in primary fibroblasts. However, an early study using rat neurons, has shown VPA (1mM and 3mM duration not specified) to induce autophagy

via an unknown mechanism that is independent of PINK/parkin pathway or mTOR (Sarkar *et al.*, 2005; Williams *et al.*, 2002). A more recent publication shows VPA treatment (3mM for 24 hours) to increase BNIP3 in Raji cells. BNIP3 is an inducer of autophagy (Cavaliere *et al.*, 2014).

1.12 Model development- What makes a good model in medical research and why use them?

The objective of any model is to study the nature of disease, progression and the effect of current and novel treatments. There are three basic choices for a study, it can either be *in vivo* or *in vitro* or a mix of both. The use of animals is highly regulated and very expensive. The Home Office 2013 report and statistics for the period 1945 to 2013 show a decline in use of all animals. In 1945, under the 1876 act there was just over a million experiments carried out, peaking in the early 1970s with over 5.5 million. Since then there has been a gradual decline in animal used for experiments and procedures under the 1986 act. This is reflecting tighter regulations, and the promotion of the 3Rs (Reduction, Replacement and Refinement) in the development of cell based *in vitro* models. According to the Home Office 2013 report, mice are far the most popular choice as an animal model in the UK with just over 3 million used in the period 2012/2013. Rodents e.g. mice, rats, guinea pigs and hamsters accounts for 82% of procedures and fish 12%. The other 6% include birds at 3%, other mammals including dogs, horses and primates at 2%, reptiles, amphibians and specially protected species accounts for 1% (Great Britain Home Office, 2013). The fruit fly *Drosophila melanogaster*, nematode *Caenorhabditis elegans*, yeast *Saccharomyces cerevisiae* are other model organisms used in biomedical research. As these are not classed as animal models, they are relatively cheaper and have far less regulation for laboratory to use.

1.12.1 Animal models

Animal models of cancer are routinely used for the investigation of novel cancer therapies. These models are commonly developed in mice such as immunosuppressed animals or xenograft models. Animal models have also been developed for the study of mitochondrial disease by engineering mutations in nuclear encoded proteins. These mouse models include point mutation in complex I subunit *NDUFS4* (Ingram *et al.*, 2009), point mutation in the mitochondrial helicase *twinkle* (Tynismaa *et al.*, 2005) and point mutation in the polymerase gamma (*PolG* mutator mouse)(Trifunovic *et al.*, 2004). Mutations in the human gene *NDUFS4* leads to Leigh syndrome and *PolG* to AHS (Chinnery, 2010).

1.12.2 Cellular models for biomedical research

The cell model used should be a reflection of the tissue or organ for example liver disease one could use primary hepatocytes or an immortalised cell line which is commercially available. Although transformed, many cancer lines retain some of the characteristic of the cell/tissue type they originated from. The advantages to working with cell lines are that they are often well characterised. Primary cells are more difficult to obtain and handle than most cell lines with many not able to proliferate in culture or requiring expensive media.

1.12.3 Primary cell models

Fibroblasts derived from skin biopsies from patients and healthy controls have been used as a disease model for mitochondrial disorders, PD and AD (Mortiboys *et al.*, 2008; Pensalfini *et al.*, 2011; Sitarz *et al.*, 2014). These cells proliferate and are easy to cultivate. Furthermore, as fibroblasts are large and flat, they are ideal for mitochondrial morphological studies by microscopy.

Primary hepatocytes have the advantage that they have the ability to express the relevant cytochrome P450 necessary for drug biotransformation however there are genetic differences between populations, as discussed previously, section 1.5 .

Primary hepatocytes do not proliferate *in vitro* and therefore need to be freshly isolated for each experiments furthermore it is difficult to obtain human hepatocytes and rats are therefore a common source (Shulman and Nahmias, 2013). In addition, human hepatocytes are rarely isolated from healthy liver tissue but from tissue collected following surgical procedures for e.g. tumour removal, often from patients who has undergone chemo and or radio therapy.

Human umbilical vein endothelial cells (HUVECS) are used in *in vitro* investigation of angiogenesis in cancer and atherosclerosis in cardiovascular disease (Lai *et al.*, 2015; Zhang *et al.*, 2015).

1.12.4 Human immortalised cell models

There are numerous immortalised human cell models, such as A549, HeLa and HepG2 which are available commercially. The A549 cell line was derived from a human alveolar cell carcinoma from 58 year old Caucasian male. This cell line was generated by Lieber *et al* and first published in 1976 and is now widely used in cancer studies. The HeLa cell line was the first commonly available immortalised human cell line. The cells were derived from Henrietta Lack's cervical biopsy and first published in 1952 by Grey and colleagues who sent cells to laboratories all over the world. It soon became the workhorse of *in vitro* research. These cells have been instrumental in the research that underpins our current understanding of cancer biology and have featured in thousands of research publications. The human derived hepatoma-derived cell line HepG2 was first published by Knowles and colleagues in 1980 and it too is widely used. The cell lines discussed above are available for purchase from American Type Culture Collection (ATCC). The ATCC maintains nearly 4,000 different cell cultures and distributes internationally (www.lgcstandards-atcc.org). Many of these cell lines have been cultured for hundreds of generations and this may have altered their original genetic and metabolic behaviour. Recently it was reported in Nature (Ledford, 2016) that the U.S National Cancer Institute (NCI) is discontinuing using the NCI-60 cell line panel as a tool for cancer drug screening. The NCI-60 is a panel of 60 human cancer representing nine different types of cancers; brain, breast, colon, kidney, leukaemia, lung, melanoma, ovary and prostate (www.cancer.gov). These cell lines have been instrumental in developing our understanding tumour biology and drug activity. After nearly 30 years of culturing, these cells have changed their metabolic and genetic properties and therefore do not necessarily represent the tissue the cells originated from. Our understanding of cancer biology and drug actions has moved on, therefore, more sophisticated *in vitro* models of diseases including cancer are needed (Ledford,

2016). The NCI phasing out the use of the NCI-60 cell model in favour of patient-derived xenografts (PDX). A section of the patient's tumour is transplanted into immunodeficient mice, either under the skin or into the organ from which the tumour originated (Richmond and Su, 2008). The PDX will allow tumour and related drug research to be carried in an environment which closer mimic to the human condition. It is estimated that the NCI will have 75 PDX cancer models ready when the new repository opens in 2016. The aim of this ambitious project is to have 1,000 PDX s including genome sequence, gene expression pattern and the donor's treatment history available to other research institutions (www.cancer.gov). There is currently a European effort to create a similar repository, EurOPDX, encompassing 16 different research institutions from across Europe (www.Europdx.eu).

1.12.4.1 Immortalised cell models of disease derived from rodents

The use of cell lines derived from animals as a model for human disease is a valuable tool and an important part of scientific discovery. There are currently 68 cell lines of rat and 138 of mouse origins covering a wide range of disease including hepatoma, diabetes and glioblastoma available from ATCC (www.lgcstandards-atcc.org).

INS-1 is an insulinoma cell line derived from rat. These cells display many important characteristics of the pancreatic beta cell including high insulin content, synthesis of proinsulin I and II. They respond to the physiological range of glucose. The INS-1 cell line was first published by Asfari *et al* in 1992. A drawback of using this cell line is that it requires the addition of β -mercaptoethanol to its growth medium to maintain insulin secretion. The Neuro2 a (N2a) neuroblastoma cell line has provided valuable insight in to the mechanism of ischaemic injury (Chi *et al.*, 2014). This cell line was derived from a spontaneous tumour in an albino strain A mouse and is commercially available. It was first published in 1969 by Klebe and Ruddel. This cell line has been used to develop a model for ischaemic stroke by oxygen and glucose deprivation followed by reperfusion in order to investigate the effect on autophagy in the development of post-stroke dementia (Zhang *et al.*, 2014). The HL-1 is a cardiac muscle cell line which was derived from the mouse atrial cardiomyocyte tumour lineage in a C57BL/6J mouse. This cell line was published by Claycombe and colleagues in 1998. According to their publications these cells can be serial passaged and still retain the ability to contract. Furthermore, after repeated passaging HL-1 cells retains its cardiac morphology, biochemical and electrophysical properties (Claycomb *et al.*, 1998). This cell line has been used in a wide range of studies such as myocardial energy metabolism and mitochondrial dysfunction caused by the anticancer drug doxorubicin (Strigun *et al*, 2011). The HL-1 cell line has also been found useful in the study of remodelling of the mitochondrial morphology in ischaemic and reperfusion injury (Ong *et al.*, 2012).

Existing animal derived cell models do not reproduce all aspects of human disease however, they have provided significant insight into disease mechanism and drug treatments. It is acknowledge that the use of improved animal and cellular models of diseases including cancer and neurodegenerative will lead to a more complete understanding of the biological foundation of disease mechanism thus enabling improved drug treatments.

1.13 Aims of this thesis

1. To investigate the effect of sodium valproate on mitochondrial function in HepG2 cell line.
2. To develop high throughput methods enabling quantifying mitophagy using known modulators of the mitochondrial network.
3. To investigate the effect of sodium valproate on mitochondrial function in primary fibroblasts from patients harbouring *PolG* mutations and non-affected controls.
4. To investigate the effect of sodium valproate *in vitro* using a mouse model with a *PolG* mutation.

Chapter 2

Materials and Methods

2 General Material and Methods

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich, UK.

2.1 General cell culture

All cell cultures were maintained at 37°C in 5%CO₂. The medium was changed every 2-3 days and cells passed when they reached 80-90% confluency. All cell lines were routinely tested for mycoplasma infection using MycoAlert™ PLUS Mycoplasma Detection Kit (Lonza, LT07-418) and used according to manufacturer's protocol.

2.1.1 General passaging and harvesting of cells

All cell lines were subjected to the same protocol when passaged or harvested for experimental procedures, as follows: spend media was aspirated and the cells were washed once with PBS, aspirated. The cells were washed once with 2ml trypsin, aspirated and 1.5ml fresh trypsin was added and the cells were incubated for 3-4 minutes at 37°C in 5%CO₂. The treatment was stopped by the addition of 5ml medium. For passaging, the cells were split 1:5. For harvesting, the cell suspension was collected and placed in a Falcon tube (Sarsted) and centrifuged at 1,300xg for 3 minutes at RT. The supernatant was aspirated and the cell pellet re-suspended in fresh media. Cells were counted, using a haemocytometer and seeded into tissue culture plates.

Primary human fibroblasts

For experiments cells were harvested at 80% confluency and cultured in Dulbecco's Modified Eagles's Medium 4.5 g/L glucose supplemented with 10% foetal bovine serum (FBS), 4mM L-glutamine, 25mg/ml uridine, 100 U/ml penicillin and streptomycin.

HepG2 cell line

For experiments cells were harvested at 60-80% confluency and cultured in 1x Minimum Essential Medium Eagle Medium with 5mM glucose supplemented with 10% FBS , 2mM L-glutamine, 5% MEM non-essential amino acids, 100 U/ml penicillin and 100mg/ml streptomycin.

Mouse Embryonic Fibroblasts

For experiments cells were harvested at 80% confluency and cultured in Dulbecco's Modified Eagle's Medium with 4,5g/L glucose powder reconstituted with distilled H₂O, additionally 1.1g sodium bicarbonate, 10% FBS, 25mg/ml uridine, 0.5mM pyruvate, 2mM L-glutamine 100 U/ml penicillin and 100mg/ml streptomycin. The medium was filter-sterilised using a Corning filtering system.

2.1.2 Galactose media preparation

10mM Galactose media; Dulbecco's Modified Eagle medium with no glucose was supplemented with 10mM galactose, 10% FBS, 0.5 mM sodium pyruvate, 100U/ml penicillin and 100ug/ml streptomycin. For fibroblast and MEFs cultures, 25mg/ml uridine was added.

2.2 General sodium valproate treatment regime for all experiments

A sodium valproate (VPA) working stock of 100mM was made by dissolving the compound in molecular grade water (Merck Millipore) and filter sterilised (Sarsted). The working stock was aliquoted and stored at -20°C, defrosted and used once only. During the experimental phase the media containing the VPA was changed every day. The addition of VPA to media was prepared fresh every time. The treatment time points were 24 hour, 48 hours and 72 hours.

2.3 Preparation of 96 well plates

The majority of experimental assays used in this thesis were carried out in 96 well plates. The table below lists the assays and plate type used, table 2.1.

Table 2.1. 96 wells plates used for assay featured in this thesis.

Plate type	Assay
Black walled with a clear bottom (BD Falcon)	Oxygen consumption
White walled with clear bottom (BD Falcon)	ATP assay
Clear plates (Nunc/Thermo Fisher)	Alamar Blue, IN Cell imaging, Hoechst, MTT

HepG2

HepG2 cells were used at 50-60% confluence and harvested as described in section 2.1.1. The supernatant was aspirated and cell pellet resuspended in fresh medium and then the cells were passed through a 100µm cell strainer (Fisher) to prevent clumping. The cells were counted using a haemocytometer and 200µl dispensed at appropriate concentration into 96 well plates, using a multichannel pipette. The cell densities used were 25,000 cells/well for 72 hour treatment, 17,500 cells/well 48 hour treatment and 15,000 cells/well for 24 hour treatment. The cells were left overnight for the cells to adhere before any treatments were carried out. Fresh media (glucose or galactose) containing appropriate concentration of VPA was added every 24 hours, as described in section 2.2. After the treatment period the media was aspirated and cells washed with warm PBS before analysis.

Fibroblasts

Patient and control skin derived fibroblasts were used at 80% confluency and harvested as described in section 2.1.1. The 96 well plates were seeded at various densities depending on rate of proliferation of the cell line. The cell densities used were between 20,000-30,000 cells/well with the aim for a final density of 50,000 for the no treatment control group after the treatment period.

2.4 Antibodies used in experiment featured in the thesis

Table 2.2. Antibody concentrations and suppliers.

Antibody	Concentration	Supplier
Rabbit monoclonal anti-LC3 (primary)	1:500	MBL International
Mouse monoclonal anti-TOM20 (primary)	1:200	SantaCruz
Goat anti-rabbit AlexaFluor488 (secondary)	1:500	Life Technologies
Goat anti-mouse AlexaFluor546 (secondary)	1:200	Life Technologies

2.5 General Assays

Flow cytometry- BODIPY 493/503

Flow cytometry was used to assess lipid accumulation in the cells after VPA treatment. The flow cytometry measures the properties of fluorescently stained cells whilst in a fluid stream.

Cells were seeded in T175 tissue culture flasks and cultured to no more than 80% confluence before VPA treatment was added. After treatment cells were harvested as described in section 2.1.1, the cells were counted and 1×10^6 cells was placed in clean falcon tubes, one tube for stain and one for unstained, centrifuged and resuspended in media contain BODIPY 493/503 (Life Technologies D-3922) or no stain control.

BODIPY dissolved in ethanol to give a stock of 1mg/ml in aliquots and stored wrapped in foil at -20°C , used at final concentration of $10\mu\text{g/ml}$. Cells were incubated in the dark at 37°C for 15 minutes. After incubation, cells were then pelleted by centrifugation and supernatant aspirated. This was followed by 2x washing with 500 μl FACS buffer (PBS+0.1% FBS), centrifugation at 1,300xg/3 minutes/RT and aspiration. After final wash and aspiration the pellet was resuspended in 500 μl FACS buffer and transferred to 5ml BD polystyrene round bottomed tube for analysis using a BD LSRII Flow Cytometer (BD Biosciences, Oxford, UK) equipped with a blue (488nm) and red (633nm) lasers. 20,000 events were collected for each sample. The data collected was analysed using FACSDiva software (BD Biosciences, Oxford, UK) and plotted as scatter plot of side scatter (SSC) vs forward scatter (FSC), figure 2.1. Where SSC is an estimation of intracellular granularity and FCS is an estimation of cell size. On these histogram plots, cells appear according to fluorescent intensity and these can be separated by a 'gate'. The term gate is a numerical or graphical boundary that is used to define the characteristics of cells for further analysis. The gate ensures that only

events of interest will be acquired. Once an appropriate gate had been established, 20,000 events were collected per sample. Figure 2.2 illustrates how the gates are set, panel A show the peak of unstained cells and panel B cells that that were stained with BODIPY.

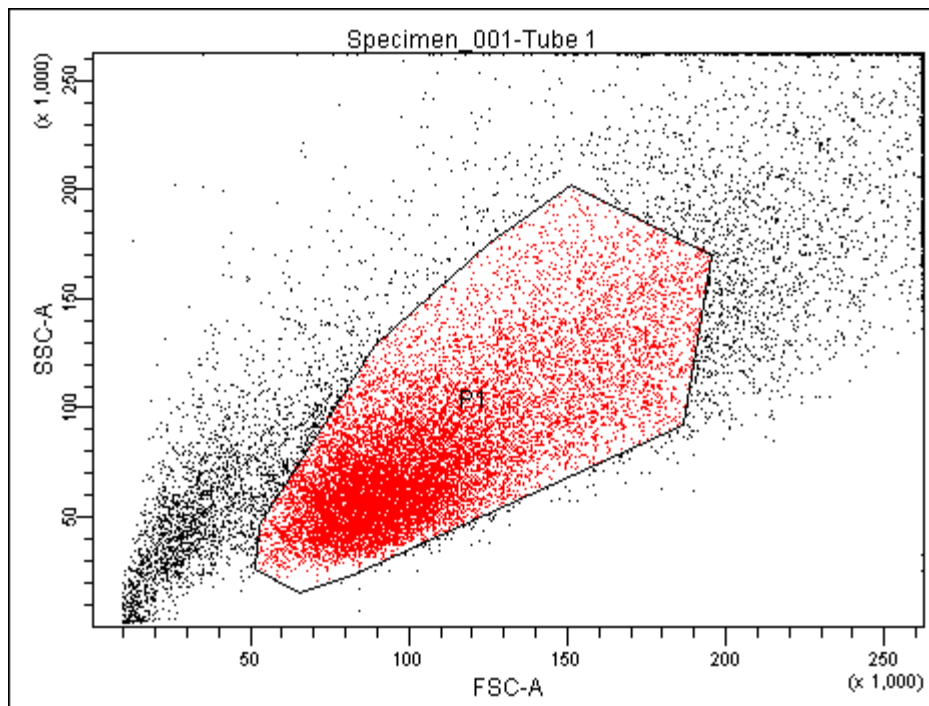


Figure 2.1. Representative flow cytometry scatter plot. The red population demarks the cell population of interest. X-axis side scatter (SSC). Y-axis forward scatter (FSC).

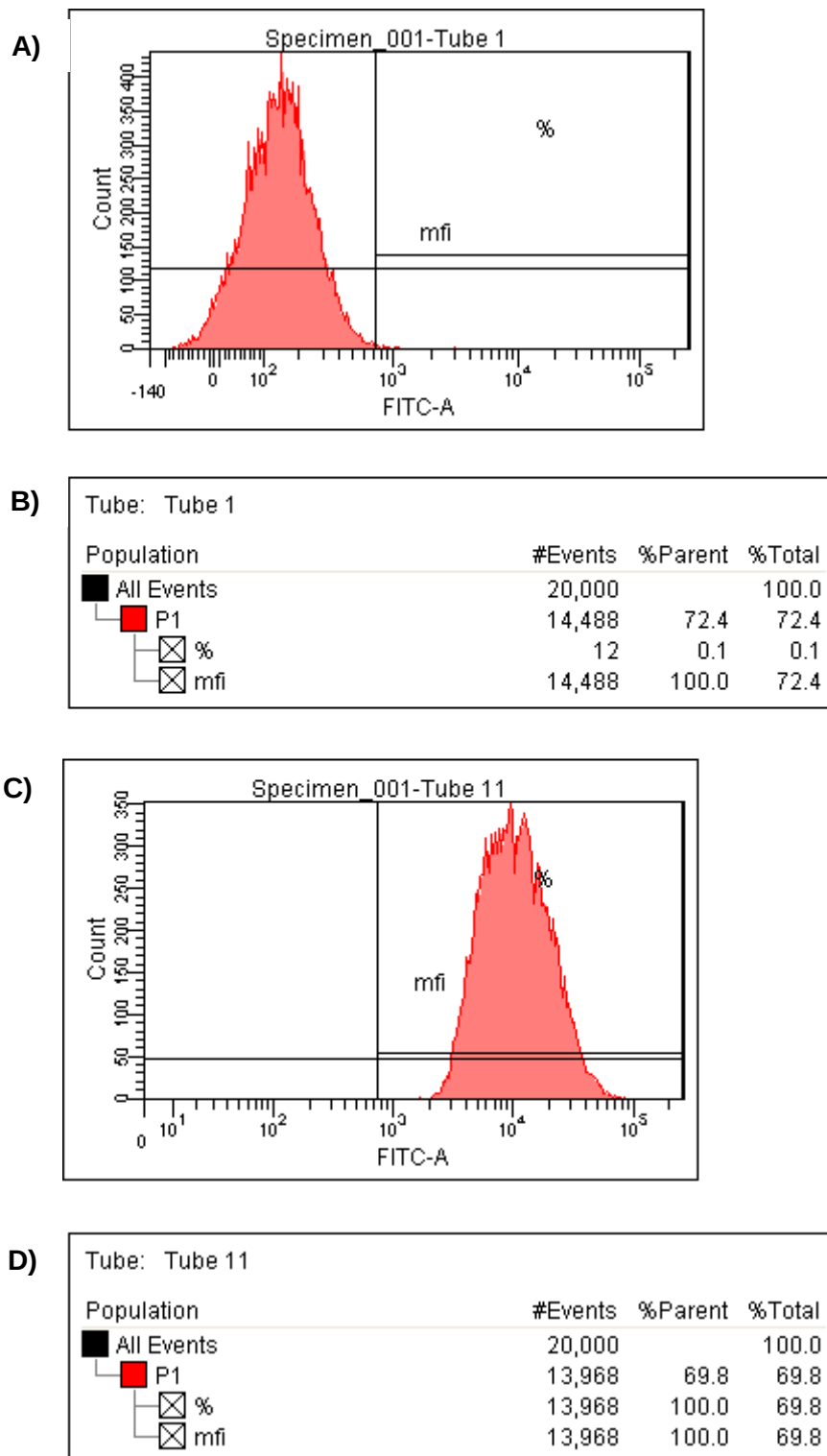


Figure 2.2. Flow cytometry, setting the gates for BODIPY. The gate is set immediately to the right of the unstained cell population, (A). The gate is marked by a vertical line. BODIPY stained cell population (C). B and D provide the number of events in the gated population as depicted in the histograms, A and C.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay

The MTT assay is a widely used method for establishing cell viability and cytotoxicity after drug treatment and was first published by Mosmann in 1983. The assay principal is colorimetric and measures the reduction of yellow tetrazolium salt (referred to as MTT) by metabolically active cells to insoluble formazan precipitate which is dark purple and can be quantified, figure 2.3.

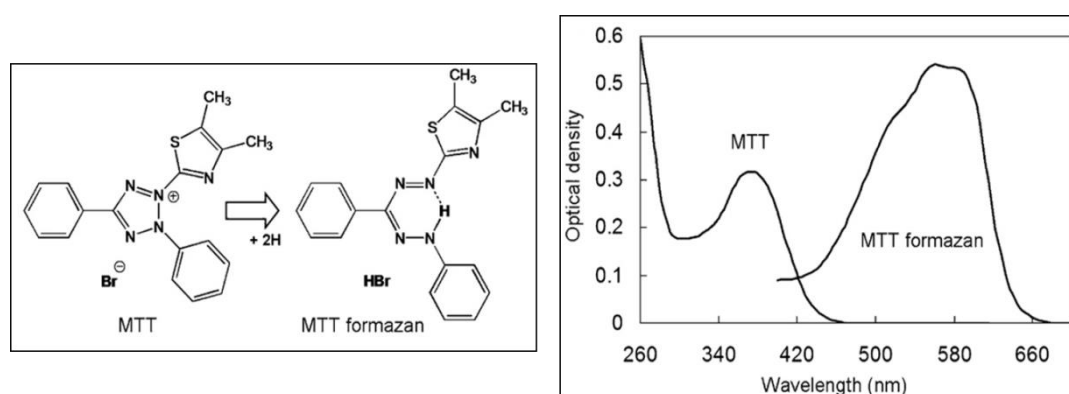


Figure 2.3. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. A) Chemical structure of MTT tetrazolium and its reduce form formazan. B) Absorption spectra of MTT and of its precipitate formazan. Adapted from Stockert *et al* 2012.

Cells were seeded at densities previously described in two 96 well plates. One plate for the MTT assay and one for cell number (Hoechst). Both plates were subjected to the same treatments. After VPA treatment the plates were washed twice with appropriate warm medium before MTT was added. A stock solution of MTT was made by dissolving it at 5mg/ml in molecular grade water, filter sterilised, aliquoted and stored at -20°C. The MTT was added at 10µl per well and 140µl of appropriate medium. Blank controls were medium and MTT only. Plates were incubated in the dark and left at

37°C in 5%CO₂ for 4 hours. Then 50ul of the medium was removed and 100µl DMSO was added to each well to dissolve the formazan precipitate. The plate was left on a microplate shaker (Stuart) for 20 minutes, plates were wrapped in foil. The absorbance was read at 570nm using a FLUOstar Omega plate reader (BMG Labtech).

Alamar Blue® (resazurin) assay

The Alamar Blue® assay (AB) uses resazurin sodium salt which is non-fluorescent and is reduced to red/pink fluorescent resorufin in metabolically active cells, figure 2.4, thus confirming cell viability.

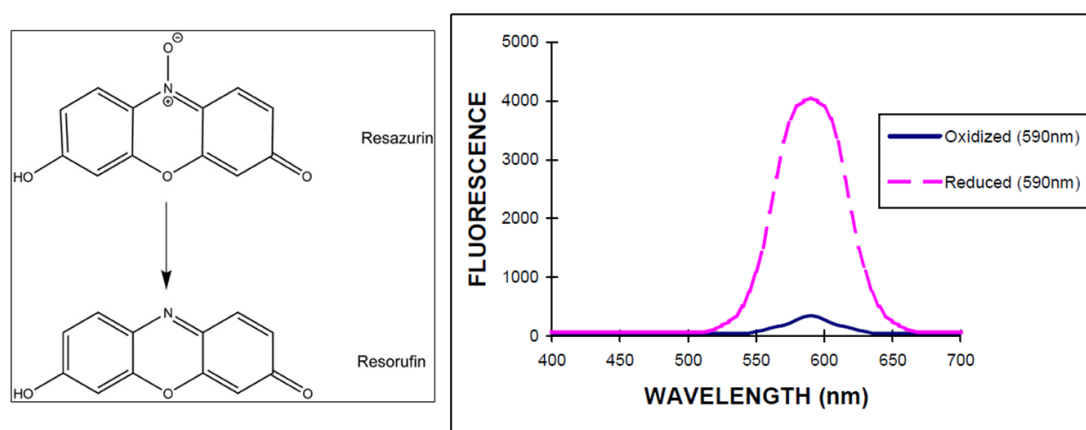


Figure 2.4. Alamar Blue assay. A) Chemical structure of resazurin and its reduced form resorufin. B) Fluorescence spectra of resazurin (oxidised) and resorufin (reduced). Adapted from Invitrogen methods and O'Brien *et al.*, 2000.

For each experiment cells were seeded in two 96 well plates and VPA treatment was carried out on all plates. Resazurin sodium salt (Sigma) was dissolved in molecular grade water, filter sterilised, aliquoted and stored at -20°C. After treatment with VPA the plate were washed twice with warm media before 100µM AB in 150µl media was added to the wells. The plate was incubated for 4 hours at 37°C in 5%CO₂ in the dark. The fluorescence of resorufin was read at Ex 540 and Em 620 (FLUOstar Omega plate reader, BMG Labtech). Control blanks were medium and AB only and fully reduced medium and AB only. Fully reduced AB was achieved by autoclaving a sample of medium and AB.

Hoechst 33342 assay

In tandem with assays such as OCR, MTT, AB a second plate was used to determine cell population. Working stock of Hoechst was prepared at 10mg/ml in molecular grade water and stored in the dark at -20°C. After VPA treatment, Hoechst 33342 was diluted in medium without VPA to give a final concentration of 0.1µg/ml. The plates were incubated in the dark at 37°C in 5%CO₂ for 20 minutes. Plates were gently washed twice with warm PBS. Finally adding 100µl of PBS, plates were read at room temperature using by BMG FluoStar Optima plate reader in fluorescent end-point mode with Ex 350nm and Em 460nm, bottom read.

Propidium iodide

To determine cell death propidium iodide (PI) was used. The fluorescent dye is impermeable to healthy cells and can only enter those which are dead/dying. PI then binds to the exposed DNA and the fluorescence increases. This assay was adopted from methods published by Dengler *et al.*, (1995). The PI (Sigma) was dissolved in molecular grade water and added to the wells at a final concentration of 50µM. The control blanks were medium and PI only. The plate was incubated for 30 minutes in the dark at 37°C in 5%CO₂. The fluorescence was measured using a FluoStar Optima (BMG) plate reader in end-point mode with Ex 540-20nm and Em 620-10nm, bottom read. To determine total cell death the plate, wrapped in foil, was then frozen overnight at -80°C, defrosted and frozen again at -80°C overnight. The following day the plate was left on the bench to reach room temperature and read again. The calculation for cell death is as follows:

$$\text{viable cells} = \text{total dead cells (2nd read)} - \text{dead cells (1st read)}.$$

Lactate measurement

To investigate the effect of treatment on glycolysis a hand held lactate meter (Accutrend, Roche) was used. As before cells were seeded in 96 well plates and treated with VPA. After treatment, 25µl of medium was removed from each well and pooled according to treatment into Eppendorf tubes. The samples were kept at 37°C until tested. 20µl of the pooled sample was added to the BM-Lactate strip (Roche) and inserted into the device. The Accutrend device can detect lactate levels at 0.7-26 mmol/L in plasma. Lactate levels below or above these values are displayed as LO and above HI. Medium with and without VPA was used as control.

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism v.6 (GraphPad Software Inc. La Jolla, CA, USA). Unless stated otherwise, statistical differences between control and treated cells were analysed using one-way analysis of variance (ANOVA) with Bonferroni correction *post hoc*. Differences were considered as statistically significant with * p-value=0.01-0.05, ** p-value=0.01-0.001, *** p-value= <0.001.

Chapter 3

Developing *in vitro* models to study the effect of sodium valproate treatment on mitochondrial function.

3 Developing *in vitro* models to study the effect of sodium valproate treatment on mitochondrial function.

3.1 Introduction

Drug metabolism (xenobiotic) by hepatocytes can be divided into three phases. Phase I involves oxidative, reductive and hydrolytic pathways which add functional groups (i.e hydroxyl). During phase II the added functional group is further modified making the drug molecule water soluble allowing its excretion in bile and urine. In phase III the bio transformed drug and its metabolite uptake occurs through the cell's transporters and excretion process via kidneys. Cytochrome P450 enzymes are involved in phase I and II and the function of these enzymes will determine the drug's efficiency and duration of compound's influence (Peng and Zhong, 2015; Wilkening *et al.*, 2003). As the majority of drug studies are carried out using animal models there is the potential for differences in human drug metabolism to impact on the relevance of the results.

Therefore, a robust human derived *in vitro* model would be very useful as a pre-clinical toxicity screen. The preferred model would be primary hepatocytes as these express the full complement of enzymes involved in drug metabolism. However, using primary hepatocytes would not necessarily provide the full picture as drug metabolism differs in males and females and also with age and ethnic groups (Zanger and Schwab, 2013). Furthermore, primary hepatocytes are difficult to source and have limited cell culturing potential~10 days. An *in vitro* alternative to primary human hepatocytes is the human hepatoma cell like HepG2. These cells have shown promise in previous studies investigating mitochondrial associated drug toxicity (Marroquin *et al.*, 2007; Kamalian *et al.*, 2015). However, HepG2 cells do not express all of the cytochrome P450 needed for total drug metabolism but do possess many of the other metabolic pathways that

are present in primary hepatocytes that can be affected by drug treatment (Wilkening *et al.*, 2003)

The drug sodium valproate (VPA) is a widely prescribed anticonvulsant for different types of epilepsy (Pourahmad *et al.*, 2012; Silva, 2008). VPA's mechanism of action and toxicity is discussed in the introduction section 1.4.3. Briefly, VPA is an eight carbon branch chain fatty acid. Several metabolic pathways are involved in the biotransformation of the parent drug including β -oxidation and cytochrome P450, microvesicular ω -oxidation and glucuronidation. The biotransformation of VPA creates 50 different metabolites some of which are highly toxic (Aries *et al.*, 2010; Luis *et al.*, 2007; Silva *et al.*, 2008).

3.2 Materials and Methods

Unless stated otherwise all materials were purchased from Sigma-Aldrich.

3.2.1 Oxygen consumption assay

The traditional method for monitoring oxygen consumption (OCR) and mitochondrial respiration (OXPHOS) has been the Clark electrode however, the technique has moved on to fluorescent oxygen probes (review by Wolfbeis, 2015). The use of fluorescent oxygen probes allows for high throughput research using whole cells or isolated mitochondria. There are now several alternative systems available, including the Seahorse Biosciences XF analyzer and Luxcel Bioscience's MitoXpress kit. Both these assays are based on fluorescent probes responding to oxygen concentrations in the surrounding cellular environment. The MitoXpress fluorescent probe is quenched

by oxygen (Will *et al.*, 2006). However, the measurement methods are very different. The Seahorse XF is a standalone instrument, using bespoke plates and pre-prepared probe assay kits whereas the MitoXpress kit can be used on standard black tissue culture plates with clear bottom and the oxygen probe signal is detected by using a standard fluorescent plate reader. Overall, both systems provide a high throughput approach to assess the effects of treatments on mitochondrial respiration.

In this study I used Luxcel Bioscience's MitoXpress extracellular oxygen sensing kit to study mitochondrial respiration in whole cells.

MitoXpress extracellular oxygen probe; theory of measurement

The Luxcel Biosciences MitoXpress probe system uses a phosphorescent oxygen sensitive probe to measure extracellular oxygen levels. Oxygen quenches the probe fluorescence which is measured as an increase in signal as oxygen is decreased in the surrounding media of actively respiring cells (Hynes *et al.*, 2009). Cells incubated in galactose medium, which drives the cells to increase OXPHOS to generate sufficient ATP have generally higher oxygen consumption rates than cells incubated in high glucose medium (Marroquin *et al.*, 2007). The assay is carried out in real time, using an oil sealed system. Changes in mitochondrial respiratory chain activity due to drug treatment can be observed by monitoring changes in probe signal over time. Assays were carried out using the time-resolved fluorescence function on a BMG Labtec FLUOstar Omega plate reader. Rates of oxygen consumption were calculated using the MARS software (BMG Labtech). The oxygen levels is measured using dual delay time of 30 μ s and 70 μ s, time resolved fluorescence (TR-F), figure 3.1 (A). The TR-F intensity is converted to phosphorescence lifetime values using the following equation; $\text{Lifetime} = t_2 - t_1 / \ln(D_1/D_2)$ where t =delay time and D =measured intensity value (HHynes *et al.*, 2005; Zhadanov *et al.*, 2011). From this equation, a lifetime curve is generated where time (x-axis) and TR-F intensity (y-axis) are plotted, figure 3.1 (B) enabling the calculation of extracellular oxygen consumption rate (OCR) to be calculated in a 96 well format. Lifetime is defined as the time in which a fluorescent molecule exists in an excited state before returning to the ground state. Furthermore, the conversion of TR-F intensity to lifetime phosphorescence is independent of fluorophore concentration, reducing variances between sample replicates and between experiments. Changes in extracellular oxygen rates reflect changes in intracellular oxygen consumption/mitochondrial respiration. Work carried out in the Morten Lab found that HepG2 non-mitochondrial respiration was between 2-5%. The OCR is not only determined by the activity of the cells but also the number present. As this is a sealed

system, cell number/viability cannot be determined on the same plate due to the addition of the oil, therefore, a clear tissue culture plate is used in parallel to estimate cell number. The parallel plate undergoes the exact same treatment as the black tissue culture plate used for the OCR assay. Hoechst 33342 or propidium iodide (Dengler *et al.*, 1995) was used to determine the relative number of cells present.

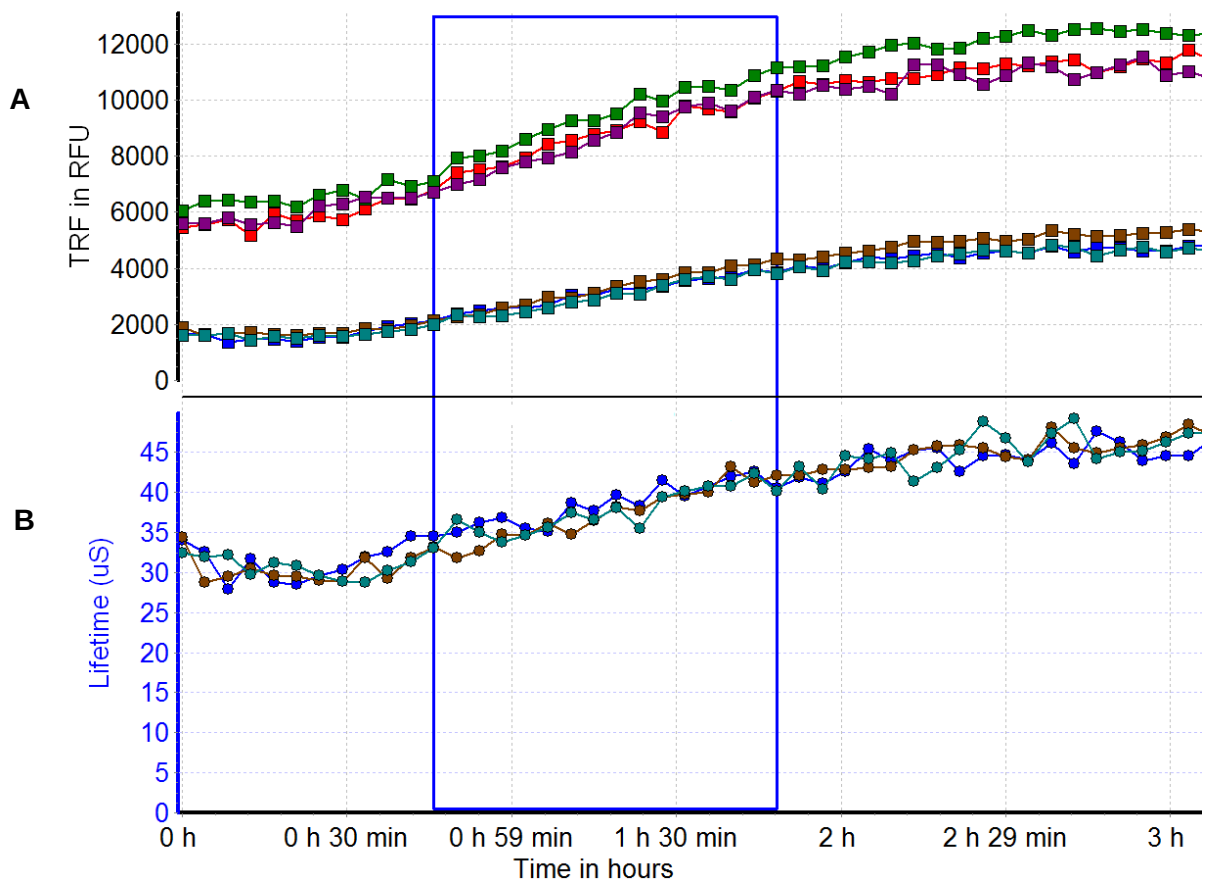


Figure 3.1. MitoXpress fluorescent oxygen sensing probe, raw data output. A) Probe signal measured using dual delay time of 30 μ s and 70 μ s in time resolved fluorescence in relative fluorescent units (TRF in RFU). B) The converted TRF to lifetime of the probe signal. The blue box is placed on the linear part of the curve to determine the range for which oxygen consumption rate (OCR) is calculated.

MitoXpress assay protocol

The cells were seeded in black plates with a clear bottom and in parallel a clear 96 well plates. The cell densities used over 72 hours varies according to the doubling time of the cell line used, see table 3.1.

Table 3.1. Cell densities per well used for the MitoXpress and cell number determination assays.

Cell line	Cells per well			Comment
	24 hours	48 hours	72 hours	
HepG2	15,000	17,500	25,000	Approximate number as difficult to count due to cell clumping
Primary fibroblasts	15,000-20,000	20,000-30,000	35,000-40,000	Range of cells/ well due to varying doubling time.
MEFs WT	15,000	17,500	-	WT fast doubling time
MEFs POLG	20,000	40,000	-	POLG very slow doubling time.

For the glucose control the lowest seeding density was used.

Cells were left to adhere overnight before any treatments were added. After VPA treatment, the 96 well plates were carefully washed once with appropriate respiration assay medium. The medium used for this assay contained no phenol red as it was found to reduce the probe signal. The respiration medium used was DMEM (Gibco) supplemented with either appropriate glucose concentration to mirror that of the normal maintenance medium of the cell line used or galactose. The respiration medium would always mirror the original medium used during treatment. As standard all the media were further supplemented with 10% FBS, 1mM sodium pyruvate, 2mM L-glutamine, 100IU/ml penicillin, 100µg/ml streptomycin. There were further additions such as 25µM uridine for primary fibroblasts and MEFs, please see materials and methods chapter, section 2.2.1 for a detailed overview of all the media used in all the studies. The MitoXpress probe was reconstituted in 1ml molecular grade water (Merck Millipore)

and 10µl was added to 140µl of respiration media per sample well. 100µl HS mineral oil was applied to each sample well. The plate was read using BMG Labtech FLUOstart Omega in time resolved fluorescence mode using Ex TR-ex filter (340nm) and Em TR-em² (650nm), at 37°C. The raw output from the probe signal and the range selected to calculate the oxygen consumption rate (OCR) is depicted in figure 3.1. The increase in cell number is reflected in the increasing oxygen consumption rate. After 50x10⁴ of HepG2 cells are loaded per well, the OCR fluctuates and plateaus as the cells per well become over confluent, figure 3.2

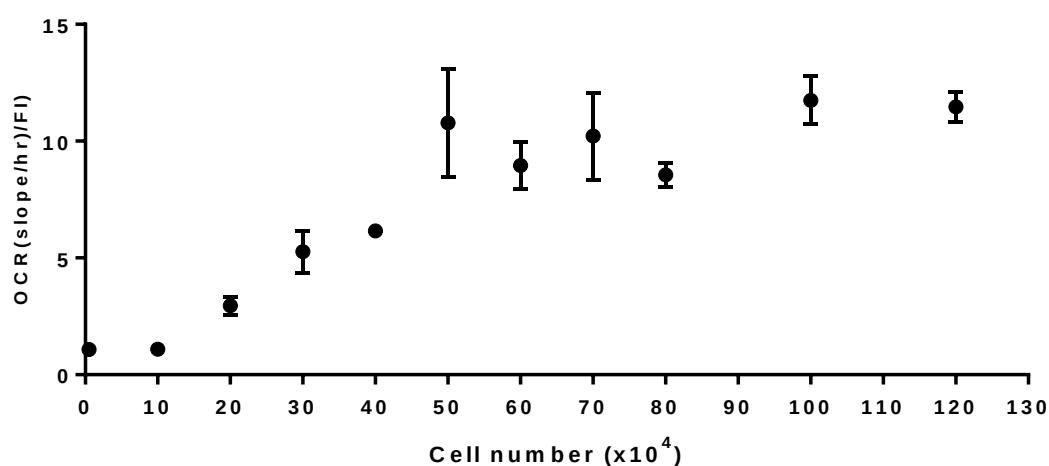


Figure 3.2. Optimising OCR assay for cell number. HepG2 cells seeded in 96 well plates and left overnight to adhere in glucose medium before the OCR was measured. Error bars represent the mean $\pm$ SD of three technical replicates, n=1.

3.2.2 Citrate synthase activity assay

Citrate synthase is found in the mitochondrial matrix and can be used as marker for mitochondrial integrity and number. Citrate synthase activity is measured via the reaction between free acetyl-coA (CoA) and DTNB (5,5-dithiobis-(2-nitrobenzoic acid) producing TNB (2-nitro-5-thiobenziolate) which can be read by measuring absorption at 412nm (Morgunov and Srere,1998). Citrate synthase forms citrate and CoA from the reaction between oxaloacetate and acetyl-CoA. 50×10^3 HepG2 cells were seeded in 6 well plates and treated for 72 hours with 2.0mM VPA in either 5mM glucose or 10mM galactose media. After treatment cells were washed in cold PBS and scraped off with a cell scraper and placed in a 15mL Falcon tube. Cells were centrifuged at 2,000 rpm for 3 minutes at room temperature. The supernatant was discarded and cells resuspended in PBS and frozen at -80°C . Cells were lysed by two freeze thaw cycles. To determine sample protein content was established using Pierce ® BCA protein assay kit (Thermo Scientific) according to manufactures protocol. Briefly, 25µl of samples and standard were added to 96 well plate in triplicate. Standards came with the kit and made according to protocol. The working reagent was added at 200µl per well and plates were incubated for 30 minutes at room temperature before being read on the plate reader. Sample protein concentrations were calculated from the standard curve generated. For the citrate synthase assay, 40µg of each sample was added in triplicate to clear 96 well plates. The final concentration of the reaction mix was 100nM Tris buffer (pH 8.0), 0.1% (v/v) Triton X-100, 0.1mM Acetyl CoA and 0.2nm DTNB. Using the plate reader's injection pumps, 220µL of the reaction mix was dispensed followed by 5µl of 20mM oxaloacetate. Oxaloacetate was prepared fresh and kept on ice.

Oil red O staining

Oil red O is a fat soluble diazot dye which stains neutral lipids and cholesteryl esters but not biological membranes (Mehlem *et al.*, 2013). 5×10^3 HepG2 cells were seeded on sterile coverslips ($\varnothing$ 13mm) and left overnight to adhere before VPA treatment commenced. After the VPA treatment had finished the cells were washed with PBS and fixed in 10% formalin for 1 hour at RT. The formalin was aspirated and cells washed twice with sterile water. 60% isopropanol was added for 5 minutes and aspirated. Before the staining procedure commenced, Oil red O stock was prepared by dissolving 0.35g of Oil red in 100ml isopropanol. On the day of staining, a working stock was prepared by adding 6ml of Oil red stock to 4ml of sterile water which was passed through a filter. Oil red O was added to the fixed cells for 10 minutes at RT. Oil red O was removed and cells were washed with tap water, until the water ran clear. This was followed by haematoxylin staining. The cells were dipped in haematoxylin for 30 seconds and rinsed in tap water until the water ran clear. The coverslips were mounted onto microscope slides, using Aqua-mount (Thermo Scientific). The cells were visualised using a Leitz DMRB microscope.

Determination of lipid content

To determine lipid content of HepG2 cells after treatment with VPA, 50×10^3 cells were seeded in 6 well plates and left overnight to adhere before adding treatment. The cells were scraped off in PBS. The cells were then centrifuged and the supernatant discarded. The cell pellet was resuspended in 200 μ l PBS and split into two 100 μ l aliquots. One aliquot was sent to Dr Leanne Hodson at Oxford Centre for Diabetes, Endocrinology & Metabolism for lipid analysis and the other one was used protein determination. The lipid analysis was performed using the kit IL Test Triglycerides with the IL ILab300 chemistry analyser system. Protein content was determined using Pierce's BCA kit according to manufacturer's protocol.

Results

3.2.3 *Evaluating the effect of sodium valproate treatment on cellular metabolism in HepG2 under normal and energetic stressed conditions*

Introduction

To study the effect of sodium valproate (VPA) treatment on mitochondrial respiration, two different culture conditions were used. The standard practice for culturing cells is in high glucose (20-25mM) which allows the cells to generate ATP through glycolysis rather than mitochondrial OXPHOS and potentially masking any drug induced mitochondrial toxicity effect. Glycolytic cells such as HepG2 have been shown to be resistant to traditional mitochondrial toxicants such as rotenone and antimycin A when incubated in high glucose (25mM) medium. However in low glucose (5.5mM) medium there was a slight sensitivity detected (Marroquin *et al.*, 2007). However, when glucose was replaced with galactose, the HepG2 cells became fully sensitised to drug treatments with ATP levels reduced due to inhibition of mitochondrial respiration (Marroquin *et al* 2007). Glycolytic cell lines such as HepG2 do not depend on OXPHOS to sustain ATP levels when glucose concentration are high (25mM) but when glucose is replaced with galactose then mitochondrial function be detected (Marroquin *et al.*, 2007; Domenis *et al.*, 2012).

By changing the medium to a more physiological glucose (5mM) level or by removing glucose and supplementing it with galactose would force the cells to rely more on the mitochondria to sustain adequate ATP levels in the event of a mitochondrial toxicant being present. Cells in galactose medium would be under energetic stress as the demand on the mitochondria function to generate ATP would be higher than in glucose medium.

3.2.3.1 VPA does affect the OCR in HepG2 cells under normal and energetic stressed conditions.

The effect of VPA treatment on mitochondrial activity in HepG2 cells was investigated using the MitoXpress oxygen probe as previously described, section 3.2.1. HepG2 cells were seeded in 96 well black walled, clear bottom tissue culture plates. Cell seeding density was as follows; 15,000 cells for 24 hour treatment, 17,500 cells for 48 hours and 25,000 cells for 72 hours treatment. The cells were left overnight to adhere before VPA treatment commenced. VPA concentrations of 0.5mM which was considered to be close to a therapeutic dose (Komulainen *et al.*, 2015), 1.0mM and high concentration of 2.0mM was administered at three time points, 24 hours (short-term), 48 hours (medium-term) and 72 hours (long-term) in 5mM glucose or 10mM galactose media. Media and VPA treatment was replaced every day.

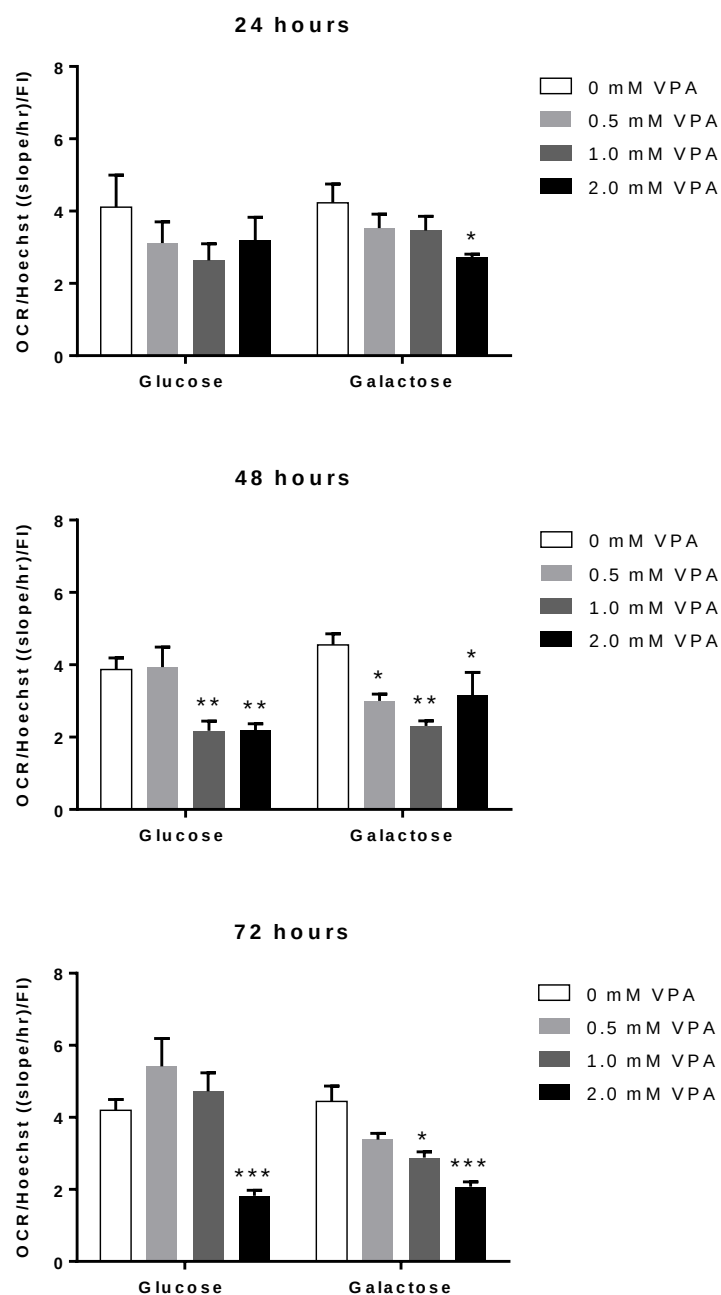


Figure 3.3. Oxygen consumption rates measured in HepG2 cells decreases after treatment with VPA under normal (5mM glucose) and energetic stress (10mM galactose) conditions. HepG2 were treated with VPA at concentrations and duration as indicated. The cells were seeded at different densities according to time point and left overnight before first VPA treatment (72hrs) was added the following morning. Control was medium and vehicle only. Data is normalised to cell number (Hoechst) and represent mean $\pm$ SD, n=3, *p<0.05, **p<0.01, ***p<0.001, ANOVA with Bonferroni correction when compared to baseline condition, glucose or galactose.

The OCR data indicates that VPA does affect OXPHOS, after treatment in both glucose and galactose media. This effect was time dependant, figure 3.3. Following short-term (24 hours) treatment there was no significant effect on OCR after VPA treatment in glucose medium, whereas, in the galactose medium only the highest concentration of VPA had a significant effect on OCR. The medium-term treatment (48 hours) resulted in inhibition at all concentrations in galactose medium and at the highest VPA concentrations (1.0mM and 2.0mM) in glucose. Interestingly, the inhibitory effect at 1.0mM VPA was not shown after 72 hours treatment of cells in glucose media. Only the highest concentration (2mM) inhibited mitochondrial respiration. After 72 hours treatment, cells under energetic stress (galactose) showed a concentration dependant decrease in OCR.

3.2.4 VPA treatment does affect the total cellular ATP levels in HepG2 cells under normal and energetic stressed conditions.

To investigate the effect of VPA on cellular ATP levels, HepG2 cells were seeded in 96 well plates, left overnight to adhere before VPA treatment commenced. The VPA treatment significantly altered the total cellular ATP levels after 24 hours incubation in at all drug concentrations under normal and energetic conditions (glucose and galactose), figure 3.4. After 48 hours, only the highest concentration of VPA (2.0mM) caused a significant decreased in ATP levels, in both media. At the 72 hours point only 0.5mM in glucose and 2.0mM in galactose impaired cellular ATP levels. After 72 hours incubation in galactose medium only, there was a small but significant, $p=0.0128$, increase in cellular ATP in cells incubated when compared to cells in normal glucose media.

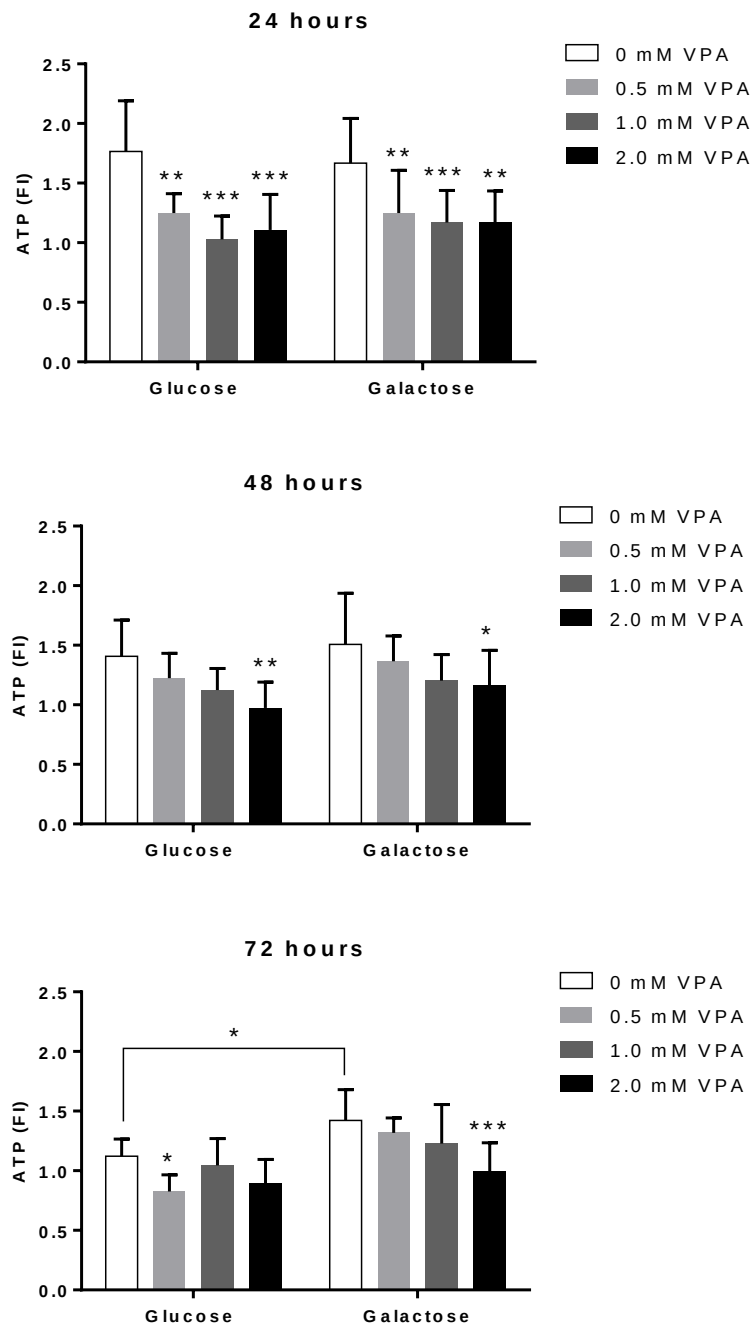


Figure 3.4. Reduced ATP levels in HepG2 cells after treatment with VPA under normal (5mM glucose) and energetic stress (10mM galactose) conditions. Data is normalised to cell number (Hoechst) and expressed as ATP/Hoechst fluorescent intensity (FI) and represent mean $\pm$ SD, n=3, *p<0.05, **p<0.01***p<0.001, ANOVA with Bonferroni correction when compared to baseline condition, glucose or galactose.

3.2.5 VPA treatment affects lactate levels in HepG2 cells under normal and energetic stress conditions

Glucose is metabolised through glycolysis to generate ATP and pyruvate. Pyruvate can either enter the TCA cycle to generate more ATP via OXPHOS or be converted to lactate. The conversion of pyruvate to lactate has an important role in glycolysis as the reaction generates NAD^+ . For continued glycolysis the NAD^+/NADH redox balance must be maintained (Lunt and Vander Heiden 2011). To investigate the effect VPA treatment has on glycolysis, lactate levels were measured in the media as an indicator of glycolysis. The media was collected from HepG2 cells seeded in 96 well plates after treatment with the VPA for 24 hours, 48 hours and 72 hours in either glucose or galactose media.

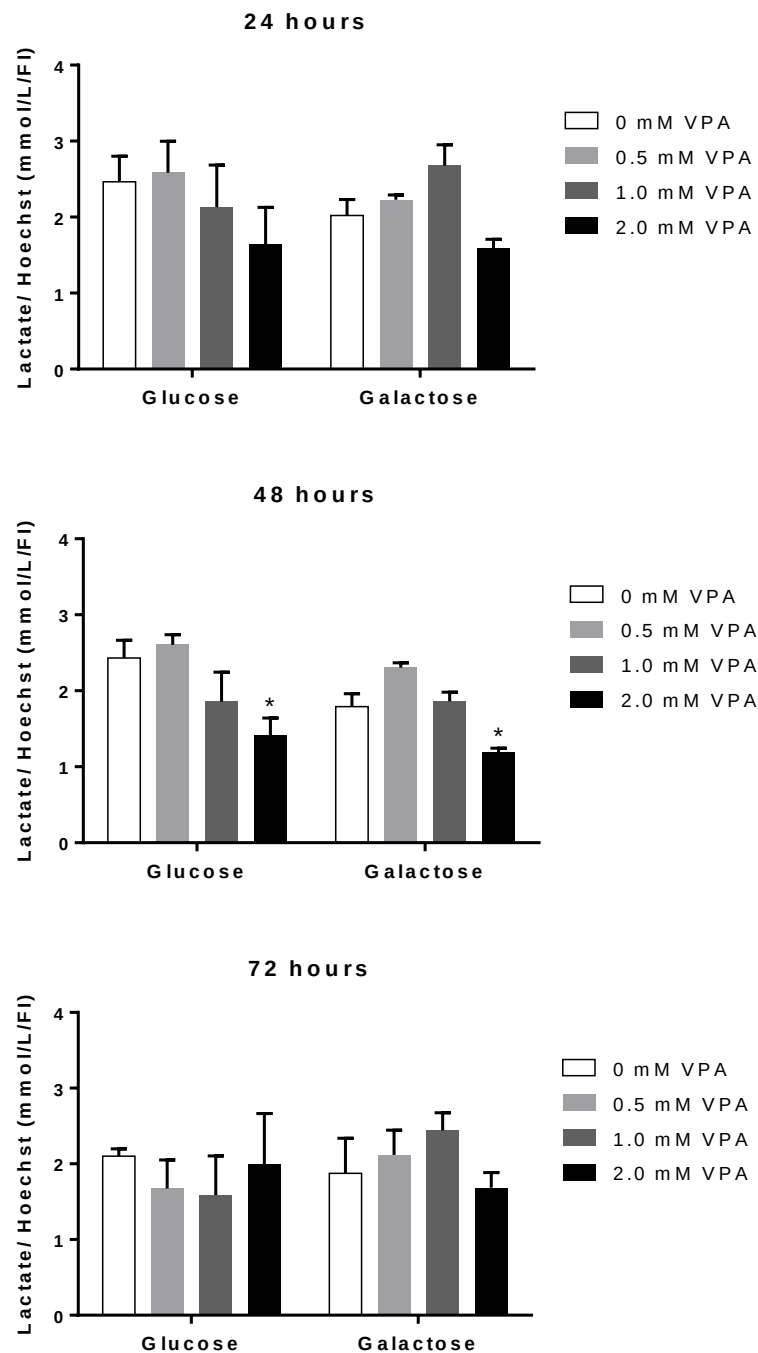


Figure 3.5. Reduced lactate levels measured after treatment with VPA. Media was collected after VPA treatment from 96 well plates seeded with HepG2 cells. Data is normalised to cell number (Hoechst) and expressed as lactate/Hoechst fluorescent intensity (FI) and represent mean $\pm$ SD, $n=3$, * $p<0.05$, ANOVA with Bonferroni correction when compared to baseline condition, 5mM glucose or 10mM galactose.

There were no changes in lactate levels detected in cells incubated for 24 hours, 48 hours and 72 hours under energetic stress condition only when compared to normal glucose condition only. After 24 and 48 hours VPA treatment in glucose or in galactose media there was an indication of an inhibitory role of VPA. Only 48 hours and at the highest VPA concentration (2mM) was significant. There were no changes in lactate levels after 72 hours VPA treatment in glucose or in galactose media, figure 3.5.

3.2.6 VPA treatment does affect citrate synthase activity in HepG2 cells under normal and energetic stress conditions

Incubating the HepG2 cells for 72 hours under energetic stress only, did not caused significant change in citrate synthase activity when compared to baseline normal glucose condition, figure 3.6. The citrate synthase activity did significantly increase after 72 hours of 2mM VPA treatment under energetic stress at the end time point 100 seconds, $p=0.00475$. VPA treatment in glucose medium did not significantly alter citrate synthase activity.

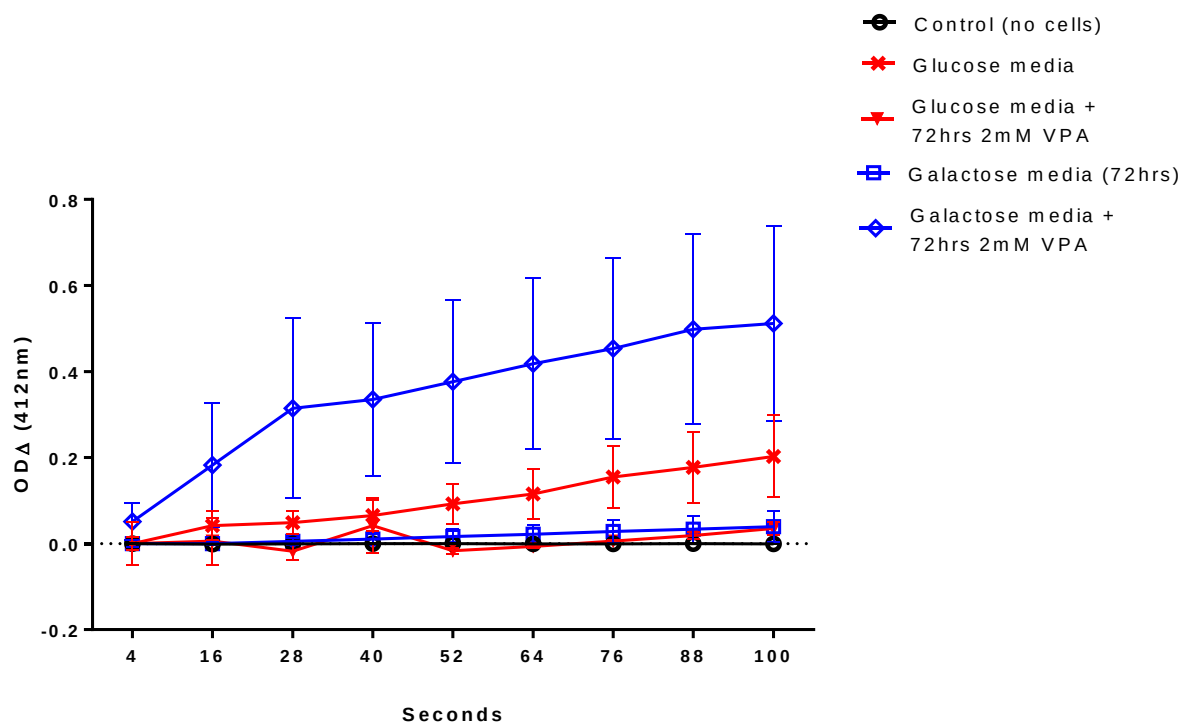


Figure 3.6. Citrate synthase activity of HepG2 cells after 72 hours VPA treatment in normal condition and under energetic stress condition. HepG2 cells were seeded in 6 well plates and treated for 72 hours with 2.0mM VPA under normal 5mM glucose or under energetic stress condition. Data presented as optical density (OD) and represent the mean $\pm$ SD of three technical replicates, n=2.

3.2.7 The cytotoxic effect of VPA treatment in HepG2 cell line under normal and energetic stress conditions

To establish the cytotoxic effect of VPA three assays were employed, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), Alamar Blue and propidium iodide (PI). The MTT assay uses the detection of the reduction of yellow tetrazolium salt to purple formazan, which can only be achieved if the cells are metabolically active. The second assay used was Alamar Blue (AB) in which resazurin is reduced by metabolically active cells to resorufin. PI is a fluorescent dye and is impermeable to healthy cells and can only enter those which are dead/dying. PI binds to the exposed DNA of the dead/dying cells and the fluorescence increases. For the MTT and AB assays, Hoechst was employed to assess cell number.

3.2.7.1 VPA treatment increases cell death in HepG2 cells

Significant increased cell death was observed after 24, 48 and 72 hours VPA treatment in glucose medium. When HepG2 cells were subjected to energetic stress in galactose, significant cell death occurred only after 72 hours VPA treatment. VPA treatment had no significant effect on cell death at 24 and 48 hours in galactose medium. However, energetic stress condition alone did significantly increase cell death after 48 and 72 hours incubation, figure 3.7.

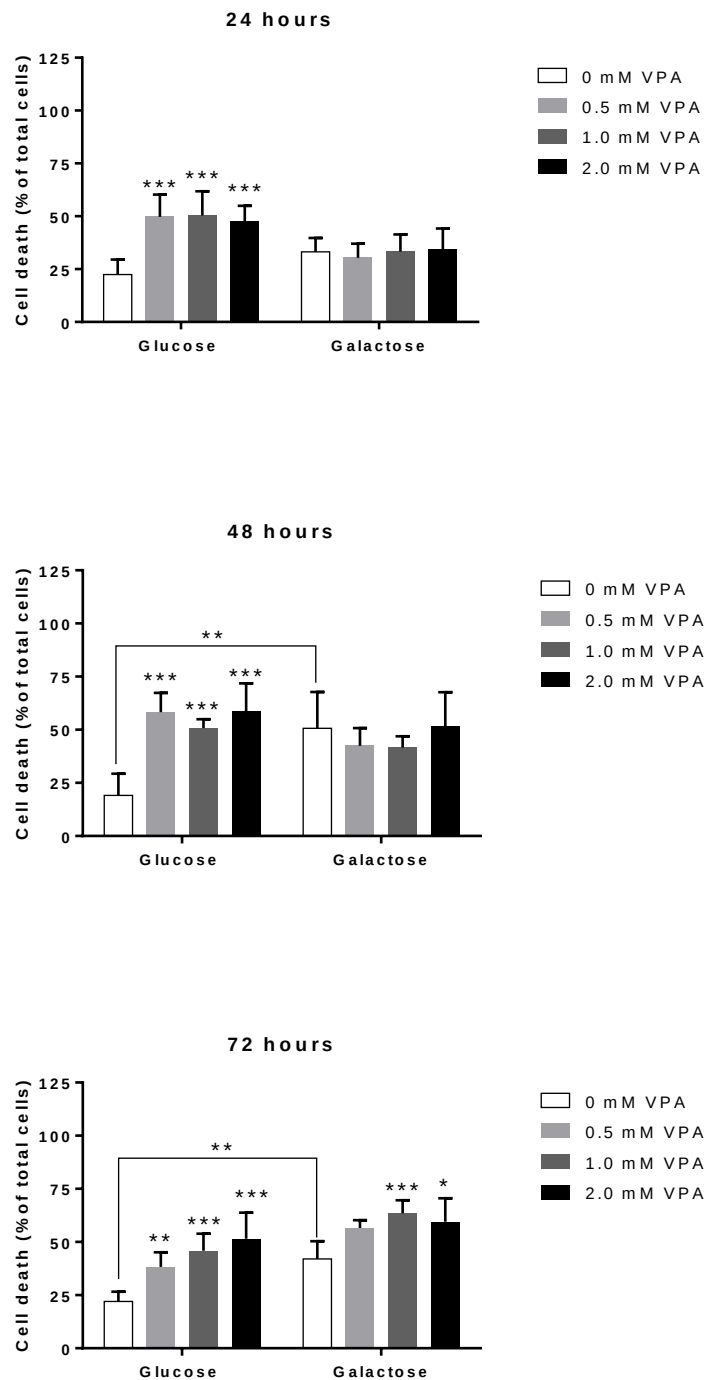


Figure 3.7. Increased cell death after VPA treatment in HepG2 cells under normal and energetic stress conditions. Cell death detected by PI assay. Data is presented as percentage of death relative to total cell. Mean $\pm$ SD, n=3, *p<0.05, **p<0.01***p<0.001, ANOVA with Bonferroni correction when compared to baseline condition, glucose or galactose.

3.2.7.2 Cytosolic metabolic activity is increased in HepG2 cells after VPA treatment.

The MTT assay detected a significant increase in the reduction of tetrazolium to formazan after 72 hours VPA treatment in both glucose and galactose media, figure 3.8. The reduction of tetrazolium to formazan can only occur in metabolically active cells. Therefore, it appears that VPA treatment does not cause cytotoxicity in HepG2 cells. This was also found to be reflected when measuring the reduction of resazurin to resorufin in the AB assay. There was significant increase in resorufin detected after 24, 48 and 72 hours of high concentration VPA (2mM) in glucose medium, figure 3.9. No significant changes were detected after VPA treatment under energetic stress.

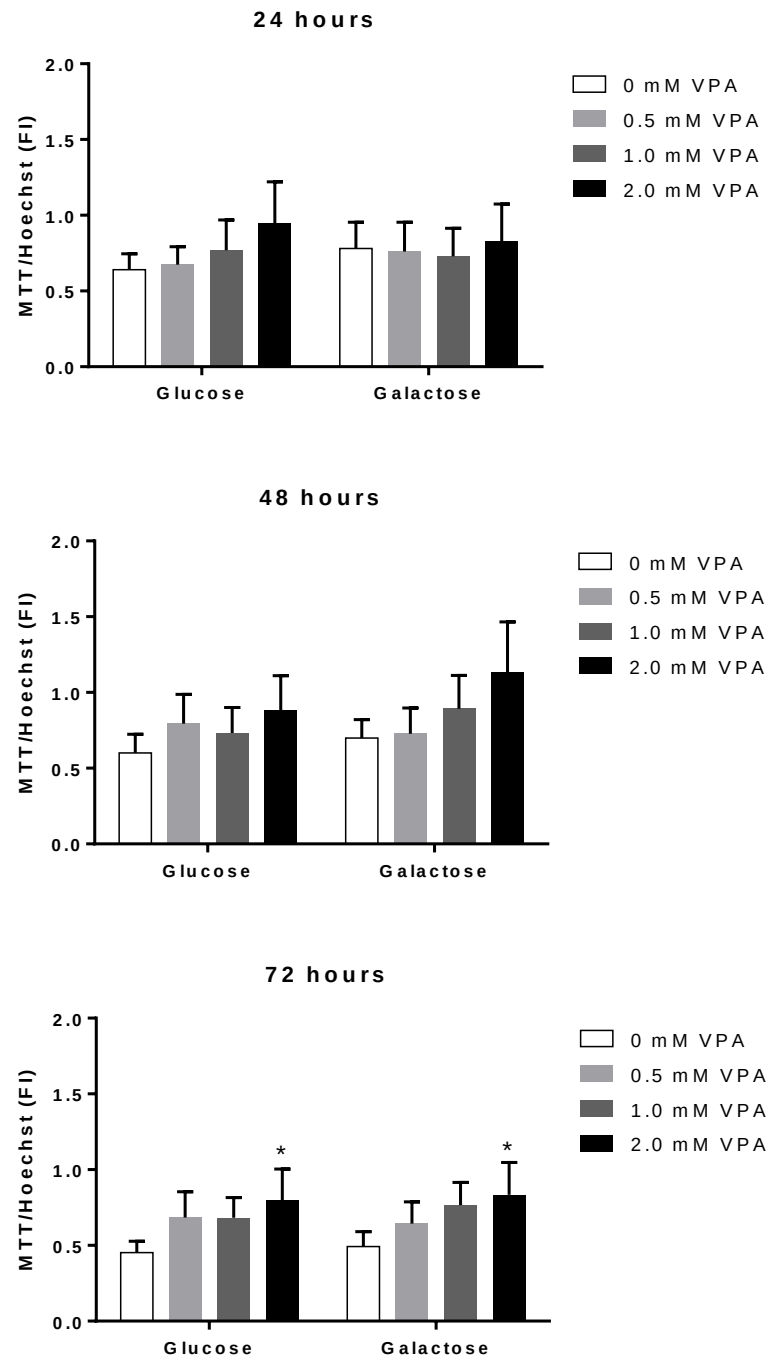


Figure 3.8. Increased MTT signal after VPA treatment in HepG2 cells under normal and energetic stress conditions. VPA treatment in normal 5mM glucose medium and in galactose medium. Data is normalised to cell number (Hoechst) and expressed as MTT/Hoechst fluorescent intensity (FI) and represent mean $\pm$ SD, $n=3$, * $p<0.05$, ** $p<0.01$ *** $p<0.001$, ANOVA with Bonferroni correction when compared to baseline condition, glucose or galactose.

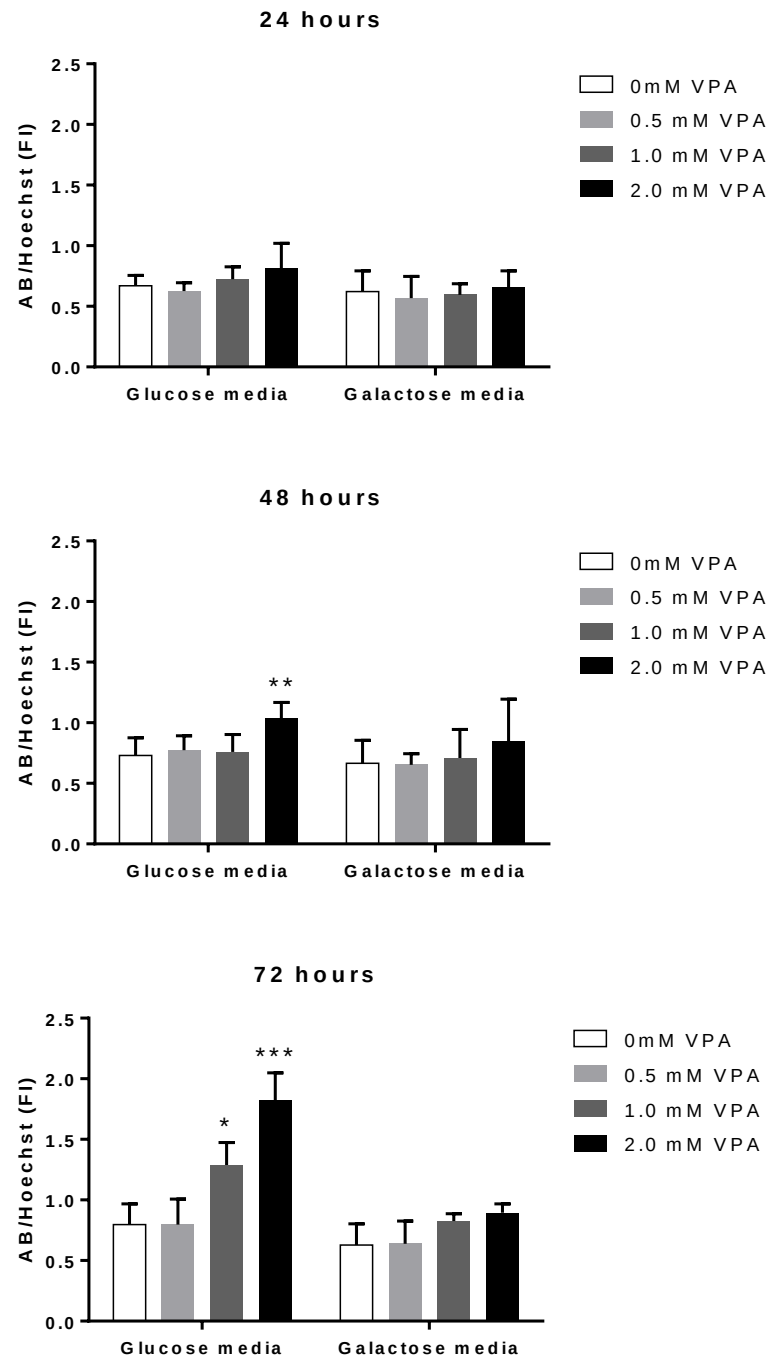


Figure 3.9. Increased Alamar Blue (AB) signal after VPA treatment in HepG2 cells under normal and energetic stress conditions using. VPA treatment in normal 5mM glucose medium and in galactose medium. Data is normalised to cell number (Hoechst) and expressed as AB/Hoechst fluorescent intensity (FI) and represent mean $\pm$ SD, n=3, *p<0.05, **p<0.01***p<0.001, ANOVA with Bonferroni correction when compared to baseline condition, glucose or galactose

3.2.7.3 VPA treatment reduces cell population in HepG2 cells as detected by Hoechst stain.

The fluorescent nucleic stain Hoechst was used to account for cell numbers after VPA treatment. This enabled the data generated from such assays as OCR, MTT and AB to be normalised to relative cell number as determined by Hoechst fluorescent. The Hoechst stain was always performed in a parallel plate and would have had the exact same treatment as the assay it was mirroring. Please see Appendix A for optimisation.

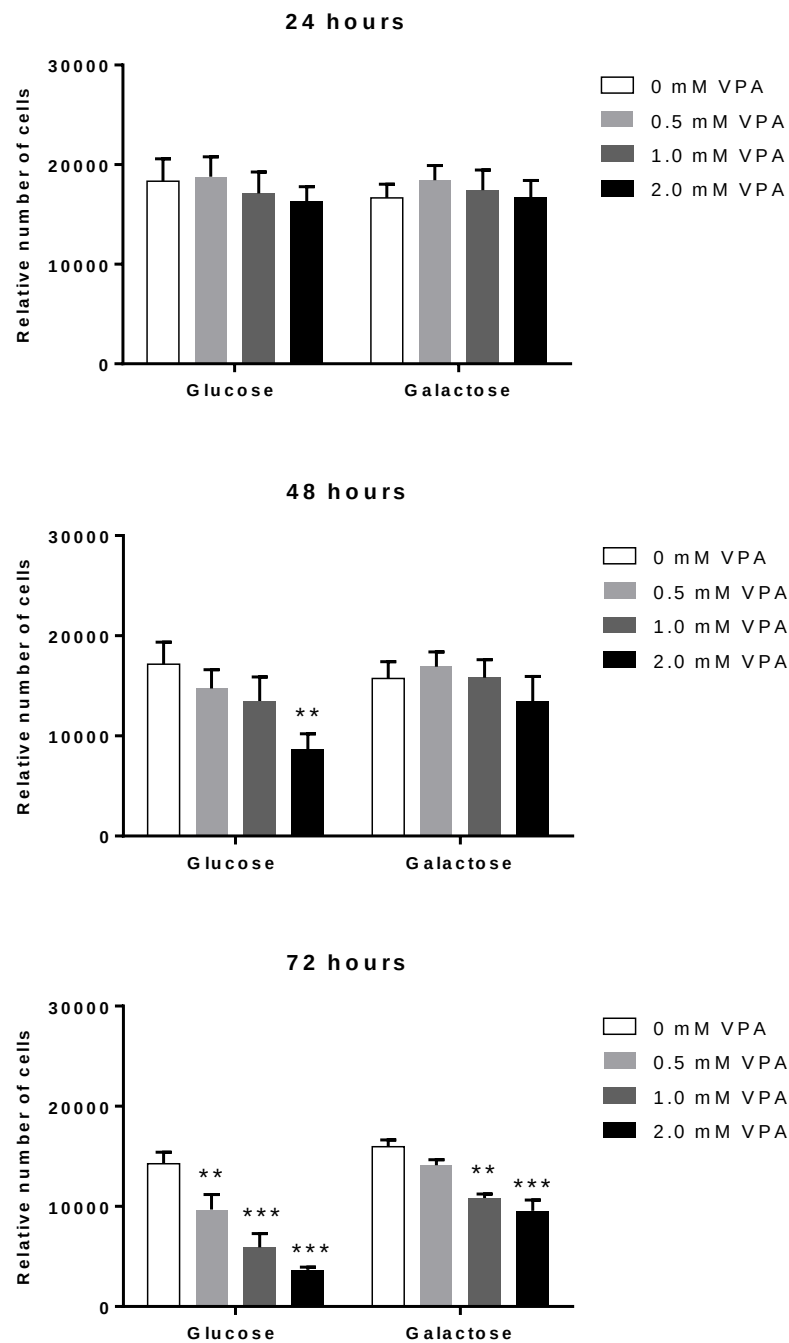


Figure 3.10. Reduced relative cell numbers after VPA treatment under normal and energetic stress conditions. Relative cell number was determined using Hoechst stain. Data represented as cell number relative to Hoechst fluorescent intensity, mean $\pm$ SD, n=3, **p<0.01***p<0.001 ANOVA with Bonferroni correction when compared to baseline condition, glucose or galactose.

VPA treatment had no effect on cell numbers after 24 hours incubation in either normal or energetic stressed conditions. Only the highest VPA concentration (2mM) caused a significant reduction under normal glucose condition after 48 hours treatment. A significant reduction in cell number was observed after 72 hours of VPA treatment in glucose and galactose media, figure 3.10. The reduction in cell number was concentration dependant. There were no statistical changes in cell number observed between cells incubated under normal or energetic stress condition alone.

3.2.7.4 Discussion – Sections 3.3.1 to 3.3.5

VPA treatment increases cell death and the remaining cell population have increased cytosolic oxidoreductase activity.

Energetic stress alone had little effect on HepG2 cells. This cell line was able to adapt when glucose was replaced with galactose. There were no significant changes to OCR or lactate levels. Interestingly, ATP levels were increased after prolonged incubation under energetic stress condition. This could indicate that HepG2 cells are well adapted to changing nutrient conditions which could be due to the low 5mM glucose concentrations in the normal medium. HepG2 cells subjected to energetic stress condition (no VPA treatment) for 48 hours and 72 hours resulted in increased cell death but it had no effect on cell proliferation as the cell population (relative number of cells) did not significantly reduce. VPA treatment in normal glucose conditions cause increased cell death at all time points and all drug concentrations but it did not significantly affect the relative number of cells present, with the exception of 2.0mM VPA after 48 hours incubation. 24 hours and 48 hours VPA treatment under energetic stressed galactose conditions did not cause cell death nor did it affect cell proliferation

as the relative number of cells did not change. The effect of VPA treatment in galactose medium on cell death and the relative number of cells was only observed after 72 hours treatment. 72 hours VPA treatment in glucose or galactose media caused increased cell death and a reduction in cell population in all the drug concentrations. Thus, HepG2 cells were unable to maintain cell number after treatment with VPA in both glucose and galactose media. Interestingly at 1.0mM VPA in glucose, the remaining cell population was able to maintain OCR and the ATP pool, suggesting the use of a compensatory mechanism. Previous studies have shown the effect of high concentration (2.0mM) and long term VPA treatment (72 hours) in HepG2 cells increased ROS (Komulainen *et al.*, 2015). It is possible that the increase in ROS is acting as a signalling molecule, stimulating pathways involved in energy metabolism.

Cytosolic oxidoreductase activity

Since the pioneering publication by Salter *et al.*, in 1963 which reported that MTT reduction to formazan was via the mitochondrial electron transfer chain, complex II/succinate dehydrogenase. The MTT assay is frequently used as a screen for drug toxicity. However, Bernas and Dobruck (2002) published results indicating that only 25-40% formazan precipitates were due to mitochondrial activity. The reduction of MTT is a reflection of not only mitochondrial activity but also of oxidoreductase enzymes and co-factors such as NADH and NADPH in the cytosol (Liu *et al.*, 1997). There is evidence that MTT reduction can be found to occur in perinuclear vesicles such as endosomes or lysosomes (Liu *et al.* 1997). Although, the MTT assay is widely used due to its suitability for high-throughput *in vitro* drug toxicity screening, it does have several drawbacks which could lead to false positive viability results. For example, under conditions of oxidative stress, superoxide has been found to reduce tetrazolium to formazan (Wang *et al.*, 2011). Furthermore, when the MTT is reduced the formazan

precipitate can form either granules or long structures which may pierce the cellular membrane causing cell death (Liu *et al.*, 1997). Therefore, the cell death observed will not necessarily be due to drug treatment. It is therefore vital to optimise MTT incubation period to ensure that these crystals do not form. Alamar Blue (AB) also known as resazurin is a redox dye and upon entering the cytosol of living cells is reduced to resorufin by mitochondrial enzymes by accepting the electrons from cytochromes, NADH, FADH and NADPH (Borra *et al.*, 2009). The advantage of the AB assay is that it is not cytotoxic and therefore lends itself to time-course studies. However, there is evidence that resazurin causes ROS and reduced cell proliferation (Rampersand, 2012). There is currently no evidence in the literature indicating that ROS can reduce resazurin. With the limitations of these two assays in mind, it is interesting to note that there was an increase in the reduction of MTT and AB after 72 hours VPA treatment in normal glucose and energetic stressed conditions. Previously published data (Komulainen *et al.*, 2015) show that only the highest concentration and the longest treatment period (2.0mM VPA and 72 hours) in glucose and galactose caused an increase of ROS in HepG2 cells. Therefore ROS could account for the increased MTT reduction after 72 hours of 2.0mM VPA treatment. What is remarkable is the difference between the effects of VPA treatment on HepG2 cells under normal glucose condition compared to galactose medium (energetic stress). Under energetic stress neither MTT nor AB was significantly changed in galactose media, with the exception for the high 2.0mM dose of VPA at 72 hours in the MTT assay. VPA treatment in glucose media caused increased cell death but cell population was maintained and together with the MTT and AB data suggests that the surviving cells have enough cytosolic enzyme activity to compensate for mitochondrial dysfunction up to 48 hours. This only applies to VPA concentrations of 0.5mM and 1.0mM and appears to be linked to glucose metabolism as both medium were supplemented with pyruvate and glutamine. After 48 hours of 2.0mM VPA treatment there was increased cytosolic enzyme activity but it was unable to compensate for OCR inhibition as there

is a significant reduction in ATP under both normal and energetic stress conditions.

What is not clear is the type of oxidoreductase co-factors involved in cytosolic reduction of MTT and AB, it may be NADH derived from glycolysis.

Results continued

3.2.8 VPA treatment does affect lipid accumulation in HepG2 cells

Patients treated with VPA have been found to suffer from a high incidence of Non-alcoholic fatty liver disease (NAFLD) and weight gain. NAFLD is defined as an excess of fat in the liver, where at least 5% of hepatocytes have accumulated lipid droplets exceeding 5%-10% of total liver weight (Leuf *et al.*, 2009). Several studies using *in vitro* cultures, human serum and rodent models have found VPA to inhibit β -oxidation and increased accumulation of triglycerides, (Huo *et al.*, 2014; Silva *et al.*, 2008; Zhang *et al.*, 2014). In this study Oil red O and BODIPY 493/503 staining was used to observe and quantify lipid droplets in HepG2 cells after 72 hours VPA treatment in normal and energetic stressed conditions.

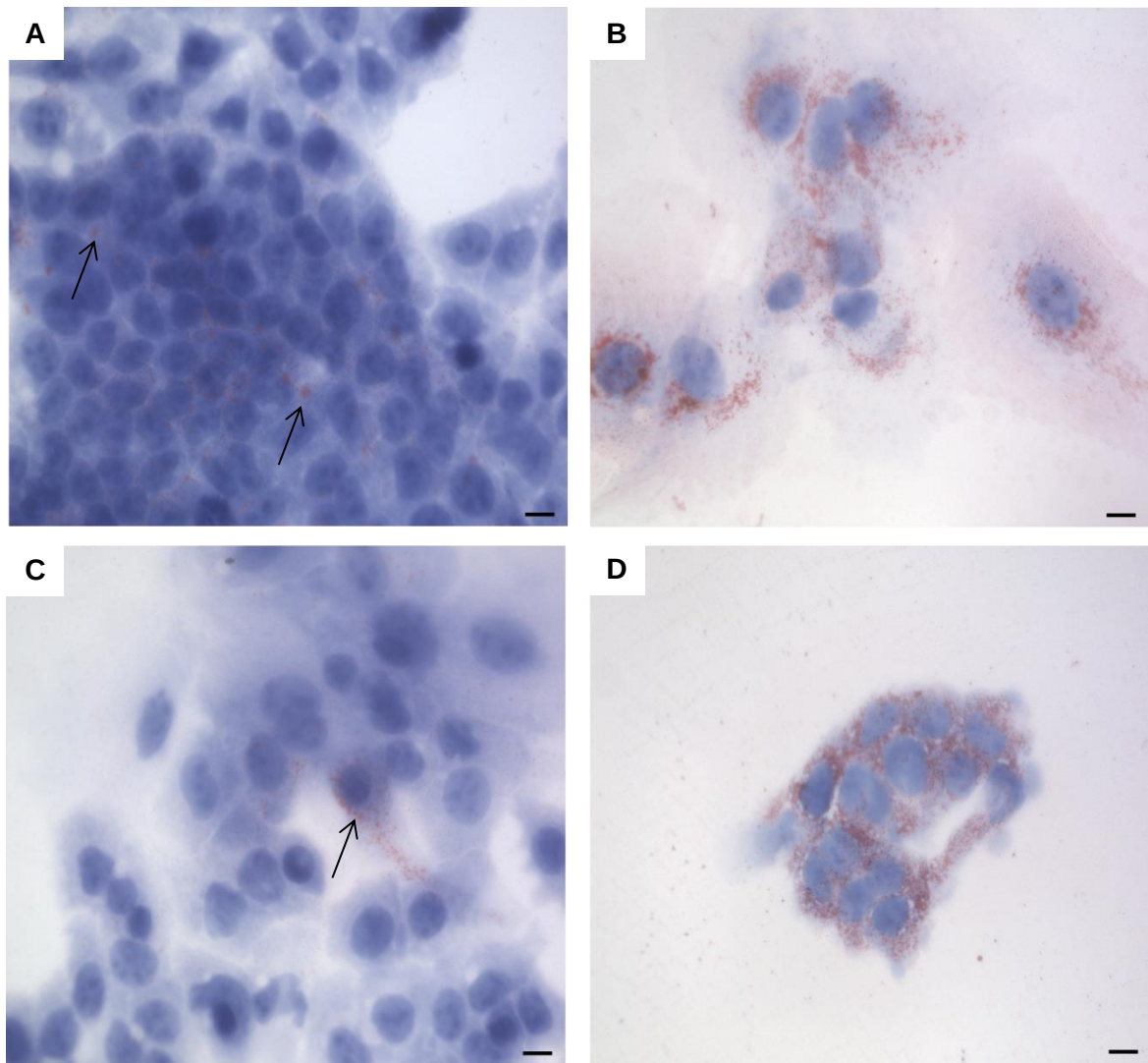


Figure 3.11. Lipid assessment in HepG2 cells after 2mM VPA treatment for 72 hours. control conditions, glucose (A) and 72 galactose (C) low levels of neutral lipids can be observed, arrows. After VPA 72 treatment in glucose (B) or galactose (D) there is increased Oil red O staining. Cells were fixed and stained with Oil red O. In Scale bar represent 10µm.

Long term treatment of high VPA concentration appeared to cause increased lipid accumulation in HepG2 cells as detected by Oil red O staining, figure 3.11.

Quantification of the lipid accumulation observed by microscopy using Oil red O was carried out by flow cytometry, using BODIPY lipid fluorescent marker. HepG2 cells

were treated for 72 hours with VPA, in glucose or galactose media. The cells were harvested and stained with BODIPY 493/503 prior to flow cytometry analysis.

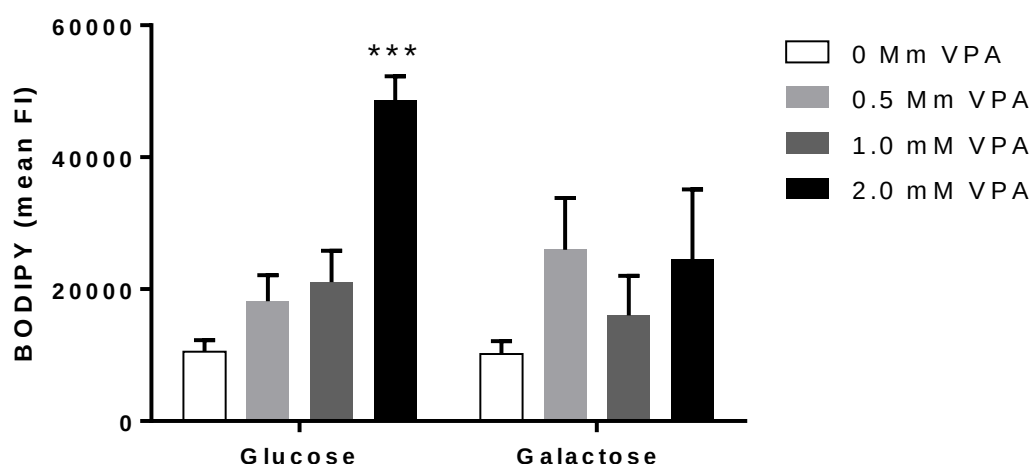


Figure 3.12. 72 hours VPA treatment increases lipid accumulation in HepG2 cells. The accumulation of neutral lipids measured by BODIPY 493/503 fluorescent (FI) intensity as detected by flow cytometry. Data represented as mean $\pm$ SD, n=3, ***p<0.001 ANOVA with Bonferroni correction when compared to baseline condition, glucose or galactose.

A significant increase in lipid accumulation was observed in cells treated for 72 hours with the highest concentration of VPA (2mM), figure 3.12. The 72 hour time point was used in further experiments to determine the effect of VPA on cellular lipid composition. HepG2 cells were seeded in 6 well tissue culture plates and treated with 2mM VPA for 72 hours in glucose or galactose media before harvesting. The cellular lipid content was determined by Leanne Hodson (OCDEM, University of Oxford) as outlined in the materials and methods section, 3.2.4.

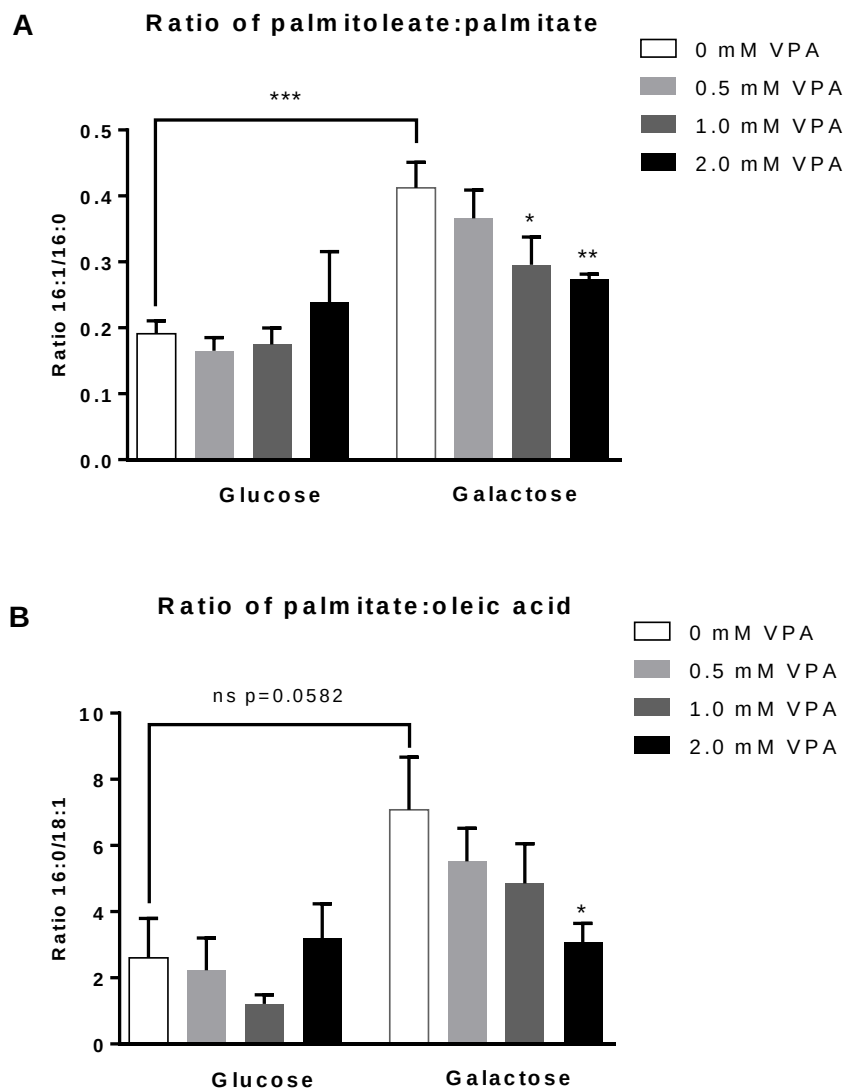


Figure 3.13. 72 hours of VPA treatment reduces fat desaturation and *de novo* lipogenesis in HepG2 cells. (A) The ratio of palmitoleate to palmitate was used to measure of desaturation. (B) The ratio of palmitate to oleic acid is a measure of *de novo* lipogenesis. Data normalised to total protein and presented as mean $\pm$ SD, $n=3$, ** $p<0.01$ *** $p<0.001$ ANOVA with Bonferroni correction when compared to baseline condition, glucose or galactose.

HepG2 cells incubated in galactose had significant increased levels of desaturation of saturated palmitate relative to the monounsaturated palmitoleate when compared to glucose media only. This increased desaturation observed in galactose was inhibited

by VPA in a concentration dependant manner, figure 3.13, (A). There was no effect observed in cells incubated glucose after VPA treatment. The conversion of palmitate to oleic acid was increased in HepG2 cell incubated in galactose media for 72 hours when compared to glucose media only, figure 3.13, (B).

3.2.8.1 Discussion Section – 3.3.6

VPA treatment in normal glucose conditions increases lipid accumulation in HepG2 cells

Several studies have reported that VPA inhibits mitochondrial beta-oxidation and increases levels of triglycerides (Bergriche *et al.*, 2011; Silva *et al.*, 2001). Therefore, it was not surprising to detect increased lipids after 72 hours of VPA treatment. However, what was interesting was that this only applied to cells treated in normal glucose conditions. There was no significant change in lipid content of cells treated with VPA under energetic stress condition. On further investigation into the types of triglycerides present in HepG2 cells it was revealed that the ratio of palmitoleate: palmitate was significantly increased in cells incubated under energetic stress compared to normal glucose condition. However, following 72 hours VPA treatment, in galactose medium, this was significantly reduced in a concentration dependant manner. HepG2 cells under energetic stress desaturate palmitate at a higher level than under normal glucose conditions. Furthermore, cells under energetic stress also have more *de novo* lipid glycogenesis than those in normal condition (5mM glucose). After 72 hours VPA treatment under energetic stress (galactose) this was reduced in a concentration dependant manner.

3.3 General chapter discussion

3.3.1 The effect of VPA treatment in HepG2 cells under normal and energetic stress conditions

HepG2 cells were used in this study as an *in vitro* model for mitochondrial toxicity as they contain numerous mitochondria are uniform in size and easy to culture. HepG2 cells in this study were always maintained in low glucose medium (5mM) as this was closer to physiological conditions than the traditional high glucose concentration (20-25mM) used for cell culture. The introduction of energetic stress condition by incubating cells with galactose for 24 and 48 hours did not alter OCR or cellular ATP levels. Previous studies have also demonstrated that HepG2 cells can maintain equivalent levels of ATP when cultured in galactose media after 24 hours incubation (Marroquin *et al.*, 2007). Interestingly, after 72 hours incubation in galactose, ATP levels were increased. This could be due to demand for ATP to maintain the cell population as increased cell death was observed under galactose condition but the relative number of cells was maintained. To compensate for the lack of glucose and the breakdown of galactose to glucose-1-phosphate is relatively, slow the cells are probably producing ATP via glutamine metabolism (Gohil *et al.*, 2010), figure 3.14. Additionally, there was no significant change in lactate at baseline glucose when compared to galactose. This is curious as one would expect lactate to be higher in the glucose incubated cells as HepG2s are highly glycolytic and therefore dependant on glycolysis for adequate ATP production. However, changes in lactate do not necessarily equate to changes in glycolysis as pyruvate can be oxidised from malate and then be reduced to lactate. Glutamine as well as glucose can be a source for pyruvate with both driving the TCA cycle and OXPHOS to maintain ATP (Lunt and Vander Heiden, 2011). In addition to feeding into the TCA cycle via glutamate,

glutamine can also be converted into pyruvate in the cytosol, figure 3.15. Please note, the ATP assay used in this study, measured total cellular ATP and not ADP/ATP ratio.

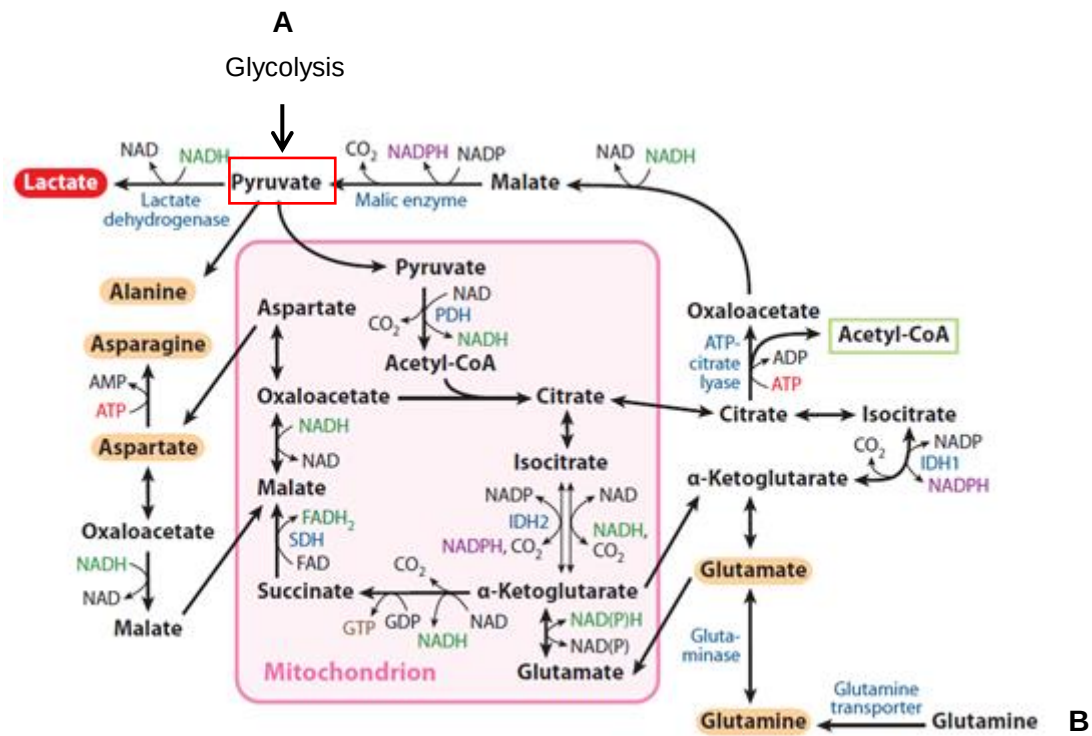


Figure 3.15. The fate of pyruvate and glutamine. The glycolytic pathway produces two pyruvate molecules (A). Pyruvate (red box) can either cross into the mitochondrion where it will enter the TCA cycle or it can be reduced to lactate in the cytosol. Pyruvate can also be transaminated to alanine. Extracellular glutamine enters the cell's cytosol where it can be converted to glutamate. Glutamate can be converted to α-ketoglutarate in the mitochondrion or in the cytosol (B). Adapted from Lunt and Vander Heiden, 2011.

3.3.1.1 VPA treatment cause mitochondrial dysfunction in HepG2 cells

When HepG2 cells were treated with VPA for 24 hours at concentrations of 0.5, 1.0 and 2.0mM, under both normal glucose (5mM) and energetic stress conditions (10mM galactose), there was no change in OCR but a reduction in ATP was detected in all drug concentrations. After 48 hours of 2.0mM VPA treatment there was significant reductions observed in OCR, ATP and lactate, table 3.2. Concentrations of 0.5mM and 1.0mM VPA reduced OCR which suggests that the electron transport chain (ETC) had been compromised but not to a level where the ATP pool was affected. The HepG2 cells under energetic stress for 48 hours and treated with 0.5mM and 1.0mM VPA were able to maintain the ATP pool either through glycolysis or by simply using less ATP as an adaptive response. Our studies are consistent with earlier findings which have shown that VPA inhibits OXPHOS and ATP synthesis in isolated rat mitochondria (Ponchaut *et al.*, 1993; Louis *et al.*, 2007) at drug concentrations of 0.5mM and 1.0mM. Mechanistically, VPA has been shown to inhibit α -ketoglutarate and pyruvate driven OXPHOS. VPA inhibits the E3 subunit (dihydrolipoyl dehydrogenase) of pyruvate dehydrogenase complex and α -ketoglutarate dehydrogenase (Louis *et al.*, 2007; Silva *et al.*, 1997), potentially inhibiting TCA cycle activity causing a reduction in available FADH, NADH and H^+ needed for optimal electron transport chain function.

Table 3.2. Summary of cellular response of HepG2 cells after VPA treatment. The arrows indicate where a significant response occurred relative to glucose baseline.

↓ significant reduction, ↑ significant increase, ↔ response not significant, – baseline glucose. Glu, normal glucose condition (5mM). Gal, energetic stress (10mM galactose) condition. AB, Alamar Blue.

	VPA conc. (mM)	OCR		ATP		Lactate		AB		Relative cell nos.		% cell death	
		Glu	Gal	Glu	Gal	Glu	Gal	Glu	Gal	Glu	Gal	Glu	Gal
24 hours	0.0	–	↔	–	↔	–	↔	–	↔	–	↔	–	↔
	0.5	↔	↔	↓	↓	↔	↔	↔	↔	↔	↔	↑	↔
	1.0	↔	↔	↓	↓	↔	↔	↔	↔	↔	↔	↑	↔
	2.0	↔	↓	↓	↓	↔	↔	↔	↔	↔	↔	↑	↔
48 hours	0.0	–	↔	–	↔	–	↔	–	↔	–	↔	–	↑
	0.5	↓	↓	↔	↔	↔	↔	↔	↔	↔	↔	↑	↔
	1.0	↓	↓	↔	↔	↔	↔	↔	↔	↔	↔	↑	↔
	2.0	↓	↓	↓	↓	↓	↓	↑	↔	↓	↔	↑	↔
72 hours	0.0	–	↔	–	↑	–	↔	–	↔	–	↔	–	↑
	0.5	↔	↔	↓	↔	↔	↔	↔	↔	↓	↔	↑	↑
	1.0	↔	↓	↔	↔	↔	↔	↑	↔	↓	↓	↑	↑
	2.0	↓	↓	↔	↓	↔	↔	↑	↔	↓	↓	↑	↑

3.4 Research highlight

- VPA treatment under normal and energetic stressed conditions resulted in mitochondrial dysfunction by inhibiting mitochondrial respiration.
- VPA treatment in physiological glucose concentration caused more cell death than treatment in stressed conditions.
- VPA treatment in glucose caused more lipid accumulation than under energetic stress conditions.

3.5 Limitations of this study

In this study I used the basal cellular oxygen consumption rates only, and did not take into account the maximum or spare respiratory capacity of the HepG2 cells. To establish these quantities, the additions of mitochondrial respiratory modulators are needed. The OCR measurements were not carried out in the presence of oligomycin (ATPase inhibitor), FCCP (mitochondrial uncoupler), antimycin A (complex III inhibitor) and therefore do not represent just OCR of mitochondrial origin nor does it take into account the role of MPTP (mitochondrial permeability transition pore) opening in circumstances where an decreased OCR was observed.

Chapter 4

**Development of a high throughput assay for
the detection of mitophagy.**

4 Development of a high throughput assay for the detection of mitophagy.

4.1 Introduction

The mitochondria's function goes far beyond the classical role of ATP production and energy metabolism. The organelle plays central roles in cell survival, signal transduction and redox homeostasis (Liesa and Shirihai, 2013). Mitochondrial fusion and fission events underpin mitochondrial network dynamics. In eukaryotic cells, mitochondrial numbers or mass and morphology are influenced by cellular needs. It has become clear that the regulation of mitochondrial morphology and mass is of great importance to cellular physiology in health and disease (Shirihai, Song and Dorn, 2015). The balance between biogenesis and degradation of the mitochondria is intricately regulated. The term mitophagy refers to the removal and recycling of damaged and unhealthy mitochondria and it is a form of quality control for the mitochondrion (Rugarli and Langer, 2012).

One of the key research questions regarding mitophagy is when stressful events occur such as nutrient deprivations and chemical assaults, is it beneficial to have more or less mitophagy? Is it better to have dysfunctional mitochondria generating more ROS but maintaining ATP production or fewer better functioning mitochondria with less ROS but also less ATP production, figure 4.1?

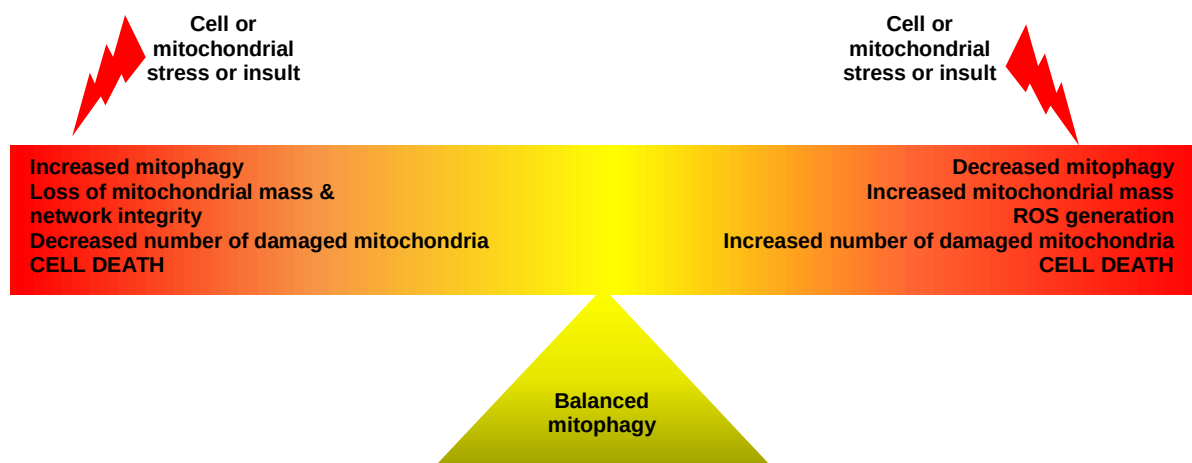


Figure 4.1. Preserving optimal mitochondrial function whilst maintaining cellular homeostasis. Maintaining a balanced level of mitophagy is essential for the survival of cells after an insult or stress such as exposure to chemical or in starvation. Increased mitophagy could lead to irrecoverable loss of mitochondrial mass which may lead to reduction of ATP. This would lead to a decrease in the number of damaged mitochondria but may also lead to cell death if the cell is unable to maintain ATP. Equally decreased mitophagy would increase mitochondrial mass thus resulting in accumulation of damaged mitochondria and potentially increasing ROS mediated cell death.

Mitophagy and mitochondrial biogenesis act together and are dependent on the coordinated expression of nuclear and mitochondrial encoded genes. Mitochondrial quality control depends on the level of mitophagy or autophagy, biogenesis and the fusion and fission of the network, as discussed earlier (chapter 1.8). Mitophagy is a heterogeneous cellular event with different degrees of mitophagy events occurring at the same time depending on the levels of mitochondrial dysfunction in specific mitochondria (Klionsky *et al.*, 2012). At present there are no reliable quantitative methods for specifically monitoring mitophagy in cell lines that have not been genetically modified i.e. overexpression of GFP- tagged proteins linked to the mitophagy pathway (Melser *et al.*, 2013). Current methods for detecting autophagy and

mitophagy rely on immunoblot analysis of hallmarks of autophagy such as LC3, in conjunction with fluorescent microscopy and electron microscopy (Klionsky *et al.*, 2012). These microscopy methods are laborious and counting co-localisation of LC3 and mitochondria by eye can be very subjective. In a recent publication by Diot *et al.*, (2015), we showed that the high throughput fluorescent imaging system IN Cell Analyzer (GE Healthcare) was a robust method for the detection and quantification of autophagy and mitophagy. In this publication, the IN Cell method was used to assess levels of autophagy by detection of fluorescent LC3 puncta, indicating autophagosomes. To monitor mitophagy events, the co-localisation between LC3 punctae and the fluorescent signal of the mitochondria was assessed.

The aim of this chapter was to develop an assay for detecting mitophagy using a 96 well tissue culture plate based high throughput fluorescent microscopy, IN Cell method. Several known pharmacological modulators of mitophagy, such as phenanthroline, were used (Park *et al.*, 2012). Additionally, the effect of sodium valproate was also investigated in comparison to known modulators of mitophagy.

4.2 Materials and Methods

4.2.1 Autophagy/mitophagy Assay

Cells were seeded in 96 well plates at various concentrations depending upon the cell type. Human fibroblasts were seeded at 2,500 cells per well while HepG2 at 1,500 cells per well and left over night to adhere before treatments commenced. After treatment plates were washed with warm PBS x1, and then fixed with 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature. The plate was then washed with PBS twice, adding 100µl PBS after last wash. Plates were stored at 4°C in the dark until required, up to a maximum of one week before staining. The plates were incubated overnight at 4°C with the following primary antibodies: fibroblasts and HepG2 cells, for mitochondrial visualisation mouse TOM20 (1:200, Santa Cruz Biotechnology). For lysosome visualisation, rabbit anti-LC3 (1:500, MBL International) was used for all cell types. Primary antibodies incubations were in 80µl permeabilisation buffer 1x (eBioscience) and plates were left on a rocker at slow speed. For the fluorescent secondary antibodies, plates were washed three times at 5 minutes with PBS and incubated with: anti-mouse Alexa Fluor® 594 (1:200, Life Technologies) and anti-rabbit Alexa Fluor® 488 goat antibodies (1:500, Life Technologies) in 80µl permeabilisation buffer 1x, on rocker at room temperature. Plates were kept in the dark throughout the staining procedure. Plates were then washed three times in PBS for 5 minutes each time. DAPI (5mg/ml) was used as nucleic stain, 1:1000 in PBS followed by previous PBS washing regime. Plates stored, in the dark at 4°C until imaging using the In Cell Analyzer 1000. Imaging took place within 48 hours of staining.

4.2.2 Image acquisition

The plates were imaged using IN Cell Analyzer 1000 (GE Healthcare) which has a epifluorescent 100w xenon lamp, Nikon microscope and a high-resolution CCD (charge-coupled device) camera. The images were captured at 250 or 500 cells per well, within 9 or 12 fields of view placed at the centre of the well, using a x20 objective. Images were acquired in fluorescent channels as follows; DAPI, Ex360/Em460. LC3, Ex480/Em535. TOM20, Ex535/Em600. The images were saved as TIF files.

4.2.3 Image processing

The true power of this technique is the speed at which images can be acquired, followed by image processing using the high-content image analysis software IN Cell Developer Toolbox v1.6 (GE Healthcare). This software enables the user to analyse the image stack acquired as it offers a range of algorithms covering a range of target areas i.e. nucleus, vesicles and cell morphology. For the detection of autophagy and mitophagy the target objects of interest were the nucleus, mitochondria and autophagosomes. However, correct and careful segmentation of cells, mitochondria and autophagosomes from the acquired images is of outmost importance and will be described in section 4.3.2.1. The accuracy of segmentation parameters will define the overall success of the experimental analysis, thus the quality of data obtain from each experiment and subsequent replicate experiments

Image segmentation by binarisation- finding the objects of interest

Image segmentation is the most important step of the image analysis. The purpose of segmentation is to separate the targeted objects of interest such as nucleus, mitochondria and autophagosomes, from each other and from their background. There are two basic criteria that need to be balanced, the kernel size (number of pixels detected) and the sensitivity of the segmentation (object's intensity relative to background).

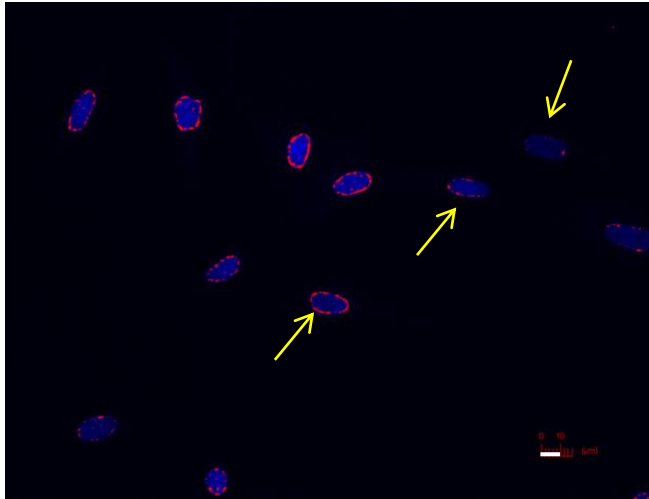
The data output is affected by several factors such as the kernel size, sensitivity and intensity threshold. The kernel size is defined as a size of 3 which is the smallest 3x3 group of pixels, where the centre pixel would be replaced by a value computed using the eight pixels that surround it. If the kernel size is too small the target of interest may be excluded, figure 4.2 (A). By increasing or decreasing the kernel size the area of targeted object (in this example the nucleus) is manipulated and it is imperative that the kernel size selected allows to cover at least the periphery of the object of interest, figure 4.2 (B and C). Increasing the kernel size results in a larger area of the targeted object being highlighted as the number of pixels is increased. In the example, figure 4.2, the ideal kernel size is illustrated in panel C, where the whole of the targeted object is covered.

The parameter for sensitivity allows the user to alter the segmentation threshold as the efficiency of the segmentation is affected by the target's (i.e. nucleus and mitochondria) intensity (fluorescence) relative to background, figure 4.3. If there is a poor signal from the object of interest in relation to background or the sensitivity threshold is too low, then the software cannot successfully detect the object as a valid target, figure 4.3 (A). By increasing the sensitivity more of the targeted object is found, figure 4.3 (B and C). The optimum sensitivity is illustrated in panel C. For a successful

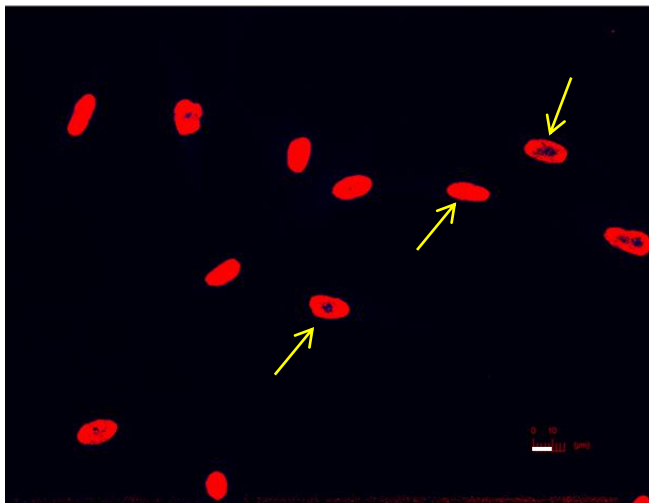
segmentation a balance between kernel size and sensitivity must be found for each targeted object.

In summary, the kernel size and segmentation sensitivity parameters searches for objects (targets) within the captured image and if they are too low or too high will leave holes in the targeted object or pick up too much background. Hence, it is important to be consistent when setting up the parameters.

A)



B)



C)

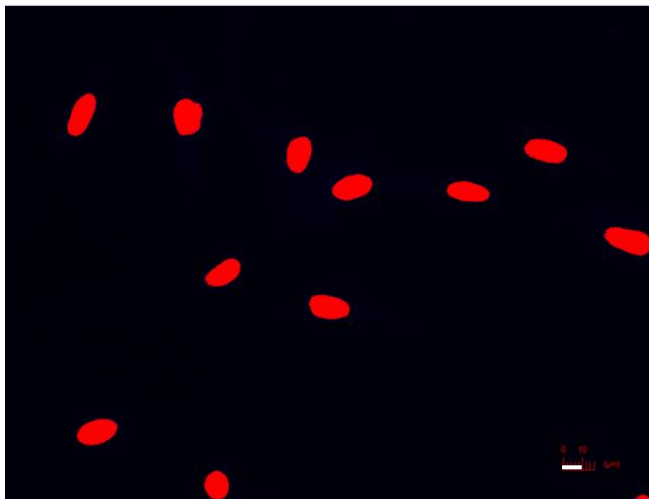


Figure 4.2. The effect of kernel size. Illustration of changes of kernel size altering the size of kernel will affect the size of the objects being highlighted for segmentation. The object in the image is nucleus, stained with DAPI.

A) Kernel size of 5 is too small as large area of the nucleus is not highlighted. Yellow arrows denotes excluded target for segmentation.

B) Kernel size 9 covers the nucleus periphery and most objects are highlighted and targets for segmentation. Yellow arrows denotes excluded target.

C) Kernel size 15 highlights the whole nucleus enabling correct segmentation. All object of interest are highlighted and are therefore valid targets.

In this illustration the sensitivity has been keep at the same level (90) and the kernel size changed. Scale bar 10 μ m.

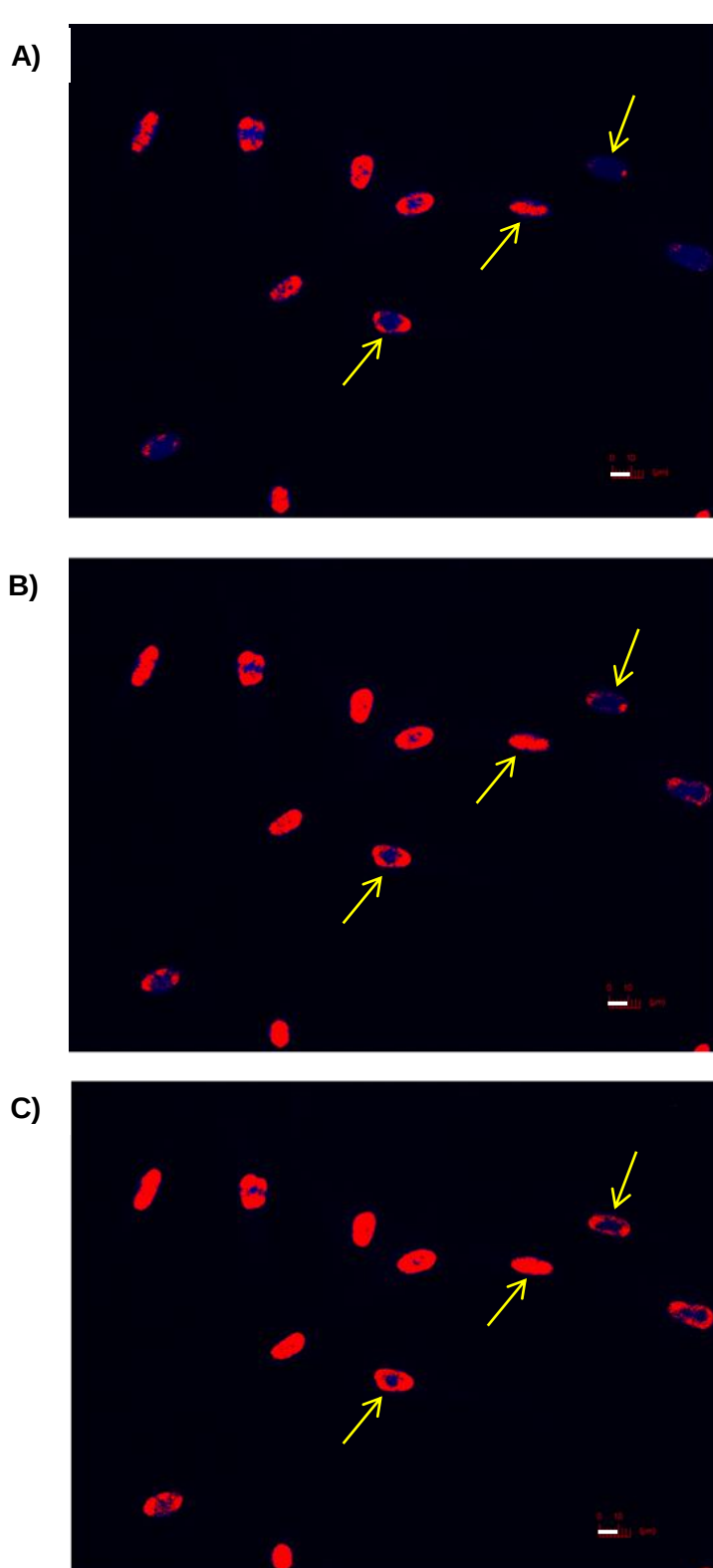


Figure 4.3. Segmentation criteria, the effect sensitivity. Illustration of how a change in sensitivity changes the targeted object validation for segmentation.

A) Low sensitivity of 10 will exclude objects, yellow arrow.

B) Medium sensitivity of 50 will exclude some objects

C) Higher sensitivity of 90 highlights the objects periphery and are valid targets for segmentation.

In this illustration the kernel size has remained at 15 and the sensitivity has been altered, Scale bar 10µm.

The kernel size and segmentation sensitivity will be different for each targeted object, a larger object such as the nucleus will have a larger area than autophagosomes and the mitochondria. The aim is to highlight as much of each objects as possible without increasing the background noise, as illustrated in figures 4.2 (C) and 4.3 (C). It is of outmost importance that the staining procedure is optimised to retain the robustness of the analysis.

A signal intensity threshold is applied to the individual objects (nucleus, mitochondria and autophagosomes), range 0 to 4095. If the intensity threshold is too low the object's signal is lost and if too high, too much signal is picked up, figures 4.4 (A and B). The optimum intensity threshold is illustrated in figure 4.4 (C). Once the intensity threshold has been set, the image is further processed by clump breaking and individual cells can then be seen, figure 4. 4 (D). In table 4.1 is an over view of the values used for kernel, sensitivity and intensity threshold for the detection of nuclei, autophagosomes and co-localisation with mitochondria.

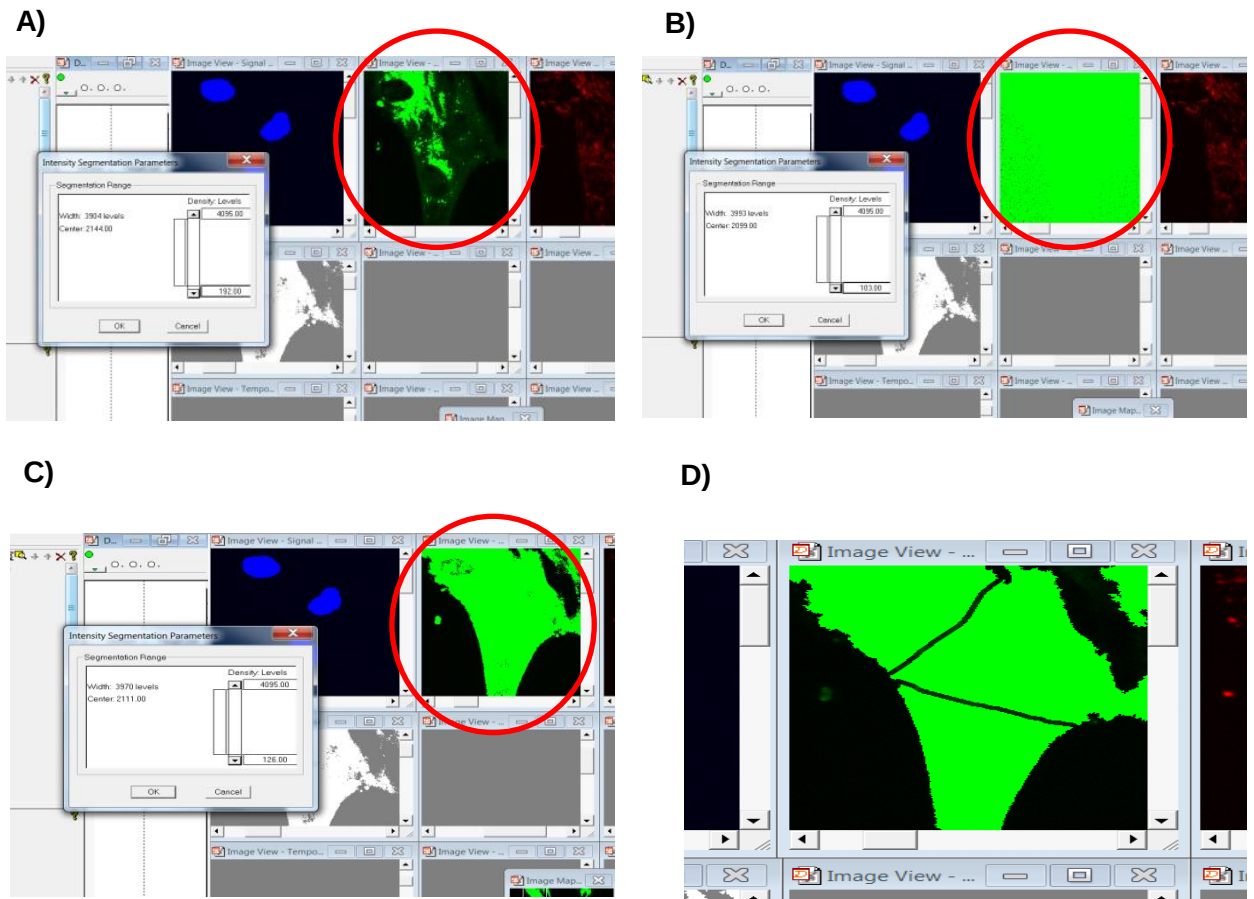


Figure 4.4. Segmentation of cell boundaries. Screen shot of the Developer Toolbox output, illustrating cell segmentation based on the LC3 signal back ground signal (A) Too low intensity threshold were signal is lost and (B) too bright. (C) Optimum intensity as all the cells' area is shaded green. (D) Cell segmentation by a declumping process.

Table 4.1. Parameters set for the detection of nuclei, mitochondria, autophagosomes and cell segmentation for fibroblasts cells. The nuclei have a larger area and therefore higher kernel size and sensitivity than the smaller objects such as mitochondria and autophagosomes. The cell boundary was first determined by using the LC3 back ground signal, applying the grey scale.

Stain	Target	Mean ($\pm$ s.d)	Mean intensity threshold ($\pm$ s.d)
DAPI	Nuclei	Kernel size: 39.33 ($\pm$ 1.51) Sensitivity: 51.50 ($\pm$ 7.01)	718.9 ($\pm$ 0)
Tom20	Mitochondria	Kernel size: 3 ($\pm$ 0) Sensitivity : 83.57 ($\pm$ 1.51)	2.76 ($\pm$ 0.62)
LC3	Autophagosome	Kernel size: 3 ($\pm$ 0) Sensitivity: 22 ($\pm$ 3.85)	3.23 ($\pm$ 0.73)
LC3 Background signal	Cell boundary	N/A	Intensity threshold, grey scale min. 1008 ($\pm$ 0.65) max. 4095 ($\pm$ 0)

Mean values from six independent experiments $\pm$ standard deviation.

Any changes to the above settings, table 4.1 were kept at a minimum. Due to stringent adherence to staining protocol followed by consistent segmentation parameters the standard deviation of the values are low. Examples of different object are illustrated in figure 4.5 for cell segmentation and autophagosomes and figure 4.6 for mitochondrial segmentation. The resulting segmentation is shown with multi-coloured mask, each colour corresponding to one target (nucleus, autophagosomes, mitochondria and cell boundary), figure 4.7.

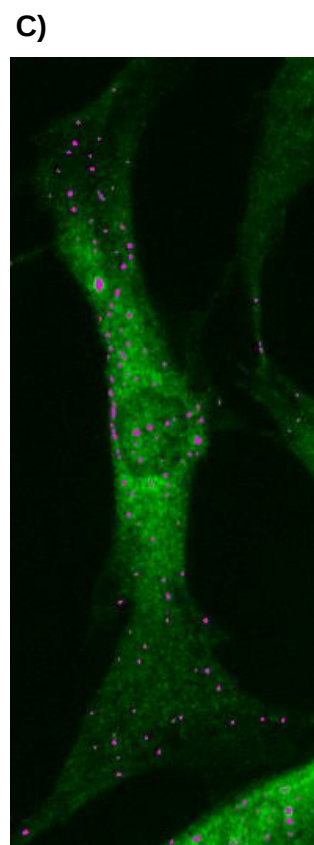
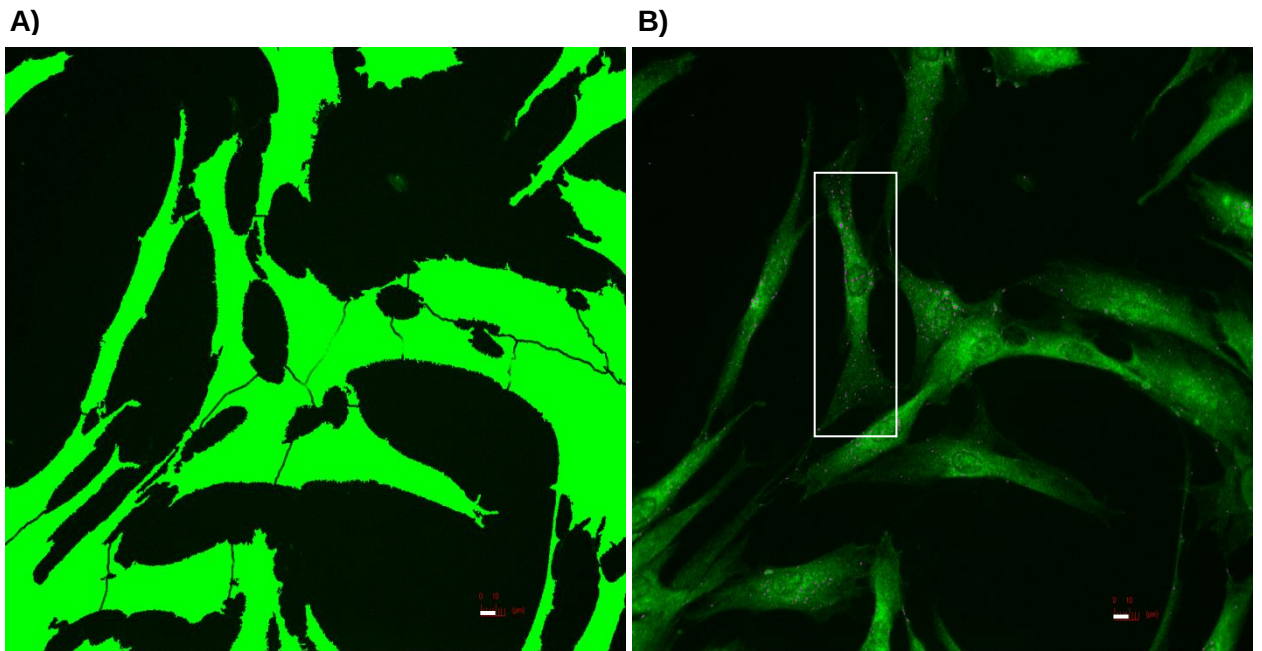


Figure 4.5. Illustration of segmentation of autophagosomes and cell boundary in control fibroblast. A) The segmentation of cell boundary is based on the background of the LC3 signal. B) and C) segmentation of autophagosomes highlighted in purple. Scale bar 10μm.

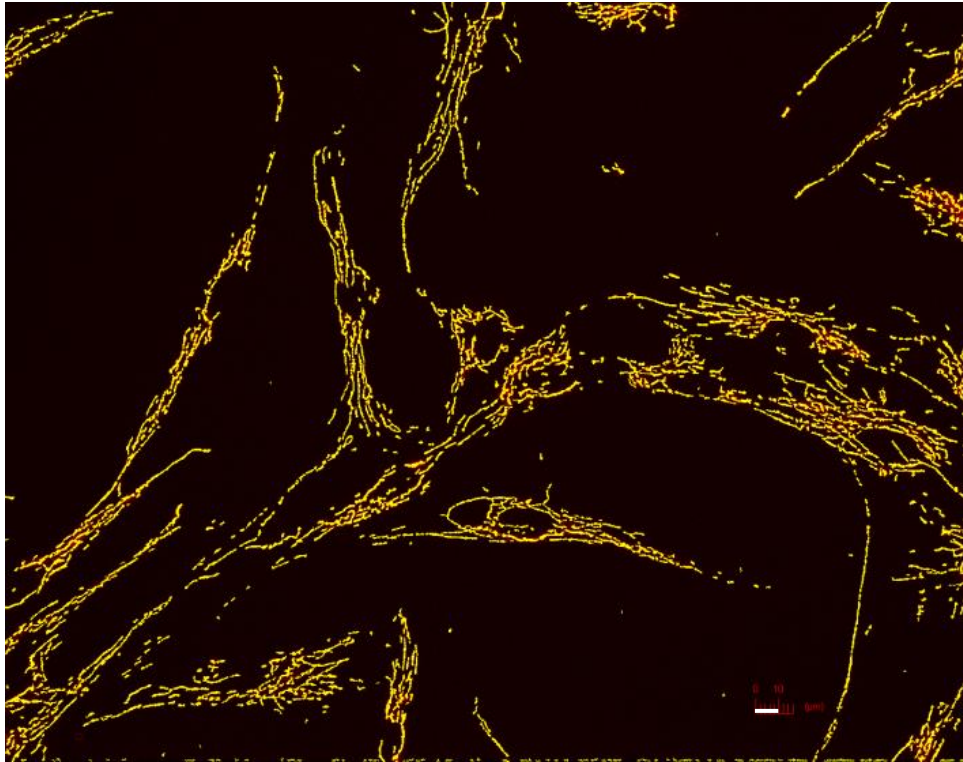


Figure 4.6. Illustration of segmentation of mitochondria. Yellow overlay represent mitochondria. This segmentation is used to calculate the total mitochondrial area per cell area and bifurcations.

Using the method illustrated in figure 4.6, the software can calculate the total area of mitochondria per cell and bifurcations.

From the segmentation of the different objects (targets) of interest a mask can be superimposed over the images, enabling the user to check that the segmentation parameters selected were correct, figure 4.7.

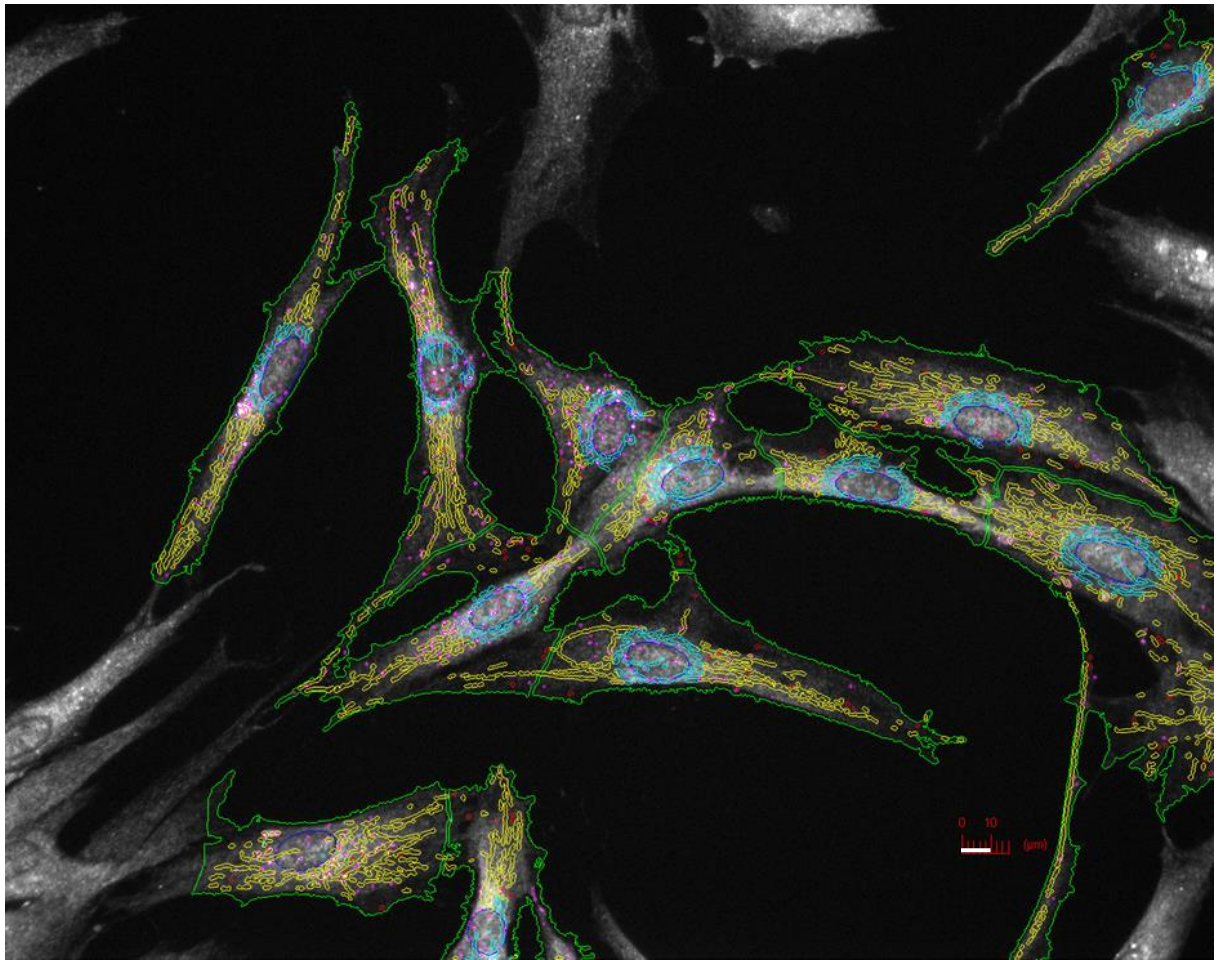


Figure 4.7. Segmentation of objects in control fibroblasts. Images are acquired in three different fluorescent channels; DAPI (nuclei), FITC (autophagosomes, LC3) and TRITC (mitochondria, TOM20). Once all the segmentation criteria have been set, a mask can be applied, enabling the illustration of the selected parameters, where green is cell boundary, dark blue is nuclei and light blue the peri-nuclear mitochondria. Yellow is long mitochondria and red, short mitochondria. Scale bar 10μm.

Most cells located on the periphery of the field of view will not be included, figure 4.7, as they may not contain a whole nucleus. After all the above parameters are established the user can generate a bespoke report. Within the report further parameters can be set which exclude incomplete cells as a valid target. For a target (cell) to be valid the cell must contain; whole nucleus, one LC3 positive punctae, one long mitochondria (>1.5μm) and one short mitochondria (<1.5μm) and a mitophagy event (co-localisation

of TOM20 and LC3). This can be reported as per cell, per field of view or per well and include statistical summaries. Furthermore, additional information can be requested such as number of cells with whole nuclei, number of perinuclear mitochondria, area of cell, mitochondria and autophagosomes. The software will apply all the set values of all the parameters to all the captured images in the image stack and produce a report in an Excel format for further analysis by the user. Should there be any anomalies within the data generated the user can then go back to the original images to check for such as cell clumping or poor staining.

4.2.4 Cell lines used for the treatment with modulators of mitochondrial morphology and the autophagy pathway.

To validate the IN Cell method for detecting autophagy and the co-localisation of autophagosomes with mitochondria (mitophagy), two control fibroblast cell lines were select, one aged 20 years (control 1) and the other aged 1 year (control 8).

Furthermore, HepG2 cells were also used to investigate if the response observed in fibroblast was cell type specific.

The cell lines were incubated with several established modulators of mitochondrial network integrity such as phenanthroline and starvation which causes network fragmentations (Park *et al.*, 2012) and induce autophagy/mitophagy. E64D and pepstatin A (E&P) treatments and bafilomycin A1 inhibit the autophagy pathway by preventing the lysosomal degradation of its cargo, please see table 4.2 for an overview of compounds used.

Table 4.2. Summary of the compounds used to either induce or inhibit the autophagic pathway.

Compound	Concentration and duration	Mode of action	Effect on autophagosomes levels
Bafilomycin A1	5 μ M over night	Inhibitor of lysosomal ATPase, the acidification of lysosomes, prevents fusion by autophagosomes with lysosomes	↑
Carbonyl cyanide m-chlorophenyl hydrazine (CCCP)	Range 5-20 μ M 4 hours	OXPHOS uncoupler, mitochondrial membrane depolarisation	↑
Colchicine	2 ng/ml 24 hours	Inhibits formation of microtubules, reduced membrane potential	↑
Chloroquine	25 μ M over night	Elevate and neutralise the lysosomal pH	↑
E64d & Pepastatin A (E&P)	10 μ g/ml over night	Acid protease inhibitors	↑
Oligomycin A	0.5 μ M 30 minutes	Inhibitor of OXPHOS via ATP synthase	↑
1,10 Phenanthroline (Phen)	10 μ M 24 hours	Mitochondrial network fragmentation	↑
Rapamycin A	100 nM 24 hours	Inducer of autophagy via mTOR pathway	↑
Trehalose	100 mM 24 hours	Inducer of autophagy independent of mTOR	↑
3-Methyladenine (3-MA)	5 mM 4 hours	Early stage inhibitor of the autophagic pathway	↓

The compounds listed in table 4.2 and used in the validation experiments are described in more detail in the introduction chapter (1.4).

In addition to chemical modulators, cells were also subjected to a 2 hour starvation by replacing the glucose medium with Hank's balanced salt solution (HBSS). Energetic stress was induced by incubation the cells in glucose-free galactose (10mM) medium for 24 hours.

4.3 Results

4.3.1 *Evaluating the response to treatment with modulators of mitochondrial morphology, autophagy and mitophagy in control fibroblasts.*

The fibroblast control cells were treated with various modulators of mitochondrial morphology and the autophagy pathway as described above, section 4.3. Based on the segmentation criteria as described in the previous section 4.2, the summed area of autophagosomes was assessed and the area reported as percentage of cell area, figure 4.8. All treatments were carried out in 25mM glucose. Following treatment with E&P and bafilomycin A1, both control cell lines showed increased levels of autophagosomes. This is expected as these compounds are inhibitors of late stage of the autophagy pathway and thus will reduce the degradation of the autophagolysosome. Control 8 showed significant increase in autophagosomes after treatment with colchicine. Colchicine prevents the fusion between lysosomes and autophagosomes thus creating a block of the autophagy pathway. Inducers of autophagy such as rapamycin A and trehalose had no significant effect on levels of autophagosomes, in high glucose medium (25mM), figure 4.8.

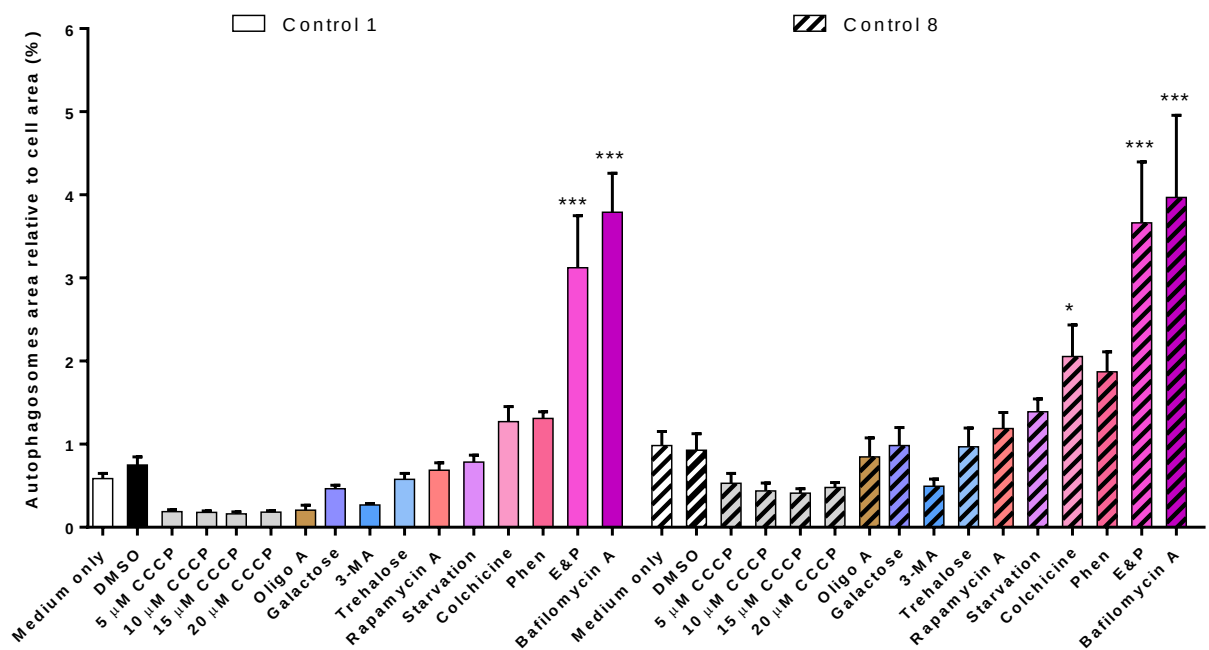


Figure 4.8. The IN Cell method for the detection of autophagosomes in control fibroblasts treated with modulators of autophagy. After treatment the levels of autophagosomes were detected based on the LC3 signal. The treatments were as follows: CCCP for 4 hours, 5mM 3-MA 4 hours, galactose 24 hours, 100nM trehalose 24 hours, 100nM rapamycin A and starvation (HBSS) 2 hours, 2ng/ml Colchicine 24 hours, 10uM 1,10 phenanthroline (phen) 24 hours, 10ng/ml E&P (E64D+pepstatin A) overnight, 0.5μM oligomycin A (oligo A) 30 minutes and 5μM bafilomycin A1 overnight. Data reported as percentage autophagosomes relative to cell area and represent the mean $\pm$ SD, ≥ 500 cells/experiment analysed, $n=3$. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$, ANOVA with Bonferroni correction, compared to medium only or DMSO. DMSO was used as vehicle control for CCCP, 3-MA, oligo A, bafilomycin A1, E&P, oligomycin A and rapamycin A.

In order to determine if the increase in autophagosomes resulted in increased co-localisation with mitochondria and resulting in potential mitophagy events, the co-localisation between TOM20 (mitochondria) and LC3 (autophagosomes) signals (using segmented images) were determined. The data is expressed as percentage co-

localisation of mitochondria and autophagosomes relative to total mitochondrial area

figure 4.9.

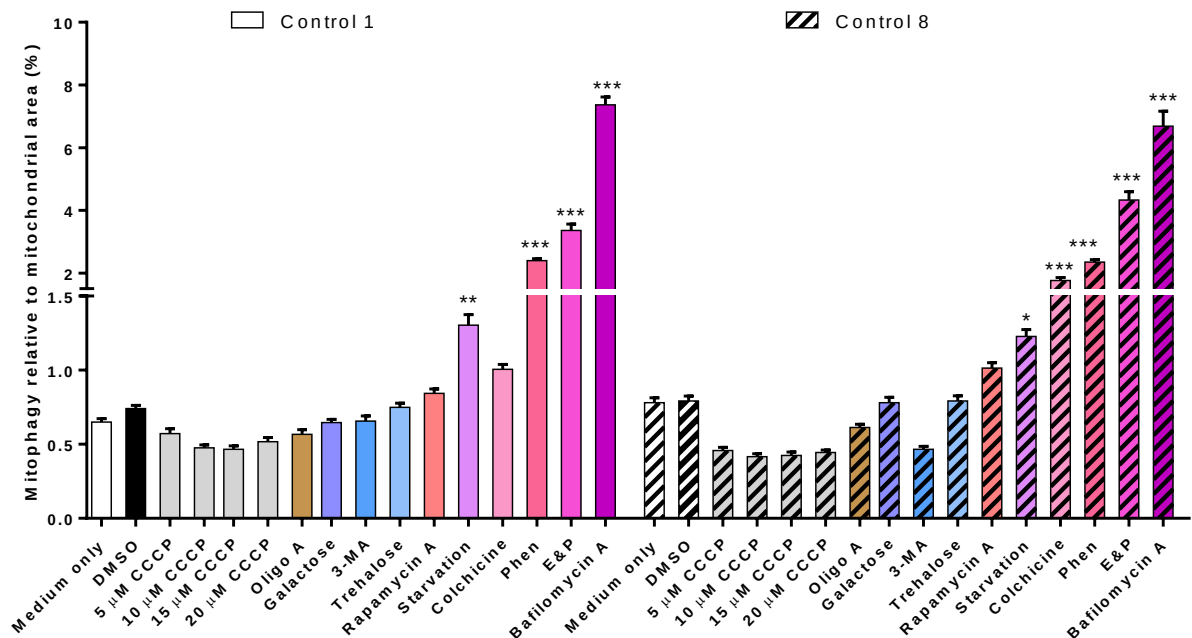


Figure 4.9. The IN Cell method for the detection of co-localisation between autophagosomes and mitochondria (mitophagy) in control fibroblasts after treatment with modulators of autophagy. After treatment the levels of autophagosomes co-localising with the mitochondria was detected based on the LC3 signal and TOM20 signal. Mitophagy is presented as proportion of the total mitochondrial area. Phen, phenanthroline, E&P, E64D+pepstatin A, oligo A, oligomycin A. Data represent the mean $\pm$ SD, ≥ 500 cells/experiment analysed, $n=3$. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$, ANOVA with Bonferroni correction, compared to medium only or DMSO. DMSO was used as vehicle control for CCCP, 3-MA, bafilomycin A1, E&P, oligo A and rapamycin A.

The co-localisation between autophagosomes and mitochondria increased after treatment with colchicine, E&P and bafilomycin A1, figure 4.9. This was not surprising due to the high levels of autophagosomes detected. Curiously, the classical mitophagy

inducer CCCP had no significant effect on the autophagosomes (figure 4.8) or the co-localisation detected. Both fibroblast controls had increased co-localisation after starvation and treatment with phenanthroline however these treatments did not elicit increased levels of autophagosomes. After phenanthroline treatment the autophagosomes co-localised to the mitochondria indicating the occurrence of mitophagy. Previous studies have found phenanthroline induces mitophagy in HeLa cells (Park *et al.*, 2012). As expected 3-methyladenine did not increase the levels of autophagosomes as this is an early inhibitor of the autophagy pathway (Wu *et al.*, 2009).

To investigate the effect treatments may have had on the mitochondrial morphology, the mitochondrial length and the number of three-way intersections (Y), bifurcations of the network was evaluated, figure 4.10 (A). The more interconnected the network the more bifurcations are expected. The number of bifurcations was detected by segmenting the image and is presented as a mean arbitrary unit (a.u) per cell, figure 4.10 (B).

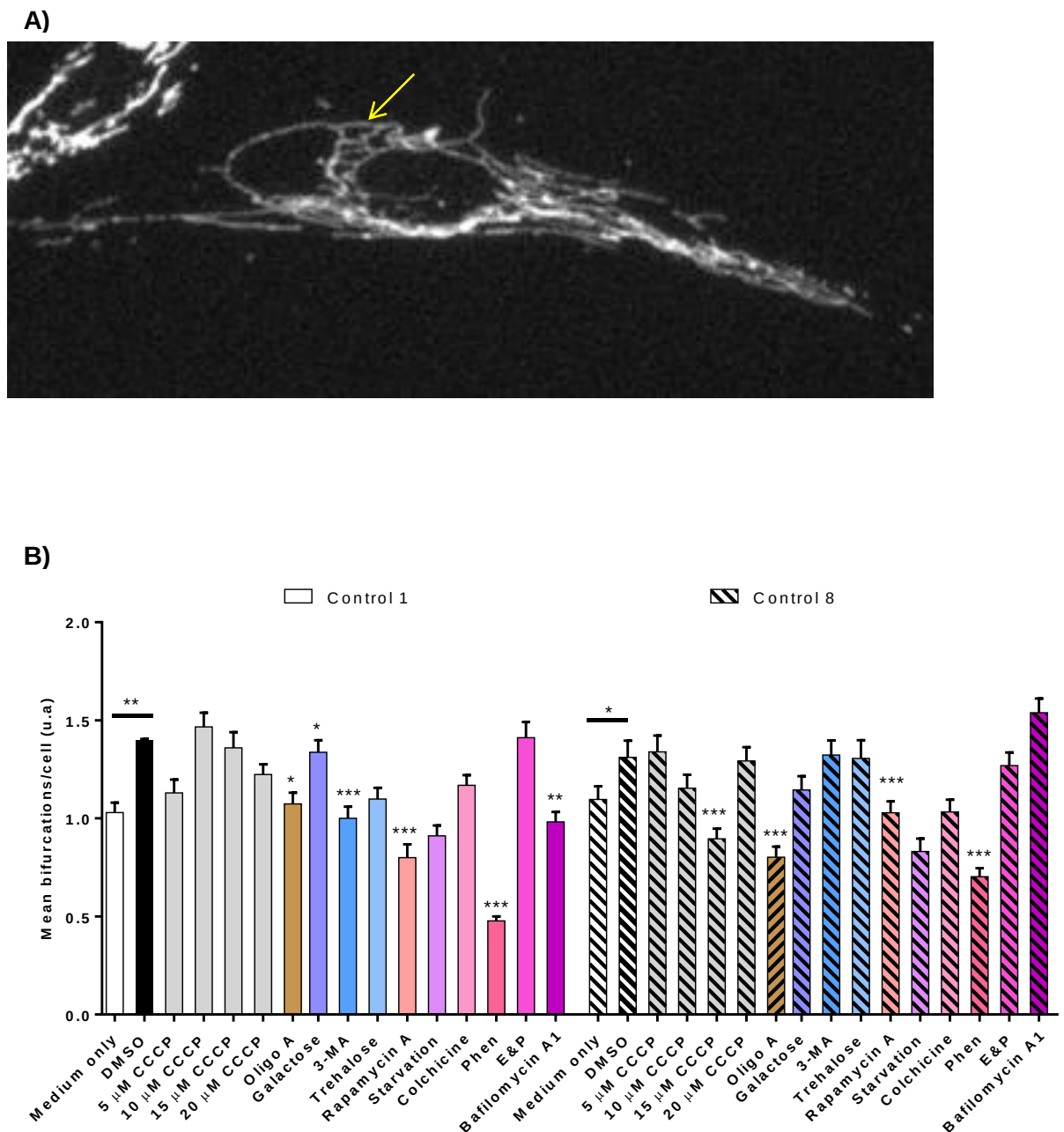


Figure 4.10. The IN Cell method for detecting the effect of treatments on the mitochondrial morphology by determine the number of bifurcations in control fibroblasts.

(A) Control fibroblast1 in normal glucose medium. Area of bifurcations marked by yellow arrow. For illustration purpose only, the image is not to scale.

(B) Mitochondrial morphology was as detected by TOM20 signal and the number of bifurcations were calculated as mean number of bifurcations per cell. Data represent the mean $\pm$ SD, ≥ 500 cells/experiment analysed, $n=3$. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$, ANOVA with Bonferroni correction, compared to medium only or DMSO. DMSO was used as vehicle control for CCCP, 3-MA, bafilomycin A1, E&P, oligo A and rapamycin A.

There were fewer bifurcations detected after phenanthroline treatment, figure 4.10 (B), which is in line with previous published results where it was shown to cause mitochondrial network fragmentation (Park *et al.*, 2012). This was further confirmed by microscopy, figure 4.12, panel F. After treatment with 3-MA and rapamycin A, control 1 had significant fewer bifurcations than the DMSO control. This was not observed in control 8. Starvation has been found to induce low levels of mitophagy but also stressed induced mitochondrial hyperfusion. Mitochondrial hyperfusion occurs under stress conditions such as starvation when the mitochondrial network morphology changes and becomes elongated and highly interlinked (Gomes *et al.*, 2011; Frank *et al.*, 2012). However, mitochondrial stress induced hyperfusion did not occur after starvation as no change to mitochondrial network morphology was detected, figure 4.10 (B). Energetic stress (galactose medium) did induce increased number of bifurcations in control 1. Thus, indicating the mitochondrial network becoming more interlinked after being subjected to energetic stress for 24 hours.

The treatment with E&P and bafilomycin A1 resulted in significant increase in autophagosomes and co-localisation with the mitochondria (figures 4.8 and 4.9) but had no effect on the mitochondrial network (figure 4.10 B). To evaluate if the co-localisation and possible mitophagy events resulted in reduced mitochondrial area, the total mitochondrial area relative to cell area was assessed, figure 4.11.

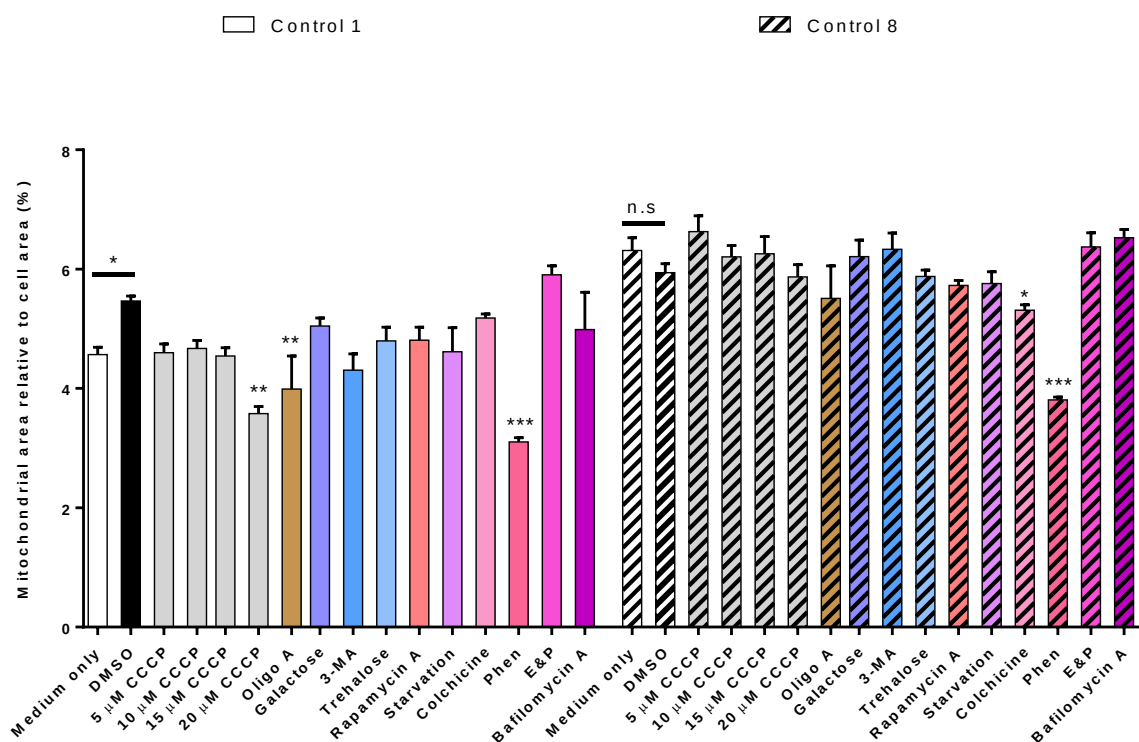


Figure 4.11. The IN Cell method for detecting the effect autophagy and mitophagy modulators on mitochondrial area in control fibroblasts. The mitochondrial area as detected by TOM20 signal. Data represent the mean $\pm$ SD, ≥ 500 cells/experiment analysed, $n=3$. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$, ANOVA with Bonferroni correction, compared to medium only or DMSO. DMSO was used as vehicle control for CCCP, 3-MA, bafilomycin A1, E&P, oligo A and rapamycin A.

The treatment of fibroblasts with E&P and bafilomycin A1 did not result in reduced mitochondrial area when compared to DMSO. This was as expected as E&P and bafilomycin A1 inhibit the autophagy/mitophagy pathway. Interestingly, cells treated with phenanthroline had significantly less mitochondrial area per cell when compared to the media only condition, figure 4.11. Starvation also caused a decrease in mitochondrial area but only in control 8. After treatment with the highest concentration of CCCP (20 μ M) the mitochondrial area was decreased in control 1 only, when compared to DMSO. It should be noted that DMSO increased the mitochondrial area in control 1 when compared to medium only ($p=0.0171$).

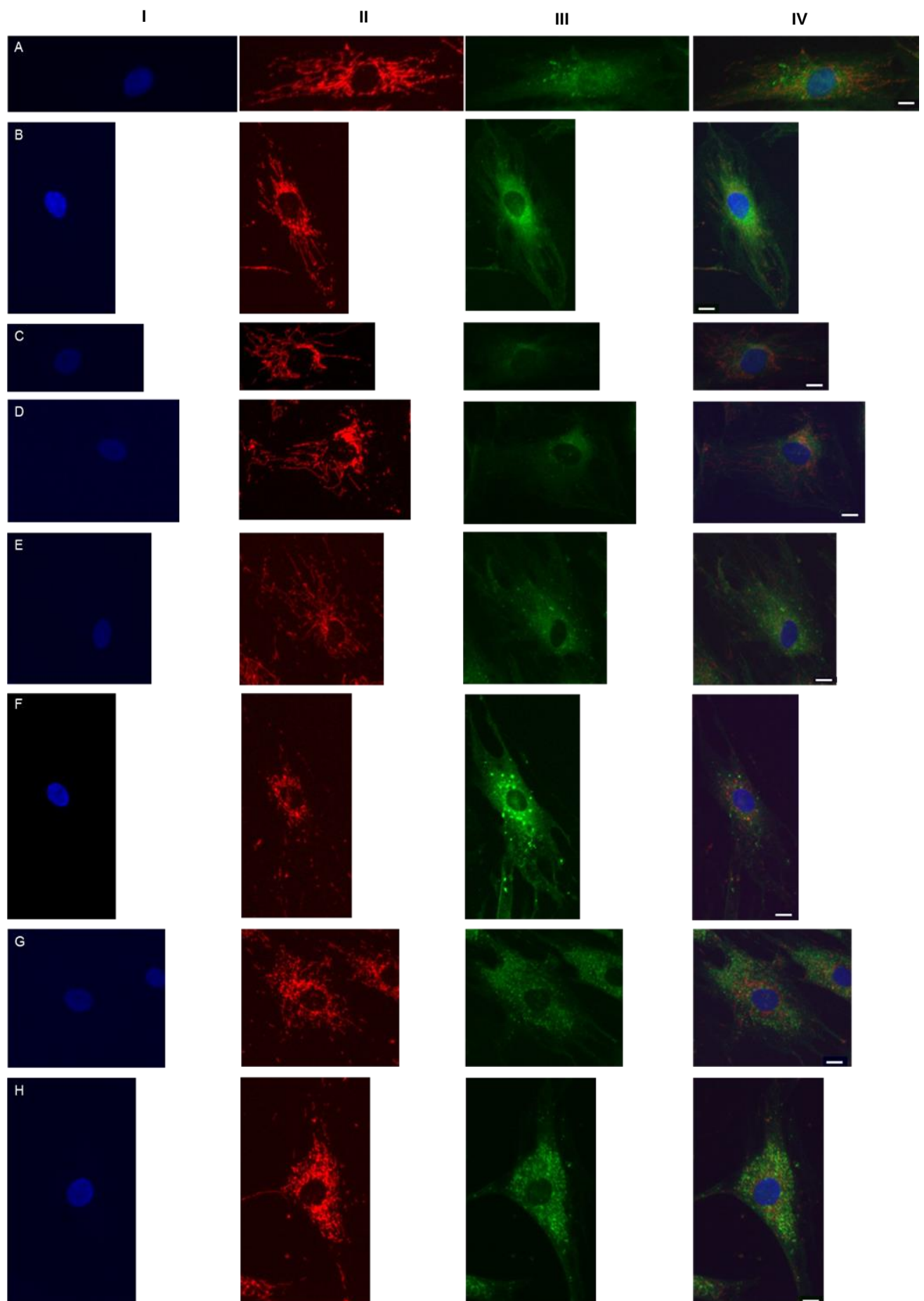


Figure 4.12. Representative microscope images of control fibroblasts after treatment with modulators of autophagy/mitophagy and mitochondrial morphology . Nucleus stained with DAPI (I), mitochondria TOM20 (II), autophagosomes LC3 (III), merge (IV).(A) Maintenance medium (B) 20 μ M CCCP (C) 3-MA (D) Starvation (E) Colchicine (F) Phenanthroline (G) E64D/pepstatin A (H) Bafilomycin A1. Scale bar 10 μ m.

There were fewer bifurcations detected with phenanthroline treatment, figure 4.10. This was further confirmed by the microscope images, figure 4.12, panel F (II). Please see appendix B, for the full field of view.

4.3.2 High-throughput analysis for detection of mtDNA in live primary control fibroblasts after treatment with modulators of mitochondrial morphology and autophagy, using the IN Cell 1000 analyser.

The fibroblast controls were treated with the same compounds as described in section 4.3.1. After treatment the cells were stained for mtDNA using PicoGreen (Quant-iT dsDNA, molecular probes). PicoGreen is a DNA specific fluorescent dye that can be used to detect mtDNA (Ashley *et al* 2005). Briefly, after treatment the cells were incubated for 20 minutes in phenol red free Opti-MEM (Gibco) containing 3µl/ml PicoGreen (Quant-iT dsDNA, molecular probes). After incubation, cells were washed x1 (Opti-MEM) and 100µl Opti-MEM was added to the wells. The cells were then imaged using the IN Cell analyser.

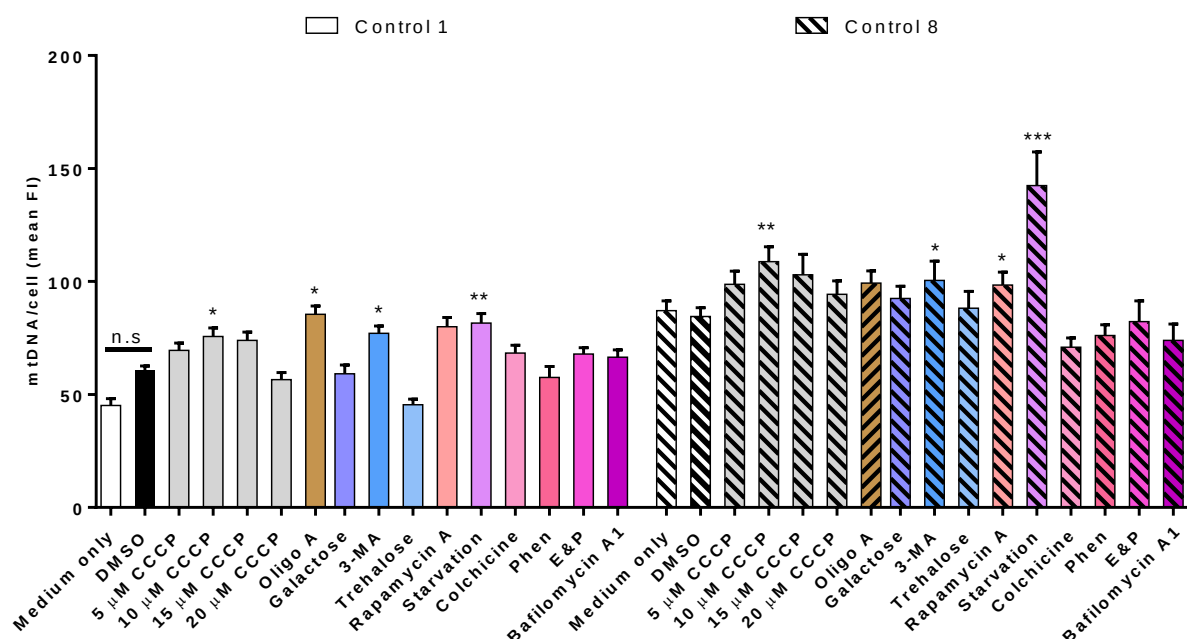


Figure 4.13 Mitochondrial DNA detected by PicoGreen staining. After treatment the cells were incubated with PicoGreen in clear media. Treatments as follows; CCCP for 4 hours, 5mM 3-MA 4 hours, galactose 24 hours, 100nM trehalose 24 hours, 100nM rapamycin A, starvation (HBSS) 2hours, 2ng/ml Colchicine 24 hours, 10uM 1,10 phenanthroline (phen) 24 hours, 10ng/ml E&P (E64D+pepstatin A) over night and 5 μ M bafilomycin A1 overnight. Data represent the mean $\pm$ SD of triplicate well of one independent experiment, ≥ 500 cells/well analysed. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, ANOVA with Bonferroni correction, compared to medium only or DMSO. DMSO was used as vehicle control for CCCP, 3-MA, bafilomycin A1, E+P (E64D/pepstatin A) and rapamycin A.

The level of mtDNA present after starvation was significantly increased in both controls, figure 4.13. Although phenanthroline treatment caused mitochondrial network fragmentation and increased mitophagy resulting in loss of mitochondrial area, this did not have an effect on mtDNA. These fibroblast controls were able to maintain mtDNA levels after treatment with phenanthroline. Furthermore, there were no changes in mtDNA after treatment with bafilomycin A1, E&P or colchicine all of which had

increased autophagosomes and auto/mitophagy. Interestingly, starvation, CCCP (10 μ M) and 3-MA all caused an increase in mtDNA when compared to control condition (medium only or DMSO).

4.3.3 Do modulators of autophagy and mitochondrial network morphology act in the same manner in HepG2 cells as in primary control fibroblasts?

In the previous section of experiment the mitochondrial uncoupler CCCP had no effect on autophagosomes or mitochondrial network morphology of control fibroblasts. This was interesting as several studies have shown that CCCP induces mitophagy (Narendra *et al.*, 2008; Lee *et al.*, 2014). Furthermore, CCCP has been found to inhibit degradation in the lysosomes by equilibration of the pH resulting in a reduction of autophagosomes clearance (Padman *et al.*, 2013).

I used HepG2 cells to investigate if the response to modulators of auto/mitophagy and mitochondrial morphology observed in primary fibroblasts was cell type specific, using the same IN Cell 1000 Analyzer methodology as in the previous experiments. However, fibroblasts are large, flat cells and have a distinct mitochondrial network and therefore suitable for high content imaging. Conversely, HepG2 cells are smaller, rounded and have the tendency to clump together which makes high-throughput analysis a challenge, figure 4.14. Hence mitochondrial morphology could not be robustly assessed in HepG2 cells.

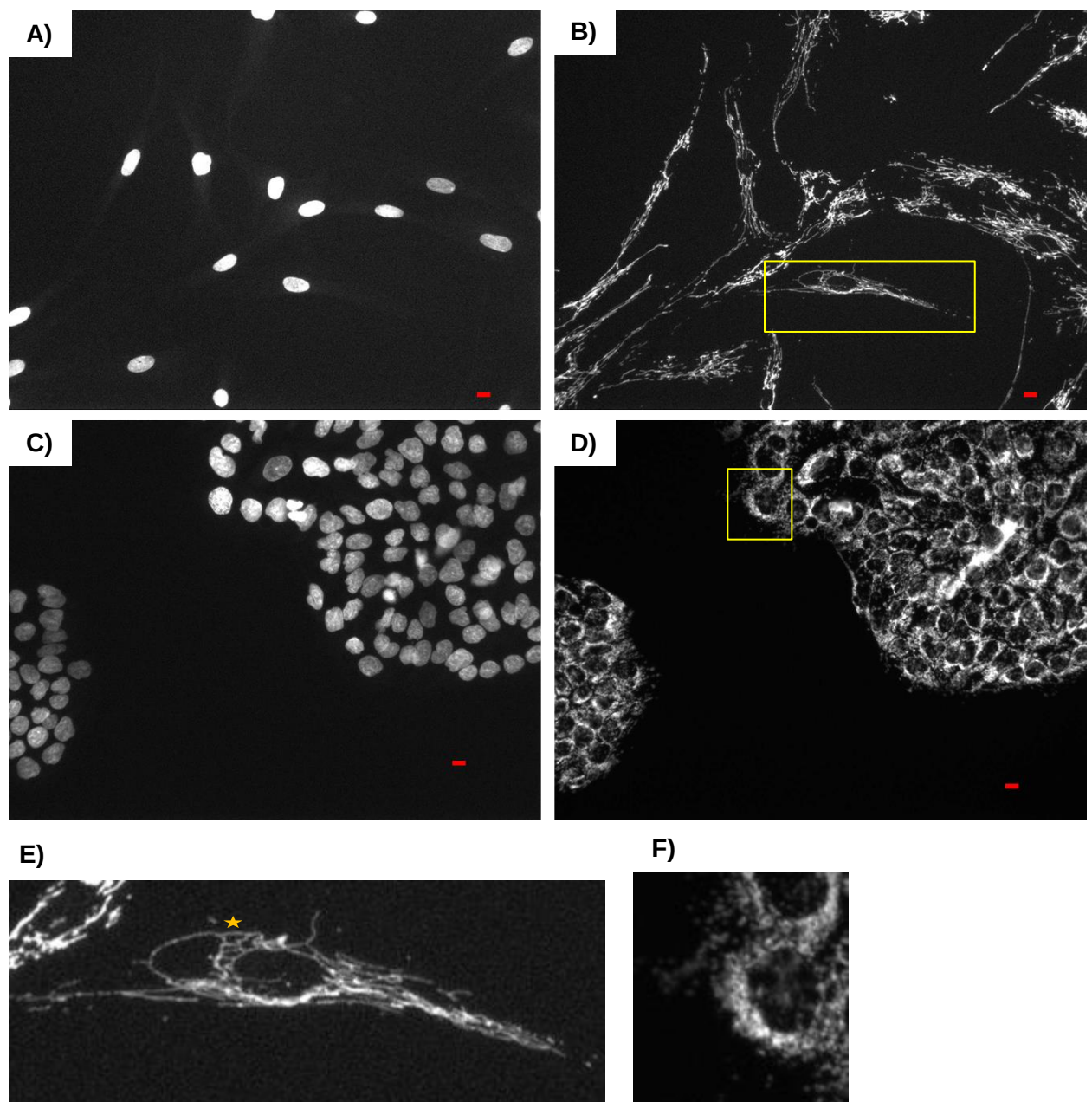


Figure 4.14. Contrasting mitochondrial network morphology of fibroblasts and HepG2. Both cell lines incubated in glucose maintenance media, no additional treatments. Nucleus stained with DAPI and mitochondria with TOM20. Top panel are of control fibroblasts, nucleus (A) and (B) mitochondria forming a network. Bottom panel, HepG2, (C) nucleus and (D) mitochondria appears densely packed. (E) and (F), images magnified as highlighted by yellow box, yellow star example of network bifurcations. Scale bar in red 10μm.

4.3.3.1 High-throughput analysis of HepG2 after treatment with modulators of mitochondrial morphology and autophagy, using the IN Cell 1000 analyser.

HepG2 cells were seeded at low density (~1,500 cells) in 96 well plates, after treatment with modulators of mitochondrial morphology and autophagy; plates were fixed and stained, as previously described, section 4.3. All compounds, with the exception of E&P (E64D/pepstatin A), were the same as with the primary control fibroblasts. E&P was replaced by chloroquine as inhibitor of late stage auto/mitophagy.

As previously observed in the fibroblasts, the treatment with phenanthroline and bafilomycin A1 caused significant increase in autophagosomes in HepG2 cells, figure 4.15.

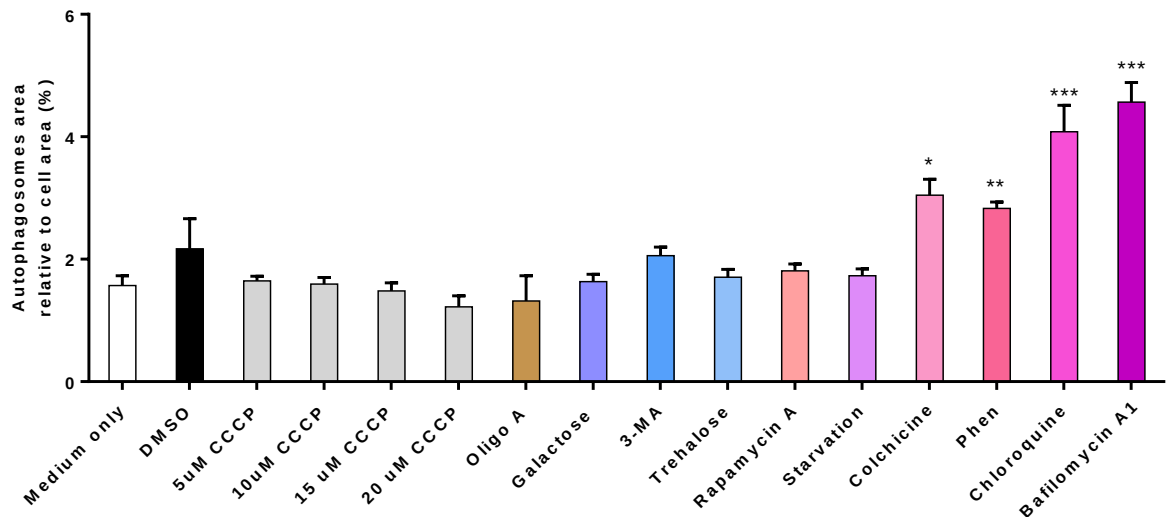


Figure 4.15. IN Cell method for the detection of autophagy in HepG2 cell line.

Autophagosomes detected based on LC3 signal, expressed as a percentage of cell total area. CCCP for 4 hours, 5mM 3-MA 4 hours, galactose 24 hours, 100nM trehalose 24 hours, oligomycin A (oligo A) 30minutes, 100nM rapamycin A 24 hours, starvation 2hours, 2ng/ml Colchicine 24 hours, 10uM 1,10 phenanthroline (phen) 24 hours, 10ng/ml chloroquine overnight and 5μM bafilomycin A1 overnight. DMSO was used as vehicle control for CCCP, 3-MA, bafilomycin A1, E&P, oligo A and rapamycin A. Data shown are the mean $\pm$ SD of four technical replicates, n=2, $\geq$ 500 cells/experiment analysed. *p<0.05, ANOVA with Bonferroni correction.

After treatment HepG2 increased levels of autophagosomes were observed after incubation with colchicine, bafilomycin A1 and chloroquine which are inhibitors of late autophagy, figure 4.15. Furthermore, phenanthroline also caused increased autophagosomes. The increase in autophagosomes were reflected in the increasing co-localisation between autophagosomes and mitochondria, figure 4.16.

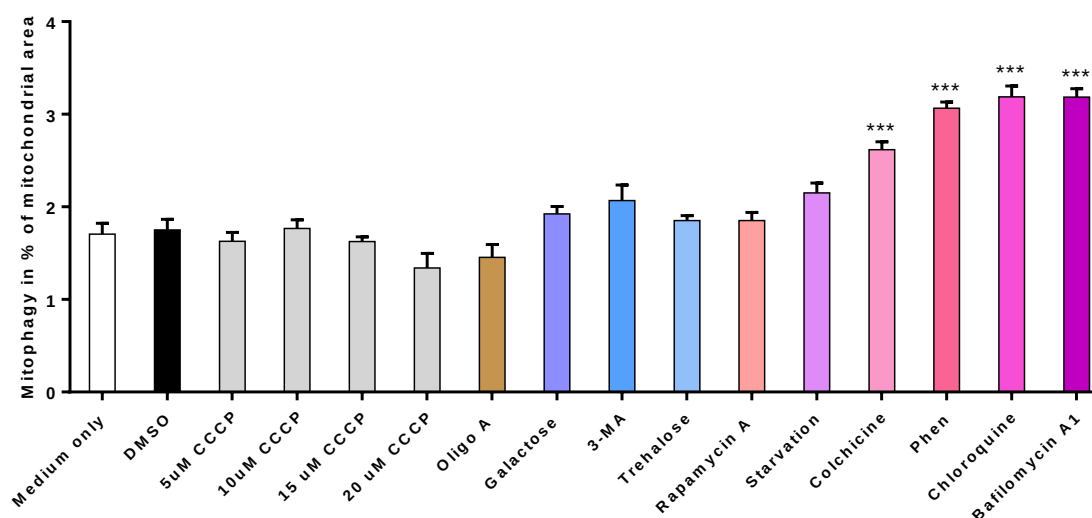


Figure 4.16. IN Cell method for the detection of co-localisation between autophagosomes and mitochondria (mitophagy) in HepG2 cell line. Co-localisation between TOM20 and LC3 (mitophagy) after treatment with modulators of autophagy and mitochondrial integrity. DMSO was used as vehicle control for CCCP, chloroquine, bafilomycin A1 and rapamycin A. Data shown are the mean $\pm$ SD of four technical replicates, $n=2$, ≥ 500 cells/experiment analysed. $p < 0.05$, $**p < 0.001$, $***p < 0.0001$, ANOVA with Bonferroni correction.

As with the control fibroblasts there were no detectable change in mitophagy levels after treatment with CCCP which is a known inducer of mitophagy, figure 4.17.

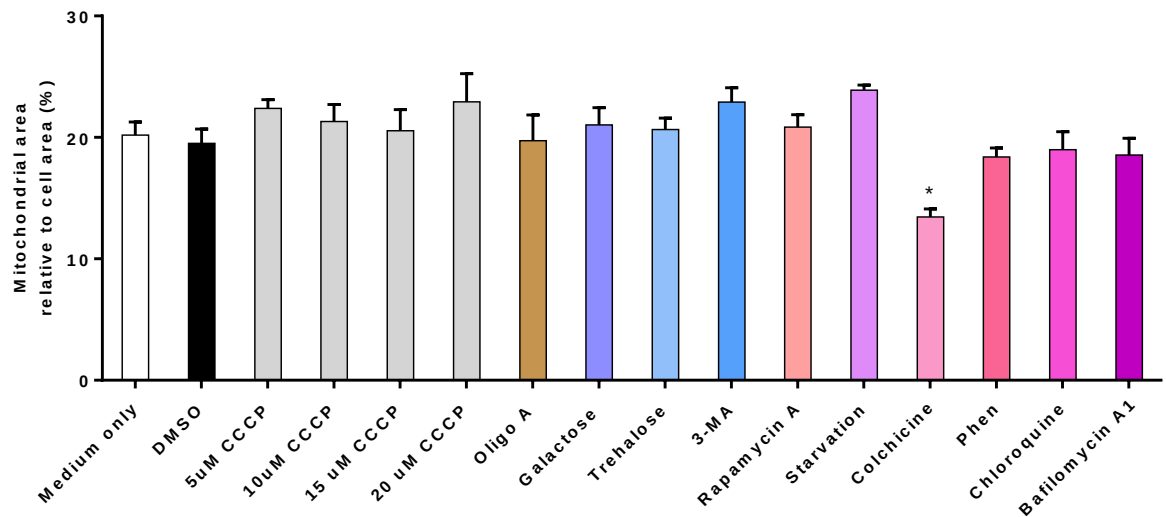


Figure 4.17. IN Cell method for detecting the effect autophagy and mitophagy on mitochondrial area in HepG2. The mitochondrial area of HepG2 after treatment with various modulators of autophagy and mitochondrial integrity. Mitochondrial area based on TOM20 signal and presented as percentage total mitochondrial area relative to total cell area. Data represent the mean $\pm$ SD of four technical replicates, $n=2$, ≥ 500 cells/experiment analysed. * $p<0.05$, ANOVA with Bonferroni correction.

The mitochondrial area of the HepG2 cells appears to be unaffected by the treatments as there were no significant changes with the exception of colchicine, which caused a reduction, figure 4.17. Phenanthroline treatment did not result in reduced mitochondrial area in HepG2 as it did for the control fibroblasts.

4.3.4 Discussion – Sections 4.4.1 and 4.4.3

4.3.4.1 High throughput quantification of mitophagy.

The ability to detect and quantify mitophagy can be challenging. Previous methods have relied on manually counting cells containing mitophagy events. However, recently we (Diot *et al.*, 2015) published an approach for detecting and quantifying mitophagy. To demonstrate proof of principle that the co-localisation of autophagosomes and mitochondria equates to mitophagy, we used the IN Cell 1000 Analyser and the IN Cell Toolbox image software (GE Healthcare Lifesciences) in conjunction with functional assays. The experiments presented in the results section, 4.4.1, formed part of this publication. In addition, I used HepG2 cells to investigate if the effect of modulators observed in control fibroblasts were cell type specific. By using high content fluorescent microscopy method in conjunction with image analysis software to segment the nucleus, mitochondria, autophagosomes and cell boundaries, as described in section 4.3.2.1, it was possible to detect mitochondria area and autophagy and/or mitophagy events. However, successful segmentation of the mitochondrial network morphology was only possible for the control fibroblasts as these cells have larger cytosolic area with a clearly defined mitochondrial network. Conversely, HepG2 cells have less cytosolic area and the mitochondria network is not distinct. Therefore, mitochondrial network morphology was not thought to be a robust measure for HepG2 cells and only the mitochondrial area was considered.

Is the response to modulators of mitochondrial morphology and autophagy cell type specific?

HepG2 cells and control fibroblasts displayed similar responses to the various modulators. Both cell types had increased autophagosomes after treatment with late inhibitors of autophagy (bafilomycin A1 and chloroquine). Both the two control fibroblasts and the HepG2 cells showed no change in levels of autophagosomes or mitophagy after treatment with CCCP at concentrations 5 μ M to 20 μ M. This is curious as CCCP is a known inducer of autophagy/mitophagy, as it was used fresh and at what was believed to be at appropriate concentrations and durations. CCCP is a protonophore and often used to uncouple mitochondrial OXPHOS. It has been reported by numerous groups (Lee *et al.*, 2014; Rossin *et al.*, 2015; Gegg *et al.*, 2010) to be an inducer of mitophagy through the PINK1/Parkin pathway. In these studies several cell lines were used; HEK293T and MEFs by Rossin *et al.*, (2015) and SH-SY5Y by Gegg *et al.*, (2010) and Park *et al* (2012) used HeLa in their study. The cells used in the above studies are cell lines that have been immortalised since originally being derived from tumour biopsies taken over 40 years ago and it is therefore not surprising that primary fibroblasts did not have the same response. Furthermore, often these studies use cells that have been transformed to overexpress such proteins as PINK1. The lack of response to CCCP treatment observed in the fibroblasts, figure 4.9, could be due to these being primary cell lines and therefore less sensitive to the concentrations of CCCP used in here in this study. However, this does not explain why the HepG2 was not affected by CCCP treatment, figure 4.16 as these cells are immortalised cancer cells. There are variations in the literature between CCCP concentrations and treatment times, ranging from 20 μ M for 5 hours to 10 μ M and 20 μ M for 18 hours and 24 hours (Lee *et al* 2014; Rossin *et al* 2015). While the response to CCCP could be cell type or concentration and exposure dependant, it is more likely that this discrepancy reflects the method for detecting mitophagy. Lemasters (2014)

postulated that there are two types of mitophagy. Type 1 mitophagy occurs during nutrient starvation and is not linked to mitochondrial membrane potential whereas type 2 happens when the membrane potential depolarises. If the IN Cell method is primarily detecting type 1 mitophagy, we would not expect to detect a CCCP dependant effect. As the cell lines used in this current study were sensitive to other inhibitors and inducers of auto/mitophagy, the CCCP effect was not explored further.

The effect of DMSO vehicle control

The primary fibroblast controls were sensitive to the DMSO treatment which resulted in a significant increase in mitochondrial network connectivity and control 1 showed increased mitochondrial area. HepG2 cells were not sensitive to DMSO treatment. DMSO is commonly used as a solvent for the dissolution of poorly soluble molecules. However, it has been found to cause mitochondrial swelling, increased network fusion and reduced cellular oxygen consumption, in a variety of cell lines (RGC-5, C2C12 and astrocytes) at concentration ranges of 1% to 8% (v/v) for 24 hours (Yuan *et al.*, 2014; Turkseven *et al.*, 2013, Galvao *et al.*, 2013). For the IN Cell validation experiments, cells were incubated with DMSO at a concentration of 0.5% (v/v) and were used as vehicle control for CCCP, rapamycin A, oligomycin A, bafilomycin A1 and E&P. It is possible that treating primary fibroblast with DMSO induces gentle mitochondrial stress which causes stress induced hyperfusion.

Results

4.3.5 Does VPA modulate autophagy and/or mitophagy in HepG2 cells?

There is currently very little literature on the effect of VPA treatment on autophagy or mitophagy. A few studies have shown VPA to be an inducer of autophagy (Sarkar *et al.*, 2005; Cavaliere *et al.*, 2014) but neither of these studies used HepG2 cells. In the previous chapter (chapter 3.3.1) VPA treatment was found to cause mitochondrial dysfunction in HepG2 cells by inhibiting respiration. To investigate if the mitochondrial dysfunction would result in increased autophagy or mitophagy, HepG2 cells were treated with VPA at concentration of 0.5mM, 1.0mM and 2.0mM for 72 hours. The treatment was either under normal 5mM glucose condition or energetic stress condition (10mM galactose). Furthermore, six modulators of mitochondrial morphology and autophagy/mitophagy were also used to elucidate VPA's effect. The concentration and treatment duration was as previously described (table 4.2).

After 72 hours treatment with VPA there was a decrease in autophagosomes detected at the higher concentration (2mM) under normal glucose and energetic stress conditions, figure 4.18. As before (section 4.4.2) chloroquine and bafilomycin A1 treatment resulted in increased autophagosomes, when compared to the vehicle control (DMSO). There was no difference in autophagosomes between normal and energetic stress conditions (glucose and galactose) or co-localisation with the mitochondria. As previously, colchicine and phenanthroline increased mitophagy. VPA treatment had no effect on mitophagy, figure 4.19.

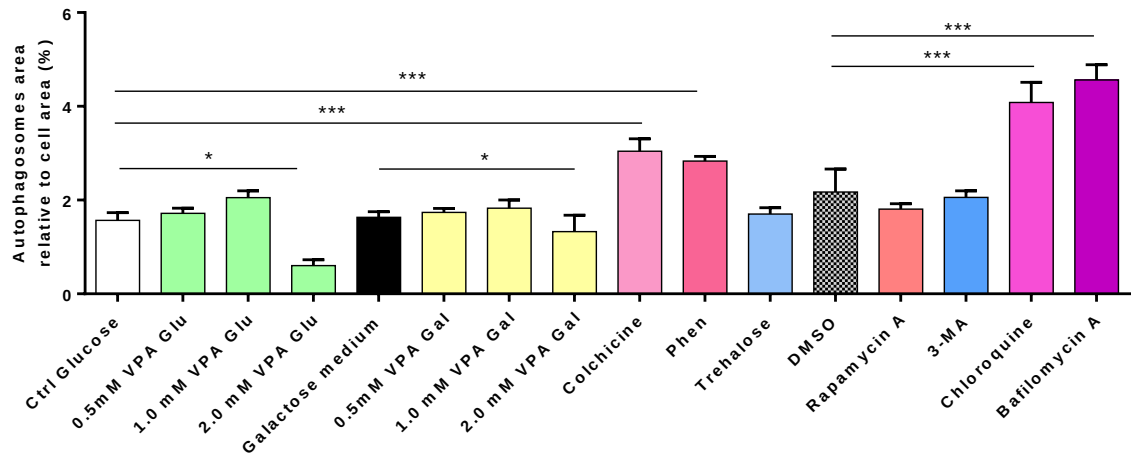


Figure 4.18. Autophagosomes present after treating HepG2 cells with VPA and modulators of autophagy. Cells were treated with VPA for 72 hours under normal glucose (5mM) or energetic stress condition (10mM galactose). Cells were treated in 5mM glucose conditions as follows: 5mM 3-MA 4 hours, 100nM trehalose, 100nM rapamycin A, 2ng/ml Colchicine, 10uM 1,10 phenanthroline (phen) for 24 hours. 10ng/ml chloroquine and 5μM bafilomycin A overnight. Data represent the mean $\pm$ SD of four technical replicates, $n=2$, ≥ 500 cells/experiment analysed. * $p<0.05$, ANOVA with Bonferroni correction, compared to control as indicated. DMSO was used as vehicle control for chloroquine, bafilomycin A and rapamycin A.

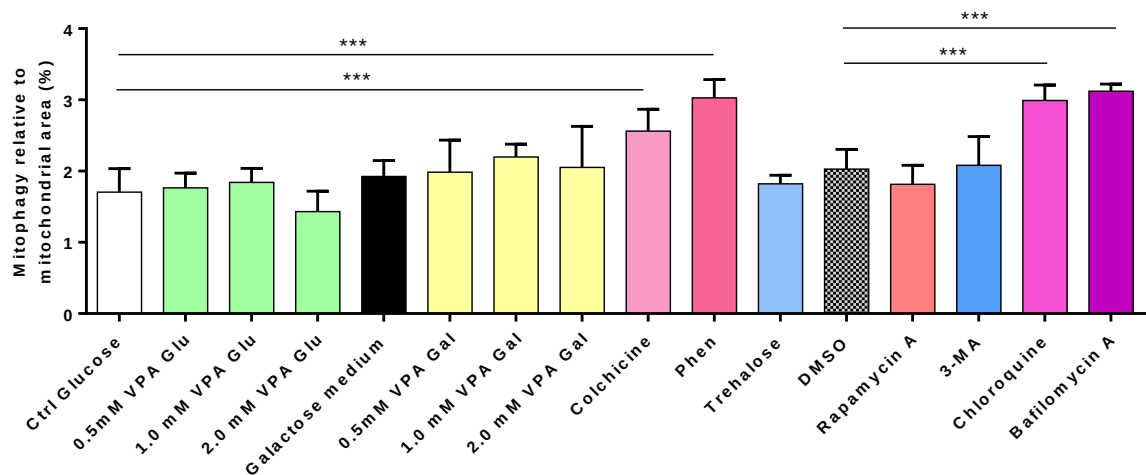


Figure 4.19. Co-localisation between autophagosomes and mitochondria (mitophagy) after treating HepG2 cells with VPA and modulators of autophagy in. Cells were treated with VPA for 72 hours under normal glucose or energetic stress condition (galactose). All other treatments were carried out under normal conditions. Data represent the mean $\pm$ SD of four technical replicates, $n=2$, ≥ 500 cells/experiment analysed. * $p<0.05$, ANOVA with Bonferroni correction, compared to control as indicated. DMSO was used as vehicle control for chloroquine, bafilomycin A1 and rapamycin A.

Interestingly, VPA treatment at high concentrations under normal and energetic stress conditions caused a decrease in autophagosomes levels but the co-localisation between autophagosomes and mitochondria remained at the same level as the glucose and galactose controls, figure 4.19.

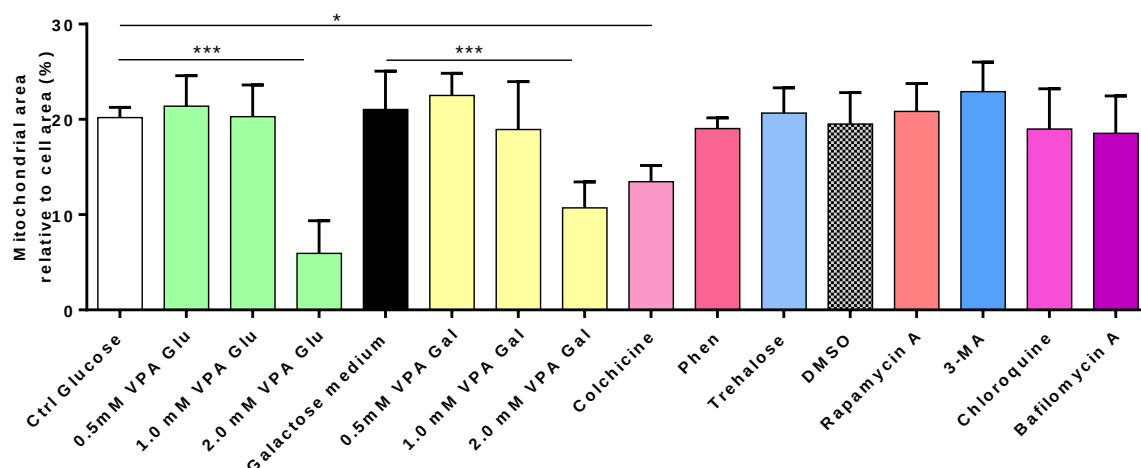


Figure 4.20. Mitochondrial area assessed after treatment with VPA and modulators of autophagy in HepG2 cells. Cells were treated with VPA for 72 hours under normal glucose (5mM) or energetic stress condition (galactose). All other treatments were carried out under normal conditions. Data represent the mean $\pm$ SD of four technical replicates, $n=2$, ≥ 500 cells/experiment analysed. * $p<0.05$, ANOVA with Bonferroni correction, compared to control as indicated. DMSO was used as vehicle control for chloroquine, bafilomycin A1 and rapamycin A.

After 72 hours VPA treatment under both normal and energetic stress conditions and at the highest concentration (2mM) caused a significant decrease in mitochondrial area. Additionally as in previous experiments, treatment with the late stage autophagy inhibitor, colchicine also resulted in significant reduction of the mitochondrial area, figure 4.20.

Discussion Section 4.4.4.1 - Did VPA modulate autophagy/mitophagy in HepG2 cells?

There are very few published studies investigating VPA and its effect on autophagy and mitophagy. Current publications report that VPA induced autophagosomes and autophagy using COS-7, SK-N-SH and Raji cells (Cavaliere *et al.*, 2014; Sarkar *et al.*, 2005). The study using COS-7 cells used standard fluorescent microscopy and assessed autophagosomes and mitochondrial mass after treatment with 3.0mM VPA for 24 hours (Sarkar *et al.*, 2005). They reported an increase in autophagosomes and decreased mitochondrial area. Here I use VPA concentrations of 0.5, 1.0 and 2.0mM for 72 hours and found that a high concentration (2.0mM) of VPA reduced autophagosomes relative to the glucose control. HepG2 cells were also subjected to VPA treatment under energetic stress condition (galactose). This resulted in a reduction of autophagosomes relative to control (galactose) condition. These findings contradict the previous studies but this could be due to the different cell lines and treatment regimes used (Cavaliere *et al.*, 2014; Sarkar *et al.*, 2005). Interestingly, the reduction of autophagosomes was not reflected by a reduction in the co-localisation between autophagosomes and the mitochondria (mitophagy). However, there was significant reduction in mitochondrial area, after treatment with 2.0mM VPA under normal and energetic stress conditions. Following 2.0mM VPA treatment under normal glucose condition, 10.73% of the total autophagosomes detected co-localised with mitochondria resulting in a 29.22% reduction of the mitochondrial area. Hence VPA treatment reduces the mitochondrial area with a significant fraction of the mitochondria remaining associated with autophagosomes. The effect of VPA treatment in primary fibroblast will be considered in the next research chapter.

4.4 General chapter discussion

4.4.1 Evaluating the IN Cell method for detecting mitophagy

The levels of autophagosomes (mitophagosomes) are dynamic as there is a balance between formation and fusion with autolysosomes and this can be assessed using compounds that inhibit or induce the autophagy pathway, e.g. the levels of autophagosomes increased when the fusion with lysosomes had been blocked by colchicine treatment. Treatment with bafilomycin A1, chloroquine or E&P prevents the lysosomes from functioning as these compounds change the pH of the vesicles. This in turn caused an increase of autophagosomes which was far greater than the increase caused by colchicine. This increase in autophagosomes was observed in both control fibroblast cell line and HepG2 cells after overnight treatment with bafilomycin A1, chloroquine and E&P. However, this increased detection of autophagosomes does not automatically mean increased autophagy but is an indication of autophagosomal flux (Mizushima *et al*, 2010). Under certain circumstances, increased autophagosome number is not due to an increased cellular need to increase autophagy because of factors such as nutrient starvation but due to a late inhibition of the pathway. Therefore, there are four points to consider before a drug/compound can be judged as an inhibitor or inducer of autophagy or mitophagy. Firstly, one needs to establish the basal levels of autophagosomes within a cell line, when cells are incubated in normal maintenance medium. Secondly, how the cells respond to an inducer of autophagy such as trehalose or phenanthroline. Thirdly, treatment of cells with an inhibitor of late stages of auto/mitophagy pathway will establish a baseline autophagosomal flux, for example E+P and bafilomycin A1. By using these three different approaches in specific cell models in conjunction with the compound of interest it should be possible to determine the impact of a specific compound on the autophagy pathway (Klionsky *et al*, 2012; Mizushima *et al*, 2010). Finally, determining the impact of a treatment on

mitochondrial morphology and mass together with mtDNA copy number will further assist in determining the drug/compound's effect on autophagy/mitophagy (Diot *et al.*, 2015).

Treatment with bafilomycin A1, chloroquine and E&P resulted in a significant increase in autophagosomes in control fibroblasts and HepG2 cells but the level of increase varied between the two cell lines. After treatment with bafilomycin A1 the levels of autophagosomes increased by over 400% in both the control fibroblast whereas in the HepG2 cells the increase was 192%. This increase was not surprising as these compounds inhibit late stage autophagy, thus creating a backlog of unprocessed autophagosomes. However, the increase in number mitophagy events observed could be the result of chance co-localisation between autophagosomes and mitochondria due to the high number phagosomes present. Had mitophagy been highly activated in these cell types, one would expect to see a decrease in mitochondrial area and/or mtDNA due to the sheer volume of auto/mitophagosomes present. This is one of the limitations of the IN Cell method when there is a large number of autophagosomes present; mitophagy could be artificially high due to false positive co-localisation. It may be that there is a mix of mitophagy and autophagy observed with treatments such as E&P and bafilomycin A1. After treatment with colchicine and phenanthroline there was an increase in the number of autophagosomes but not to the level of E&P or bafilomycin A1 treatment. Interestingly, Colchicine has been found to be an inhibitor and phenanthroline an activator of the auto/mitophagy pathway however, after treatment both compounds resulted in increased levels of autophagosomes and mitophagy events with reduced mitochondrial area. Furthermore, both of these treatments caused the mitochondria to fragment, i.e. a reduction in the number of bifurcations, three-way intersections of the mitochondrial network was observed. Therefore, the fragmented mitochondria become a more likely target for mitophagy after phenanthroline treatment, as bafilomycin A1 or E&P did not

result in changes to the mitochondrial network or the mitochondrial area, please see table 4.3 for summary overview.

Table 4.3 Summary overview of the effect of treatment with modulators of autophagy in control fibroblasts. ↑ significant increase. ↓ significant decrease. ↔ no significant change. Rows highlighted in blue denotes that the treatment had no effect on mitophagy. Rows highlighted in pink are treatments which induced mitophagy.

Treatment	Autophagosomes (autophagy)		Autophagosomes co-localisation with mitochondria (mitophagy)		Mitochondrial network fragmentation		Mitochondrial area	
	Ctrl 1	Ctrl 2	Ctrl 1	Ctrl 2	Ctrl1	Ctrl 2	Ctrl 1	Ctrl 2
Bafilomycin A	↑	↑	↑	↑	↔	↔	↔	↔
CCCP	↔	↔	↔	↔	↔	↔	↓	↔
Colchicine	↔	↑	↔	↑	↔	↔	↔	↓
E & P	↑	↑	↑	↑	↔	↔	↔	↔
Galactose	↔	↔	↔	↔	↓	↔	↔	↔
Oligomycin A	↔	↔	↔	↔	↑	↑	↓	↔
Phenathroline	↔	↔	↑	↑	↑	↑	↓	↓
Rapamycin A	↔	↔	↔	↔	↔	↑	↔	↔
Starvation	↔	↔	↑	↑	↔	↔	↔	↔
Trehalose	↔	↔	↔	↔	↔	↔	↔	↔
3-MA	↔	↔	↔	↔	↑	↔	↔	↔

Furthermore, Diot *et al.*, (2015) found that by using modulators of mitochondrial network morphology and auto/mitophagy in fibroblasts from patients harbouring the mtDNA mutation m.3243A>G, the mutant load could be reduced. Using mutant load

quantification by qPCR enabled the confirmation that the co-localisation of LC3 and TOM20 was indeed engulfed mitochondria in autophagosomes rather than chance overlay. The mutant load was reduced in accordance with increased co-localisation between the LC3 and TOM20 signal thus confirming that mitophagy had occurred. This approach would not be appropriate for control fibroblasts used in my study as these cells do not carry an easily identifiable mutation. Instead mtDNA was assessed using PicoGreen staining in control fibroblast after treatment but phenanthroline did not have an effect on mtDNA levels detected, although mitochondrial area was reduced. It appears that these control fibroblasts are able to compensate for any loss of mtDNA that may have occurred due to mitophagy. To assess changes in mtDNA due to auto/mitophagy the use of real-time PCR to detect mtDNA copy number would be suitable.

4.5 Research highlights.

- IN Cell high throughput image acquisition and image analysis can detect co-localisation between autophagosomes and the mitochondria.
- Treatment with phenanthroline and colchicine increased mitophagy in both control fibroblasts and HepG2 cells.
- Treatment at high concentration of VPA reduced the levels of autophagosomes and mitochondrial area.

4.6 Limitations of this study

The data presented using IN Cell as a method for detecting mitophagy represents a snapshot only and autophagosome/mitochondria co-localisation could result from either an increase in mitophagy or a block of late stage mitophagy. Furthermore, the IN Cell method cannot differentiate between the fluorescence of LC3I the unbound cytosolic form and LC3II the membrane-bound lipidated form. Therefore, the level of autophagosomes could be overestimated. Immunoblotting probing for LC3I and LC3II would confirm that the increase in LC3 fluorescence is due to autophagosomes formation. There are however several publications supporting that the increase in LC3 fluorescent signal observed after treatment with e.g. chloroquine, E&P and phenanthroline is due to LC3II as established by Western blotting (Melser *et al.*, 2013; Mizushima *et al.*, 2010; Park *et al.*, 2012). However, due to stringent application of the image analysis method and by segmenting only the bright punctae, the possible over estimation would be at the same level across all experiments. Furthermore, to validate that the co-localisation represent altered auto/mitophagy this assay in conjunction with mtDNA copy number and appropriate qPCR mutant load detection

Chapter 5

***In vitro* models of mitochondrial disease: Primary
fibroblasts derived from patients with mitochondrial
disorders and PolG mutator MEFs**

5 *In vitro* models of mitochondrial disease: Primary fibroblasts derived from patients with mitochondrial disorders and PolG mutator MEFs

5.1 *Introduction*

Mitochondrial disease is highly variable in its clinical presentation as it can affect any organ, either singularly or multiple and can occur at any age. The genetic origins of the diseases are complex as mutations in over 100 genes have been found (Tucker *et al.*, 2010). The complex inheritance of potential mutations coupled with variable clinical presentations due to mtDNA heteroplasmy and tissue specific mutation makes the diagnosis of mitochondrial disease very challenging.

Creating animal models of mitochondrial disease will increase our understanding of the pathogenic mechanisms in human mitochondrial disease as well as creating a disease model in which preclinical testing of potential therapeutic interventions can be carried out. Several mouse models are now available which express mutations in mitochondrial nuclear encoded proteins known to cause mitochondrial disease. These include complex I subunit NDUFS4 (Ingram *et al.*, 2009), mitochondrial helicase twinkle (Tyynismaa *et al.*, 2005) and polymerase gamma (PolG mutator mouse)(Trifunovic *et al.*, 2004). The PolG mutator mouse developed by Trifunovic expresses a proofreading-deficient version of the nuclear encoded mitochondrial DNA polymerase gamma (PolG). This model accumulates point mtDNA mutations in a variety of tissues (Edgar *et al.*, 2009). The mitochondrial free radical theory of aging postulated by Harman proposed that the accumulation of mtDNA mutations over time is linked to the regeneration of mitochondrial reactive oxygen species which accelerate the aging process (Harman, 2006). Interestingly, in the PolG mutator mouse the

accumulation of mitochondrial mutations does not correlate with increased levels of reactive oxygen species (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005). Studies by Morten *et al.*, (2005) suggest that if mitochondrial defects and respiratory chain function are very severe, superoxide levels may not increase. This occurred in heart mitochondria from a mouse model lacking SOD2 with severely impaired respiratory chain function. Models such as the PolG mutator mouse give insights into the effects of mitochondrial mutations in mitochondrial disease and are likely to further our understanding into the role of mitochondrial dysfunction in chronic disease.

Primary fibroblasts established from patients with mitochondrial disease have proved useful in studying the pathogenesis of mitochondrial associated mutations (Morais *et al.*, 2014; Mortiboys, *et al.*, 2008; Sitrazz *et al.*, 2014). However, fibroblasts under standard culture conditions tend to rely on glycolysis to generate ATP (Guillery *et al.*, 2008). In addition, fibroblasts from patients are not derived from the affected organ such as the brain in the case of familial forms of Parkinson's disease. In mitochondrial disease, affected organs include the more energetic tissue such as muscle, brain, heart, β -cells and liver (Boczonadi and Horvath, 2014). However, patients' derived fibroblasts are used for many studies and have shown to be a promising model for the disease but they do have their limitations (Morais *et al.*, 2014; Mortiboys, *et al.*, 2008).

The cell's ability to adapt its mitochondrial content and network morphology is important in the regulation of cellular energetic homeostasis in periods of stress, during glucose starvation or drug insults (Melser *et al.*, 2013; Willems *et al.*, 2015). To investigate the effect of sodium valproate (VPA) on mitochondrial function and network morphology in this study, I use fibroblasts derived from skin biopsies taken from patients harbouring PolG mutations and non-affected controls and MEFs from the PolG mouse model. To drive cells to use their mitochondria in cell culture an element of energetic stress was introduced to challenge these cells to use their mitochondria for energy production. Energetics stress can be induced by changing the glucose to

galactose in the medium (Lemasters *et al.*, 2005 and Marroquin *et al.*, 2007) this forces the cells to use oxidative phosphorylation as a means of energy production which in some cell types can also trigger mitophagy (Lemasters *et al.*, 2005; Melser *et al.*, 2013).

5.2 Methods and Materials

See sections general methods and materials chapter, section 2.1.1 and 2.2 for detailed tissue culturing and chapter 3, section 2.1 for OCR assay. For OCR detection in fibroblast, the volume of MitoXpress probe and media was reduced from 150µl to 140µl.

Please see chapter 4.2 (IN Cell validation) for detailed methods of staining, image capture and analysis.

PolG patients and non-affected controls fibroblasts

Two patients were selected from Professor J. Poulton's library of primary fibroblast collected from her clinical patients. The PolG patients were selected because they both had the mutation p.Trp748Ser, found in the linker region of PolG, table 5.1. Both patients suffered from seizures and were prescribed the anti-seizure drug VPA. However, the patient outcome from VPA treatment was very different. Patient 1 suffered acute liver failure and died at the age of ~4 years. Patient 2 has been on VPA therapy for a number of years, seemingly without any side-effects and is relatively healthy at the age of ~43 years. The non-affected control primary fibroblasts were a kind gift from Dr Simon Olpin (Sheffield Children's Hospital, UK). There was no clinical information regarding these controls and it is believed that they do not have mitochondrial disease.

Primary fibroblasts were obtained from participants with informed consent (parental consent for children) and with approval from the UK National Research Ethics Service (South Central Berkshire). All samples were anonymised.

Table 5.1. Overview of patient and control primary fibroblast used in this study.

Patient (P) Control (C)	Age*	Sex	Sequence analysis	Affected protein domain	Clinical manifestation and drug treatment
P1	2 years	M	c.2125C>T (p.Arg709X) c.2243G>C (p.Trp748Ser)	Linker region Linker region	Seizures, nystagmus ataxia, hypotonia. ALF on VPA administration
P2	42 years	F	c.1399G>A (p.Ala467Thr) c.2243G>C (p.Tyr748Ser)	Linker region Linker region	Seizures, ophthalmoplegia, neuropathy. Currently on VPA treatment
C1	20 years	M			
C2	20 years	M			
C3	1 year	F			
C4	15 years	F			
C5	1 year	M			
C6	1 year	F			
C7	6 months	F			
C8	1 year	F			

*Age of the donor at the time the skin biopsy was taken. ALF- acute liver failure

5.3 Results

5.3.1 Mitochondrial function and network morphology of non-affected control fibroblast in response to normal and energetically stressed conditions.

It has been established that mitochondrial function deteriorates as part of the human ageing process, especially in organs that have a high energy demand such as cardiac tissue (Tocchi *et al.*, 2015). In the forthcoming experiments I used the seven controls listed in table 5.1 to investigate the effect of galactose induced mitochondrial energetic stress response in control fibroblasts established from individuals under the age of 21 years.

The controls were used to establish the variability of oxygen consumption rates, autophagy/mitophagy levels and mitochondrial network morphology when subjected to energetics stress within a control population. As outlined previously, energetic stress was induced by reducing the amount of glucose or replacing glucose with galactose thus, forcing the cells to rely on OXPHOS for energy. Under normal conditions i.e. where glucose is available as a carbon source fibroblasts rely on glycolysis to meet energy demand (Mortiboys *et al.*, 2008). Cells were incubated in media containing either, high glucose media (25mM), low glucose (5.5mM) or galactose (10mM) and assayed at 24, 48 and 72 hours. It should be noted that high glucose was deemed to be the baseline control as this was the normal maintenance medium. It is common laboratory practice to maintain human fibroblasts in 20 to 25mM glucose.

5.3.1.1 *The effect of glucose concentration and energetic stress on mitochondrial function and network morphology in control fibroblasts.*

The age range of the seven control fibroblasts used was between 6 months and 20 years of age, both male and female. It is understood that these controls were from skin biopsies of donors which did not have any known mitochondrial dysfunction.

Cells were seeded in 96 well plates for the OCR and Hoechst assays as previously described sections 2.2.1.2 and 2.5. The data showed that at baseline (high glucose) OCR levels in the controls were similar, only C2(20yr) and C6(1yr) being statistically different ($p= 0.0237$) from each other, figure 5.1. When the controls were subjected to low glucose condition (5.5mM), OCR increased in cell lines compared to high glucose medium (baseline).

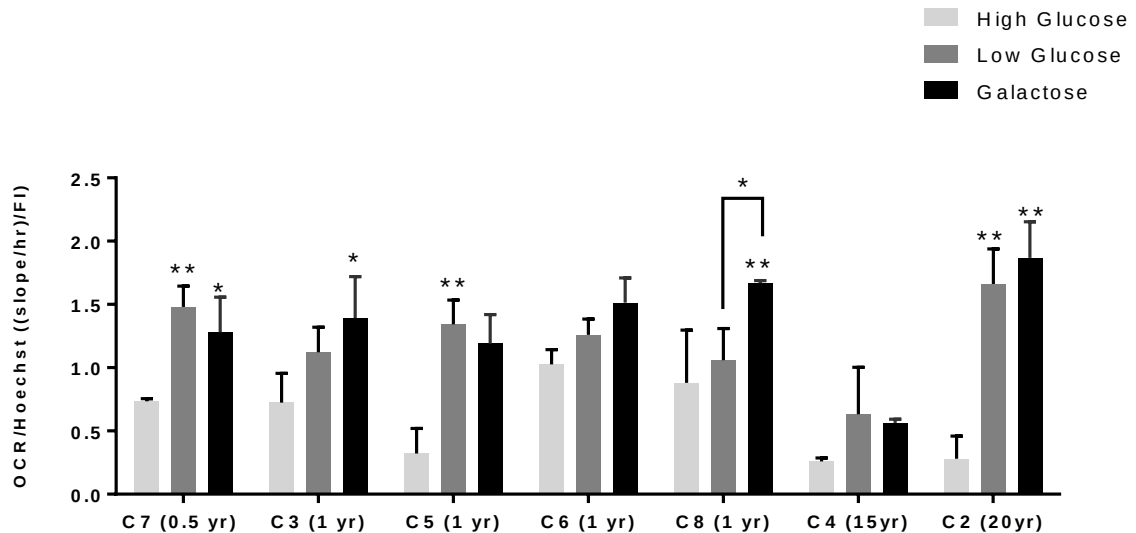


Figure 5.1. Control fibroblasts after 72 hours under energetic stress have increased oxygen consumption rates. 25,000 cells were seeded and left to adhere before the media was changed to high glucose (normal maintenance, 25mM), low glucose (5mM) and galactose (10mM). Data normalised to cell number as determined by Hoechst staining. Data represent mean $\pm$ SD of four technical replicates, n=2. *p<0.05, **p<0.001, ANOVA with Bonferroni correction when compared to baseline high glucose.

When the controls fibroblasts were further challenged by growth in galactose medium, all cell lines attempted to compensate for the lack of glucose by increasing OCR/OXPHOS. All the control cell lines responded similarly to the galactose challenge with only the C4(15yr) control line being unable to increase its OCR, figure 5.1.

In a tandem plate, Hoechst stain was used to ascertain the effect on the cell population doubling time after the incubation in high and low glucose and galactose, figure 5.2. Cell number was determined as relative to Hoechst fluorescence.

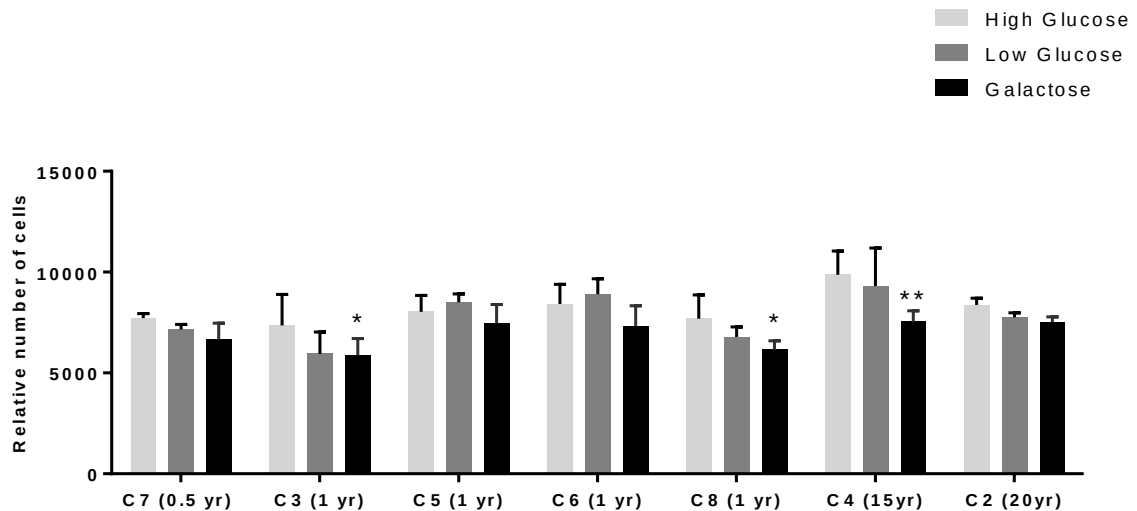


Figure 5.2. Control fibroblasts under normal conditions and energetic stress, relative number of cells present after 72 hours incubation. 25,000 cells were seeded and left to adhere before the media was changed to high glucose (normal maintenance), low glucose (5mM) and galactose. Cell number as determined by Hoechst staining. Data represent mean $\pm$ SD of four technical replicates, n=2. *p<0.05, **p<0.001, ANOVA with Bonferroni correction when compared to baseline high glucose.

After 72 hours incubation cell number in low glucose (5.5mM) was very similar to cells cultured in high glucose (25mM). However, under galactose energetic stress there was a reduction in cell number in some of the lines with C3(1yr), C4(15yr) and C8(1yr) showing a statistically significant reduction compared to high glucose medium baseline. Furthermore, there were no significant differences between control fibroblasts in low glucose or galactose, figure 5.2. Interestingly, control C4(15yr) with the lowest OCR grew the fastest under all media growth conditions.

5.3.1.2 *Establishing levels of autophagy/mitophagy events and network morphology within a group of fibroblast controls under normal and energetic stress condition*

To investigate autophagy/mitophagy and mitochondrial network changes in response to galactose induced energetic stress, 2,500 cells were seeded per well in 96 well plates under high glucose (normal maintenance), low glucose or galactose media conditions. Detailed methods can be found in chapter 4.

The first element of autophagy/mitophagy to be investigated was the numbers of autophagosomes as indications of autophagy, in the control cell lines after 24, 48 and 72 hours incubation with the various media, figure 5.3. The level of autophagosomes present was established using the LC3 signal and reported as number of autophagosomes per cell.

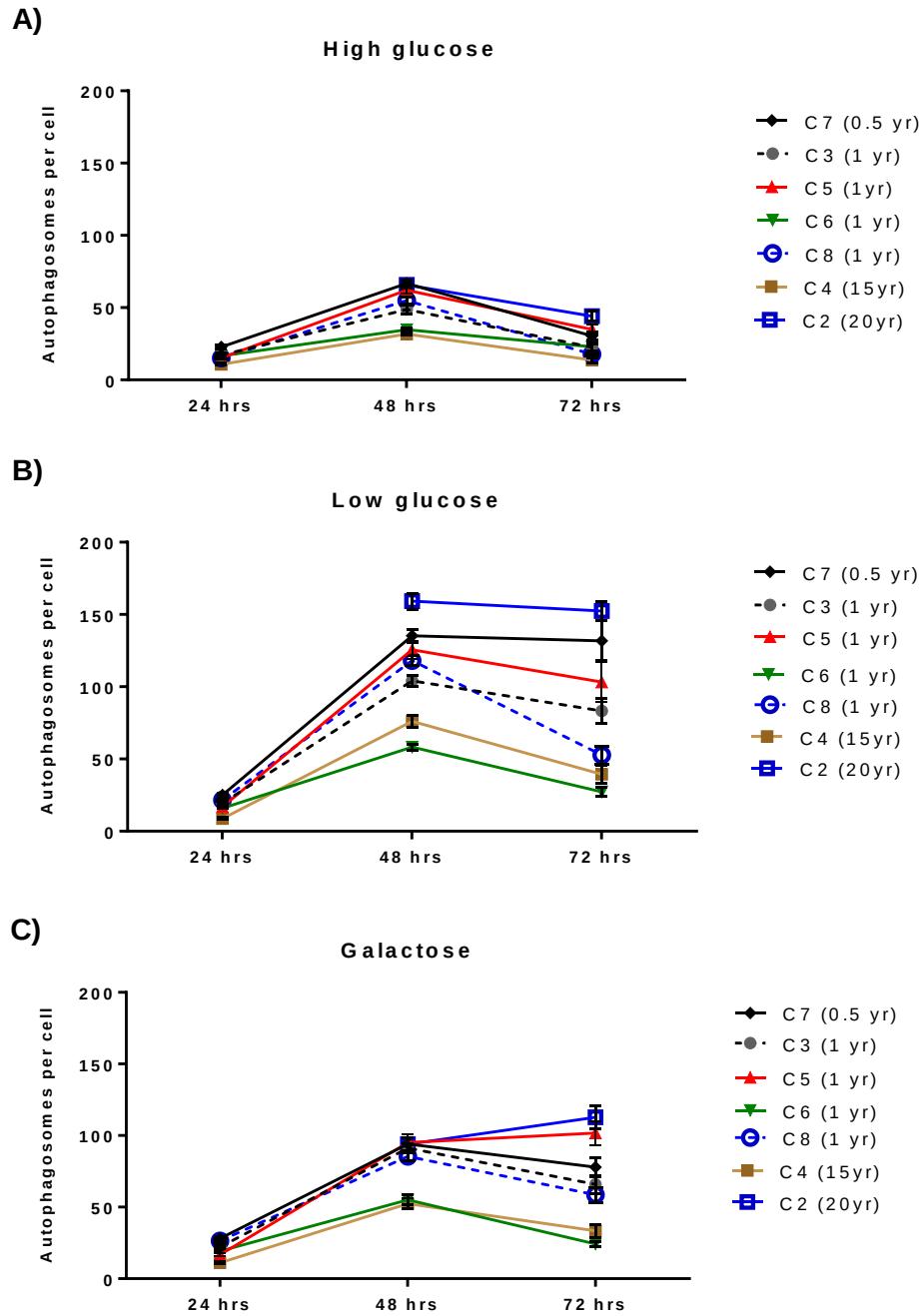


Figure 5.3. Control fibroblasts under energetic stress increases the number of autophagosomes (autophagy). Cells incubated in (A) 25mM glucose (high), (B) 5.5mM glucose (low) and (C) 10mM galactose for 24, 48 and 72 hours. Autophagosomes detected by LC3 signal. Data represent mean $\pm$ SEM of four technical replicates, $n=3$. ≥ 250 cells/experiment analysed.

As expected there was no change in autophagosomes observed after incubation in normal fibroblast media conditions (25mM glucose), figure 5.3 (A). Changing the concentration of glucose from 25mM to 5mM increased the number of autophagosomes was observed, after 48 hours incubation, figure 5.3 (B). A number of cell lines sustained this over 72 hours although there was a significant decrease in the number of autophagosomes present in three of the control lines, C4(15yr), C6(1yr), C8(1yr) ($p < 0.0001$). No data is available for C2(20yr) as it was lost to contamination. Further challenging the cells by switching the carbon source from high glucose to galactose also significantly increased numbers of autophagosomes after 48 hours incubation of all the control lines ($p < 0.001$), figure 5.3 (C). Two of the control lines increased autophagosomes further after 72 hours. Whereas the majority showed a decrease in numbers, figure 5.3 (C).

To establish levels of possible mitophagy events, the number of autophagosomes co-localising with the mitochondria was determined. The protocol used is discussed in detail, chapter 4. The data is presented as percentage of mitophagy relative to mitochondrial area rather than the whole cell. This was considered to be the most robust method as it takes into account the area of mitochondria and not only the co-localisation events. Thus, reflect the potential for mitophagy events.

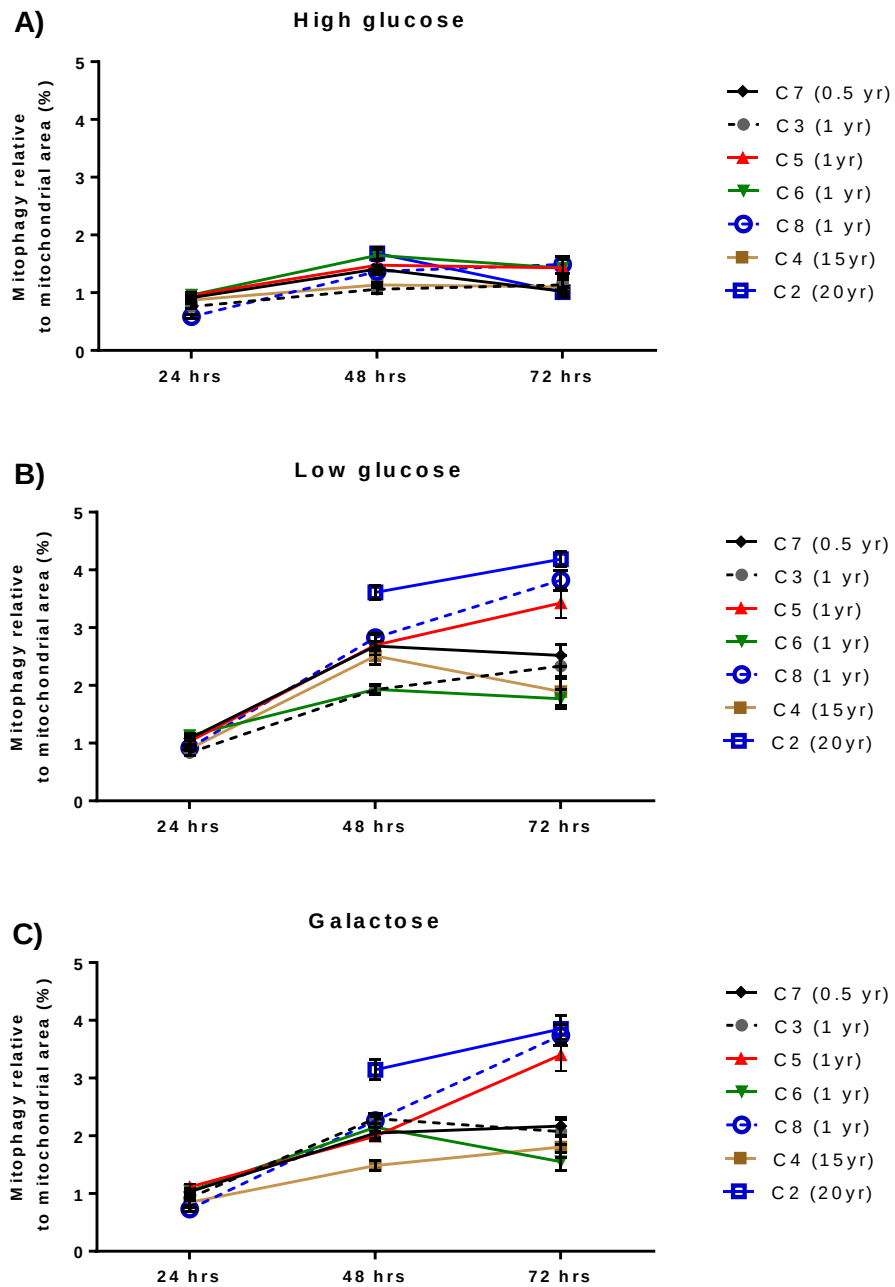


Figure 5.4. Control fibroblasts under energetic stress increases co-localisation between autophagosomes and the mitochondria (mitophagy). Cells incubated in (A) 25mM glucose (high), (B) 5.5mM glucose (low) and (C) 10mM galactose for 24, 48 and 72 hours. Data represent mean $\pm$ SEM of four technical replicates from three independent experiments, ≥ 250 cells/experiment analysed.

As expected there was little variation in the co-localisation between the autophagosomes and mitochondria under normal conditions over the three time points, figure 5.4 (A). However, after incubation in low glucose and under energetic stress condition (galactose), there was significant ($p=0.043$) increases in co-localisation after 48 hours, figure 5.4 (B) and (C).

5.3.1.3 Mitochondrial network morphology

As the ultimate goal was to investigate how VPA treatment impacts on mitochondrial morphology we developed approaches using the IN Cell 1000 analyzer to investigate changes in mitochondrial structure. Applying the same energetic stressed conditions as used in the previous section, parameters including mitochondrial length and the number of three-way intersections (Y) bifurcations of the network was established and is discussed in detail in chapter 4. The more interconnected the network is the more bifurcations are expected.

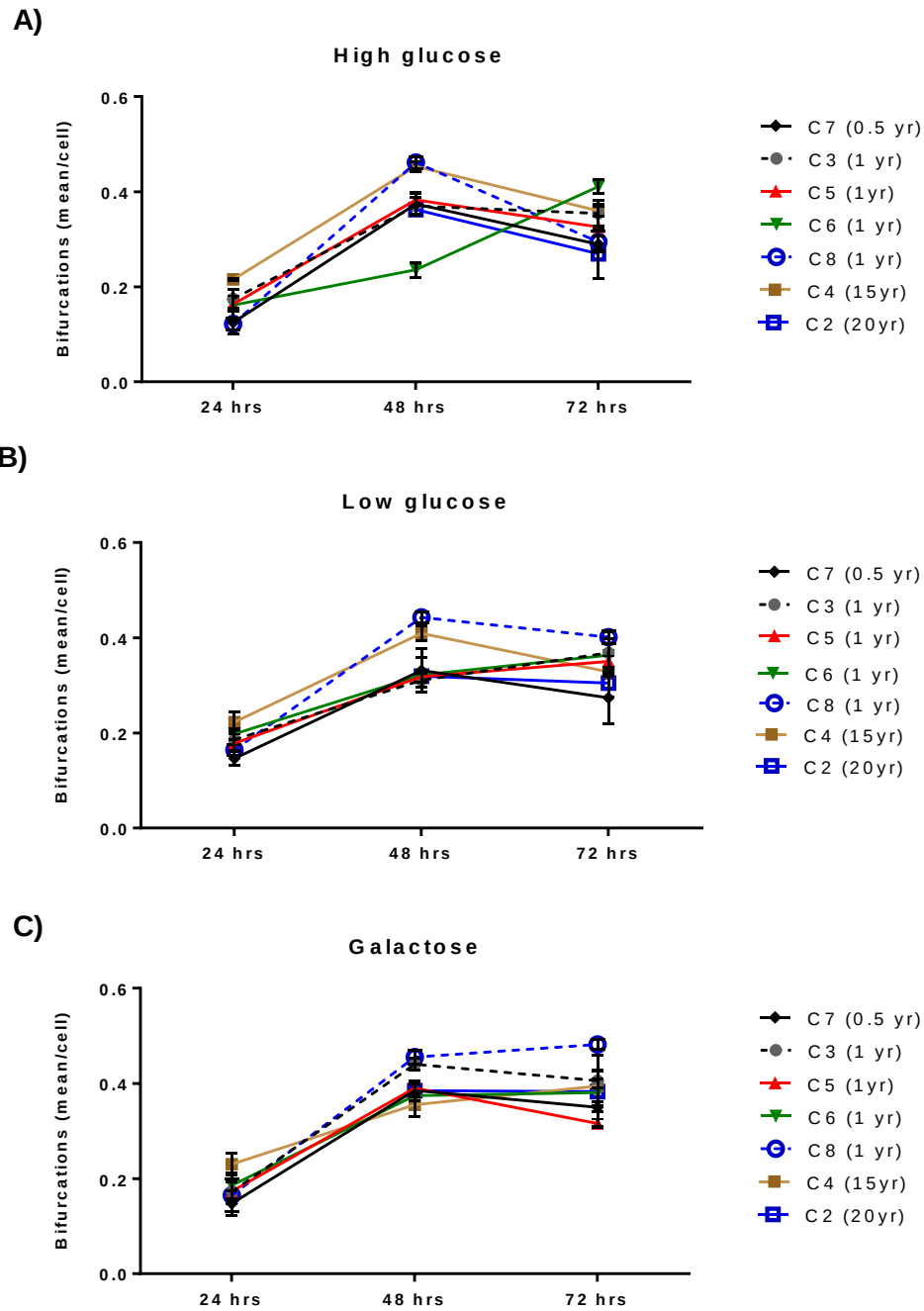


Figure 5.5. Control fibroblasts under galactose induced energetic stress increases the number of bifurcations of the mitochondrial network. Cells incubated in (A) 25mM glucose (high), (B) 5.5mM glucose (low) and (C) 10mM galactose for 24, 48 and 72 hours. Data represent mean $\pm$ SEM of four technical replicates from three independent experiments, ≥ 250 cells/experiment analysed.

The number of bifurcation detected increased after 48 hours in all media conditions, figure 5.5. The increased number of bifurcations observed under normal conditions (high glucose) was not expected as neither the levels of autophagosomes or potential mitophagy events changed. It is possible that the increased number of bifurcations is a reflection of culture conditions as the mitochondrial network becomes more interlinked after high glucose media change.

5.3.1.4 Discussion - Sections 5.3.1.1 to 5.3.1.3

Selecting non-affected mitochondrial disease control fibroblasts

Overall there was no significant difference between the seven controls under normal glucose medium (high glucose) in oxygen consumption rates, levels of autophagosomes, mitophagy or the mitochondrial network morphology. An overview of the results from the OCR assay and changes to mitochondrial morphology after 72 hours incubation in low glucose and under energetic stress (galactose) can be found in table 5.2. Interestingly, after 72 hours incubation in low glucose and under energetic stress (galactose) controls C4(15yr) and C6(1yr) did not show significant changes to OCR, levels of autophagosomes or network morphology when compared to high glucose (normal condition), table 5.2. This suggests that these two control lines do not have the ability to adjust their mitochondrial response to changes in glucose concentration (high to low) or to energetic stress (galactose). From henceforth I refer to this ability as 'plasticity'. There was however an increase in mitophagy in C4(15yr) and C6(1yr). Energetic stress induced by galactose media has previously been shown to induce increased mitophagy (Melser *et al.*, 2013).

Table 5.2. Summary overview of responses in control fibroblast after 72 hours incubation with low glucose (5.5mM) and galactose (10mM). Mitochondrial function detected by OCR, autophagy detected by levels autophagosomes, mitophagy (co-localisation between autophagosomes and mitochondria, bifurcations (mitochondrial network morphology) and relative cell population. The arrows indicate where significant response occurred when compared to baseline high glucose (25mM). ↑ significant increase, ↓ significant decrease, ↔ no significant change.

Ctrl	OCR			Autophagosomes			Mitophagy			Bifurcations			Relative cell nos.		
	High Glu	Low Glu	Gal	High Glu	Low Glu	Gal	High Glu	Low Glu	Gal	High Glu	Low Glu	Gal	High Glu	Low Glu	Gal
C7(0.5yr)	–	↑	↑	–	↑	↑	–	↔	↔	–	↔	↑	–	↔	↔
C3(1yr)	–	↔	↑	–	↑	↑	–	↑	↑	–	↔	↑	–	↔	↓
C5(1yr)	–	↑	↔	–	↑	↑	–	↑	↑	–	↔	↔	–	↔	↔
C6(1yr)	–	↔	↔	–	↔	↔	–	↑	↑	–	↔	↔	–	↔	↔
C8(1yr)	–	↔	↑	–	↑	↑	–	↑	↑	–	↑	↑	–	↔	↓
C4(18yr)	–	↔	↔	–	↔	↔	–	↑	↑	–	↔	↔	–	↔	↓
C2(20yr)	–	↑	↑	–	↑	↑	–	↑	↑	–	↔	↔	–	↔	↔

Furthermore, regression analyses performed (data not shown) showed no significant correlation was found when respiration rates (OCR) versus mitophagy or OCR versus bifurcations were compared. In summary, most of the control cell lines, with the exception of C4(15yr) and C5(1yr) had significantly increased levels of autophagosomes, mitophagy and bifurcations after 48 and 72 hours incubation in low glucose and galactose energetic stress conditions. Within the age range of the control cell lines there were no clear effect of age on mitochondrial respiration, levels of autophagosomes, mitophagy and bifurcations.

To investigate the change in network morphology linked to energetic stress correlated with changes in OXPHOS a regression analysis was performed on bifurcations and OCR data. No significant correlation was observed, appendix B, supplementary figure 2.

Results

5.3.2 *Establishing how patients with PolG and non-affected control fibroblast differ in their response to VPA treatment under normal and energetic stress conditions*

Introduction

As it was not possible to carry out all subsequent assays with the whole control cell line series, two controls were selected which reflected the control population. As a previous study by Diot *et al* (2015) showed a correlation between age and a decline in mitophagy in fibroblasts from controls aged between 0 and 81 years. Controls C2(20yr) and C8(1yr) were initially selected as the fibroblast cultures with ages as close as possible to the two PolG patients ages 2 and 42. Unfortunately, C2(20yr) was later lost to contamination therefore another control aged 20 (C1) was selected as the closest age matched control. The control fibroblast C1(20yr) was the same control used in the IN Cell validation experiments, chapter 4.

In an attempt to further determine the mitochondrial effects of sodium valproate (VPA) treatment. PolG patient fibroblasts and non-affected controls were incubated under normal and energetic stressed conditions with VPA concentrations at 0.5mM, 1.0mM and 2.0mM for 72 hours. As in previous experiments normal culture medium (25mM glucose) and galactose medium (energetic stress) was used.

VPA is highly toxic to a small group of mitochondrial disease patients. VPA therapy can induce acute liver failure in patients carrying the pathogenic autosomal recessive *POLG* mutation (Silva *et al.*, 2008). Two PolG patients were selected who showed different outcomes following VPA anti-seizure therapy. Patient 1 (P1(2 yr)) was heterozygous for PolG mutations at c.2243G>C (p.T748S) and c.2125C>T (p.A709X). These mutations are rare examples of autosomal recessive pathogenic variant, which

cause Alper Huttenlocher's syndrome when found together in the same patient. Patient 1 (P1(2yr) died age ~4 due to liver failure associated with VPA treatment. The second PolG patient (P2 (42 yr)) also harbours the *POLG* mutation c.2243G>C (p.T748S) but with a second heterozygous mutation at c. 1399G>A (p.A467T). This patient has a mild form of the disease and is currently on VPA treatment for epilepsy. These two patients and two controls were used to investigate the effect of VPA treatment on cellular respiration and mitochondria network morphology. The controls were selected as described in the earlier section 5.3.1.4, two controls selected that were as close in age as possible to the patients. The two patients were chosen due to the shared c.2242G>C (p.T748S) mutation and the different outcomes of VPA treatment.

5.3.2.1 Establishing how patients with PolG and non-affected control fibroblast differ in their response to normal and energetic stress conditions before VPA treatment

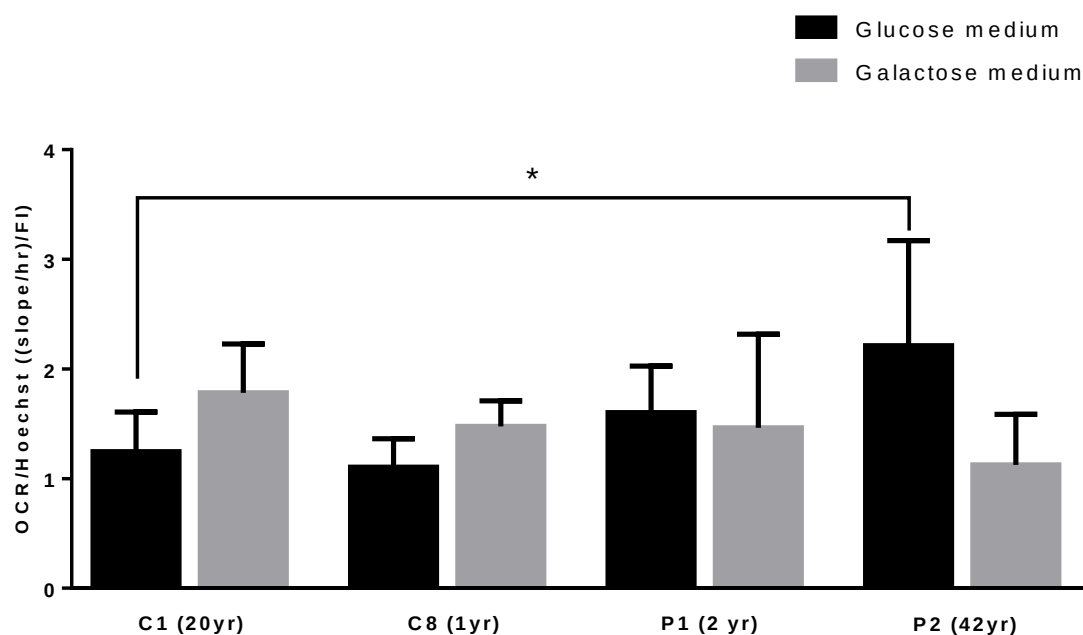


Figure 5.6. The effect of energetic stress on cellular respiration. Oxygen consumption rates of PolG patients and control fibroblasts under normal condition and after 72 hours energetic stress condition. 25,000 cells were seeded in 96 well plates and left to adhere before incubated in normal 25mM glucose condition (black bars) or under energetic stress galactose condition (grey bars). Control fibroblasts are C1(20yr) and C8 (1yr), patients are P1(2yr) and P2(42 yr). Cell number was determined by Hoechst. Data represent $\pm$ SD of three technical replicates, $n=3$, $*p<0.05$, ANOVA with Bonferroni correction.

Treatment of controls and patients with energetic stress for 72 hours did not significantly alter the OCR, figure 5.6. Surprisingly patient P2(42yr) in 25mM glucose culture medium (normal) had a statistically significant higher OCR than its aged

matched control, figure 5.6. Furthermore, P2(42yr) also grew significantly slower than the older control C1(20yr) in normal culture medium (equal number of cells seeded), figure 5.7. Only P1(2yr) showed a significant reduction in growth rate under energetic stress condition when compared to normal cell culture conditions, figure 5.7.

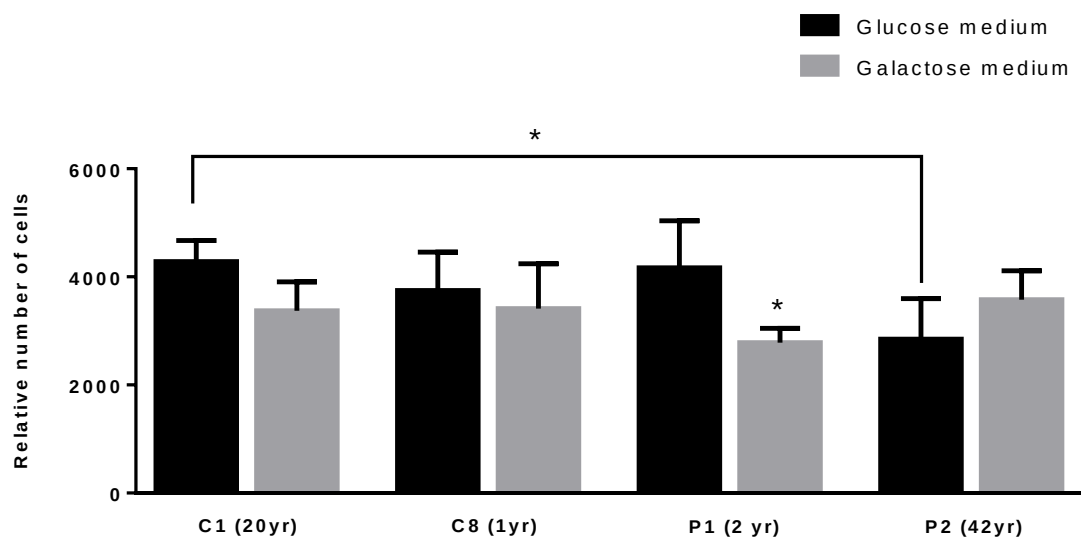


Figure 5.7. The effect of Energetic stress on cell population. The cells were incubated in normal 25mM glucose condition or under energetic stress galactose condition for 72 hours. The relative numbers of cells were detected using Hoechst. Control fibroblasts are C1(20yr) and C8 (1yr), patients are P1(2yr) and P2(42 yr). Data represent $\pm$ SD of three technical replicates, $n=3$, $*p<0.05$, ANOVA with Bonferroni correction.

Incubating the fibroblasts cell lines under energetic stress did not affect ATP levels in the controls or patient P1(2yr) but in P2(42yr) ATP levels were significant increased. In addition, patient P1(2yr) had significantly higher ATP levels under normal conditions when compared to control C1(20yr) and patient P2(42yr), figure 5.8.

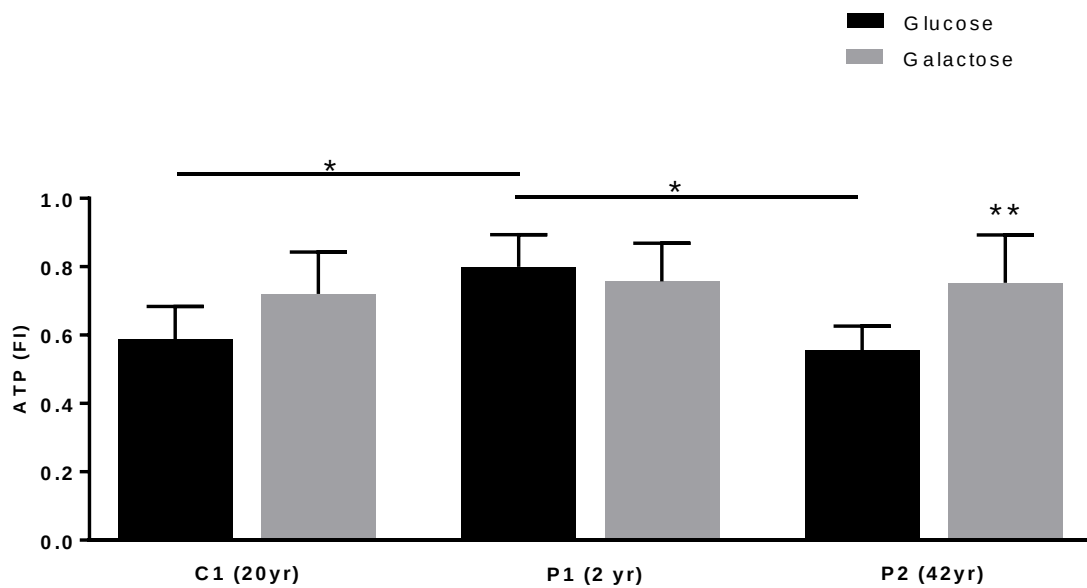


Figure 5.8. ATP levels after 72 incubated under energetic stress. The cells were incubated in normal 25mM glucose condition or under energetic stress galactose condition for 72 hours. ATP was normalised to cell number by Hoechst staining. Data represent $\pm$ SD of three technical replicates, $n=2$, * $p<0.05$, ** $p<0.001$, ANOVA with Bonferroni correction.

In summary, under high glucose conditions P2(42yr) had a higher rate of OCR and reduced growth rate when compared to its age matched control but no difference in ATP. However, in glucose condition P2(42yr) had significantly lower ATP than P1(2yr). Interestingly, P2(42yr) was the only cell line to have significant increased ATP after 72 hours incubation under galactose induced energetic stress.

5.3.2.2 Establishing how patients with PolG mutations and non-affected control fibroblast differ in their mitochondrial response to normal and energetic stress conditions after VPA treatment

In order to investigate the effect of VPA treatment in patients with PolG mutations and control fibroblasts a 72 hours incubation period was selected as previous experiments, section 5.3.1.2, showed this to be the most appropriate incubation period.

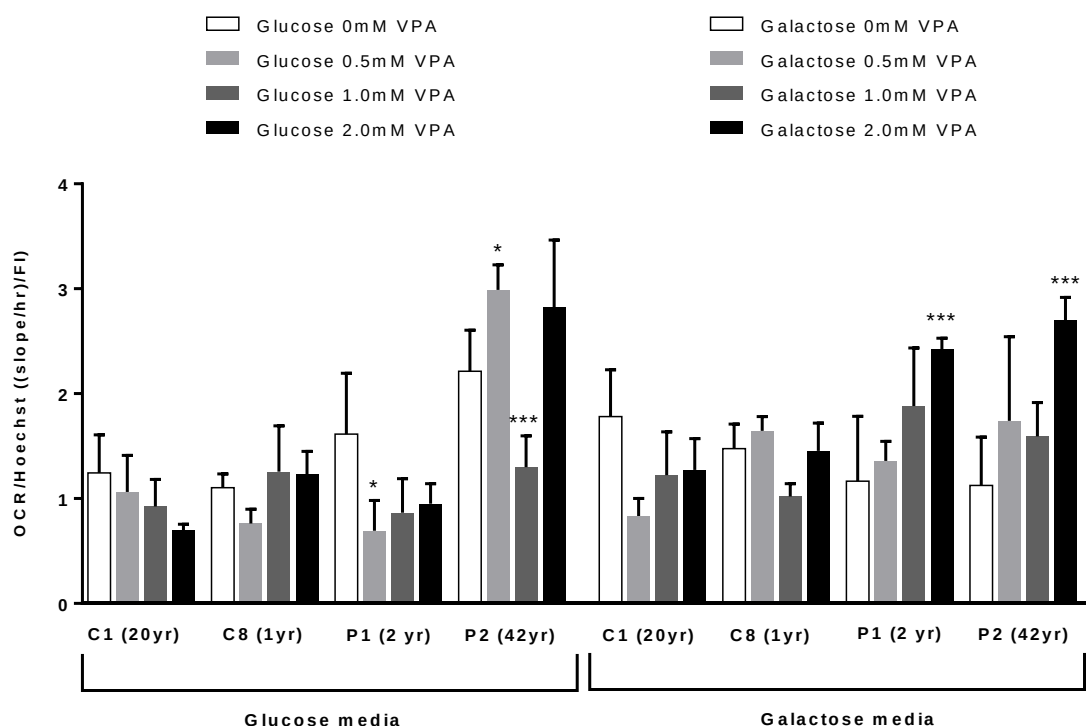


Figure 5.9. Oxygen consumption rates of PolG patient and control fibroblasts after 72 hours treatment with VPA under 25mM glucose and energetic stress conditions. 25,000 cells were seeded in 96 well plates and left to adhere before treatment commenced. Energetic stress induced by incubation in 10mM galactose. Cell number was determined by Hoechst. Data represent mean $\pm$ SD of three technical replicates, $n=3$, $*p<0.01$, $***p<0.0001$, ANOVA with Bonferroni correction when compared to baseline (glucose).

After 72 hours VPA treatment in 25mM glucose media, the effect on OCR appears to be inhibitory in the most severe PolG patient (P1(2yr)) but had no effect on the controls, figure 5.9. In patient P2(42yr), the glucose response to VPA treatment was interesting, a significant decrease in OCR was consistently found following incubation with 1mM VPA but the higher 2mM dose was no different from the control, figure 5.9. Under galactose energetic stress conditions VPA had no effect on control cells but both patient lines showed a significant increase in OCR at the high 2mM concentrations compared to non VPA treated galactose treatment.

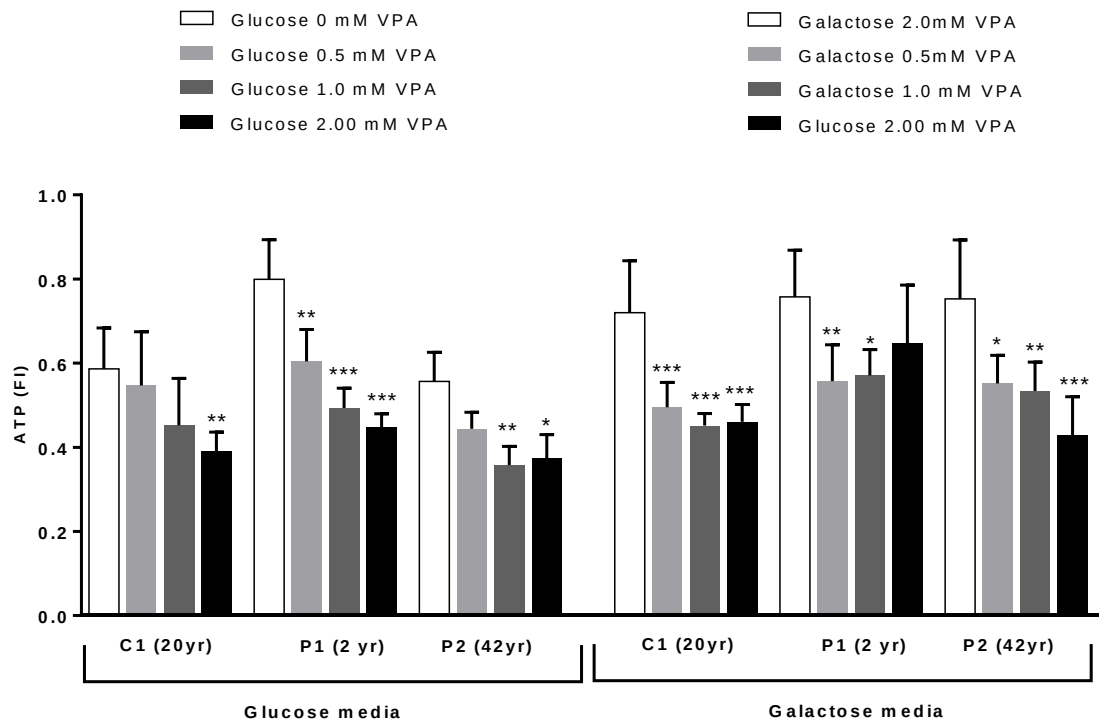


Figure 5.10. ATP levels of PolG patient and control fibroblasts after 72 hours treatment with VPA under normal and energetic stress conditions. ATP was normalised to cell number as determined by Hoechst. Data represent mean $\pm$ SD of three technical replicates, $n=3$, * $p<0.01$, *** $p<0.0001$, ANOVA with Bonferroni correction when compared to baseline (25mM glucose).

After 72 hours of VPA treatment under normal condition cellular ATP was reduced in a concentration dependant manner for C1(20yr) and P1(2yr), figure 5.10. The reduction in P2(42yr) was not dose dependant. When the cells were treated with VPA under energetic stress there was significant reduction in ATP, across all drug concentrations.

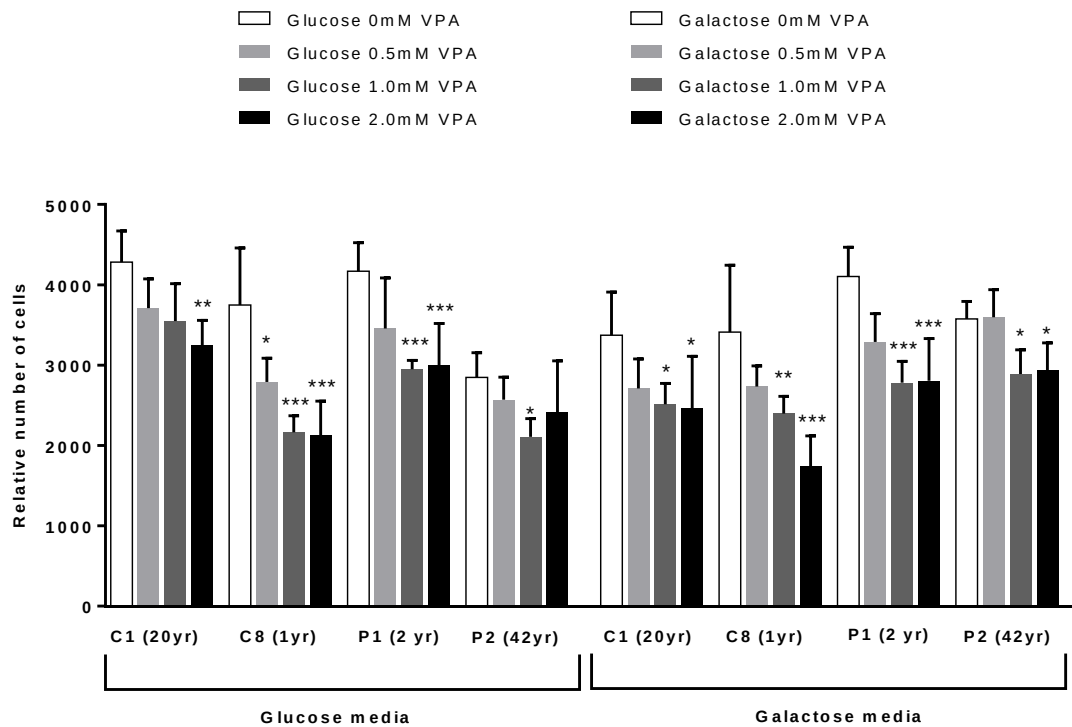


Figure 5.11. Relative number of cells present of PolG patient and control fibroblasts after 72 hours treatment with VPA under normal and energetic stressed conditions. Under normal conditions cells were incubated with 25mM glucose. To induce energetic stress, galactose replaced glucose, striped bars. Cell number was determined by Hoechst. Data represent mean $\pm$ SD, $n=3$, $*p<0.01$, $***p<0.0001$, ANOVA with Bonferroni correction when compared to baseline (glucose).

VPA treatment for 72 hours caused a reduction in cell number, either by inhibition of proliferation or increased cell death, figure 5.11. A reduction in relative cell number was observed in a VPA concentration dependant manner in controls and patients. Under normal glucose condition there were no difference between the two controls and P1(2yr) however P2(42yr) had a significantly slower cell growth rate than C1(20yr) ($p=0.0247$). All cell lines were seeded at the same density. It appears that P2(42yr) has a slower doubling time than the other cell lines under normal glucose conditions. With energetic stress and VPA treatment cell population growth slowed or increased death occurred in all cell lines, in a dose dependant manner. From figures 5.9 and 5.10

it seems that patient P2(42yr) fibroblasts can greatly increase OCR in response to stressors such as VPA but have a slower proliferation rate.

5.3.2.3 *Modulation of autophagy/mitophagy and mitochondrial network morphology in control and PolG patient's with and without sodium valproate treatment.*

Cells were prepared for autophagy/mitophagy and mitochondrial network morphology as described previously. Cells were then treated with glucose or galactose media with and without VP treatment. After treatment plates were fixed and stained as previously described section 4.2.

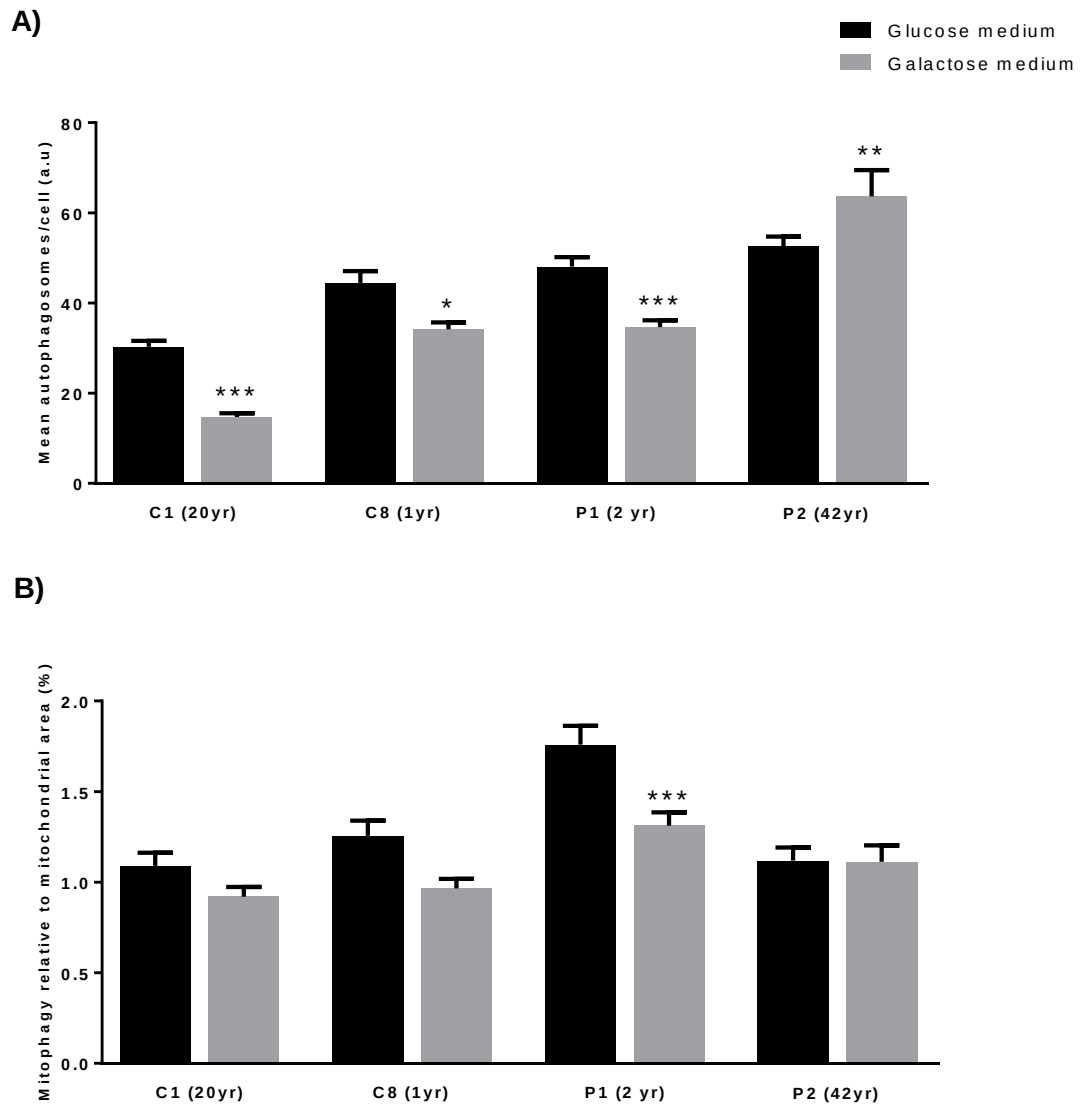


Figure 5.12. Levels of autophagosomes and mitophagy after 72 hours under energetic stressed condition. Stressed conditions were induced by incubation in galactose medium, grey bars. (A) Mean number of autophagosomes per cell as detected by LC3 signal. (B) Mitophagy detected by co-localisation of LC3 and TOM20 (mitochondrial) signal. Data represent mean $\pm$ SD of three technical, $n=3$, ≥ 500 cells/experiment analysed. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$, ANOVA with Bonferroni correction when compared to baseline (25mM glucose).

Both controls and P1(2yr) showed a significant decrease in autophagosomes following 72 hours of energetic stress in galactose medium. P2(42yr) however showed a significant increase in autophagosomes, figure 5.12, (A). Although both controls

showed a tendency to also reduce autophagosomes under energetic stress conditions only P1(2yr) had significantly reduced levels of mitochondrial/autophagosomes co-localisation resulting in mitophagy, figure 5.12 (B). Patients P2(42yr) and the controls showed no evidence of reduced mitophagy.

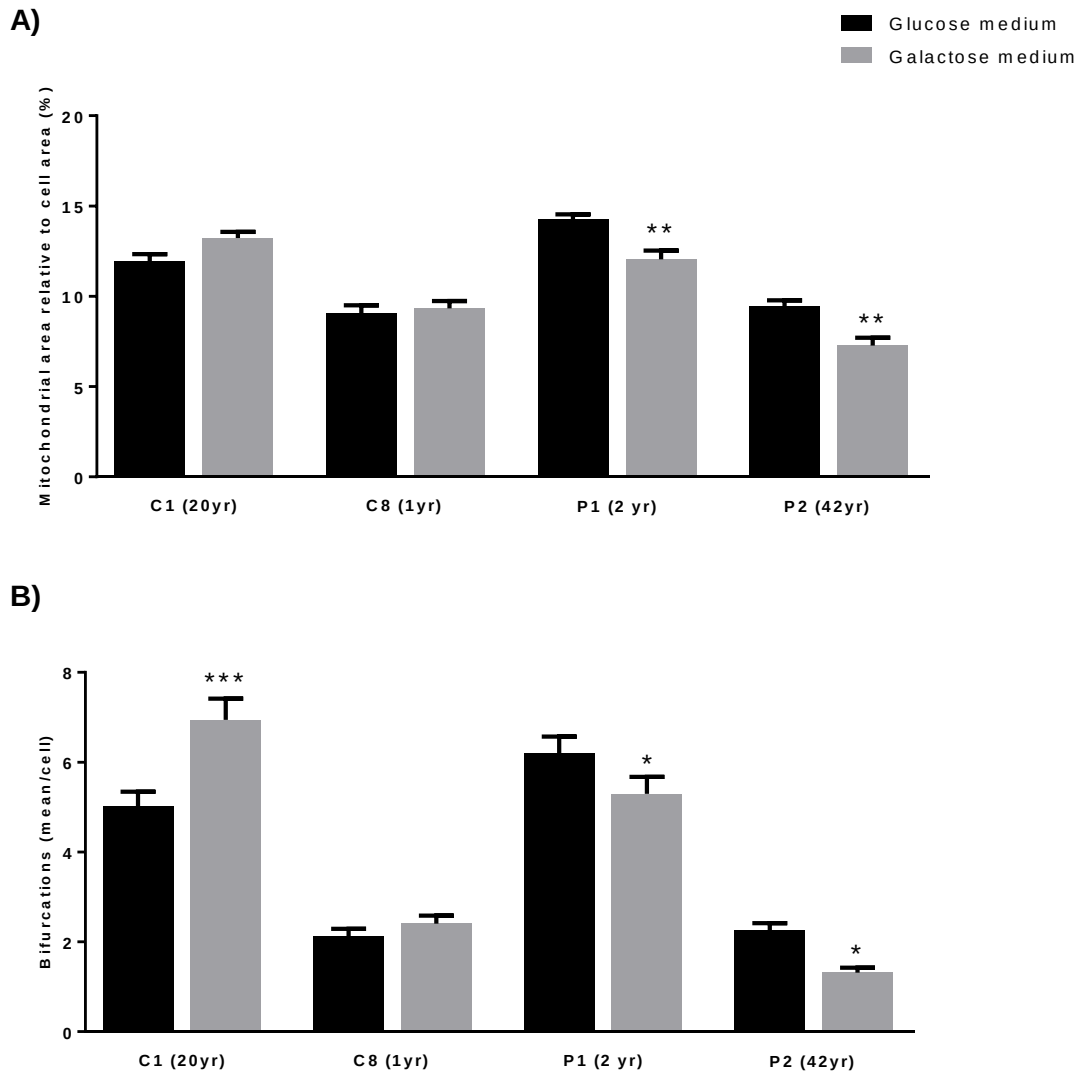


Figure 5.13. Mitochondrial area and network morphology in PolG patients and control fibroblasts after 72 hours incubation under 25mM glucose and stressed conditions. Stressed conditions were induced by incubation in galactose medium, grey bars. (A) Mitochondrial area as detected by TOM20 signal. (B) Mitochondrial network morphology detected by the number of bifurcations. Data represent mean $\pm$ SD of three technical replicates, $n=3$, ≥ 500 cells/experiment analysed. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$, ANOVA with Bonferroni correction when compared to baseline (glucose).

There was significant reduction in the mitochondrial area after 72 hours incubation under energetic stress condition in both patients but not in controls, figure 5.13 (A). Furthermore, under normal glucose conditions, patient P1(2yr) had a significantly larger mitochondrial area compared to the other cell lines, figure 5.13 (A). The number of bifurcations of the mitochondrial network was increased in C1(20yr) after 72 hours incubation under energetic stress however both patients cell lines had decreased number of bifurcations, figure 5.13 (B). This indicated that in the control C1(20yr) the mitochondrial network became more interlinked or fused under energetic stress whereas in control C8(1yr) and the patients the network became more fragmented.

Following 72 hours VPA exposure in normal glucose (25mM) medium, the levels of autophagosomes in C8(1yr), P1(2yr) and P2(42yr) were increased in a dose dependant manner. In control C1(20yr) no changes in levels of autophagosomes were detected, figure 5.14(A). VPA treatment under energetic stress increased levels of autophagosomes in C8(1yr), C1(20yr) and P1(2yr) and in a dose dependant manner. However, in P2(42yr) which interestingly showed elevated levels of autophagosomes under energetic stress showed a reduction in autophagosomes when also incubated with VPA, figure 15.14 (A).

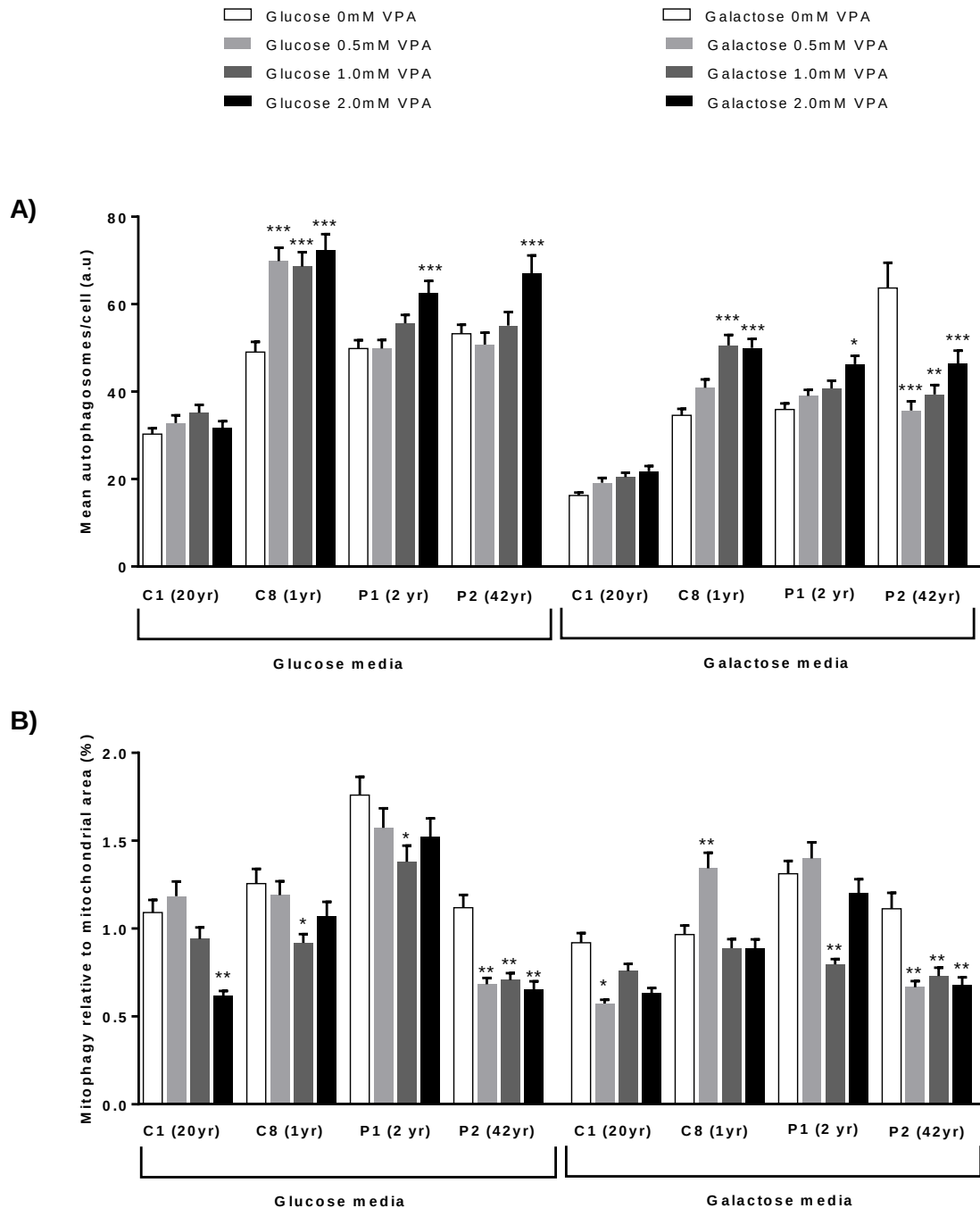


Figure 5.14. Levels of autophagosomes and mitophagy after 72 hours treatment with VPA under normal and energetic stressed conditions. Energetic stress conditions were induced by incubation in galactose medium, right. (A) Mean number of autophagosomes per cell as detected by LC3 signal. (B) Mitophagy detected by co-localisation of LC3 and TOM20 signals. Data represent mean $\pm$ SD of three technical replicates, $n=3$, ≥ 500 cells/experiment analysed. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$, ANOVA with Bonferroni correction when compared to baseline (25mM glucose).

The effect of VPA on mitophagy levels shows the opposite effect to autophagosomes with an overall reduction in mitophagy in all cell lines as VPA concentration increased on normal glucose medium. When the cell lines were treated with VPA under energetic stress there was a decrease in mitophagy in all cell lines, with the exception of C8(1yr) where there was an increase at 0.5mM VPA, figure 5.14 (B).

There were no changes to the mean mitochondrial area relative to cell area after VPA treatment under normal glucose condition, with the exception of C8(1yr) which had reduced mitochondrial area after treatment with 2mM VPA, figure 5.15 (A). Under energetic stress condition only P1(2yr) showed significant increase in mitochondrial area after exposure to 0.5mM and 1mM VPA.

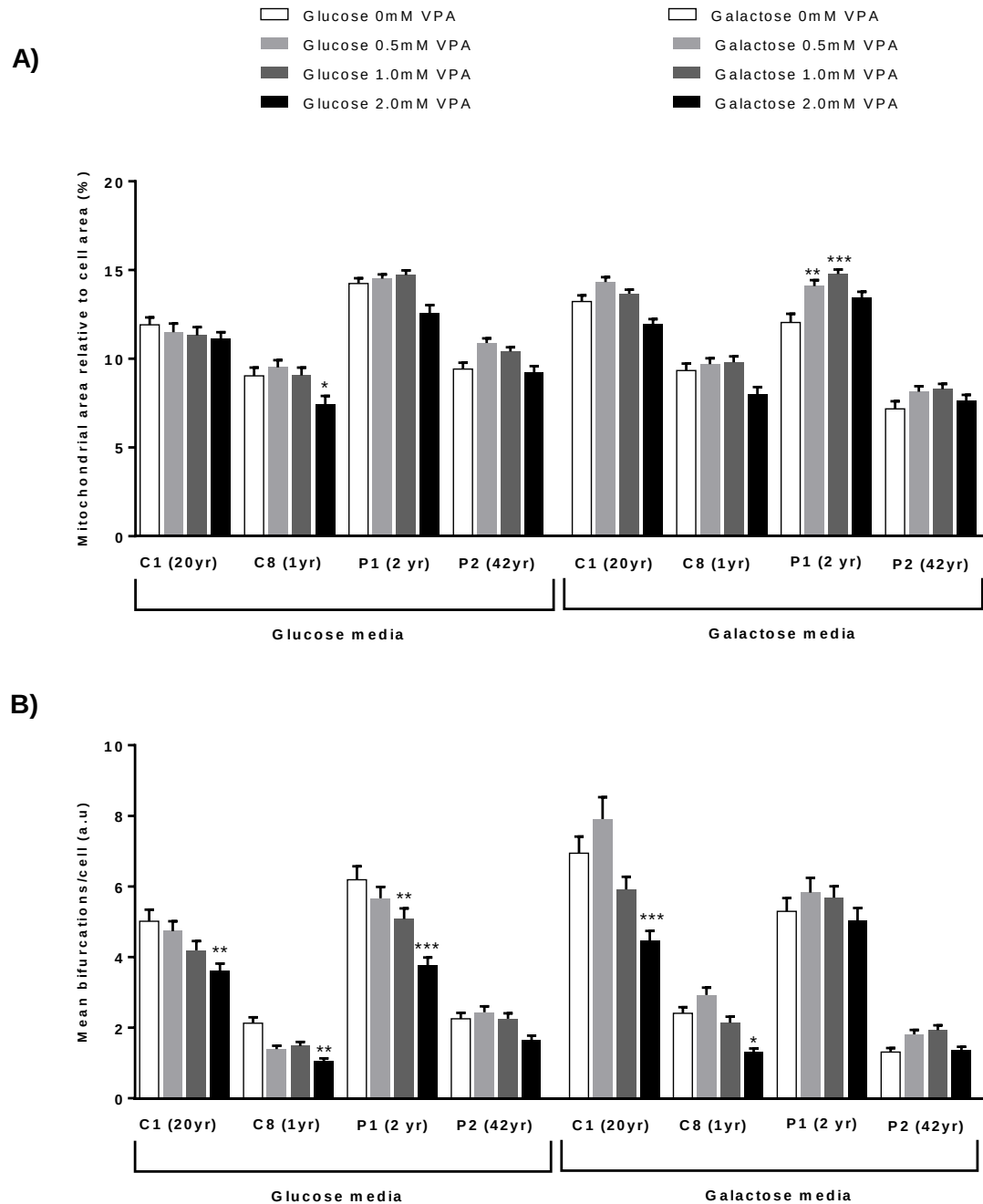


Figure 5.15. Mitochondrial area and network morphology in PolG patients and control fibroblasts after 72 hours treatment with VPA under normal and stressed conditions. Stressed conditions were induced by incubation in galactose medium, striped bars. (A) Mitochondrial area as detected by TOM20 signal. (B) Mitochondrial network morphology detected by the number of bifurcations. Data represent mean $\pm$ SD of three technical replicates, $n=3$, ≥ 500 cells/experiment analysed. * $p<0.05$, ** $p<0.001$, *** $p<0.0001$, ANOVA with Bonferroni correction when compared to baseline (25mM glucose).

VPA treatment under normal conditions did affect the mitochondrial network morphology, figure 5.15 (B). The number of bifurcations were decreased in both controls and patient P1(2yr) however, only the highest concentration of VPA was statistically significant. The reduction in bifurcations, imply that the mitochondria became less networked and more fragmented, figure 5.15 (B). VPA treatment under energetic stress caused network fragmentation in the controls at the highest VPA concentration only. Interestingly, there was no change to the network morphology in either of the patients.

5.3.2.4 Discussion – Sections 5.3.2.1 to 5.3.2.3

The effect of VPA treatment on metabolism

It is difficult to obtain a clear view of the effect of VPA treatment has on the OCR of fibroblasts. In the previous chapter (3.3.1) using HepG2 cells, VPA had a clear inhibitory effect in dose dependant manner under energetic stress condition and at the highest concentration, after 72 hours treatment. In patient fibroblast P1(2yr) VPA treatment at low dose (0.5mM) in normal glucose condition inhibited OCR where as in P2(42yr) it was increased. This could be due P2(42yr)'s ability to increase OCR as it is responding to depleting ATP levels. However, as the VPA concentration increases (1mM), in glucose condition, the OCR is inhibited. Both patients had increased OCR after treatment with 2mM VPA under energetic stress, indicating a stress response that did not result in increased ATP levels. In the control fibroblasts, VPA treatment had no significant effect in OCR in either glucose media or under energetic stress conditions. However, steady state ATP levels were reduced across all concentrations under both glucose and galactose conditions. One of the reasons behind this is that measuring OCR in fibroblast is challenging as they have very low respiration which makes it

difficult to detect. Therefore, ADP/ ATP ratio may be a more robust method for detecting mitochondrial function in primary fibroblasts. The majority of research published relating to VPA and patients with PolG mutations are clinical case studies, reporting novel mutations and its effect on for example mtDNA content and ETC complex activity of muscle or liver biopsies. In fibroblast cultures, it appears that PolG mutations does not affect ETC function but this may be patient and tissue dependant as Scaller *et al.*, (2011) showed that fibroblasts, muscle and liver biopsy from one patient with Alpers-Huttenlocher syndrome had varying degrees of reduced complex function. Liver was the most affected but skin derived fibroblasts and muscle homogenates were no different to healthy control.

The effect of VPA treatment on mitochondrial morphology

Results suggest that the mitochondrial network shows an adaptive response to energetic stress or toxicants such as VPA. To determine if changes in the mitochondrial network morphology associated with mitochondrial OXPHOS under normal and energetic stress conditions, bifurcations and OCR levels were plotted, figure 5.16.

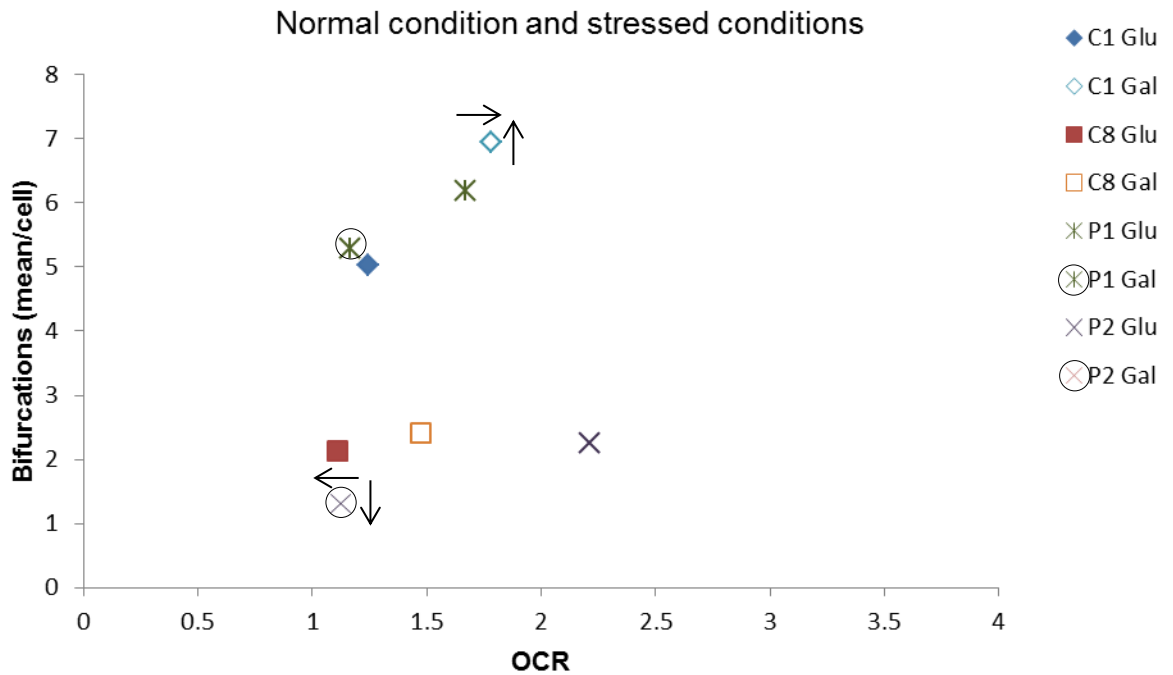


Figure 5.16. Bifurcations vs OCR after 72 hours incubation under normal and energetic stressed conditions. Normal condition was 25mM glucose (Glu) and energetic stress 10mM galactose (Gal). Arrows denotes shifting response to galactose induced energetic stress. Data for controls and PolG patients' fibroblasts were extracted from IN Cell and OCR experiments, to illustrate mitochondrial network and respiration plasticity when subjected to changing energy substrates.

It appears there are subtle differences between controls and patients in how they response to energetic stress, figure 5.16. For example C1(20yr) responds to energetic stress by increasing mitochondrial network interconnectivity and OCR thereby shifts up and to the right on the graph, figure 5.16. This does not affect the ATP levels as no change was detected. Whereas the patient P2(42yr) does the opposite. The P2(42yr)'s adaptive response is to fragment the mitochondrial network and reduce OCR. This response could potentially be conserving the ATP pool as P2(42yr) had significantly higher ATP levels after incubation under galactose induced energetic stress. Furthermore, P2(42yr) showed less cell proliferation which may be due to a slowdown of metabolic activity as a stress response.

5.3.3 Establishing how the PolG mutator and wild type MEFs differ in their response to VPA treatment under normal and energetic stress conditions

Introduction

The polymerase gamma (PolG) plays a pivotal role in the maintenance of the mitochondrial genome. Any genetic mutations which lead to impairment of the protein's functionality will be detrimental to the cell. The PolG mutator MEFs has a proofreading deficient knock-in mutation in the polymerase gamma (PolG). This mutation causes mitochondrial dysfunction (Kujoth *et al* 2005; Trifunovic *et al.*, 2004; Saleem *et al.*, 2015). Treatment of the cells with VPA could result in further cellular and mitochondrial impairment. To assess the PolG mutator's mitochondrial function under normal and energetic stress conditions, galactose medium was used to induce energetic stress. MEFs are routinely maintained in high glucose (25mM) and therefore the normal condition. The cells were seeded in 96 well plates and left overnight to adhere before medium change and VPA treatment commenced. As before the VPA concentrations used were 0.5mM, 1.0mM and 2.0mM. The treatment duration was 24 and 48 hours.

5.3.3.1 Establishing how PolG mutator and wild type MEFs response to normal and energetic stress conditions

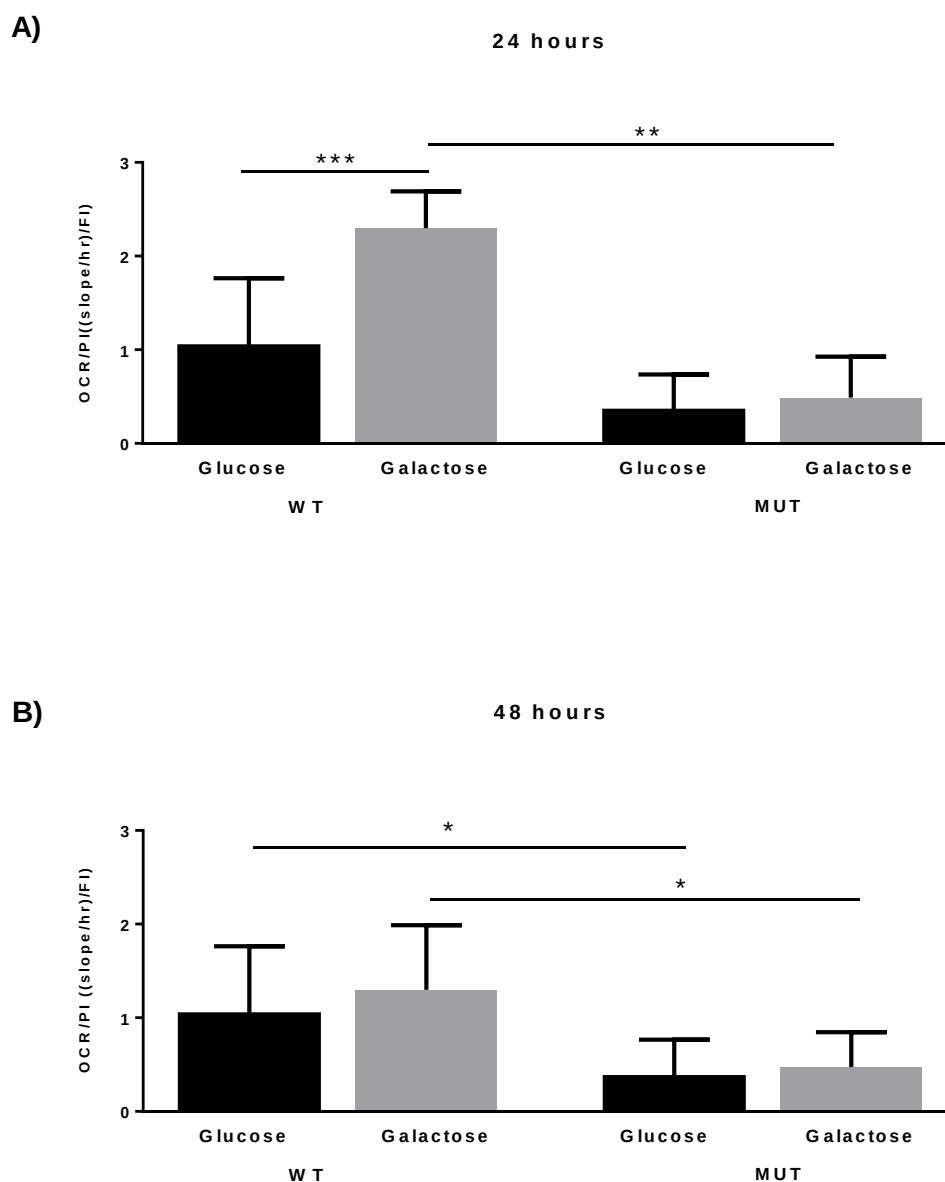


Figure 5.17. Oxygen consumption rates of MEFs. PolG MEFs (MUT) and wild type (WT) were incubated in normal glucose condition (25mM) and energetic stress condition (galactose) for 24 hours (A) and 48 hours (B). Cell number was determined by propidium iodide. Data represent mean $\pm$ SD, n=3. **p<0.001, ***p<0.0001, one-way ANOVA .

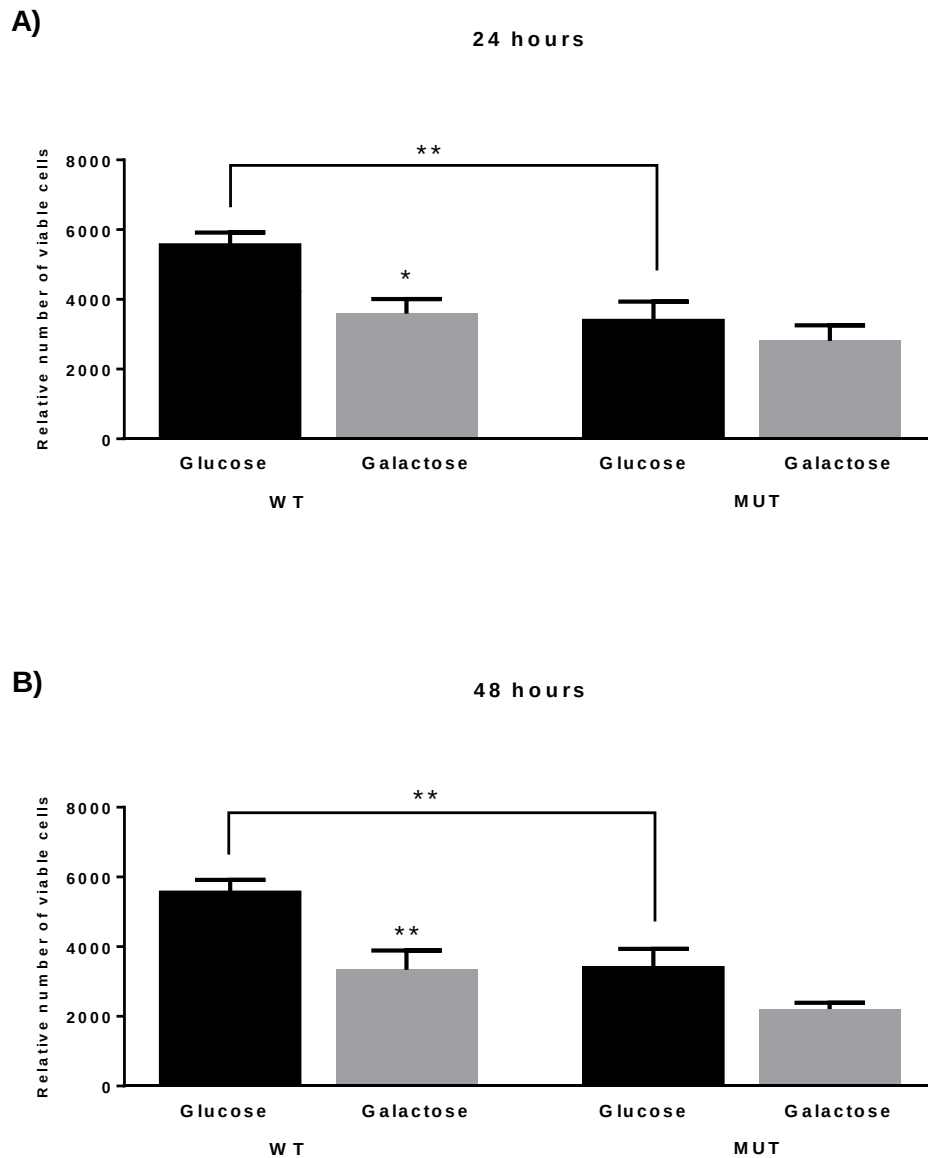


Figure 5.18. Viability of PolG and wild type MEFs' cell population after incubation under normal and energetic stress conditions. Energetic stress does affect wild type (WT) MEFs but not PolG (MUT) MEFs. Relative cell viability was assessed using propidium iodide. Cells were incubated in normal glucose condition (25mM) and energetic stress condition (galactose) for 24 hours (A) and 48 hours (B). Data represent mean $\pm$ SD, n=3. *p<0.05, **p<0.001, one-way ANOVA.

Incubating the PolG mutators under energetic stress for 24 and 48 hours did not alter the OCR ($p=0.07089$) or cell viability ($p=0.0893$), when compared to normal glucose condition, figures 5.17 and 5.18. Under energetic stress the wild type MEFs showed a significant increase in OCR after 24 hours incubation this was no longer significant after 48 hours, figure 5.18 (A and B). Energetic stress also caused decreased cell viability in WT after 24 and 48 hours incubation, figure 5.17. The mutator MEFs had significant lower OCR than WT under normal and energetic stress conditions after 48 hours incubation.

5.3.3.2 *The effect of VPA exposure on mitochondrial function in PolG mutator and wild type MEFs*

Under both normal glucose and galactose energetic stress condition the OCR in the PolG mutator MEFs was significantly lower than that of the WT MEFs. The next step was to determine if VPA treatment had a great impact on the PolG mutator MEFs compared to wild type MEFs. VPA treatment of the PolG mutator MEFs did not alter OCR at 24 or 48 hours, figure 5.19. However, treatment of the wild type MEFs at high concentration (2mM) significantly increased OCR at 24 hours under normal condition. After 48 hours, lower doses of VPA, 1mM in glucose and 0.5mM under energetic stress significantly increased wild type MEF's OCR. Interestingly, after 48 hours exposure to highest concentration (2mM) VPA failed to stimulate OCR in both glucose and galactose (energetic stress) condition, figure 5.19. VPA treatment did not affect cell growth of either cell line following 24 and 48 hours exposure, figure 5.20. It should be noted that the wild type MEFs grow less well in galactose media at 24 and 48 hours while the PolG mutator MEFs after 48 hours.

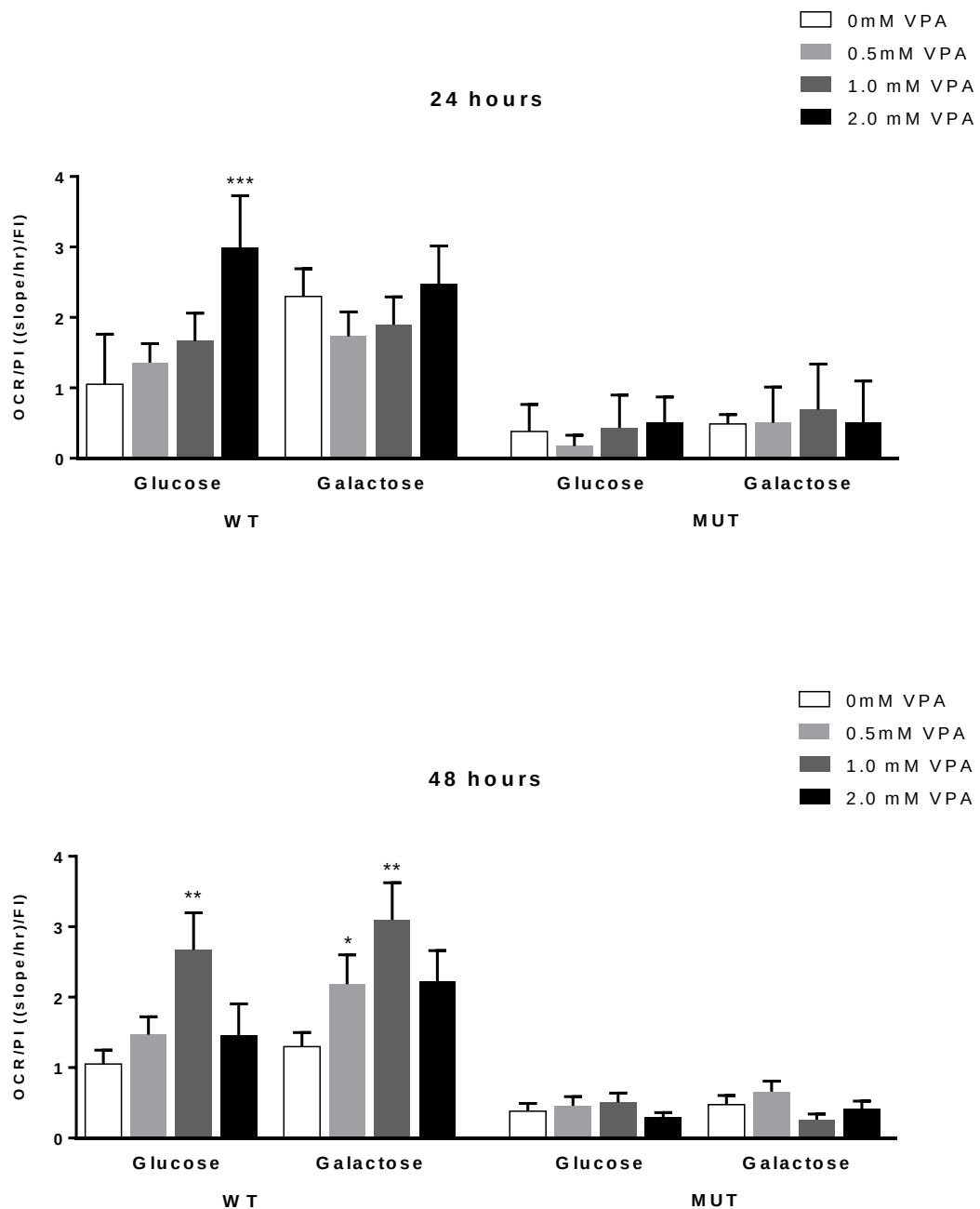


Figure 5.19. Oxygen consumption rates measured in PolG and wild type MEFs after treatment with VPA under normal and energetic stress conditions. VPA treatment in normal glucose medium and in galactose medium causing energetic stress. Wild type (WT) and Mutant (MUT) MEFs show increased OCR after 2mM VPA treatment. Data is normalised to cell number (PI), represent mean $\pm$ SD, n=4, *p<0.05, **p<0.01***p<0.001, ANOVA with Bonferroni correction when compared to baseline condition, glucose or galactose.

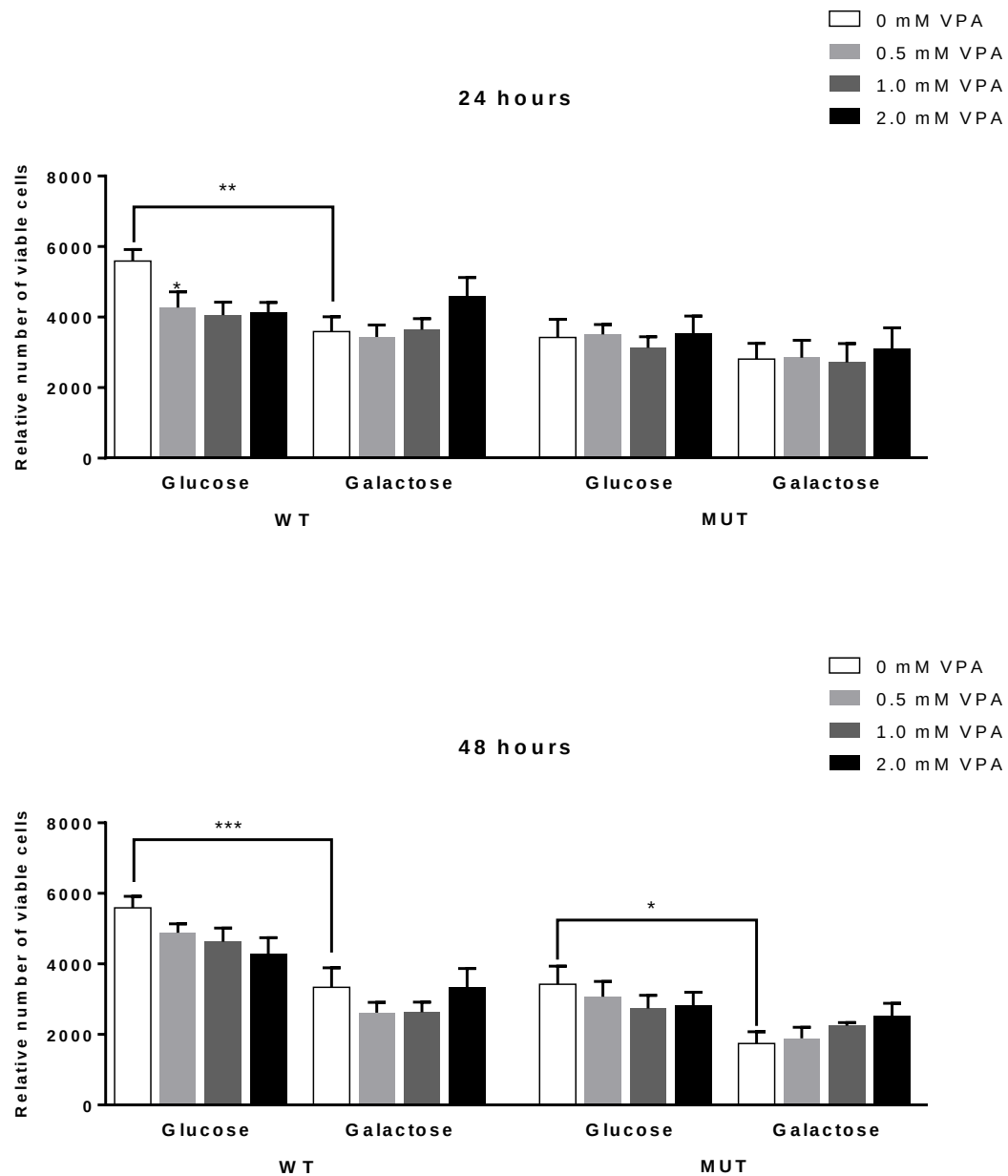


Figure 5.20. Viability of the cell population assesse after VPA treatment of wild type and PolG mutator MEFs, under normal and energetic stress conditions. . Data represented as cell number relative to PI fluorescent intensity, mean $\pm$ SD, n=4, *p<0.05**p<0.01***p<0.001 ANOVA with Bonferroni correction when compared to baseline condition, glucose or galactose

5.3.3.3 Cytosolic metabolic activity in PolG mutator MEFs and wild type MEFs

The MTT assay is often used to assess cell viability and proliferation however, as previously discussed it also reflects the activity of oxidoreductase enzymes in the cytosol. The reduction of tetrazolium to formazan can also be due to oxidative stress as well as cytosolic oxidoreductase enzymes and mitochondrial activity (Bernas and Dobrucki, 2002).

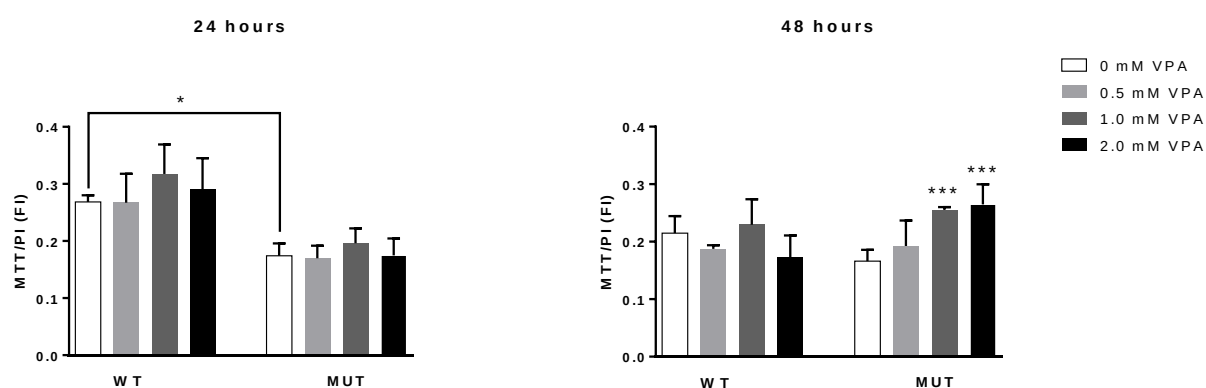


Figure 5.21. Increased MTT signal after VPA treatment under normal conditions in PolG mutator MEFs. The VPA treatments had no effect on the WT or the PolG MUT after 24 hours VPA treatment. After 48 hours incubation the PolG MUT show a concentration dependant increase in MTT signal. Data represented as MTT FI corrected for cell number using PI fluorescent intensity (FI), mean $\pm$ SD, $n=3$, * $p<0.05$, *** $p<0.0001$ ANOVA with Bonferroni correction when compared to baseline condition, no treatment glucose.

Over 24 hours VPA did not affect metabolic activity in either mutator or wild type MEFs, figure 5.21. However, after 48 hours VPA exposure metabolic activity increased in the

PolG mutator MEFs in a dose dependant manner. No effect was observed in the wild type MEFs. The increase in the PolG MEFs after 48 hours may be due to the activity of cytosolic enzymes as a compensatory mechanism for the dysfunctional mitochondria and/ or signifies a reaction to oxidative stress via superoxide.

5.3.3.4 The effect of VPA treatment on ATP production under normal conditions in PolG MEFs

The PolG MEFs have impaired OXPHOS function and are heavily reliant on glycolysis to maintain adequate ATP levels. Saleem *et al.*, (2015) found that key glycolytic enzyme phosphofructokinase was three times more abundant in the PolG mouse than in the WT. In addition, the pyruvate dehydrogenase kinase isoform four expression was also much higher in the PolG mouse than the WT. This enzyme is known to inhibit the utilisation of pyruvate in the TCA cycle thus conserving ATP (Kukat *et al.*, 2014).

In the following section, the aim was to verify if the mutator cells could sustain ATP level by glycolysis as the OCR was clearly reduced in the PolG mutator compared to WT. PolG and WT MEFs were seeded at; PolG MUT cell densities of 20,000 and 40,000 and WT 15,000 to 17,500. Left overnight to adhere before treatment commenced. Steady state ATP levels were determined using Promega Cell Titer-Glo luminescent kit.

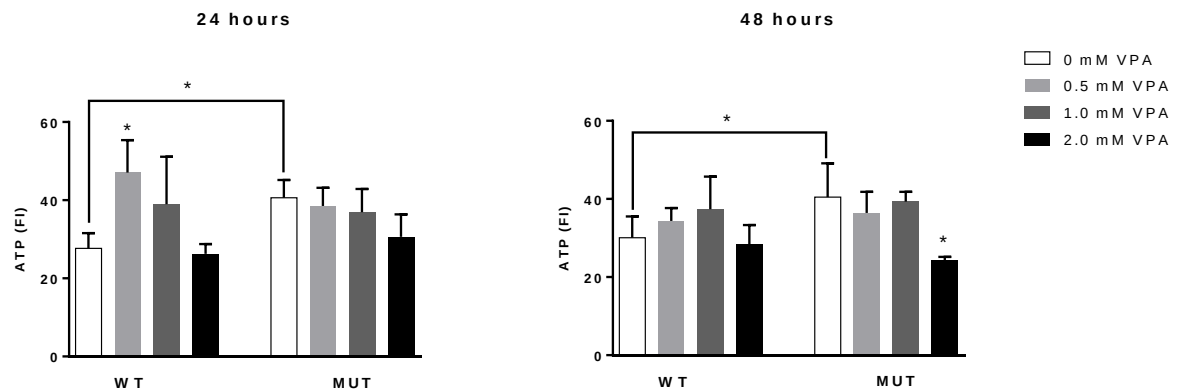


Figure 5.22. ATP assessed in WT and PolG mutator MEFs after treatment with VPA under normal 25mM glucose conditions. The PolG mutator (MUT) had increased ATP at 24 and 48 hours compared to WT. MEFs cells were seeded as per OCR protocol in a white walled parallel plate. Data represented was corrected for cell number using PI, mean $\pm$ SD, n=3, *p<0.05 ANOVA with Bonferroni correction when compared to no treatment condition.

Under normal conditions and without VPA treatment (base line) steady state ATP levels in the PolG mutator MEFs were found to be significantly higher ($p=0.0003$) than wild type, figure 5.22. After 24 hours VPA treatment there was no significant effect on ATP levels in the PolG mutator, at 2.0mM ($p=0.0821$). However, the wild type MEFs following 24 hour of 0.5mM VPA treatment showed a significant increased ATP levels with higher doses having no effect. Further incubation of wild type MEFs with VPA for 48 hours did not affect ATP levels at any concentration. However, PolG mutator MEFs incubated in 2mM VPA for 48 hours showed a significant reduction on steady state ATP levels, figure 5.22.

As discussed in the introduction, there have been several reported results where mtDNA depletion has resulted in increased ATP levels (Sitraz *et al.*, 2014; Qian and Van Houten, 2010). To determine mtDNA levels in PolG and WT MEFs PicoGreen

staining was used to detect mtDNA using live cell fluorescent and the IN Cell Analyzer. PicoGreen is a DNA specific fluorescent dye that is used to detect mtDNA (Ashley *et al.*, 2005).

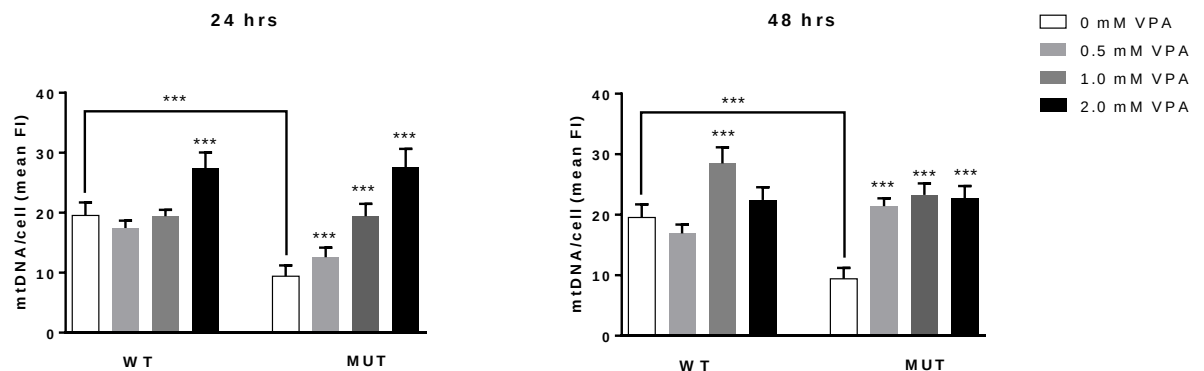


Figure 5.23. mtDNA assessed using PicoGreen and the In Cell Analyzer in wild type (WT) and PolG mutator(MUT) MEFs after treatment with VPA under normal condition conditions. After treatment the cells were incubated with PicoGreen in clear media. After incubation the stain was removed and fresh clear glucose media was added. Data presented as mean $\pm$ SD, ≥ 250 cells/experiment analysed, *** $p < 0.001$ when compared to no VPA treatment.

The mtDNA result shows that as expected, the PolG mutator MEFs had significantly lower mtDNA than the WT, figure 5.23. This is due to the poorly functioning PolG which causes mtDNA depletion (Sarzi *et al.*, 2007). What was surprising was the effect of the VPA treatment. In the PolG MEFs there was a VPA concentration dependant increase in mtDNA after 24 hours VPA treatment. An increase in mtDNA was also observed in WT MEFs after 24 hours VPA treatment but only in the highest concentration of VPA. VPA treatment for 48 hours also increased the mtDNA in the PolG MEFS, all concentrations. For the WT MEFs, only 1.0mM VPA increased mtDNA while the higher 2.0mM dose failed to increase mtDNA levels.

5.3.3.5 *Modulation of autophagy/mitophagy and mitochondrial network morphology in PolG mutator and wild type MEFs under normal and energetic stress condition*

In previous section, 4.3.2.3, subjecting PolG patients' fibroblast cells to energetic stress caused a reduction in both autophagosomes and mitochondrial area.

Furthermore, after being subjected to energetic stress the mitochondrial network became fragmented. To investigate if energetic stress would have similar enhanced effects in PolG mutator compared to wild type MEFs the same methodology was employed in the next series of experiments.

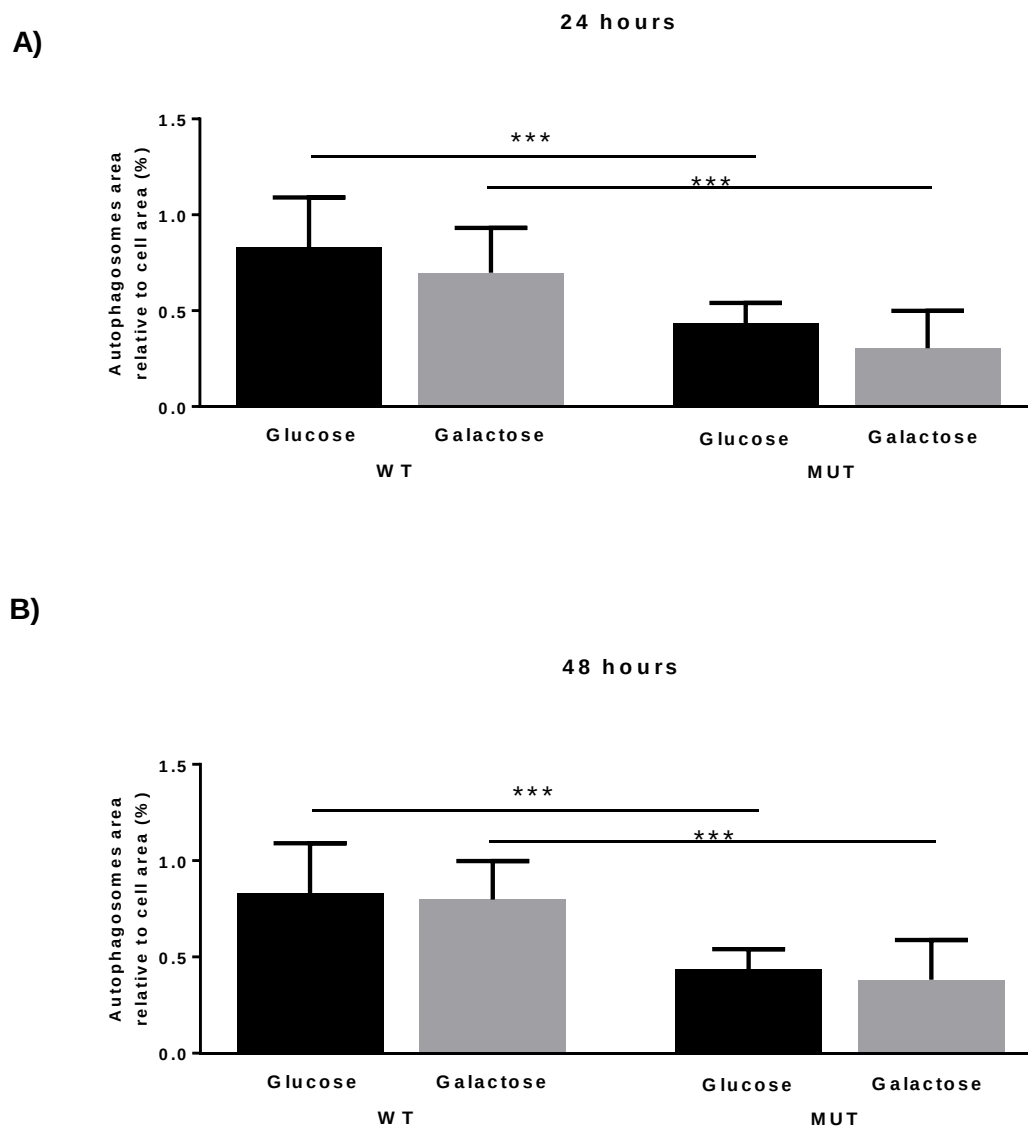


Figure 5.24 Autophagosomes detected in PolG mutator and wild type MEFs after 24 and 48 hours incubation under normal and stressed conditions. Energetic stress does not affect the levels of autophagosomes. Stress condition induced by galactose, grey bars. Autophagosomes detected by LC3 signal. Data presented as mean $\pm$ SD, $n=3$, *** $p<0.0001$, one-way ANOVA.

Although the PolG mutator MEFs had significantly lower autophagosomes than the WT MEFs under normal glucose conditions, incubation of PolG and WT MEFs under energetic stress for 24 or 48 hours did not further affect the levels of autophagosomes, figure 5.24.

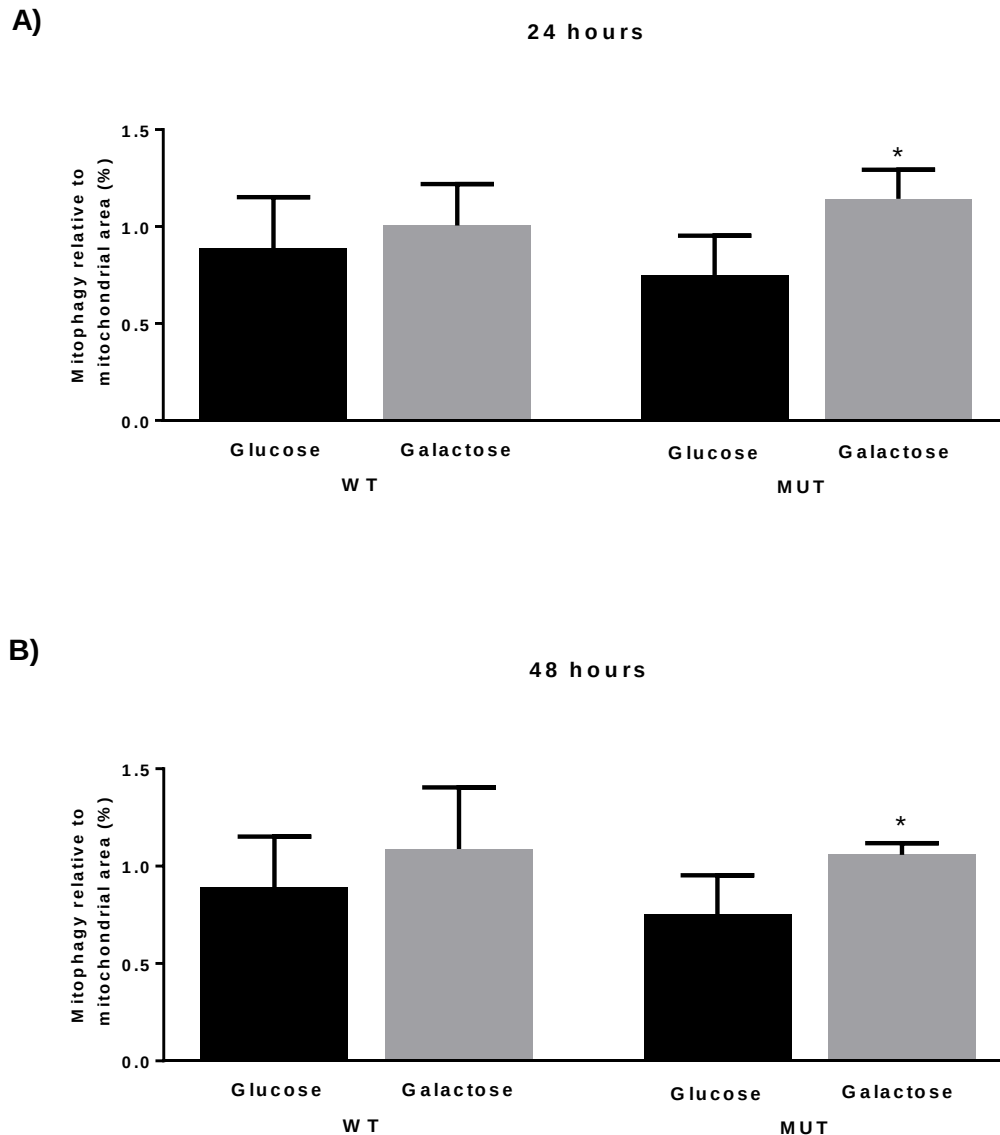


Figure 5.25. Mitophagy in PolG mutator and wild type MEFs after 24 and 48 hours incubation under normal and stressed conditions. Mitophagy assessed by co-localisation of LC3 and TOM20 signals. Data presented as mean $\pm$ SD, $n=3$, * $p<0.05$, one-way ANOVA.

By using the co-localisation of autophagosomes and the mitochondrial as an indication of potential mitophagy events data indicates that mitophagy increases when PolG MEFs are incubated for 24 and 48 hours under energetic stress, figure 5.25. The WT MEFs did not show a change in mitophagy when exposed to energetic stress.

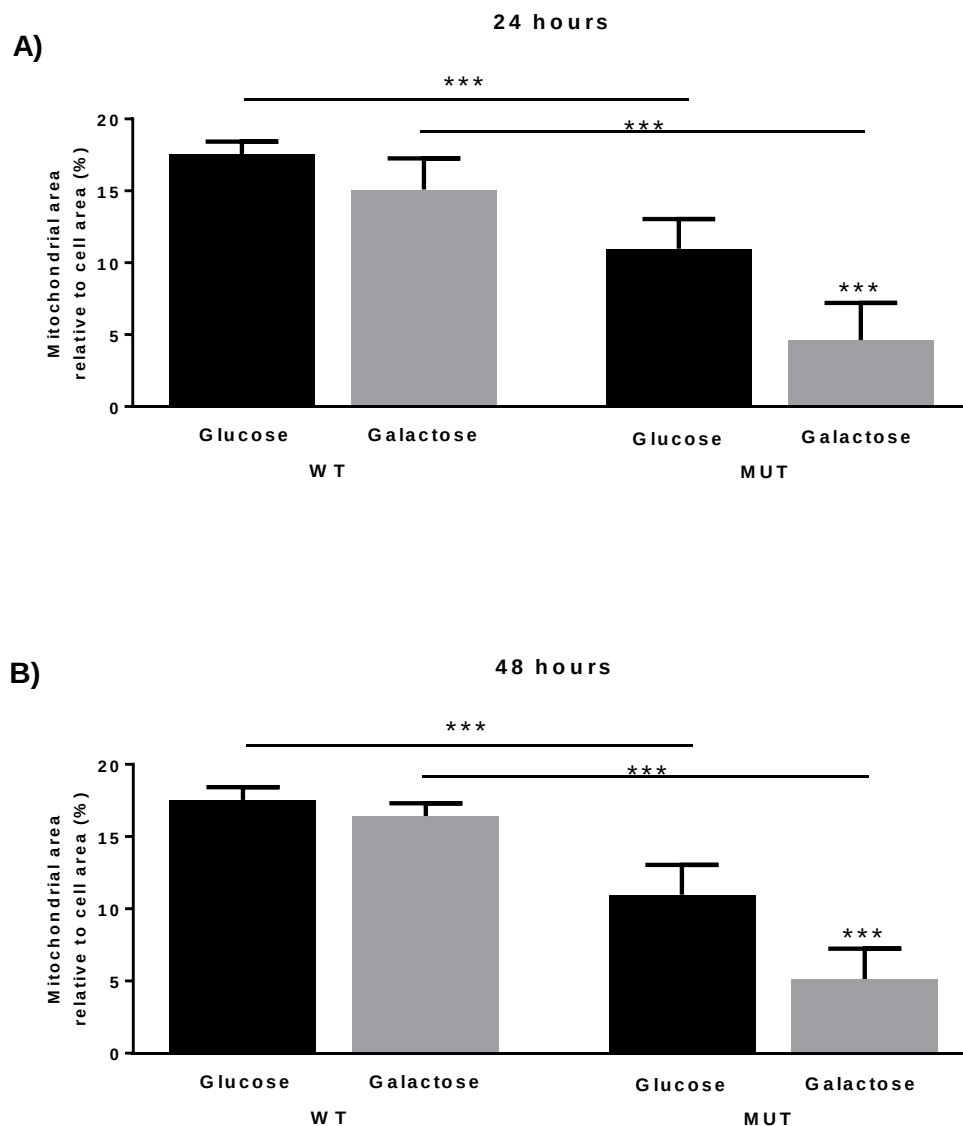


Figure 5.26. Mitochondrial area in PolG mutator and wild type MEFs after 24 and 48 hours incubation under normal and stressed conditions. Stressed conditions were induced by incubation in galactose medium, grey bars. Mitochondrial area as detected by TOM20 signal. Data presented as mean $\pm$ SD, $n=3$, *** $p<0.0001$, one-way ANOVA.

In normal culture medium (25mM glucose) there was significantly less mitochondrial area in the PolG MEFs compared to WT MEFs, figure 5.26. After 24 and 48 hours of energetic stress PolG MEFs showed significant decrease in mitochondrial area compared to normal medium while the WT MEFs showed no change.

Although the mitochondrial area of WT MEFs was not affected by energetic stress, the mitochondrial network showed significant changes. After 24 and 48 hours incubation under energetic stress the mitochondrial network in the WT MEFs became fragmented as indicated by a reduction in the number of bifurcations, figure 5.27. The PolG MEFs were also affected by energetic stress as shown by mitochondrial network fragmentation. Levels of fragmentation in the PolG MEFs were very high following energetic stress with very few bifurcations present.

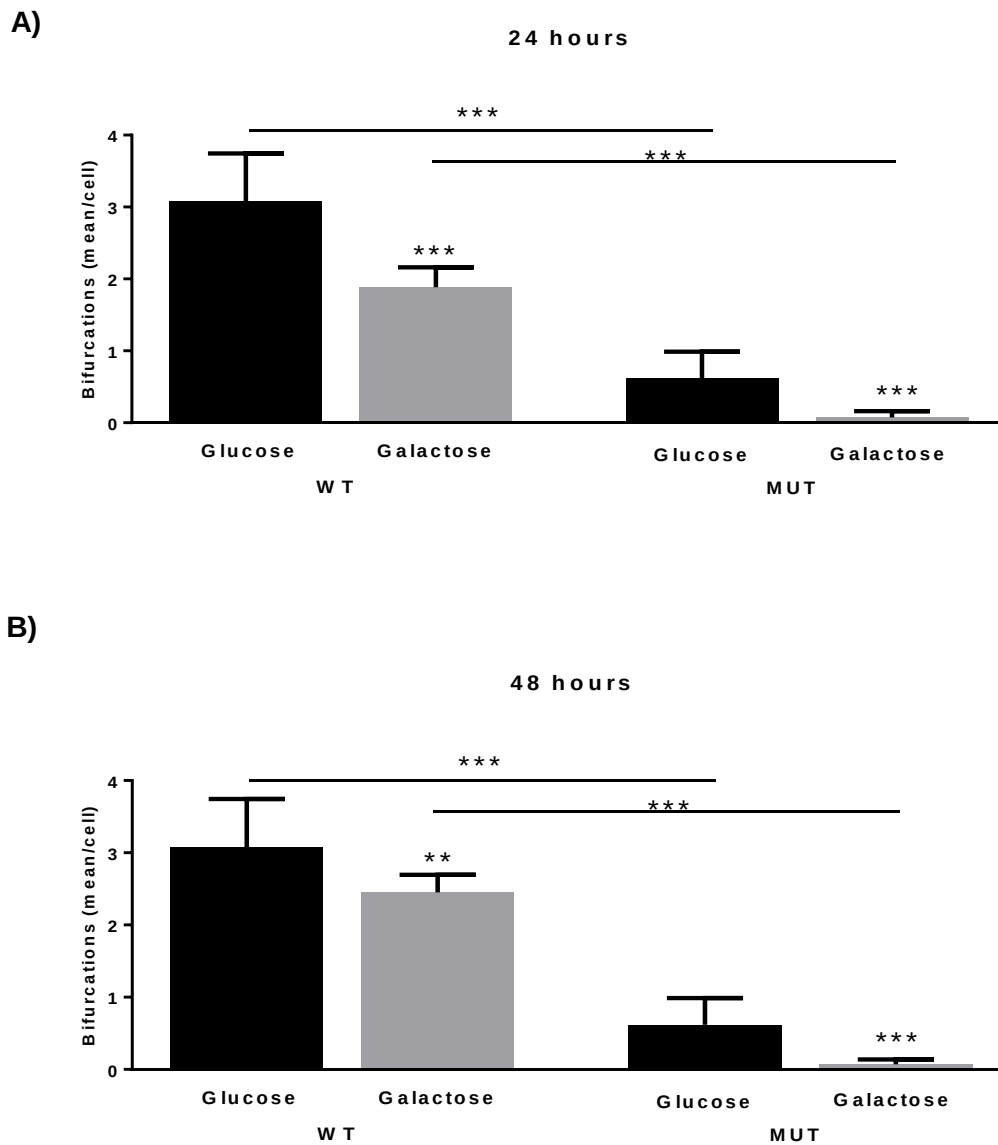


Figure 5.27. Mitochondrial network morphology in PolG mutator and wild type MEFs after 24 and 48 hours incubation under normal and stressed conditions. Stressed conditions were induced by incubation in galactose medium, grey bars. Bifurcation detected by TOM20 signal. Data presented as mean $\pm$ SD, n=3, **p<0.001, ***p<0.0001, one-way ANOVA.

5.3.3.6 Modulation of autophagy/mitophagy and mitochondrial network morphology in PolG mutator and wild type MEFs following treatment with sodium valproate under normal and energetic stress condition

In the previous section 5.3.2.3, VPA treatment caused increased autophagosomes and mitochondrial network fragmentation in fibroblasts from patients harbouring PolG mutations. To assess if VPA would have the similar effect in MEFs, the same treatment regime was followed as previously described.

In figure 5.28 the wild type (WT) cells cultured under normal glucose conditions showed a larger number of autophagosomes per cell than PolG mutator MEFs. VPA treatment had no effect on the level of autophagosomes in either WT or PolG MEFs when incubated under normal glucose condition for 24 or 48 hours. The effect of VPA treatment under energetic stress after 24 and 48 hours did not result in any changes for the WT cells. Only the PolG mutator MEFs showed any significant change by increasing the autophagosomes levels after 24 hours treatment at 0.5mM in galactose, when compared to no treatment galactose, figure 5.28 (A).

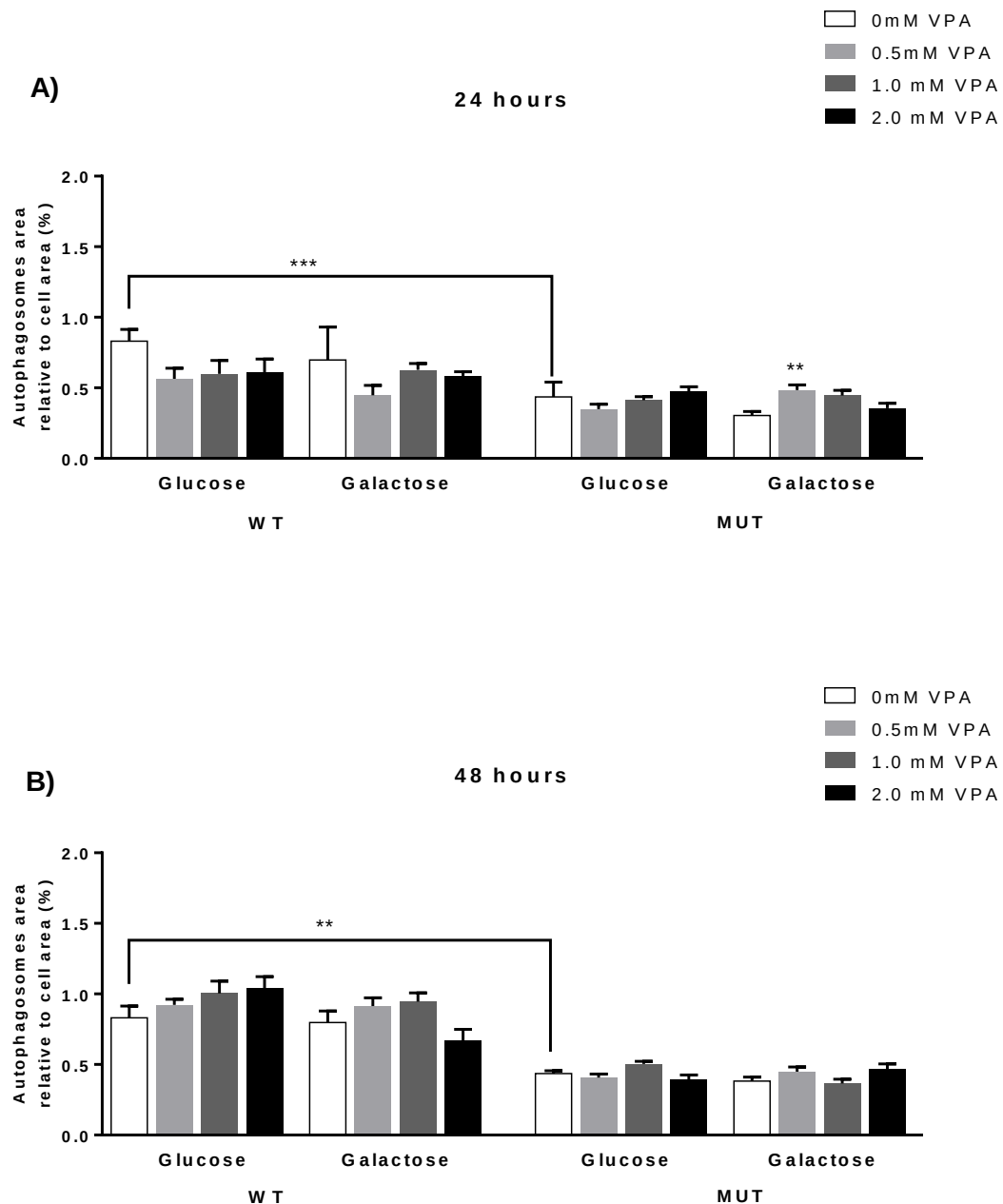


Figure 5.28. Levels of autophagosomes after 24 and 48 hours treatment with VPA under normal and energetic stressed conditions in wild type and PolG mutator MEFs. Cells were treated with VPA at various concentrations for 24 (A) and 48 (B) hours before fixing. Autophagosomes detected based on LC3 signal. Data presented as mean $\pm$ SD, ≥ 250 cells/experiment analysed $n=3$, ** $p<0.01$, *** $p<0.001$ ANOVA with Bonferroni correction when compared to baseline, no VPA treatment.

The next question to be addressed was, did the autophagosomes present co-localise with mitochondria, indicating mitophagy? The co-localisation between autophagosomes (LC3) and mitochondria (TOM20) was assessed as described in previous section.

Incubation of MEFs under energetic stress alone did not affect the level of autophagosomes present in either WT or PolG MEFs. Only PolG mutator MEFs had increased mitophagy after 24 and 48 hours incubation under energetic stress. VPA treatment of PolG MEFs for 24 and 48 hours under normal conditions, did not affect the level of mitochondrial and autophagosome co-localisation, figure 5.29. When energetic stress was applied to PolG MEFs for 48 hours at high concentration (2mM) of VPA this significantly increased mitophagy. Interestingly, the wild type MEFs appear sensitive to the mitophagy modifying effects of VPA treatment. After 24 hours incubation in normal medium containing 1mM VPA wild type MEFs significantly increased mitophagy, figure 5.29. The higher VPA concentration failed to increase mitophagy. However, under energetic stress the wild type MEFs response to VPA was quite different, over 24 hours 0.5mM VPA reduced mitophagy with higher doses having no effect. When the WT MEFs were subjected to longer VPA incubation of 48 hours a general trend of increased mitophagy was observed in both glucose and galactose media. Only the 1mM VPA concentration on galactose reached significance with the higher 2mM dose showing a reduction back to control levels. PolG mutator MEFs only showed a VPA effect on mitophagy when incubated for prolonged period on 2mM VPA for 48 hours under energetic stress condition (galactose). These cell types seem able to increase mitophagy up to a point and then there is a sharp drop back to base line level at the highest VPA concentration.

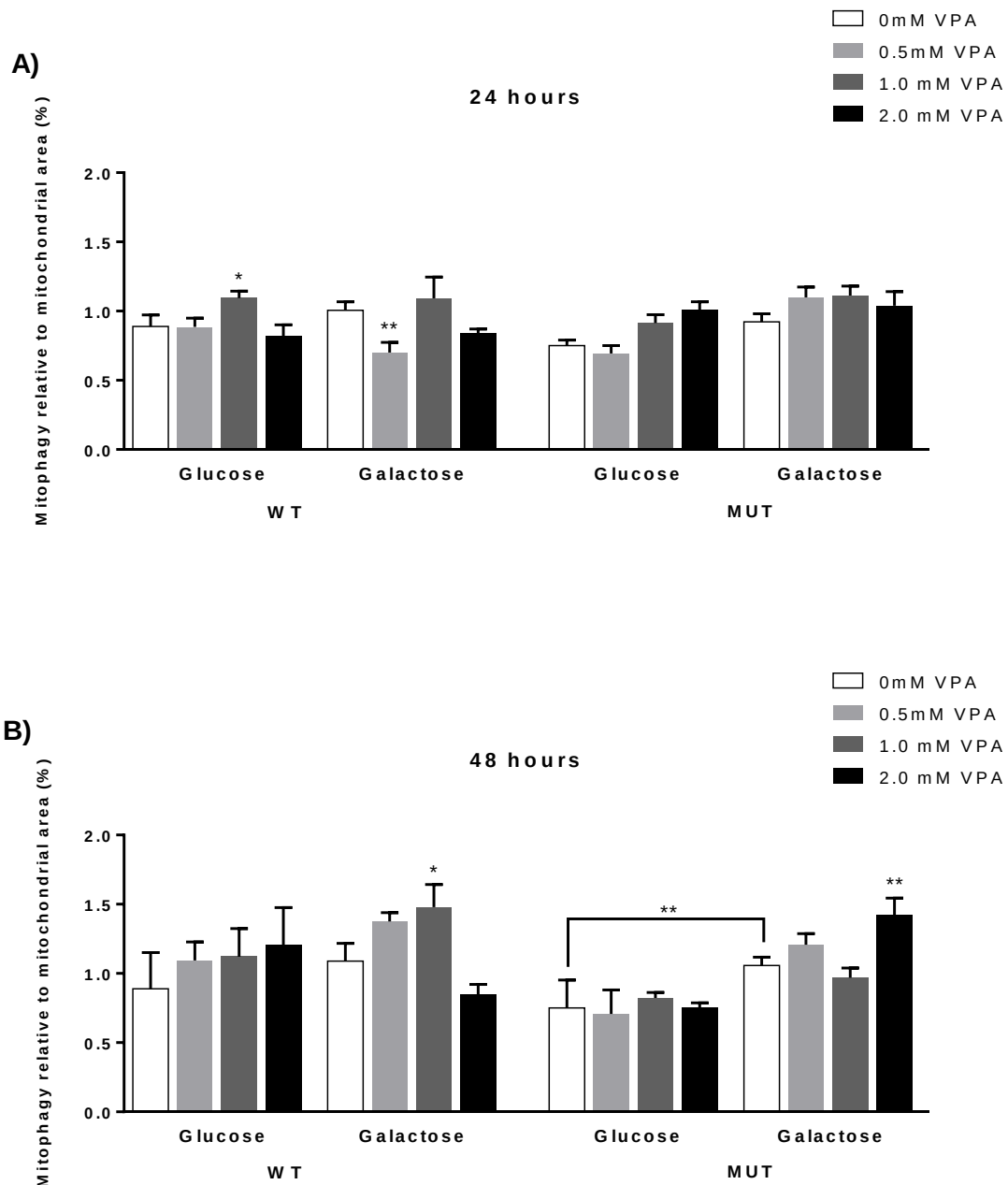


Figure 5.29. Mitophagy in PolG mutator and wild type MEFs after VPA treatment under normal and stressed conditions. Mitophagy assessed using the co-localisation of LC3 (autophagosomes) and TOM20 (mitochondria). WT and PolG MUT MEFs after treatment with VPA under normal glucose culture conditions and in galactose for 24 hours (A) and 48 hours (B). Data presented as mean $\pm$ SD, $\geq$ 250 cells/experiment analysed, $n=3$, * $p<0.05$ ** $p<0.01$ ANOVA with Bonferroni correction when compared to no VPA treatment.

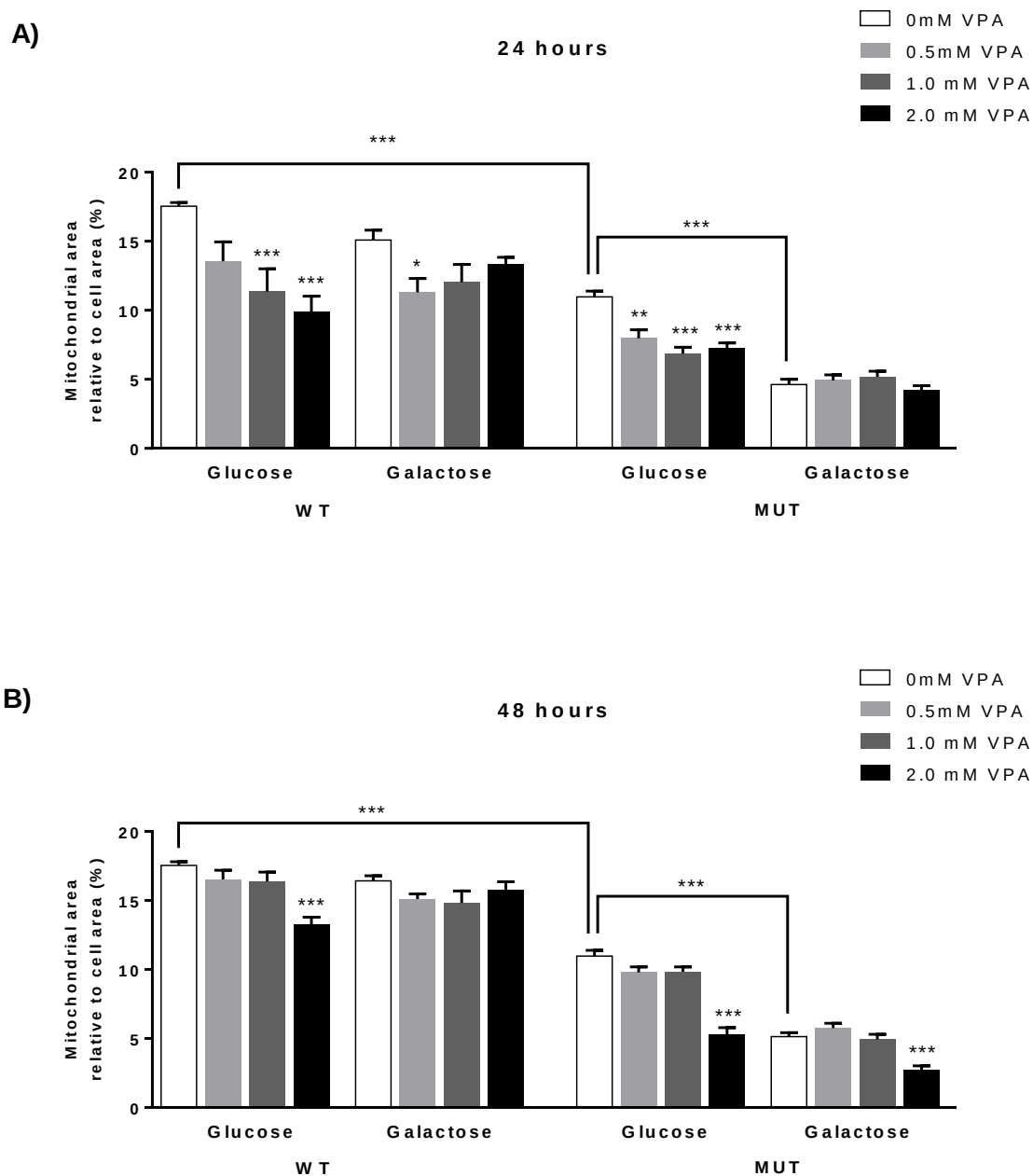
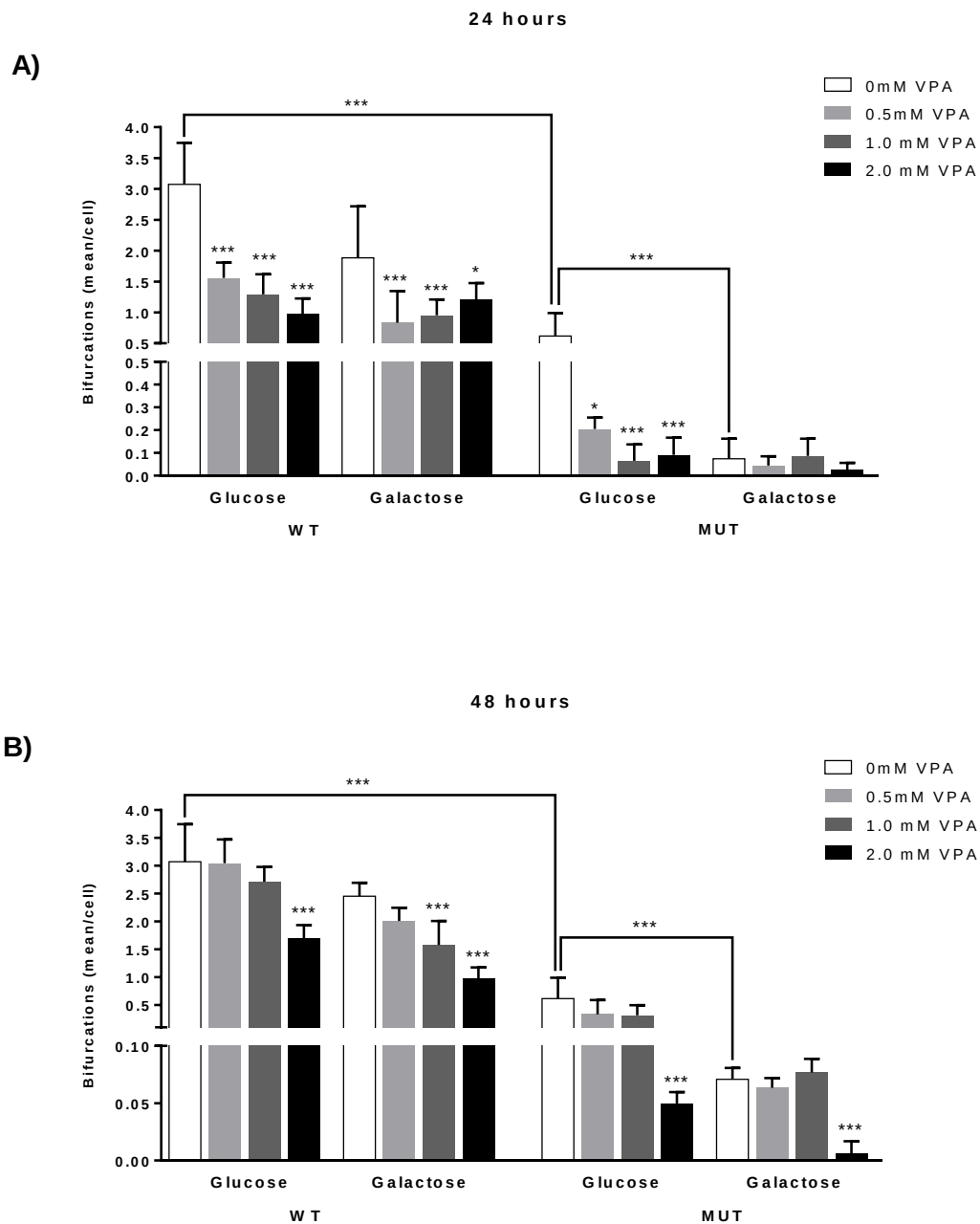


Figure 5.30. Mitochondrial area assessed using TOM20 and the In Cell Analyzer in WT and PoIG MUT MEFs after treatment with VPA under normal glucose culture conditions and in galactose. Data presented as mean $\pm$ SD, ≥ 250 cells/experiment analysed, *** $p < 0.001$ ANOVA with Bonferroni correction when compared to baseline, glucose no treatment condition.

After 24 hours VPA treatment under normal glucose conditions resulted in reduction of the mitochondrial area of both the WT and PolG mutator MEFs, figure 5.30. VPA treatment under energetic stress condition did not significantly reduce the mitochondrial area of the PolG mutator MEFs. Only lowest VPA concentration showed a reduction in the wild type MEFs. The effect of VPA treatment diminished after 48 hours incubation as only the highest concentration resulted in the reduction of mitochondrial area in both cell cultures, figure 5.30 (B).

The configuration of the mitochondrial network of the wild type (WT) is similar to that previously observed in HepG2, 4.4.2, where the bifurcation data was deemed not robust due to clumping of the cells. The WT although they have a similar cell size to that of HepG2s they do not clump on top of each other but form a uniform monolayer. With careful segmentation during the image analysis it was possible to investigate the mitochondrial network morphology.



Under normal glucose conditions the number of bifurcations was significantly higher in the WT compared to PolG mutator, figure 5.31. Under energetic stress condition there was a further decrease in the number of bifurcation in the PolG MEFs. Energetic stress condition had no significant effect in the number of bifurcation in the WT at both time points figure 5.31 (A and B). VPA treatment for 24 hours of WT and PolG MEFs under normal glucose conditions caused significant decrease in bifurcation at all drug concentrations. Indicating the mitochondrial network had become more fragmented after VPA treatment. Interestingly, VPA treatment under energetic stress condition for 24 hours had no significant effect on PolG mutator MEFs but the WT MEFs showed reduced bifurcations at all VPA concentrations. After 48 hours treatment only highest concentration of VPA showed a significant reduction of bifurcations in both PolG mutator and WT MEFs.

It must be noted that the large number of bifurcations present in the WT may be overstated due to compact mitochondrial network although the optimisation of the segmentation criteria was carefully adjusted to compensate for this.

5.3.3.7 Cell and mitochondrial network morphology in wild type and PolG

Mutator MEFs

When assessing the mitochondrial area and network morphology it was observed that the cell morphology of WT and PolG mutator MEFs were very different. The WT had a relatively small cytosolic area and the mitochondria were predominantly clustered around the nucleus figures 5.32 (A) and 5.33 (B). Whereas, the PolG mutators had a much larger cytosolic area and the mitochondria were fragmented throughout the cytosol, figure 5.32 (B). The WT MEFs nucleus is significantly smaller than the PolG mutator MEFs, figure 5.33 (B).

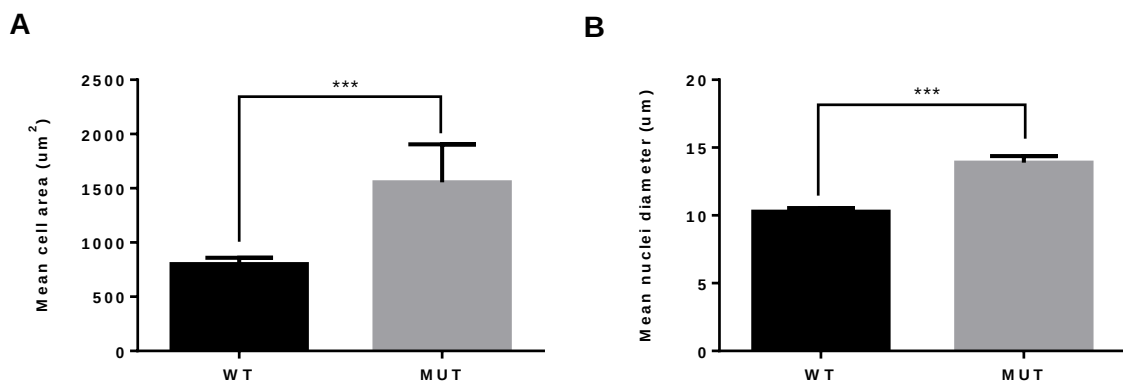


Figure 5.32. Cell area of WT and PolG mutator MEFs in normal 25mM glucose medium. (A) Mean nuclei diameter measured using DAPI signal. (B) The average cytosolic area per cell was measured by In Cell Analyzer 1000, using LC3 background signal to determine cell boundaries. Cells were incubated in normal glucose (25mM) medium before fixing and staining. Data presented as mean $\pm$ SD, ≥ 250 cells/experiment analysed, $n=3$, *** $p<0.001$, Student's T-test.

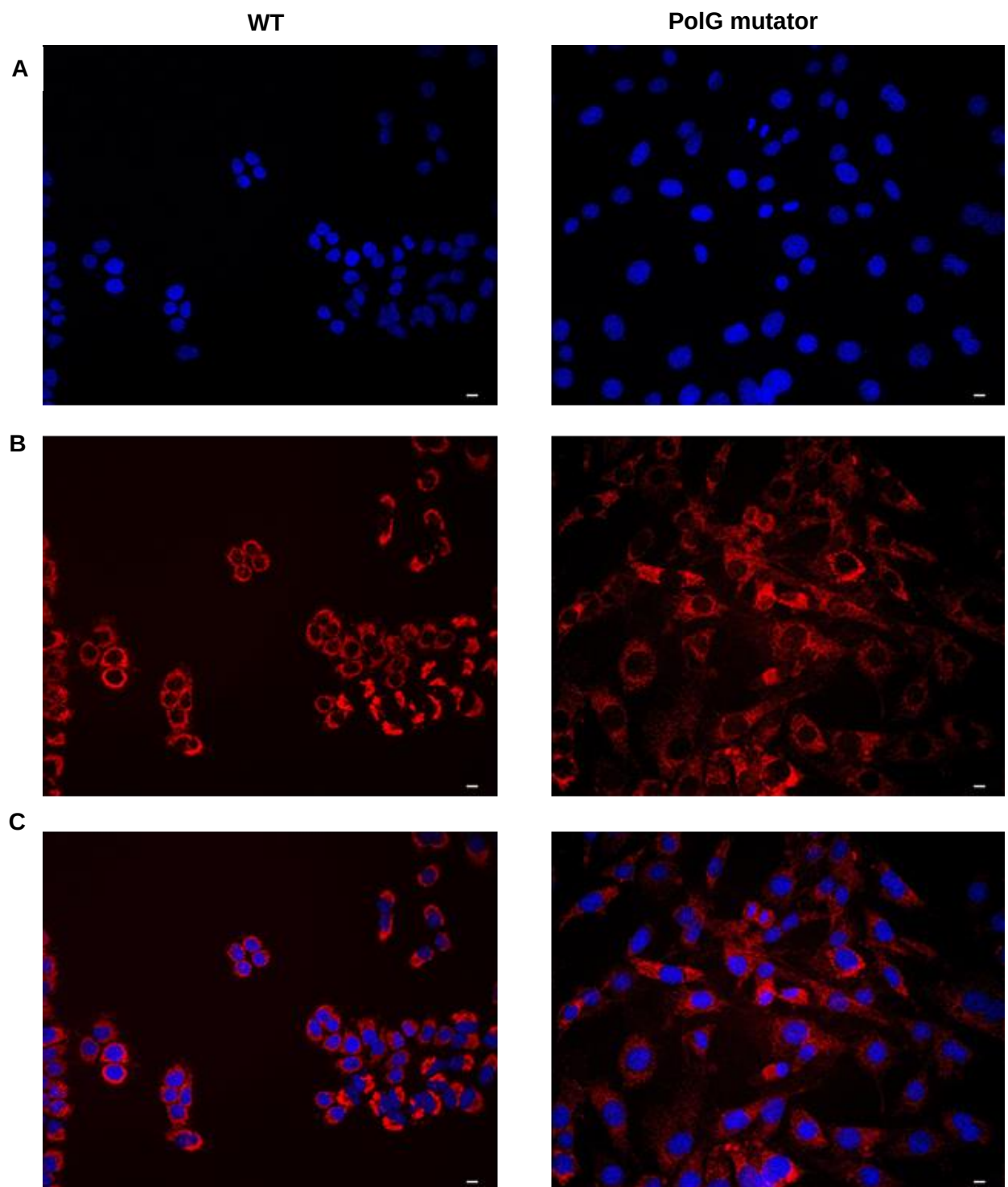


Figure 5.33. Nucleus size and mitochondrial network morphology of wild type and PolG mutator MEFs. Cells were incubated in normal glucose medium before being fixed and stained.(A) Nuclei stained with DAPI. (B) Mitochondria visualised with TOM20. (C) DAPI and TOM20 channels merged. Raw images captured by the In Cell Analyzer 1000 and subsequently colour enhanced. Scale bar 10µm.

To investigate the effect VPA treatment may have on mitochondrial network morphology, the mitochondrial length and the number of three-way intersections (Y), bifurcations of the network were determined. The more interconnected the network is the more bifurcations are expected. The wild type (WT) mitochondrion appears tightly packed around the nucleus due to the small cell area, figure 5.34 (A). This gives the appearance of a highly networked mitochondrion compared to the PolG mutator which appears more fragmented, panel (B).

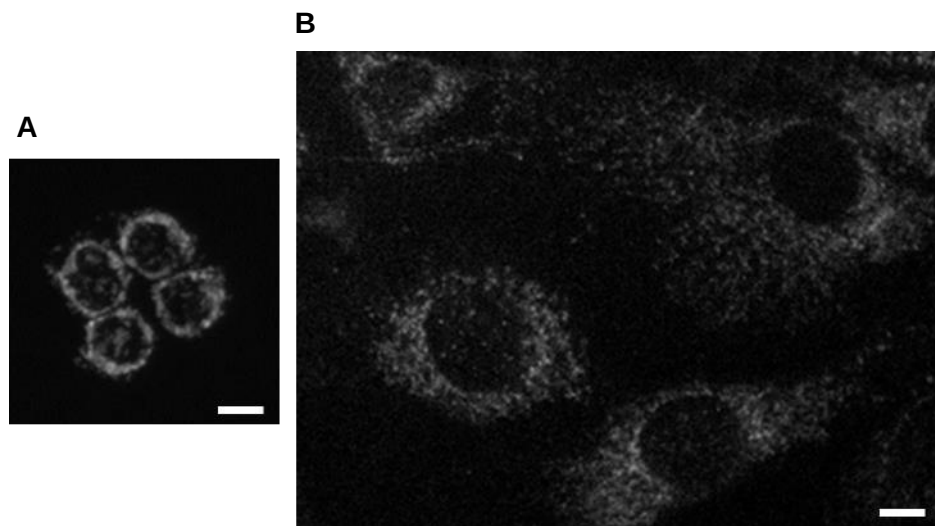


Figure 5.34. Mitochondrial network morphology of wild type and PolG mutator MEFs. Cells were incubated in glucose maintenance medium before being fixed and stained with TOM20. (A) wild type and (B) PolG mutator. Raw images captured by the In Cell Analyzer 1000. Scale bar 10 μ m.

5.3.3.8 Discussion- sections 5.3.3.1 to 5.3.3.7

Wild type and PolG Mutator MEFs response to VPA treatment

In the previous section 5.3.2, VPA treatment of PolG patient fibroblasts resulted in a mixed OCR response but increased levels in autophagosomes and mitochondrial network fragmentation both patients' cell lines. However, VPA treatment of PolG mutator MEFs showed no significant effect on OCR under normal or energetic stress conditions. The lack of response to VPA could be due to PolG mutator MEFs inability to increase respiration in response to the drug treatment as it is already working at maximum rate. Considering that the ATP level of the PolG mutator MEFs at glucose baseline were significant higher than that of the WT MEFs. This could be a reflection of reduced metabolic activity as the MTT data showed reduced oxidoreductase activity and there was also a slowdown of cell population growth. Previous studies showed that the PolG mutator MEFs were reliant on glycolysis to maintain ATP levels (Saleem *et al.*, 2015). The PolG MEFs may be conserving the ATP by slowing down metabolic activity and population expansion as glycolysis alone is not able to fully compensate for inadequate OXPHOS function.

5.4 General chapter discussion

5.4.1 The effect of energetic stress in cell lines with PolG mutation and non-affected controls; could replacing glucose with galactose be too severe?

The introduction of energetic stress by replacing glucose with galactose is widely used as a means to force cells to use the mitochondria to meet energy demand (Gohil *et al.*, 2010; Marroquin *et al.*, 2007; Rana *et al.*, 2011). In this study I used low glucose (5mM) as well as galactose and normal glucose (25mM) to investigate the cellular response to energetic stress in seven control fibroblasts.

By reducing the glucose concentration from 25mM to 5mM the response was similar to galactose. All the controls had increased OCR after 72 hours incubation with low glucose but this was statistically significant in only three cases. With less glucose present cells may have switched to glutamine driven OXPHOS as if under energetic stress (galactose). Furthermore, incubation with low glucose had a similar impact on autophagosome levels and autophagy/mitophagy as energetic stress. Low glucose did not affect the mitochondrial network to the degree that galactose did. The network became much more interconnected in galactose condition than low glucose. Low glucose appears to induce an element of stress on normal mitochondrial activity but not to the extent of galactose. Low glucose (5mM) is closer to physiological (blood) levels and could be consistent with *in vivo* mitochondrial activity. There was some variability between control cell lines in their response to energetic stress and low glucose but by combining oxygen consumption rates and mitochondrial imaging data it became clear that one of the controls lacked the plasticity displayed by the others, appendix C. The fibroblasts used in this study have been maintained in high glucose conditions through many passages. This could have resulted in the fibroblasts adapting over time to a high glucose environment and becoming reliant on glycolysis as a result.

In low glucose the mitochondrial response detected is likely a glucose reduction effect, whereby the cells can no longer rely on glycolysis alone for ATP production but need to combine it with OXPHOS. It may be that galactose is too severe as a means to induce energetic stress as it does eventually cause cell death in control fibroblasts and in HepG2 cells (table 3.2, section 3.4.1.1). Incubation with low glucose for 72 hours did not decrease the cell number of control fibroblasts when compared to high glucose. Furthermore, the most severely affected PolG patient (P1(2yr)) also had significant reduction in cell population after 72 hours under energetic stress although this did not impair OCR or ATP. Interestingly, P2(42yr) had significantly higher OCR under normal conditions than both controls and P1(2yr) but the ATP was not higher or lower than the controls or the other patient. This suggests that P2(42yr) had greater capacity to adapt to energetic stress as the ATP was increased when compared to normal conditions. This does not necessarily mean that ATP production increased but reflects conservation of the ATP pool as the mitochondrial area was decreased.

5.4.1.1 Does the type of PolG mutation affect the cellular response to VPA treatment?

The two PolG patients fibroblast ability to drive mitochondrial respiration after 72 hours treatment with VPA, under normal glucose conditions, was noticeably different at low concentration of the drug (0.5mM). P1(2yr) showed a 57%(± 0.012) reduction in OCR but P2(42yr) had a 35% (± 0.021) increase in OCR. This suggests that the impact on mitochondria of VPA treatment under normal glucose conditions was far worse in patient P1(2yr) than P2(42yr). Although P2(42yr) had less mitochondrial area than P1(2yr) and a more fragmented network, this did not appear to impair mitochondrial function. This could be a reflection of the severity of the disease observed in these two

individuals. While P2(42yr) has a common combination of two prevalent linker mutations, P1(2yr) shares one of these along with a stop mutation in the linker region. The latter predicts a protein truncated for the catalytic domain of PolG thus reducing polymerase efficiency (Ashley *et al.*, 2008). P1(2yr) was diagnosed with Alper's Huttenlocher's syndrome and suffered acute liver failure following VPA treatment, whereas, P2(42yr) is currently under VPA treatment. Interestingly, this difference was more pronounced when cells were treated under normal glucose conditions. Under energetic stress conditions both patients' cell lines responded to VPA treatment in a similar manner. There is emerging evidence from the Morten laboratory that glucose concentrations alter the response of cancer cell lines reaction to drug treatments targeting metabolism. When cancer cell lines are treated with drugs in glucose concentrations of physiological relevance a greater drug efficacy was observed compared to treatments under non-physiological high glucose concentrations (20-25mM). Therefore, it would be interesting to follow up the above study using 5mM glucose to investigate the VPA effect under more physiological conditions.

5.4.1.2 What does the PolG mutator mouse model tell us about the effect of VPA treatment?

The effect of VPA treatment under energetic stress on OCR was only observed in the wild type MEFs (WT) and not in the PolG MEFs. After 24 and 48 hours incubation, the WT OCR was significantly increased however the PolG mutator MEFs showed no effect. This is likely because the PolG mutator MEFs have significantly lower OCR than WT, indicating dysfunctional mitochondria under normal conditions. The low OCR detected in the PolG MEFs compared to WT is not surprising due to the genetic mutation it harbours. These cells were derived from a mouse with a proofreading deficit in mitochondrial polymerase gamma (PolG). Incorrect bases are incorporated into mtDNA as result of poor proofreading, compromising the mtDNA maintenance and stability, resulting in electron transport chain dysfunction (Trifunovic *et al.*, 2004, Kolesar *et al.*, 2014). The recent study published by Saleem *et al.*, (2015) reported that PolG MEFs have a reduced OCR when compared to WT. Furthermore, they found that the PolG mutator mice were dependant on glycolytic flux to maintain energy levels. Additionally, MEFs originating from these mice showed no significant OCR response when exposed to traditional mitochondrial modulators such as FCCP, oligomycin A or rotenone. This supports the PolG MEFs inability to use the mitochondria due to severe dysfunction and is unable to respond to these insults (Saleem *et al.*, 2015). The PolG MEFs show no significant response to energetic stress or VPA treatment. Interestingly, I found a dose dependant increase in OCR in WT MEFs after 24 hours incubation under normal conditions but a reduction at high concentrations. This contrasts with previously published data where VPA has an inhibitory effect (Silva *et al.*, 1997; Koumulainen *et al.*, 2015). It appears that the effect of VPA is cell type dependant as previous studies used isolated rat mitochondria and the HepG2 cell line. The lack of response in the PolG mutator MEFs is likely a reflection of severe OXPHOS dysfunction. What is

already established is that PolG mutator MEFs can compensate for mitochondrial dysfunction by increasing glycolysis and it could be the increase in OCR at 2.0mM VPA is an uncoupling effect. The OCR observed in the PolG mutator MEFs is low and could be due to other cellular oxygen usage. The mitochondria use ~90% of cellular oxygen (Obre and Rossignol, 2014). What is curious is the higher level of steady state ATP observed in the PolG mutator MEFs under normal glucose condition, this could be due to highly upregulated glycolysis and/or these cells are conserving the ATP pool by reducing cellular process (i.e. cell growth) that require ATP. The reason for these cells to have such an increase in steady state ATP when compared to WT has yet to be elucidated. The classic response to mitochondrial dysfunction is one of decreased ATP levels and there have been very few reported cases where an increase in ATP has been recorded. However, this anomaly has been found in rho0 MCF7 cells when compared to the parental cell. When these cells were depleted of mtDNA by ethidium bromide treatment it causes mitochondrial fragmentation, depletion and dysfunction. This caused a much lower OCR in the rho0 than the parental line however, the ATP levels were much higher in the rho0 than the parental line cells (Qian and Van Houten, 2010). Furthermore, I used PicoGreen staining to establish mtDNA levels of WT and PolG mutator MEFs. PicoGreen is a DNA specific fluorescent dye that is used to detect mtDNA (Ashley *et al.*, 2005). PolG MEFs had significantly lower mtDNA, as detected by PicoGreen signal, compared to WT. However, after VPA treatment the mtDNA was significantly increased in the PolG MEFs. These results are somewhat consistent with a publication by Sitraz *et al.*, 2014. They found that in fibroblasts from patients harbouring a PolG mutation, after treatment with VPA the mtDNA content was markedly increased. There has been a report that VPA treatment increased the levels of proteins involved in mitochondrial biogenesis such as PGC-1 α (Sitraz *et al.*, 2014). PGC-1 α is a transcriptional coactivator associated with mitochondrial biogenesis. However, in the PolG MEFs there was no increase in the mitochondrial area after treatment with VPA. The PolG MEFs had less mitochondrial area under normal

glucose conditions than WT. Following energetic stress the mitochondrial area was further decreased in the PolG MEFs but there were no differences between normal and energetic stress conditions in the WT. After 24 hours treatment with VPA under normal conditions there was a concentration dependant decrease of mitochondrial area in the WT and in the PolG MEFs. When the PolG MEFs were incubated under energetic stress and VPA for 24 hours, no changes in mitochondria area was observed. However, after 48 hours treatment there was a reduction in area at the highest concentration (2mM) in PolG MEFs only. It appears that the VPA effect on mitochondrial area is cell type dependant as treatment did not produce an increase as presented in previous publication and that observed in P1(2yr).

5.4.1.3 *When is a control not a control?*

Finding suitable control cell lines is a challenging task when investigating mitochondrial disease and dysfunction. It is not always possible to find age and sex matched control fibroblasts derived from healthy individuals. The younger controls are often sourced from children investigated for non-mitochondrial related diseases. Therefore, the term healthy relates to individuals who do not harbour any known DNA mutations pertaining to mitochondrial function. Previous studies have established that mitochondrial function declines with age but very little is known about differences between male and females. For a robust study several controls should be included but this is not always possible due to time, cost or space limitations of the assays used. I investigated the differences between seven controls in their ability to adapt to changing glucose and energetic stress conditions. Although, the seven controls had similar responses when combining the results from two different methods i.e. OCR and mitochondrial network morphology there was one control, C4(15yr), which clearly did not have the same energetic plasticity. This participant maybe in good health but has some underlying and unknown mitochondrial dysfunction and therefore should not be used as a control. This only became apparent when it was compared to several other controls.

In this chapter it was demonstrated that the PolG mutation changed cell morphology when compared to the wild type counterpart. The PolG mutator MEFs cells are larger and the mitochondrial mass is spread throughout the cell's cytosol whereas in the wild type the mitochondria are clustered around the nucleus due to the much smaller cytosolic area. Cell size can play a part in the demand for energy therefore, it would seem detrimental to the cell to expand the cytosolic area when already its means of producing ATP through OXPHOS had been compromised.

With this in mind, is the wild type MEFs a true control? The wild type MEFs were derived from the same strain of mice as the PolG mutator but the cell morphologies are vastly different from the Mutator so only in the sense that it does not harbour the PolG mutations. Although every precaution has been taken to account for the difference in cell morphology, one must be mindful when interpreting the data and perhaps consider the WT and PolG mutator as separate entities. There were no differences in morphology observed between control and PolG fibroblasts. How the PolG mutation is linked to the change in cell morphology in MEFs has yet to be investigated.

5.5 Research highlight

- VPA treatment at high concentrations increased OCR in PolG patients' fibroblasts but only under energetic stress condition. However, ATP levels were decreased.
- The OCR of PolG mutator MEFs is not affected by VPA treatment under normal or energetic stress conditions.
- MEFs doubling time is significantly reduced under energetic stress conditions alone. VPA treatment did not cause further reduction.
- PolG mutator MEFs have higher ATP level under normal conditions than wild type.
- PolG mutator MEFs are morphologically different from wild type

5.6 Limitations of this study

As in chapter 3, only basal cellular oxygen consumption was measured and spare or maximal respiratory capacity was not accounted for. I used a very limited number of fibroblast cultures derived from PolG patients and only one mutant MEF line. The impact of the morphological differences between PolG mutator and wild type MEFs has not been fully accounted for. It is not clear what effect VPA has on mitophagy.

Chapter 6

Thesis conclusion

6 General conclusion

The work presented in this thesis centred on the study of mitochondrial dysfunction caused by sodium valproate treatment in three different cell models; HepG2 cells, primary fibroblasts from patients with *PolG* related mitochondrial disease and non-affect controls and MEFs derived from a mouse model harbouring *PolG* mutations. *PolG* mutations are a risk factor for VPA-induced hepatotoxicity and acute liver failure however, the underlying mechanism is poorly understood.

6.1 Key achievements from this thesis

VPA causes mitochondrial stress by inhibiting mitochondrial respiration resulting in depletion of ATP and ultimately cell death. However, this was cell type specific. The HepG2 cell line showed reduced OCR while control fibroblasts were unaffected. Wild type MEFs on the other hand actually showed an increased OCR with VPA treatment. VPA treatment under energetic stress (galactose containing, glucose free medium) had no effect on *PolG* MEFs but greatly increased the OCR in *PolG* patient's fibroblast while the control fibroblasts were unaffected.

Interestingly, when MTT and AB assays were used as a method for assessing cytosolic metabolic activity, VPA treatment increased activity in the surviving cells in both HepG2 cells and *PolG* MEFs. This suggests that these cells were able to compensate for mitochondrial dysfunction caused by VPA treatment by upregulating other pathways. Furthermore, HepG2 cells showed increased citrate synthase activity after prolonged VPA treatment suggesting increased TCA cycle activity could reduce or prevent the effects of VPA. As VPA is a known inhibitor of the TCA enzyme α -ketoglutarate dehydrogenase, increasing citrate synthase activity could help to

maintain flux through the TCA cycle, maintaining levels of ATP and key metabolic intermediates.

This thesis also provided proof of principle for a new high-throughput assay for the detection of mitophagy, using high-content imaging and analysis software. As the VPA treatment had varying effect on cell lines, it is therefore challenging to draw firm conclusions as to whether VPA inhibits or increases mitophagy. In order to determine whether the detrimental effects of drugs like VPA are specific to particular mitochondrial disorders, it would have been beneficial to perform a VPA treatment time course study on different cell lines to monitor mitophagy events. Manipulation of mitophagy could be a useful strategy for ameliorating mitochondrial disease.

Work from this thesis contributed to the following publications:

Komulainen, T., Lodge, T., Hinttala, R., Bolszak, M., Pietila, M., Koivunen, P., Hakkola, J., Poulton, J., Morten, K. and Uusimaa, J., 2015. Sodium valproate induces mitochondrial respiration dysfunction in HepG2 *in vitro* cell model. *Toxicology* 331:47-56.

Diot, A., Hinks-Roberts, A., Lodge, T., Liao, C., Dombi, E., Morten, K., Brady, S., Fratter, K., Carver, J., Muir, R., Davies, R., Green, C.J., Johnston, I., Hilton-Jones, D., Sue, C., Mortiboys, H. and Poulton, J., 2015. A novel quantitative assay of mitophagy: Combining high content fluorescence microscopy and mitochondrial DNA load to quantify mitophagy and identify novel pharmacological tools against pathogenic heteroplasmic mtDNA. *Pharmacological Research* 100: 24-35.

6.1.1 Proposed VPA toxicity

VPA induced hepatotoxicity generally occurs in the first 5 to 9 days of treatment but it can also cause delayed hepatic injury after several months (Schmid *et al.*, 2013; Lee *et al.*, 2003; Koenig *et al.*, 2006). The exact mechanism of valproate-induced hepatotoxicity is still unclear. Currently, there are no studies of VPA toxicity using whole cells as much of the work has been performed using isolated mitochondria. Previous work and this current study suggest that hepatic cells under normal glucose conditions are able to adapt their metabolism in response to mitochondrial associated VPA toxicity. A possible and speculative mechanism for this compensation is as follows; after the initial VPA treatment (24 hours) which causes mild impairment to glycolysis and β -oxidation but this could be off set through increased glutamine oxidation, figure 6.1 (A). In the long term this causes a reduction in the ATP. However, after 48 hours the glycolytic pathways is severely impaired and the TCA cycle and β -oxidation stalled through the inhibition of pyruvate dehydrogenase complex and/or α -ketoglutarate dehydrogenase. Possibly further ATP depletion. This will cause reduction in the pool of NADH and FADH causing inhibition or reduction of the ETC function. In turn this can increase ROS and eventually lead to cell death. This can be offset by the aspartate-malate shuttle which can bring in NADH from the cytosol where the NADH/NAD ratio is higher, figure 6.1 (B). After 72 hours VPA treatment there are potential compensatory mechanisms in place to preserve the ATP pool possibly through restoration of glycolysis or OXPHOS, as observed by the MTT and AB data presented in previous chapters figure 6.1 (C). The findings also suggest a mechanism to explain why PolG cells are particularly susceptible to VPA toxicity. Increased mitophagy, under normal conditions, along with mtDNA depletion suggests that mtDNA turnover is greatly increased in PolG cells. The low mtDNA levels may represent a compromise between; the disadvantage to OXPHOS from low mtDNA and the

advantage of improved respiratory chain quality. Exposure to VPA increased both mtDNA copy number and co-localisation of autophagosomes and mitochondria, potentially indicating attenuated mitophagy. Inhibited mitophagy might explain how VPA increased the accumulation of ROS as reported by Komulainen *et al.*, 2015. Increased ROS could potentially impair coupling by increasing the existing damage complex 1 and perhaps increase OCR.

Both of these proposed mechanisms of VPA toxicity are drawn on the data presented in this thesis and are highly speculative.

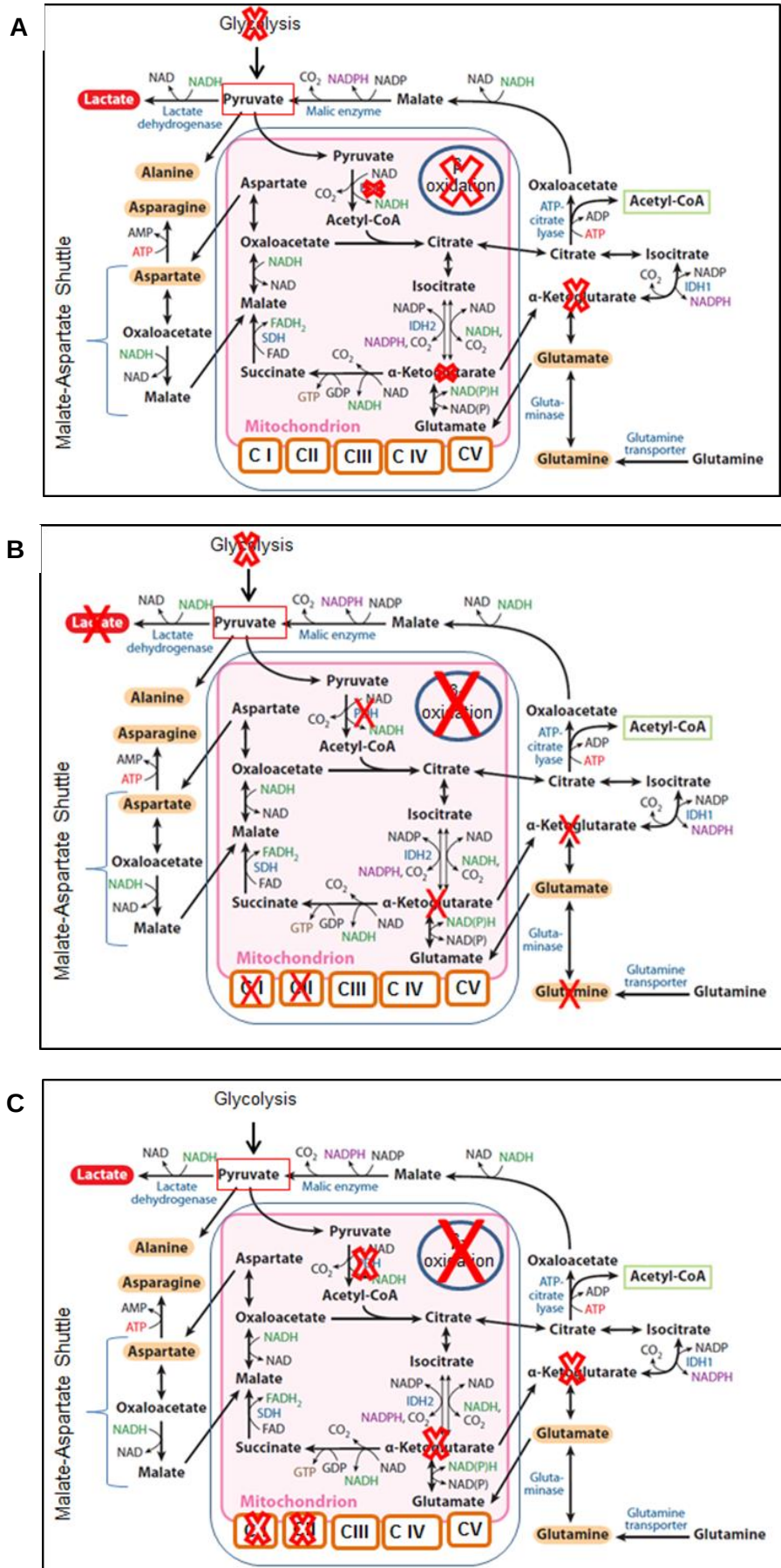


Figure 6.1. Schematic representation of the proposed inhibitory effect of high concentration of VPA treatment under normal glucose conditions over time. The schematic represent the proposed mechanism underlying the hepatotoxicity observed in cells treated with VPA. **(A)** 24 hours VPA treatment causing mild impairment of glycolysis and β -oxidation and moderate inhibition of pyruvate dehydrogenase complex (PDH) thereby impairs the TCA cycle. Cells relying on glutamine oxidation **(B)** 48 hours VPA treatment moderate impairment of glycolysis and inhibition of β -oxidation and/or α -ketoglutarate dehydrogenase and PDH. No glutamine oxidation. Reduced OXPHOS due inhibition of ETC complex I and II as there is a reduction of NADH and FADH pool. **(C)** 72 hours VPA treatment showing recovery of glycolysis and OXPHOS but α -ketoglutarate dehydrogenase and PDH are still impaired. Recovery of OXPHOS due to increased Malate-Aspartate activity providing electrons to the ETC. The mitochondrial outer membrane is in blue and the inner membrane is pink. Orange boxes are CI complex I, CII complex II, CIII complex III, CIV complex IV and CV complex V ATP synthase. Solid crossed denotes inhibition and open crosses mild to moderate deficiency. Abbreviations: ADP, adenosine diphosphate, ATP, adenosine triphosphate. FAD, flavin adenine dinucleotide. FADH₂, reduced flavin adenine dinucleotide. GDP, guanosine diphosphate. GTP, guanosine triphosphate. IDH2, isocitrate dehydrogenase 1. NAD, nicotinamide adenine dinucleotide. NADH, reduced nicotinamide adenine dinucleotide. NADP, nicotinamide adenine dinucleotide phosphate. NADPH, reduced nicotinamide adenine dinucleotide phosphate. PDH, pyruvate dehydrogenase complex. SDH, succinate dehydrogenase. Adapted from Lunt and Vander Heiden, 2011

6.2 Main limitations of this thesis

In this study I used the basal cellular oxygen consumption rates only, and did not take into account the maximum or spare respiratory capacity of the different cell lines used. Therefore, the OCR data presented does not take into account non-mitochondrial respiration. It must be noted that it was challenging to obtain OCR data for the primary fibroblasts and the PolG MEFs as these cells have very low respiration rates. This may be due to the high glucose medium used to maintain the cell cultures in, conditioning these cell lines to generate energy through glycolysis. High glucose could in itself compound the toxicity of VPA and this has not been accounted for in the work using primary fibroblasts and MEFs. Conversely, galactose medium used to induce energetic stress may be too stringent for these cell lines thus, increasing VPA toxic effect.

Due to time constraints VPA's effect on mitochondrial membrane potential and β -oxidation was not investigated.

6.3 Future work

The results presented in this thesis are far from exhaustive. Interesting research areas to explore would be repeating the primary fibroblast work using physiological glucose concentrations in perfused models. VPA's effect on the efficiency and configuration of the ETC complexes has not been explored in the cell lines used in this work. The IN Cell method for detecting mitochondrial network morphology could be developed to investigate changes to the endoplasmic reticulum morphology.

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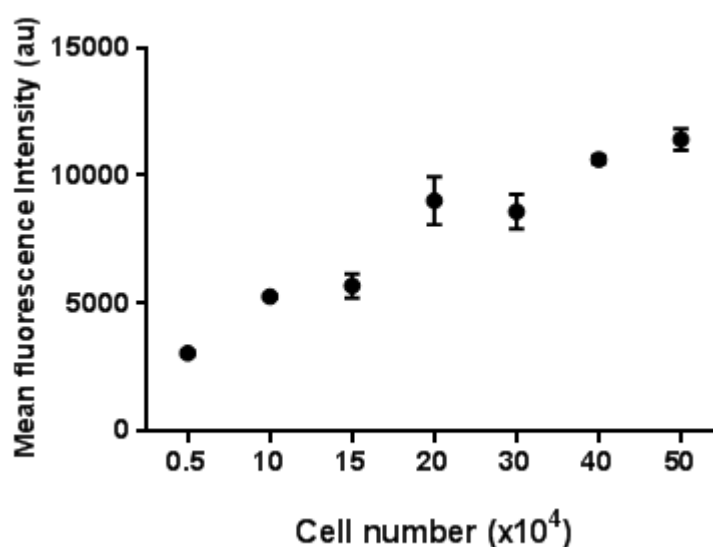
Invitrogen (Thermo Scientific). Alamar Blue® Assay protocol. Available on line
[https://tools.thermofisher.com/content/sfs/manuals/MAN0011850_alamarBlue_Cell_Vi
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Appendices

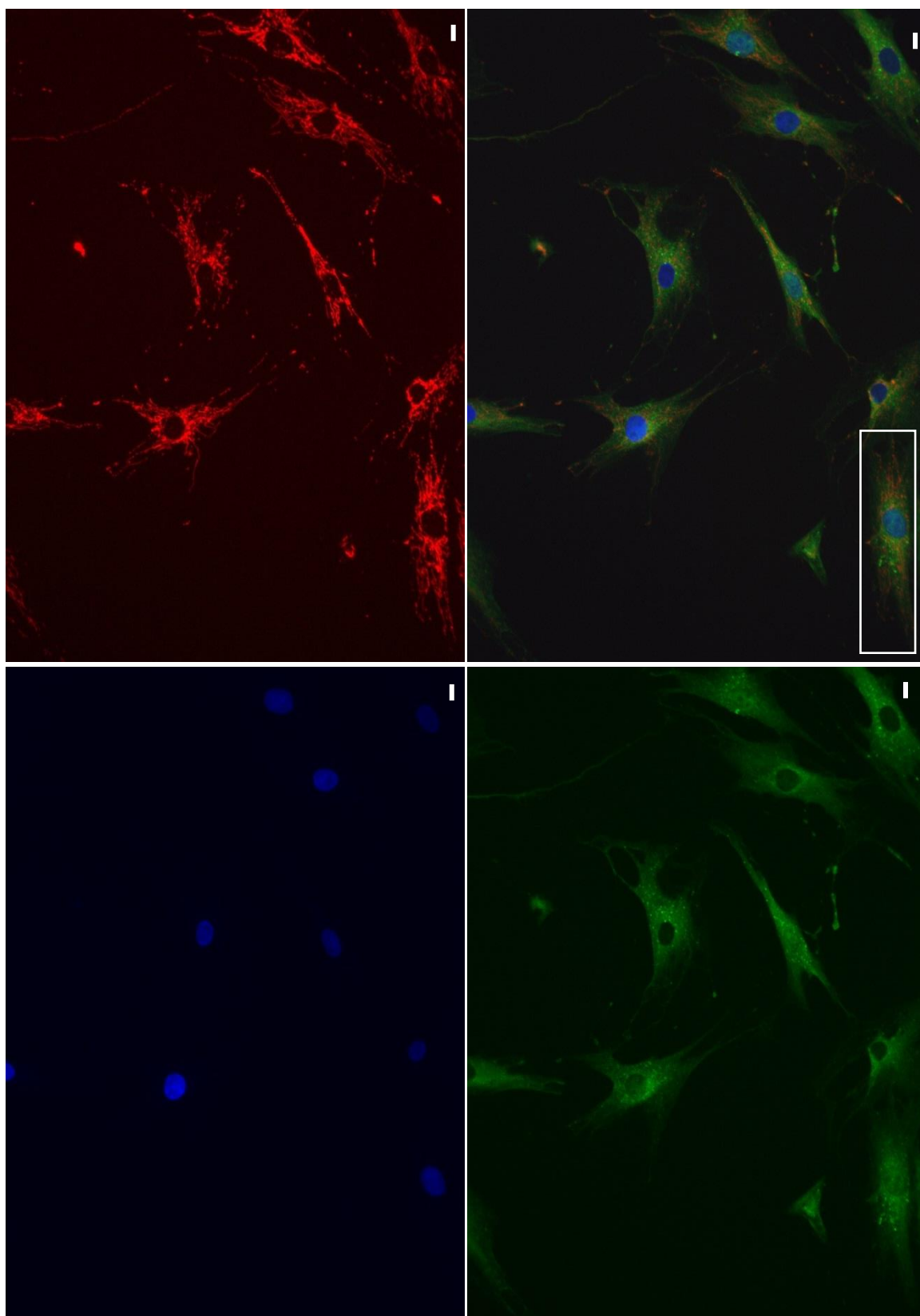
Appendix A Optimisation of Hoechst assay

To ensure that the Hoechst stain was used at the correct concentration and would report an increase in fluorescence as cell numbers increased, HepG2 cells were seeded in 96 well plates at increasing densities.

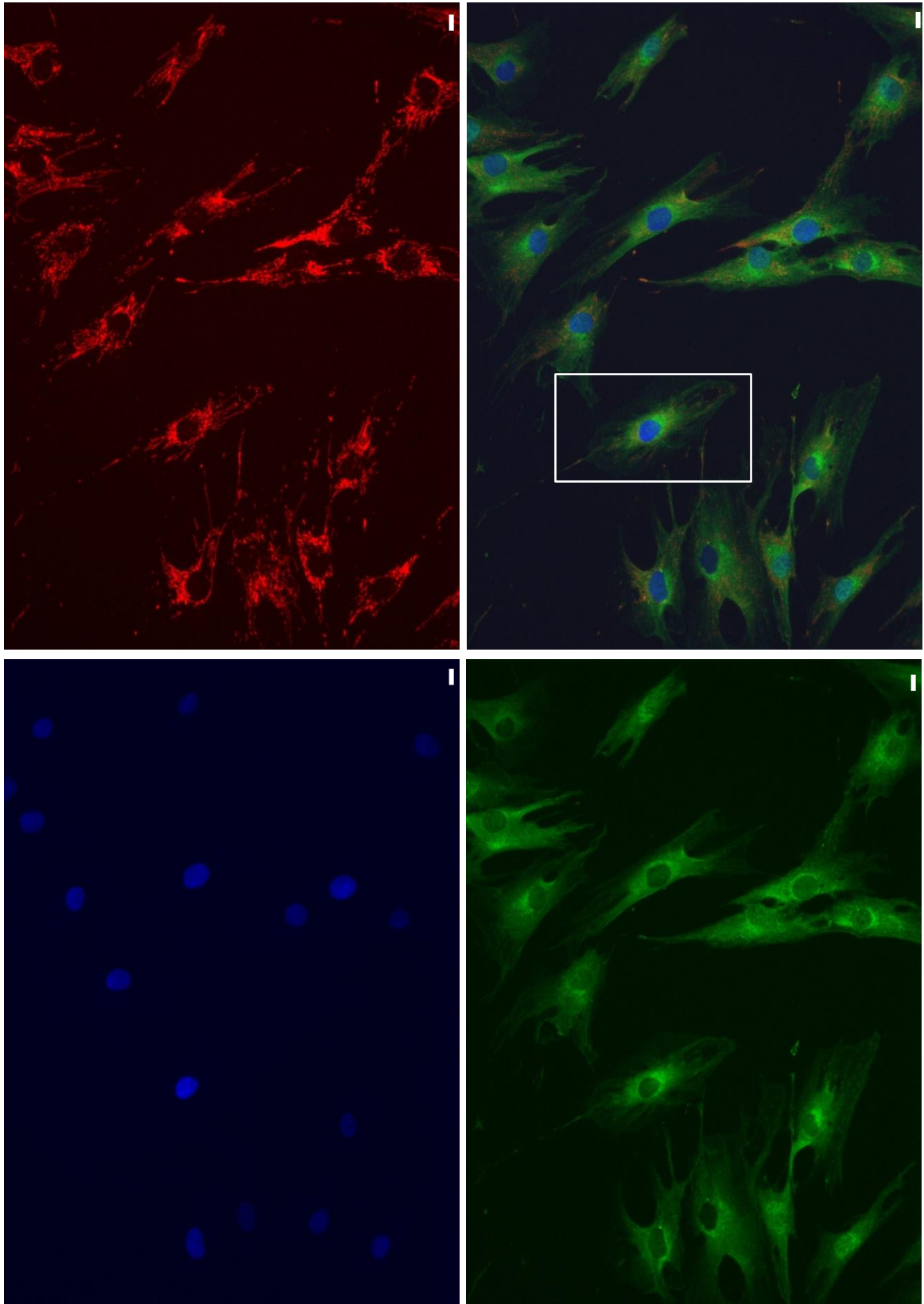


Supplementary Figure 3.1. Increasing cell number increases Hoechst fluorescence. Determine cell number relative to Hoechst fluorescence. HepG2 cells were seeded in 96 well plates and left overnight to adhere before the stain was added. Cells were incubated for 20 mins at a final concentration of 0.1µg/ml. Cells were incubated in normal glucose conditions (5mM). Error bars represent the mean $\pm$ SD of three technical replicates, n=1.

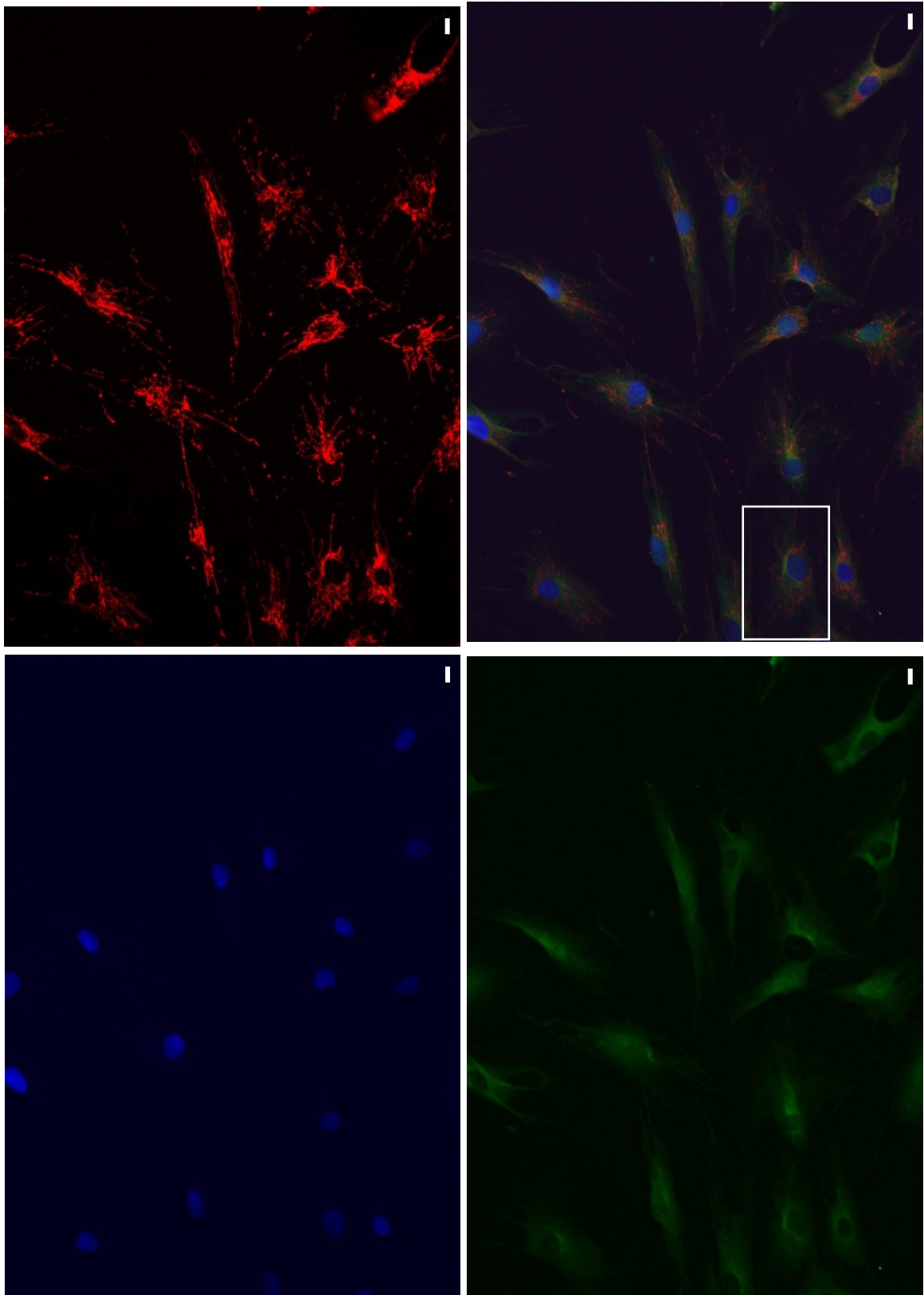
Appendix B complete field of views supplementary to figure 4.12



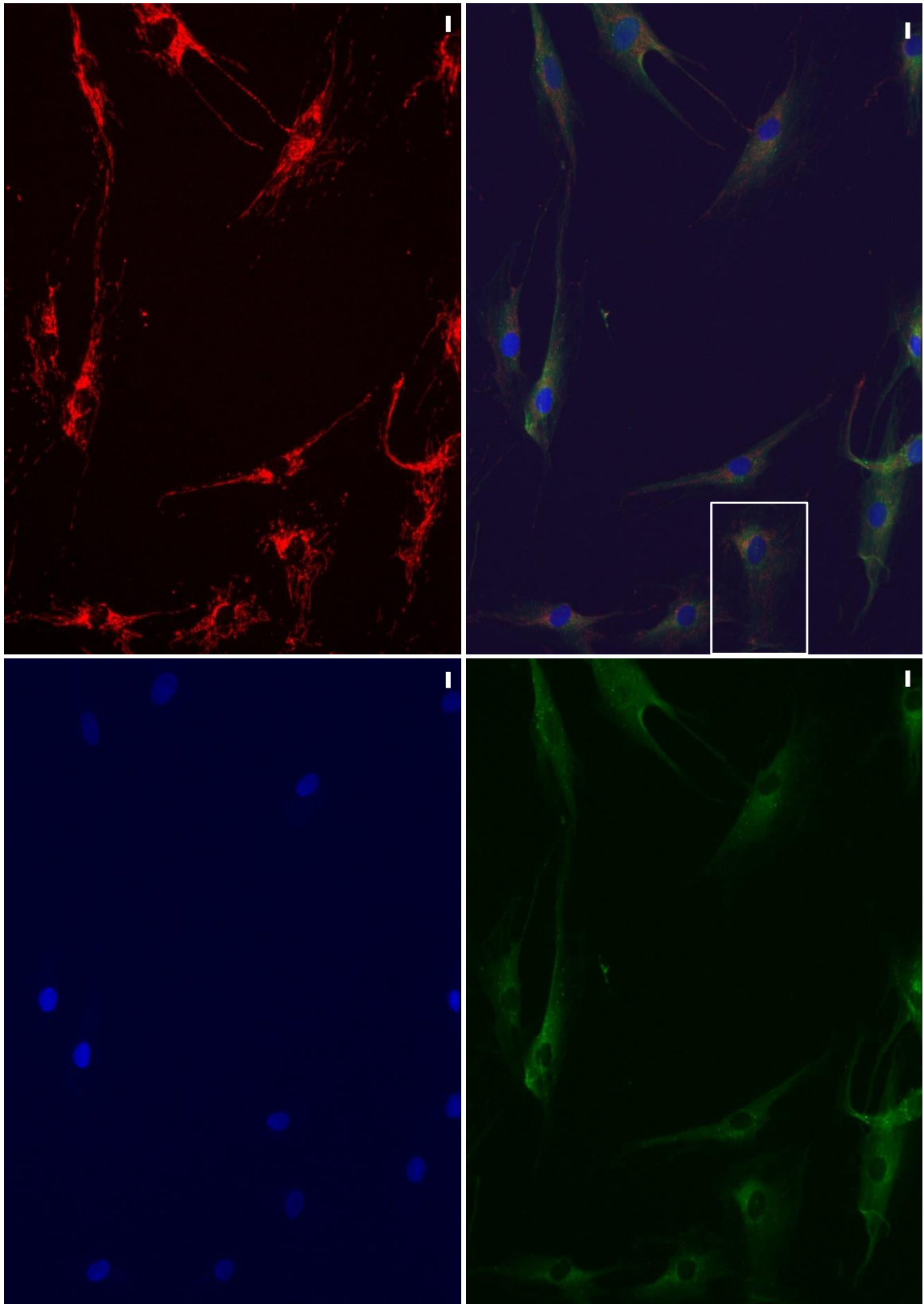
Supplementary figure 1. Control fibroblast incubated in 25mM glucose (normal condition)



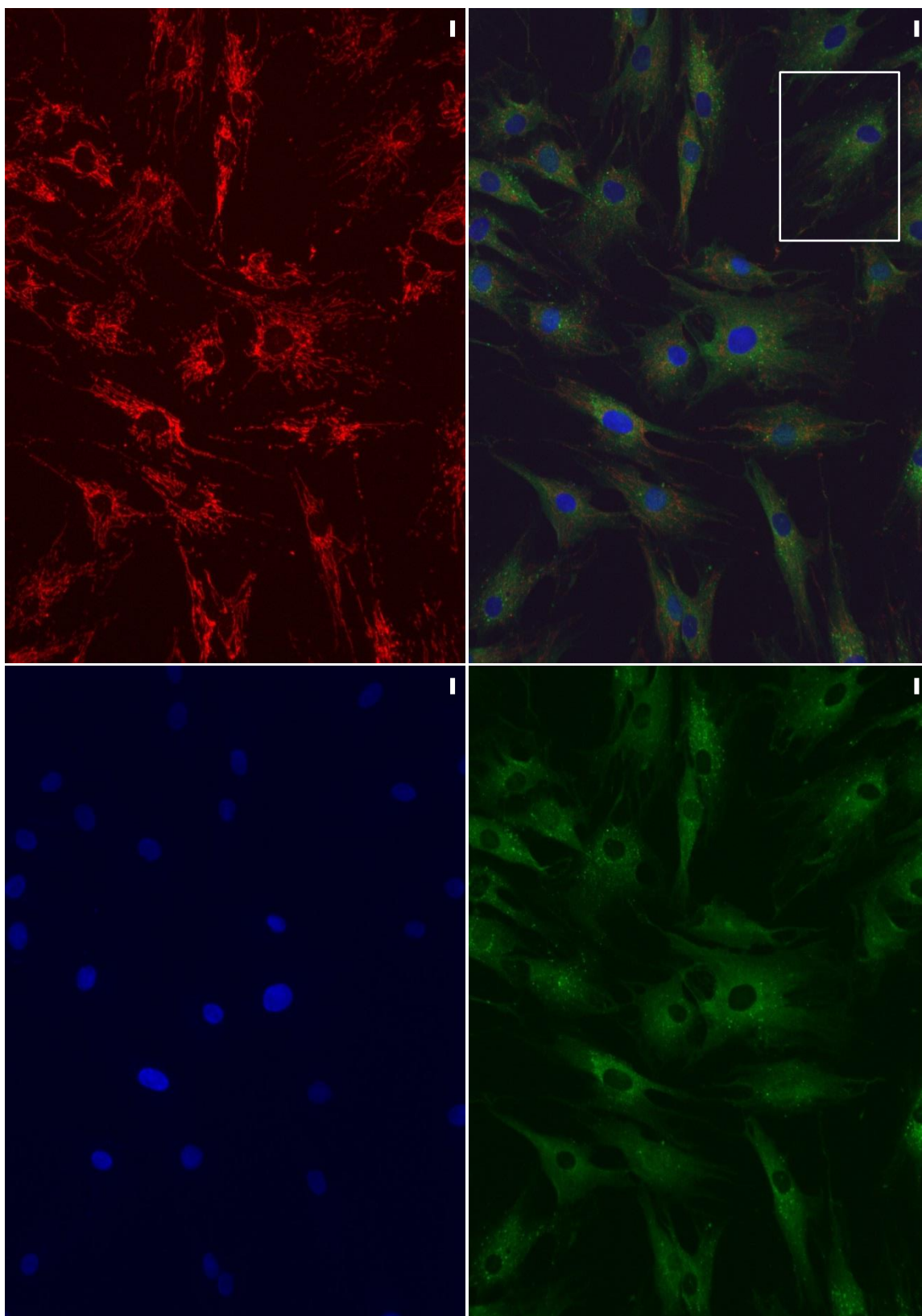
Supplementary figure 2. Control fibroblast treated with 20μM CCCP.



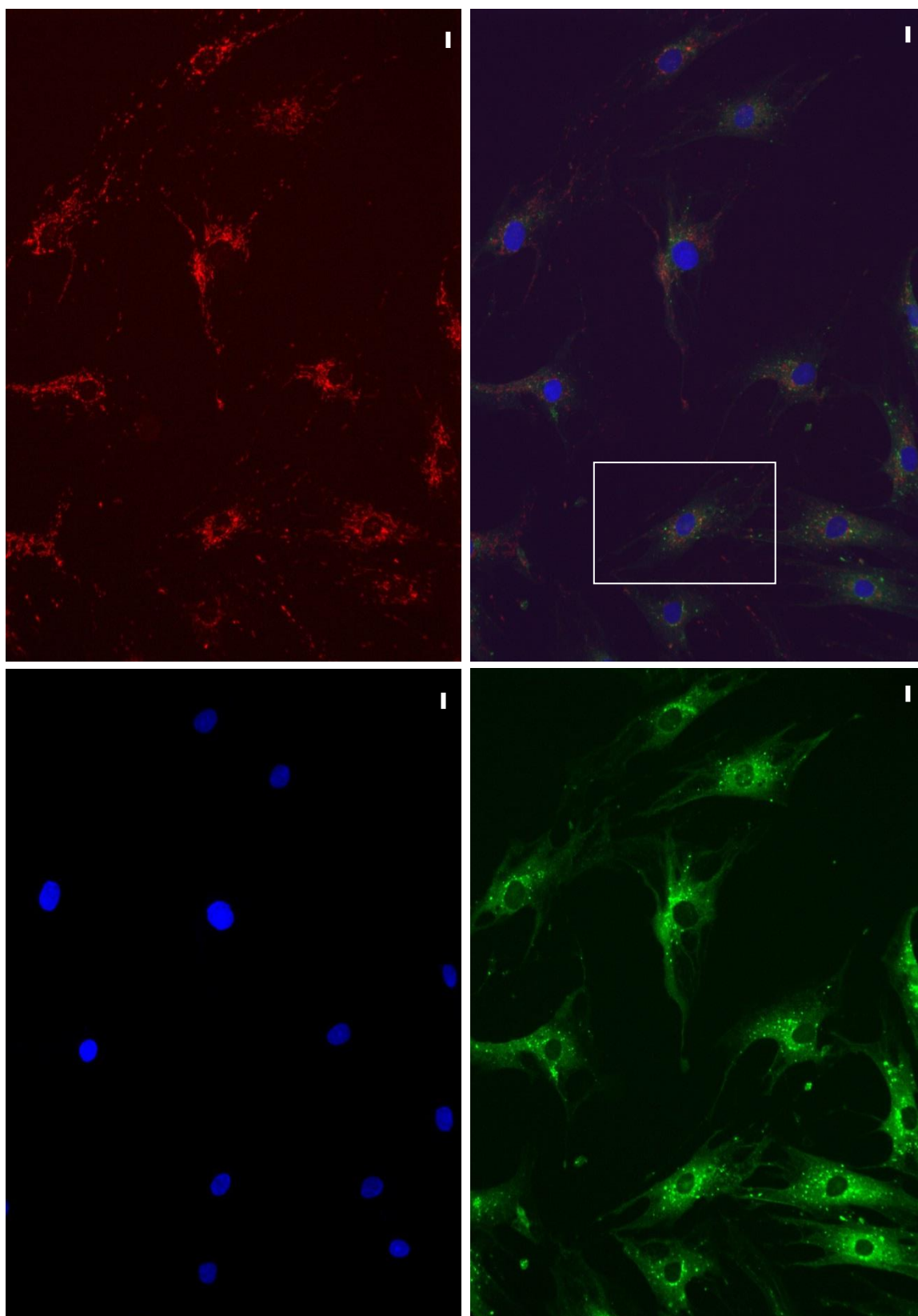
Supplementary figure 3. Control fibroblast treated with 3-MA.



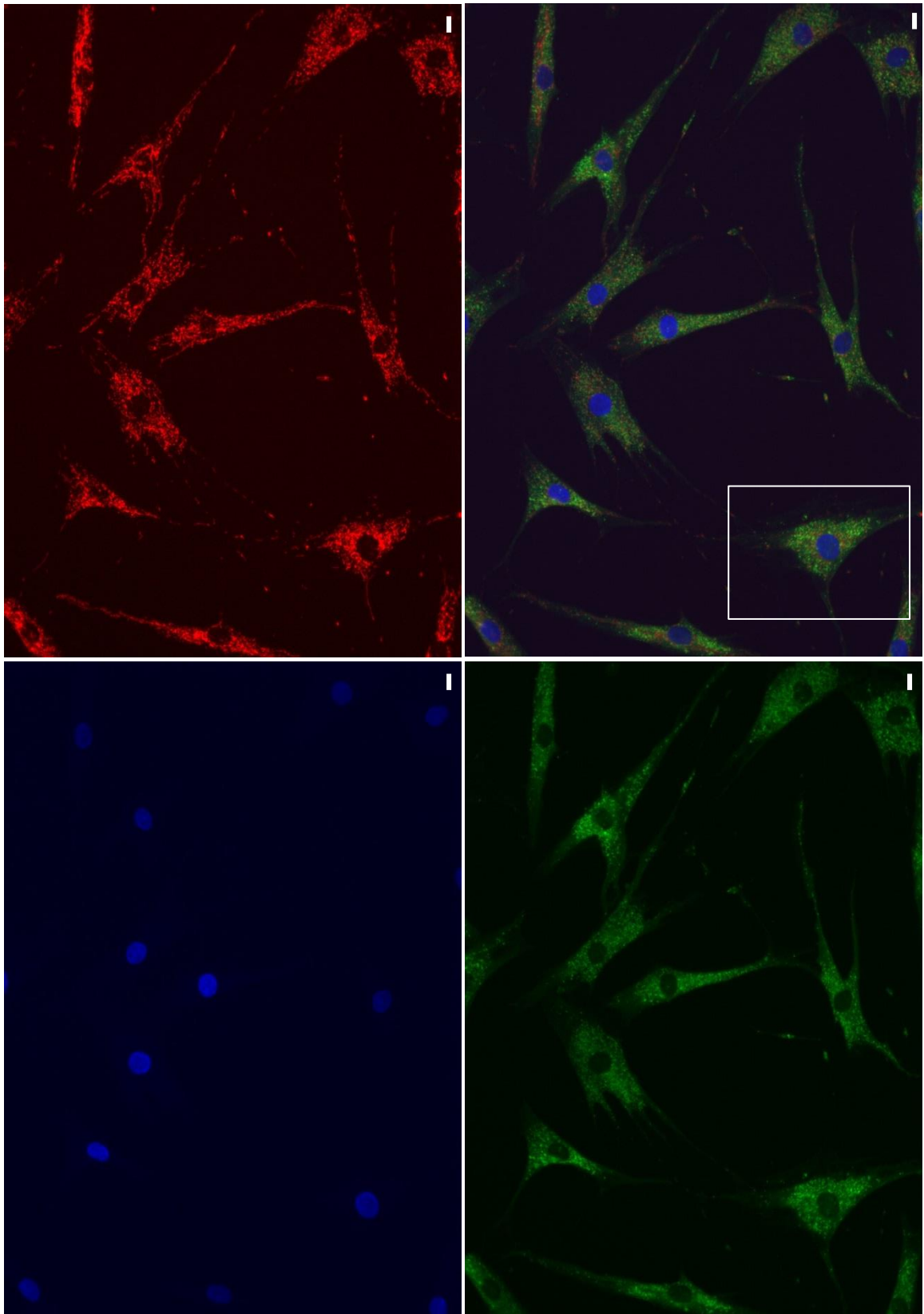
Supplementary figure 4. Control fibroblast treated with HBSS (starvation).



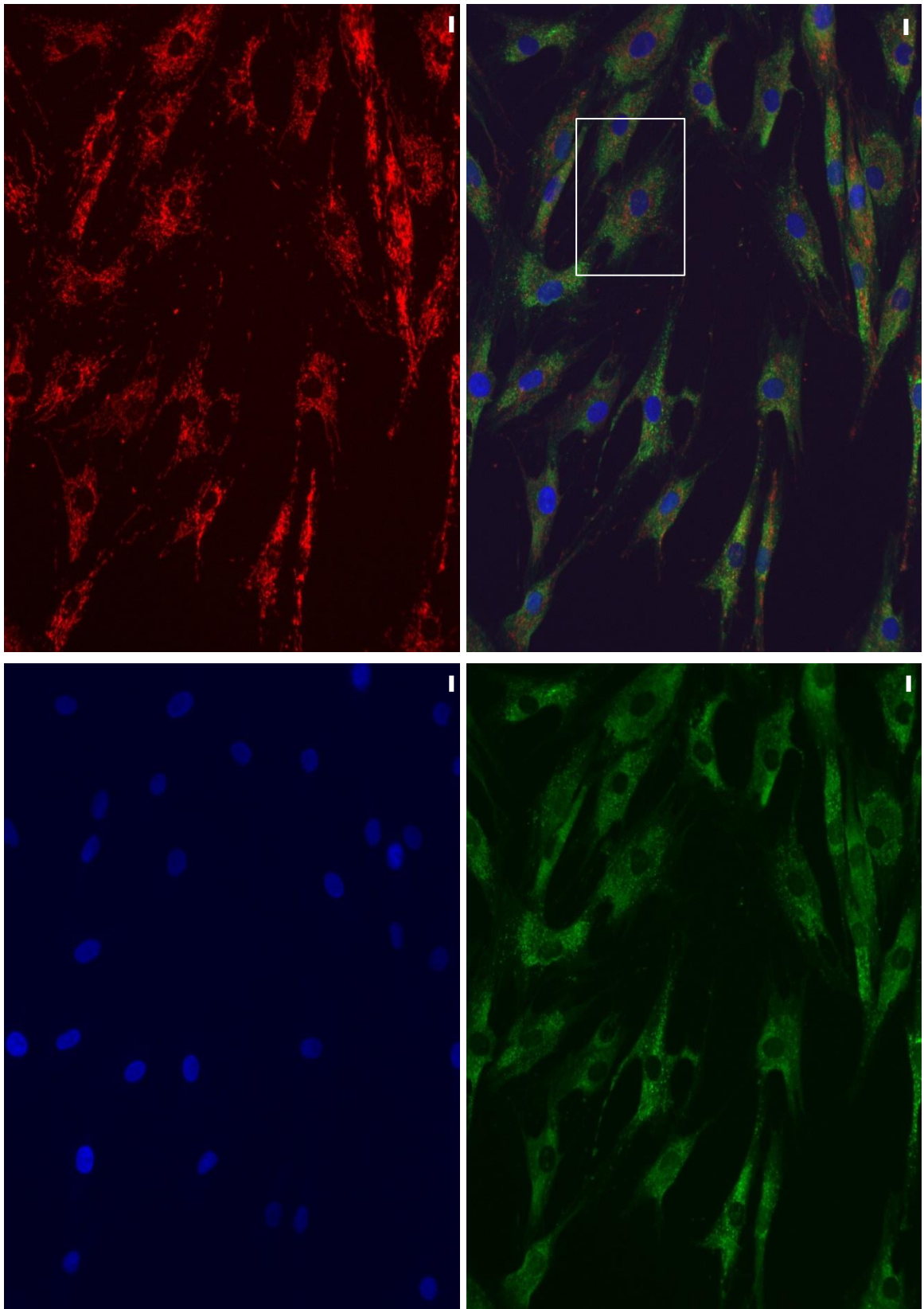
Supplementary figure 5. Control fibroblast treated with colchicine.



Supplementary figure 6. Control fibroblast treated with phenanthroline.

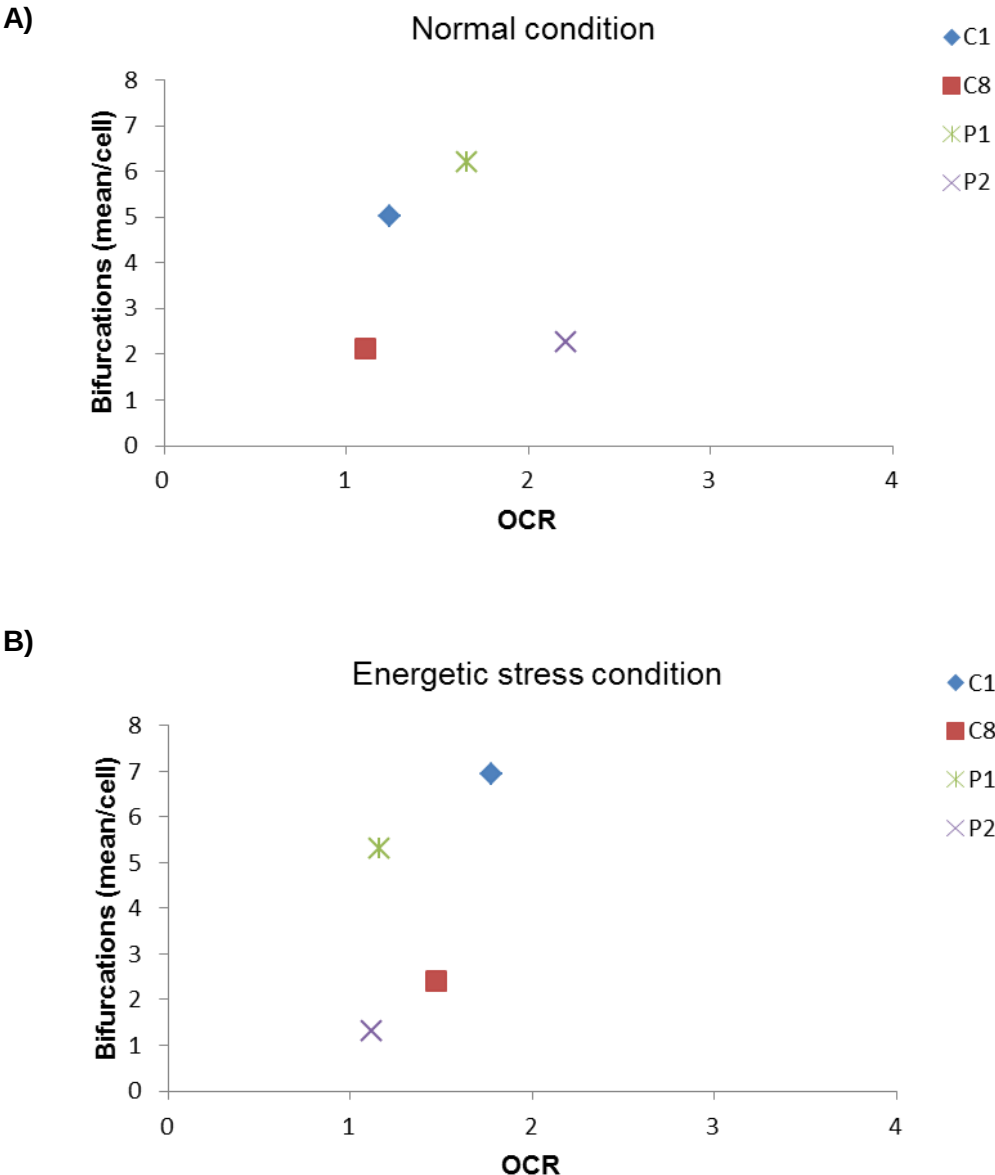


Supplementary figure 7. Control fibroblast treated with bafilomycin A1.



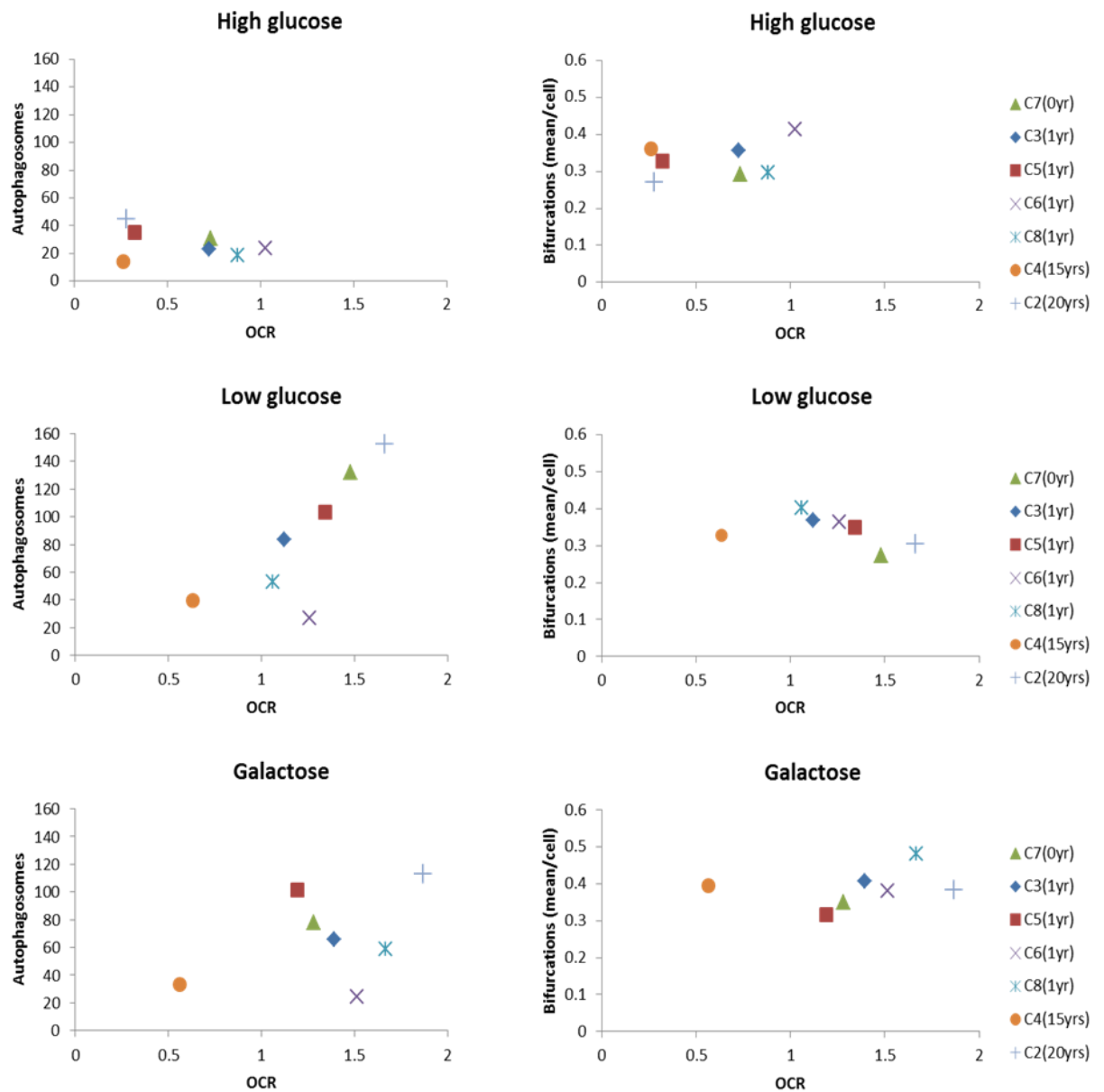
Supplementary figure 8. Control fibroblast treated with E&P.

Appendix B. The cells can adapt the mitochondrial network in response to energetic stress or toxicants such as VPA.



Supplementary figure 2. Bifurcations vs OCR after 72 hours incubation under normal and energetic stressed conditions. Data for control fibroblasts extracted from IN Cell experiments and OCR experiments to illustrate mitochondrial network and respiration plasticity when subjected to changing energy substrates. (A) High glucose (25mM), and (B) energetic stress (galactose).

There are subtle differences between controls and patients in response to energetic stress, supplementary figure 2. For example C1(20yr) responds to energetic stress by increasing mitochondrial network interconnectivity and OCR thereby shifts up and to the right on the graph. This does not affect the ATP levels. Whereas the patient P2(42yr) does the opposite. The P2(42yr)'s adaptive response is to fragment the mitochondrial network and reduce OCR. This response potentially increased ATP by conserving the ATP pool.



Supplementary figure 3. Autophagosomes and bifurcations vs OCR after 72 hours incubation under normal and energetic stressed conditions. Data for control fibroblasts extracted from IN Cell experiments and OCR experiments to illustrate mitochondrial network and respiration plasticity when subjected to changing energy substrates. (A) High glucose (25mM), (B) low glucose (5mM) and (C) energetic stress (galactose).