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I. Abstract

Diabetes is one of the most prevalent diseases globally; within the UK alone, there are 3.8 million diagnosed. The main characteristic of type 1 and type 2 diabetes is hyperglycemia, which is defined as high blood glucose concentrations. Diabetes is also thought to be a risk factor for the development of other diseases, including cancer. The World Health Organization estimates that 20% of cancers worldwide are attributable to environmental risks such as chemical exposure and air pollution. With the incidence of diabetes increasing rapidly, understanding how hyperglycemia may alter the effects of these environmental factors could provide a better understanding of the increased incidence of cancer in people with diabetes. This pilot study used a multiple-endpoint approach to monitor phenotypic and genotypic changes within the mitochondria to assess the damage induced by hyperglycaemia combined with environmental carcinogens. Mitochondria were chosen due to their smaller size compared to nuclear DNA as well as the increased evidence mtDNA is more sensitive to damage; this could assist with detection of low-level or early stage damage and allow more targeted approach. Additionally, while essential processes for life such as oxidative phosphorylation occur within the mitochondria, the effects of such chemicals on mtDNA are not well established. Four well-characterised xenobiotic carcinogens, hydrogen peroxide, diethyl hexyl phthalate, acetaldehyde, and 1-nitropyrene were chosen while two differing glucose conditions, "normal" or "high" (10mM and 29mM respectively), were used. Within this study, high glucose in the presence of these environmental carcinogens had an additive disruptive effect compared to high glucose alone. High glucose did this by increasing variations within the mitochondrial genome and decreasing the ability of cells to meet energy demand or respond to stress which may result in causation or acceleration of the development of cancer.

II. Declaration

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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STATEMENT 1

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Contents

Acknowledgements	7
Tables and Figures	8
Abbreviations	13
 Chapter 1- Introduction	
1.1 Diabetes Mellitus	15
1.2 Diabetes and Cancer	16
1.3 Mitochondria	18
1.3.1 Mitochondrial Biogenesis	20
1.3.2 The Mitochondrial Genome	22
1.3.3 Mitochondrial fusion	23
1.3.4 Mitochondrial fission	24
1.3.5 Mitochondria and Diabetes	25
1.3.6 Mitochondria and Cancer	26
1.3.7 Metformin	27
1.4 Environmental toxins	29
1.4.1 Acetaldehyde	29
1.4.2 Hydrogen peroxide	30
1.4.3 1-nitropyrene (1-NP)	30
1.4.4 Di(ethylhexyl) phthalate (DEHP)	31
1.5 Reactive oxygen species (ROS)	31
1.5.1 ROS and mtDNA damage	32
1.5.2 Oxidative stress and diabetes	33
1.5.3 Oxidative stress and Cancer	34
1.6 Aims & Objectives	34
 Chapter 2- Methodology	
2.1 Reagents	35
2.2 Cell Types	35
2.2.1 Sub-culturing TK6 cells	35
2.2.2 Sub-culturing MCL-5 cells	35
2.3 Treatment with glucose	36
2.3.1 Cell seeding and glucose treatment day one.	36
2.3.2 Glucose treatment day two	36
2.4 Carcinogen Treatment	37
2.4.1 TK6 Carcinogen Treatment	37
2.4.2 MCL-5 Carcinogen Treatment	38
2.5 Recovery of cells	39
2.6 DNA extraction	40
2.7 Analysis of mtDNA copy number using qRT-PCR	40
2.8 Fluorescence Microscopy	43
2.8.1 Staining cells for image analysis of mitochondria	43
2.8.2 Fixing Cells	44
2.8.3 Imaging cells	44
2.9 Long-Range PCR for sequencing	45
2.9.1 Amplification of DNA samples	45
2.9.2 Gel Electrophoresis (pre-run staining)	47
2.9.3 Gel Electrophoresis (post-run staining)	47
2.9.4 PCR product purification	48

2.9.5 Optimisation of amplicon 1	48
2.10 Next Generation Sequencing	48
2.11 Seahorse Bioanalyzer	50
2.12.1 Day 1- Plate preparation	50
2.12.2 Day 2- Seahorse Run	50
2.12 Statistical Analysis	52
Chapter 3. The effect of environment chemical exposure and hyperglycaemia on the mitochondrial genome	
3.1. Next Generation Sequencing	
3.1.1. Comparison of all TK6 samples for significance	53
3.1.2. The effect of high glucose within the TK6 control groups	54
3.1.3. The effect of high glucose in the presence of acetaldehyde	57
3.1.4. The effect of high glucose in the presence of DEHP	60
3.1.5. The effect of high glucose in the presence of Hydrogen Peroxide	62
3.1.6. Comparison of all sites with a VAF of 1.00% in all MCL-5 samples	64
3.1.7 The effect of high glucose within the MCL-5 control groups	65
3.1.8. The effect of high glucose in the presence of 1-NP	67
3.1. 9. Types of mutations within the sample groups	68
3.1.9.1 Absolute variant counts within samples	68
3.1.9.2 The type of variants present	69
3.1.10 Summary of NGS results discussed	71
3.1.10.1 Summary of results discussed within the TK6 samples	71
3.1.10.2 Summary of results discussed within the MCL-5 samples	73
3.2. qt-PCR	74
3.2.1. <i>MT-ND1</i> copy number change within TK6 control groups	74
3.2.2. <i>MT-ND1</i> copy number change within acetaldehyde	76
3.2.3. <i>MT-ND1</i> copy number change within DEHP	78
3.2.4. <i>MT-ND1</i> copy number change within hydrogen peroxide	80
3.2.5. <i>MT-ND1</i> copy number change within MCL-5 samples	82
Chapter 4. The effect of environment chemical exposure and hyperglycaemia on the mitochondrial genome	
4.1 Fluorescence Microscopy	84
4.1.1 Effect of high glucose on intensity changes within the mitochondria in TK6 control groups	84
4.1.2 Effect of high glucose on intensity changes within the mitochondria in acetaldehyde groups	87
4.1.3 Effect of high glucose on intensity changes within the mitochondria in DEHP groups	89
4.1.4 Effect of high glucose on intensity changes within the mitochondria in hydrogen peroxide groups	92
4.1.5 Effect of high glucose on intensity changes within the mitochondria in 1-nitropyrene groups	95
4.2 Seahorse Bioanalyzer	
4.2.1 Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in TK6 control groups	100
4.2.2 Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in acetaldehyde groups	102

4.2.3 Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in DEHP groups	103
4.2.4. Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in hydrogen peroxide groups	105
4.2.5 Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in MCL-5 control groups	107
4.2.6 Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in 1-nitropyrene groups	109
4.2.7. Effect of varying glucose concentrations on energy state within TK6 cell line groups	109
4.2.8. Effect of varying glucose concentrations on energy state within MCL-5 cell line Groups	111
Chapter 5. Discussion	113
5.1. The effect of high glucose on the mitochondria within the TK6 groups	
5.1.1. The effect of high glucose on the genome	113
5.1.2 The effect of high glucose on the phenotype	114
5.2 The effect of DMSO on the mitochondria	116
5.3 The effect of acetaldehyde on the mitochondria	116
5.3.1 The effect of acetaldehyde glucose on the genome	116
5.3.2 The effect of acetaldehyde on the phenotype	117
5.4 The effect of DEHP on the mitochondria	117
5.5 The effect of hydrogen peroxide on the mitochondria	118
5.6 The effect of 1-nitropyrene on the mitochondria	118
5.7 Limitations	119
5.8 Future work	119
5.9 Overall Conclusions and findings	119
Chapter 6. Appendix	124
Chapter 7. Glossary	158
Chapter 8. Bibliography	159

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V. List of Tables and Figures

Figure 1: The pathological events which lead to both Type 1 and Type 2 diabetes	15
Figure 2: The potential relationship between diabetes and cancer	17
Figure 3: The circular structure of the human mitochondrial genome	18
Figure 4: The electron transport chain is located within the mitochondria	19
Figure 5: The morphological effects of fusion and fission on the mitochondria	21
Figure 6: A) Glucose metabolism within a healthy cell. B) Glucose metabolism within a cancerous cell	26
Figure 7: The (mTOR) pathway including insulin activation mechanism and both direct and indirect mechanisms of AMPK inhibition.	28
Figure 8: All the potential sites within the electron transport chain at the mitochondrial membrane for ROS production which contribute to oxidative stress.	32
Table 1: The dilutions and final dosing concentration of the carcinogens used to treat TK6 cells	38
Table 2: The dilutions and final dosing concentration of the carcinogen used to treat MCL-5 cells	39
Figure 9: The sequence that was amplified during qRT-PCR that encodes for the genetic region of interest	41
Figure 10: An example of the layout of a plate and the position of each triplet for samples and serial dilutions	41
Figure 11: The serial dilution done to create a standard curve for the analysis of RT-PCR results	42
Table 3: The reagents and volumes used to produce the master mixes for GOI and β -actin	42
Table 4: Primer sequence for the mitochondrial genome of interest and the housekeeping β -actin gene	42
Table 5: The protocol used for the qt-PCR reaction	43
Table 6: The steps and volumes required to dilute the MitoTracker Green staining solution	44
Table 7: Varying protocols used for long-range PCR negative control	45
Table 8: The primer sequence of each of the primers used for amplicon 1 (9kb) and amplicon 2 (11kb) regions	46
Table 9: A. Shows the protocol used for the Long-Range PCR reaction in the thermal cycle	46
Table 10: The dilutions required to prepare the Seahorse injection solutions	51

Figure 12: The appropriate solution and the required volume loaded into each of the cartridge ports on the XFe24 plate	51
Figure 13: Comparisons of the average VAF value above 1% of TK6 sample groups	53
Figure 14: A) Comparison of Variant Allele Frequency (VAF) values above 1% within the TK6 control samples. B) Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of TK6 water control samples grown under a high or normal glucose concentration.	55
Figure 15: Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of TK6 DMSO control samples grown under a high or normal glucose concentration.	56
Figure 16: A) Comparison of Variant Allele Frequency (VAF) values above 1% within the samples grown under a normal or high glucose concentration within the water control groups or under acetaldehyde. B) Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of samples grown under acetaldehyde and either high or normal glucose concentration.	58
Figure 17: Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of samples grown under normal glucose conditions treated with either acetaldehyde or grown under a water control.	59
Figure 18: Comparison of Variant Allele Frequency (VAF) values above 1% within the samples grown under a normal or high glucose concentration within the DMSO control groups or under di(ethylhexyl) phthalate (DEHP). B) Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of di(ethylhexyl) phthalate (DEHP) samples grown under a high or normal glucose concentration.	61
Figure 19: A) Comparison of Variant Allele Frequency (VAF) values above 1% within the TK6 samples grown under a normal or high glucose concentration within the water control groups or under the hydrogen peroxide (H ₂ O ₂). B) Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of hydrogen peroxide (H ₂ O ₂) samples grown under a normal or high glucose concentration.	63
Figure 20: Comparisons of the average VAF value above 1% of MCL-5 sample groups grown under normal or high glucose concentration with DMSO control or with the test chemicals 1-nitropyrene (1-NP).	64
Figure 21: A) Comparison of Variant Allele Frequency (VAF) values above 1% within the MCL-5 samples grown under a normal or high glucose concentration within the DMSO control groups or under the test chemical 1-nitropyrene (1-NP). B) Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of MCL-5 DMSO control samples grown under a normal or high glucose concentration.	66
Figure 22: Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of 1-nitropyrene (1-NP) samples grown under a normal or high glucose concentration.	67

Figure 23 Comparison of the absolute variant counts within samples with a VAF value above 1% of TK6 sample groups grown under normal or high glucose concentration with a water or DMSO control or with one the test chemicals, acetaldehyde, di(ethylhexyl) phthalate (DEHP) or hydrogen peroxide (H_2O_2) and of MCL-5 sample groups grown under normal or high glucose concentration with DMSO control or with the test chemicals 1-nitropyrene (1-NP). 68

Figure 24: A) Comparison of the Single nucleotide polymorphisms (SNPs) count within samples with a VAF value above 1%. **B)** Comparison of the Insertion and Deletion (INDEL) counts within samples with a VAF value above 1% 70

Table 11: Summary of the comparisons of interest illustrated within chapter 3 sections 3.3.1- 3.3.6 and 3.3.10 in all TK6 samples 71

Table 12: Summary of the comparisons of interest illustrated within chapter 3 sections 3.3.7- 3.3.10 in all MCL-5 samples 73

Figure 25: A) Representation of the fold change of the *MT-ND1* genetic region within the high glucose water control (HG) against normal glucose water control (NG). and normal glucose DMSO control (NG + DMSO) samples respectively. B) Representation of the fold change of the *MT-ND1* gene within the high glucose DMSO control (HG + DMSO) samples when compared against normal glucose DMSO control group (NG + DMSO). C) Representation of the fold change of the *MT-ND1* genetic region within the normal glucose DMSO when compared against normal glucose (NG) D) Representation of the fold change of the the *MT-ND1* gene within the high glucose DMSO control (HG + DMSO) samples when compared against high glucose water control (HG) samples respectively 75

Figure 26: A) Representation of the fold change of the *MT-ND1* genetic region within the high glucose water control (HG) when compared against normal glucose acetaldehyde (NG + Ace) samples. **B)** Representation of the fold change of the genetic region within the normal glucose acetaldehyde (NG + Ace) when compared against normal glucose (NG). **C)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose acetaldehyde (HG + Ace) samples when compared against high glucose water control (HG) samples 77

Figure 27: A) Representation of the fold change of the *MT-ND1* genetic region within the high glucose DMSO control (HG + DMSO) samples when compared against normal glucose di(ethylhexyl) phthalate (NG + DEHP) samples. **B)** Representation of the fold change of the *MT-ND1* genetic region within the normal glucose di(ethylhexyl) phthalate (NG + DEHP) when compared against normal glucose DMSO control (NG + DMSO) samples. **C)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose di(ethylhexyl) phthalate (HG + DEHP) samples when compared high glucose DMSO control (HG + DMSO) samples 79

Figure 28: A) Representation of the fold change of the *MT-ND1* genetic region within the high glucose hydrogen peroxide (HG + H_2O_2) samples when compared normal glucose hydrogen peroxide (NG + H_2O_2) samples. **B)** Representation of the fold change of the *MT-ND1* genetic region within the normal glucose hydrogen peroxide (NG + H_2O_2) samples when compared against normal glucose (NG) sample. **C)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose hydrogen peroxide (HG + H_2O_2) samples when compared against high glucose water control (HG) samples. 81

Figure 29: A) Representation of the fold change of the *MT-ND1* genetic region within the high glucose DMSO control (HG + DMSO) samples when compared against normal glucose DMSO control (NG + DMSO) samples. **B)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose 1-nitropyrene (HG + 1-NP) samples when compared against normal glucose 1-

nitropyrene (NG + 1-NP) samples **C)** Representation of the fold change of the *MT-ND1* genetic region within the normal glucose 1-nitropyrene (NG + 1-NP) samples when compared against normal glucose DMSO control samples. **D)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose 1-nitropyrene (HG + 1-NP) samples when compared high glucose DMSO control (HG + DMSO) samples 83

Figure 30: Average fluorescence intensity for normal glucose and high glucose water controls (NG and HG respectively) and the normal glucose and high glucose DMSO control (NG + DMSO and HG + DMSO). 84

Figure 31: Fluorescence microscope image taken from experiments carried out on A) normal glucose water control (NG). B) normal glucose + DMSO (NG + DMSO) control. C) high glucose water control (HG) treatment. D) high glucose + DMSO (HG + DMSO) treatment. 85

Figure 32: The intensity changes seen within the data obtained for normal glucose control in comparison to the high glucose controls (NG and HG respectively) B) The intensity changes seen within the data obtained for normal glucose and DMSO control in comparison to the high glucose and DMSO control (NG + DMSO and HG + DMSO) 86

Figure 33: Average fluorescence intensity for normal glucose and high glucose water controls (NG and HG respectively) and the normal glucose and high glucose treated with acetaldehyde (NG + Ace and HG + Ace respectively). 87

Figure 34: Fluorescence microscope image taken from experiments carried out on A) normal glucose and water control (NG). B) normal glucose treated with acetaldehyde (NG + Ace). C) high glucose and water (HG). D) high glucose + acetaldehyde (HG + Ace). 88

Figure 35: The intensity changes seen within the data obtained for normal glucose treated with acetaldehyde (NG + Ace) in comparison to high glucose and acetaldehyde (HG + Ace) 89

Figure 36: Average fluorescence intensity for normal glucose and high glucose DMSO controls (NG + DMSO and HG + DMSO respectively) and the normal glucose and high glucose treated with diethyl (hexyl) phthalate (NG + DEHP and HG + DEHP). 90

Figure 37: Fluorescence microscope image taken from experiments carried out on A) normal glucose and DMSO control (NG + DMSO) within TK6 cells. B) normal glucose + diethyl hexyl phthalate (NG + DEHP) C) high glucose and DMSO control (HG + DMSO). D) high glucose + diethyl hexyl phthalate (HG + DEHP). 91

Figure 38: The intensity changes seen within the data obtained for normal glucose treated with diethyl hexyl phthalate (NG + DEHP) in comparison to high glucose and diethyl hexyl phthalate (HG + DEHP) 92

Figure 39: Average fluorescence intensity for normal glucose and high glucose water controls (NG and HG respectively) and the normal glucose and high glucose treated with hydrogen peroxide (NG + H₂O₂ and HG + H₂O₂ respectively). 93

Figure 40: Fluorescence microscope image taken from experiments carried out on A) normal glucose and water control (NG). B) normal glucose with treated with hydrogen peroxide (NG + H₂O₂). C) high glucose and water (HG). D) high glucose with treated with hydrogen peroxide (HG + H₂O₂). 94

Figure 41: The intensity changes seen within the data obtained or normal glucose treated with hydrogen peroxide (NG + H ₂ O ₂) in comparison to the high glucose and hydrogen peroxide (HG + H ₂ O ₂)	95
Figure 42: Average fluorescence intensity for normal glucose and high glucose DMSO controls (NG + DMSO and HG + DMSO respectively) and the normal glucose and high glucose treated with 1-nitropyrene (NG + NG + 1-NP and HG + 1-NP respectively).	96
Figure 43: Fluorescence microscope image taken from experiments carried out on A) normal glucose and DMSO control (NG + DMSO). B) normal glucose with treated with hydrogen 1-nitropyrene (NG + 1-NP). C) high glucose and DMSO (HG + DMSO). D) high glucose with treated with 1-nitropyrene (HG + 1-NP).	97
Figure 44: A)) The intensity changes for MCL-5 cells grown under normal glucose and DMSO control in comparison those under high glucose and DMSO control (NG = DMSO and HG + DMSO respectively) B) The intensity changes seen within the data obtained for MCL-5 ceils grown under normal glucose and treated with 1-nitropyrene (NG + 1-NP) in comparison to ceils grown under high glucose and treated with 1-nitropyrene (HG + 1-NP)	98
Figure 45: A) The intensity changes seen within the data obtained for MCL-5 ceils grown under normal glucose and treated with 1-nitropyrene (NG + 1-NP) in comparison to the normal control (NG + DMSO). B) The intensity changes seen within the data obtained for MCL-5 ceils grown under high glucose and treated with 1-nitropyrene (HG + 1-NP) (n=3) in comparison to the high glucose and DMSO controls (HG + DMSO).	99
Figure 46: Summary of all TK6 control samples seahorse data	100
Figure 47: Summary of all acetaldehyde samples seahorse data	102
Figure 48: Summary of all DEHP samples seahorse data	104
Figure 49: Summary of all hydrogen peroxide samples seahorse data	106
Figure 50: Summary of all MCL-5 samples seahorse data	108
Figure 51: Energy Map produced using the average Oxygen Consumption Rate (OCR) values and the average Extracellular Acidification Rate (ECAR) values for the same time points as the OCR values for TK6 cells.	110
Figure 52: Energy Map produced using the average Oxygen Consumption Rate (OCR) values and the average Extracellular Acidification Rate (ECAR) values for the same time points as the OCR values for MCL-5 cells.	112
Figure 53: The effects of high glucose in the presence of Acetaldehyde, diethyl hexyl phthalate and Hydrogen Peroxide	121

VI. Abbreviations

NG- Normal glucose (10mM)

HG- High glucose (29mM)

DMSO- Dimethyl sulfoxide

NG + DMSO- normal glucose treated with Dimethyl sulfoxide

HG + DMSO- high glucose treated with Dimethyl sulfoxide

ROS- reactive oxygen species

OXPHOS- oxidative phosphorylation

Ace- Acetaldehyde

DEHP- diethyl hexyl phthalate

H₂O₂ – hydrogen peroxide

1-NP- 1-nitropyrene

MCECs- Mouse coronary endothelial cells

RBC- Erythrocytes

PCR- Polymerase chain reaction

qt-PCR- Real-time polymerase chain reaction

ATP- adenosine triphosphate

Cyt c- cytochrome c

DNA- deoxyribose nucleic acid

RNA- ribose nucleic acid

mtDNA- mitochondrial DNA

nDNA- nuclear DNA

NADH- Nicotinamide adenine dinucleotide

FADH₂- Flavin adenine dinucleotide

RR- risk ratio

BMI- Body Mass Index

HR- hazard ratio

MIM- mitochondrial inner membrane

MOM- Mitochondrial Outer Membrane

IMS- Intermembrane Space

Drp-1- Dynamin-Related Protein 1

MFN1/2- Mitofusins 1/2

OPA1- Optic Atrophy Protein 1

PRC- PGC-1 related coactivator

ER α - estrogen receptor- α

c- *Myc*- cellular-*Myc*

D-loop displacement-loop

MOM- mitochondrial outer membrane

GTP- guanosine triphosphate

8-OHdG- 8-hydroxydeoxyguanosine

AGEs- advanced glycation end products

PGC-1- peroxisome proliferator-activated receptor gamma coactivators

MEHP- Mono-2-ethylhexyl phthalate

MAPK1/2- Mitogen-activated protein kinase 1/2

ERK1/2- Extracellular signal-regulated protein kinases 1/2

PI3K- Phosphoinositide-3-kinase

Akt- Protein kinase B

ECAR- Extra Cellular Acidification Rate

OCR- Oxygen Consumption Rate

CREB- cAMP-response element binding protein

NRF-1- Nuclear Respiratory Factor 1

mtROS- Mitochondrial ROS

SSB- Single strand breaks

DSB- Double strand breaks

VAF- Variant Allele Frequency

MPTP- 1-methyl-4phenyl-1, 2, 3, 6-tetra-

mTOR- Mammalian target of rapamycin

CL- Cardiolipin

PE- Phosphatidylethanolamine

PA- Phosphatidic acid

DAG- Diacylglycerol

PKD- Protein Kinase D

Chapter 1- Introduction

1.1 Diabetes Mellitus

Diabetes Mellitus is one of the most prevalent diseases globally, in the UK there are currently 3.8 million diagnosed cases (Diabetes UK, 2019), while in America, if current rates continue, it is expected that 33% of all adults will be living with diabetes by 2050 (Boyle et al., 2010). Diabetes is mainly characterised by hyperglycaemia, a fasting blood glucose level above 7.8 mmol/L compared to normal blood glucose levels which are considered 5 mmol/L (Bouma et al., 1999; Evans, 2000). Diabetes is caused by defects in insulin secretion, action or both (Kharroubi & Darwish, 2015). There are two types of diabetes with different pathological events (Fig.1), the first is Type 1 which is due to autoimmune destruction via an inflammatory response by T- & B-cells (Devendra, Liv & Eisenbarth, 2004). The second is Type 2, which is primarily a result of lifestyle factors such as increased calorie intake, physical inactivity, and sedentary lifestyle, as well as some genetic factors (Ripsin et al., 2009; Hu et al., 2001; Piko et al., 2020).

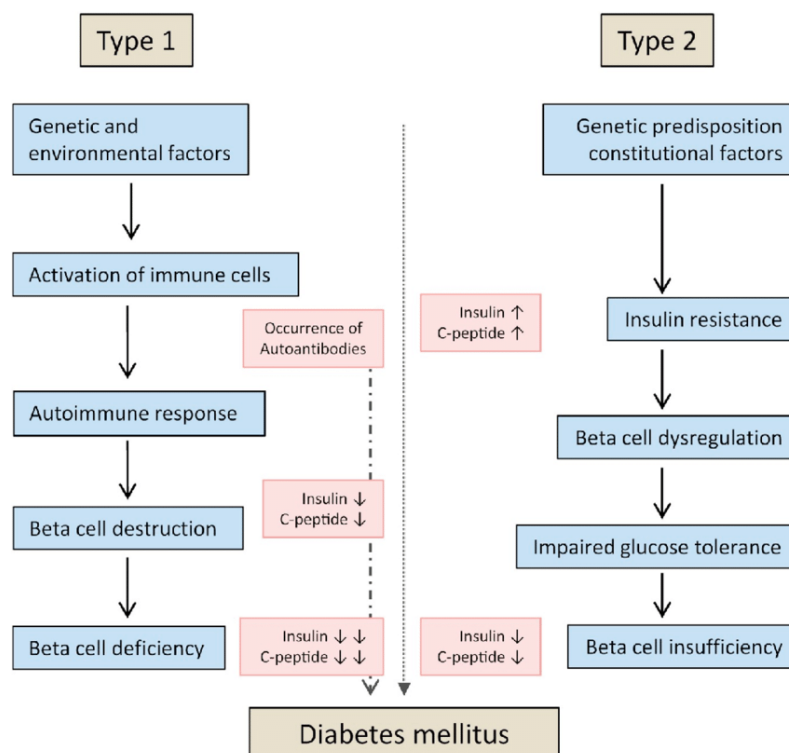


Figure 1: The pathological events which lead to both Type 1 and Type 2 diabetes (Hörber, Achenbach, Schleicher & Peter, 2020).

Type 1 development is thought to be linked to both genetic and environmental factors. Activation of immune cells is the first known event of Type 1, however why these cells become activated is unknown. This autoimmune response is thought to result in a deficiency of insulin because of the destruction of pancreatic β -cells. Type 2 also has a genetic predisposition, in this case, insulin resistance which results in dysregulation of the β -cells, which in turn, causes an impairment of glucose tolerance. Finally, this manifests as Type 2 diabetes because of eventual inadequacy of the pancreatic β -cells (Hörber, et al., 2020).

While there is a genetic predisposition in Type 1, there are several environmental factors that have been implicated in the disease which often develops suddenly with symptoms such as polyuria,

sudden weight loss and recurrent infection (Canivell & Gomis 2014; Couper & Donaghue, 2009; Zorena et al., 2022). However, the genetic predisposition of Type 2 is considered stronger than that seen in Type 1, as seen in the case of the *TCF7L2* gene which is strongly associated to Type 2 diabetes (Chandak et al., 2007). An argument proposed by Wilkin and colleagues in 2009 suggests that both forms of diabetes are the same disease that are separated by the genes associated to each. While there are mainly two types of diabetes, there are other groups to consider such as gestational diabetes, which is a form of diabetes that occurs during pregnancy, as well as rarer cases that are a result of genetic defects, disease of the pancreas or because of drugs and/or chemicals (American Diabetes Association, 2009). Diabetes has been associated with microvascular complications such as neuropathy, nephropathy, and retinopathy, it is also associated with macrovascular complications with long-term damage and eventual failure of multiple organs (Mazidi, Toth & Banach, 2017; American Diabetes Association, 2010).

1.2 Diabetes and Cancer

Diabetes is a high risk-state for the development of cancer; it is estimated that 20% of those diagnosed with diabetes will also develop some form of cancer (Habib & Rojna, 2013; Macmillan, 2017). There is data that suggests that cancer patients with pre-existing diabetes have a higher mortality rate compared to those who do not, however it is difficult to determine whether this is a result of the reduced survival rates within both diseases or whether there is an interaction between the two co-morbid disorders result in a worse prognosis for patients (Carstensen, Jørgensen & Friis, 2014). Within Fig. 2, both scenarios are illustrated alongside common risk factors for both cancer and diabetes such as ageing and weight; they also share many other risk factors such as smoking, while diabetes itself is considered a risk factor for the development of cancer (Garg et al., 2014).

While diabetes increases the risk of all cancer sites, there are several site-specific cancers that patients with diabetes have a higher risk of developing compared to other types in those same patients. These site-specific cancers include liver, pancreatic and endometrial cancers, with risks increased by up to 50%, while the risk of breast and bladder is believed to increase by 20-50% (DeNoon, 2010). A study investigating the most common malignancies associated to diabetes has found that in women, the most common is breast at 20.7%, while in men the most common is colorectal cancer at 16.3% (Dąbrowski et al., 2016). A contradiction to this has been found where those with well managed Type 2 diabetes have a reduced risk of developing prostate and lung cancer (Feng et al., 2020). However, there are discrepancies between these conclusions. While for decades the link between diabetes and an increase in overall cancer incidence has been agreed upon, no consistent increases of site-specific cancers, possibly due to different site-specific prevalence within different populations (Habib & Rojna, 2013).

Despite this, it is agreed upon that there is a link between diabetes and cancer. This link can also be seen in those with 'prediabetes', a condition where a patient has an impaired fasting glucose or impaired glucose tolerance, therefore increasing their risk of developing Type 2 diabetes (Kharroubi & Darwish, 2015). Currently, cancer is considered a genetic disease which involves somatic mutations occurring in multiple genes including oncogenes and tumour suppressor genes (Hanahan & Weinberg, 2011). These mutations are thought to occur randomly in healthy cells due to both exogenous and endogenous sources, causing genomic instability, a characteristic in most tumour cells (Tomasetti & Vogelstein, 2015). It is thought the respiratory insufficiency within the mitochondria such as aerobic glycolysis observed within cancer cells is the potential origin of the disease and the somatic mutations that are linked are a direct or indirect consequence (Warburg,

1956; Seyfried, 2012; Seyfried, 2015). Therefore, it could be said that both cancer and diabetes are both metabolic diseases (Tudzarovaa & Osman, 2015).

It has also been found that the hyperglycaemia commonly associated with diabetes or prediabetes can increase expression of certain genes such as *PIN 1* and *BRD4* that have roles in cell proliferation and migration (Stattin et al., 2007; Ramteke, et al, 2019; Yu et al., 2022a). A Korean study investigating the relationship between fasting glucose, diabetes and risk of all and specific cancers, found that elevated fasting glucose levels and diabetes are independent risk factors for several cancer types, with the risk increasing with higher levels of fasting glucose (Jee et al., 2005). In this study, there was approximately 1,298,385 Koreans (829,770 men and 468,615 women) aged between 30 and 95 years with an average Body Mass Index (BMI) of 23.2kg/m². Of this cohort, 5.1% of men and 4.5% of women had a diabetes diagnosis while the average fasting glucose levels (mmol/L) were 5.16 and 5.03. respectively. During the 10 years of follow-up, there were 20,566 cancer deaths in men and 5,907 in women. The highest fasting glucose levels (above 7.8 mmol/L, which is considered hyperglycaemic) were associated with higher death rates from all cancers combined (hazard ratio [HR]: 1.29 in men and 1.23 in women) compared to lower levels of glucose. Of the sites investigated, in both men and women, pancreatic cancer had the highest association with death rates due to higher fasting glucose, with a HR of 1.91 and 2.05 in men and women respectively. Other cancer sites associated with higher glucose levels were oesophagus, liver, and colon in men while in women, they were liver and cervix (Jee et al., 2005)

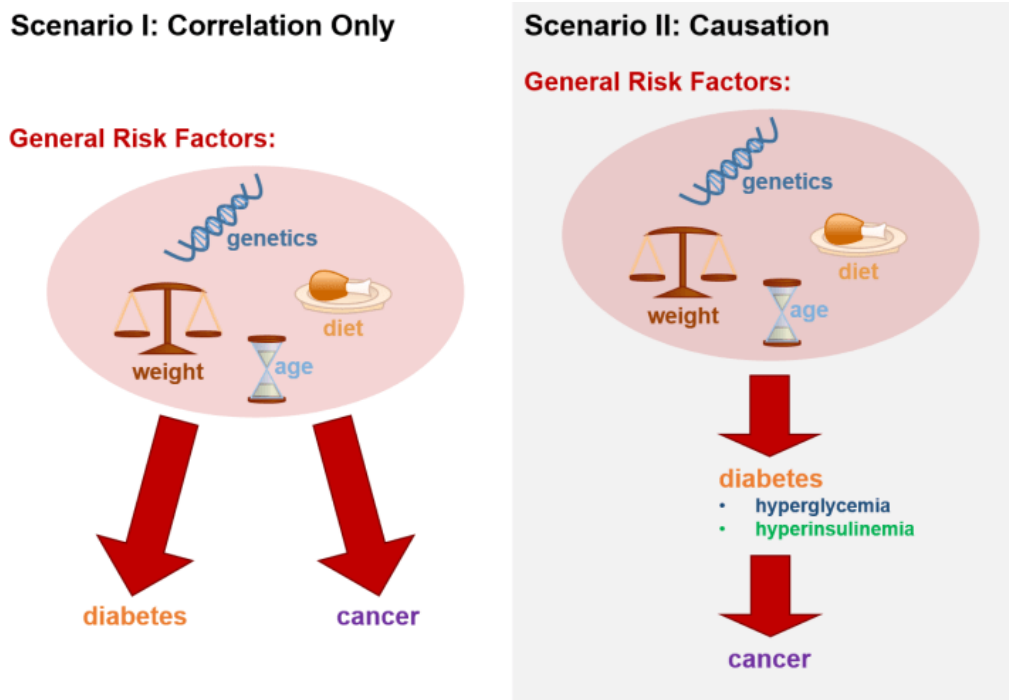


Figure 2: The potential relationship between diabetes and cancer. The left-hand side suggests that the common risk factors seen in both diseases are the reason for increased occurrence of cancer as they are also susceptible to develop diabetes. The right-hand side suggests that conditions such as hyperglycaemia, which are characteristic of diabetes, are responsible for the increased risk of cancer occurrence (Wierbowski, 2017).

While the first study was done in a predominantly leaner population, another study conducted in 6 European cohorts from Norway, Austria, and Sweden, found abnormal glucose metabolism was associated with an increased risk of overall cancer development, as well as at several specific sites (Stocks et al., 2009). In the study, 8% of men and 6% of women had impaired glucose levels, and 4% of men and 3% of women had glucose levels indicative of diabetes. It was observed that an increase

of fasting glucose levels by 1mmol/L led to an increased risk ratio (RR) of 1.05 in men and 1.11 in women for incidental cancer, while for all-site cancer the RR increased to 1.37 and 1.42 respectively (Stocks et al., 2009). This could be due to high blood glucose levels increasing the oxidative stress, ultimately causing DNA damage, another risk factor for cancer (Tatsch et al., 2015). Some of these common factors result in altered metabolic states at the mitochondria within those with obesity and diabetes which can appear to promote cancer development (Shikata et al., 2013; Giovannucci et al., 2010). While these studies gave us an insight, the biological link between these two diseases is not fully understood (Giovannucci et al., 2010).

1.3 Mitochondria

Mitochondria are eukaryotic bacterium-sized organelles that contain their own genome which is inherited exclusively through the maternal source and are present in every cell at varying numbers depending on the bioenergetic demand of the tissue (Schnon, DiMauro & Hirano, 2012; Taanman, 1998; García-Rodríguez, 2007). The mitochondrial genome (mtDNA), as shown in Fig.3, is a circular structure 16,569-base pairs in size and contains 37 genes, consisting of 2 ribosomal RNAs and 22 transfer RNAs, while the remaining 13 encode for proteins components of the electron transport chain (Anderson et al., 1981; Yoon et al., 2010). Unlike nuclear DNA (nDNA), mitochondrial DNA (mtDNA) has a lack of protection of histones compared to nDNA which limits the proofreading capacity of the of DNA polymerase and therefore makes it more susceptible to damage (Alexeyev, et al, 2013). Oxidative phosphorylation is one of the main processes occurring in the mitochondria, as its primary role is to supply the energy required by the body through the metabolism of organic substrates such as glucose to produce adenosine triphosphate (ATP) (Trotta & Chipuk, 2017; McInnes, 2013).

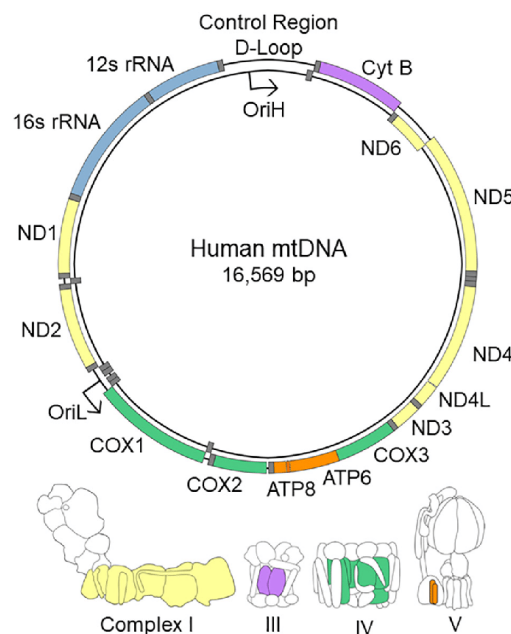


Figure 3: The circular structure of the human mitochondrial genome. The genes are colour coded with the complex of the electron transport chain they are associated with (Chocron et al., 2019).

Oxidative phosphorylation, also known as the electron transport chain, is a process where, the synthesis of ATP is coupled with the flow of electrons from Nicotinamide adenine dinucleotide (NADH) or Flavin adenine dinucleotide (FADH₂) to O₂ using a proton gradient across the inner

mitochondrial membrane (MIM) (Berg et al., 2002). The MIM is heavily folded to produce cristae to increase the surface area available for oxidative phosphorylation to occur (Paumard et al., 2002). It contains multiple copies of the chain complexes involved in this process, from complex I through IV, together with complex V, an ATP-synthesizing complex channel called ATP synthase. All five complexes form the chain which results in ATP production (Chaban et al., 2014).

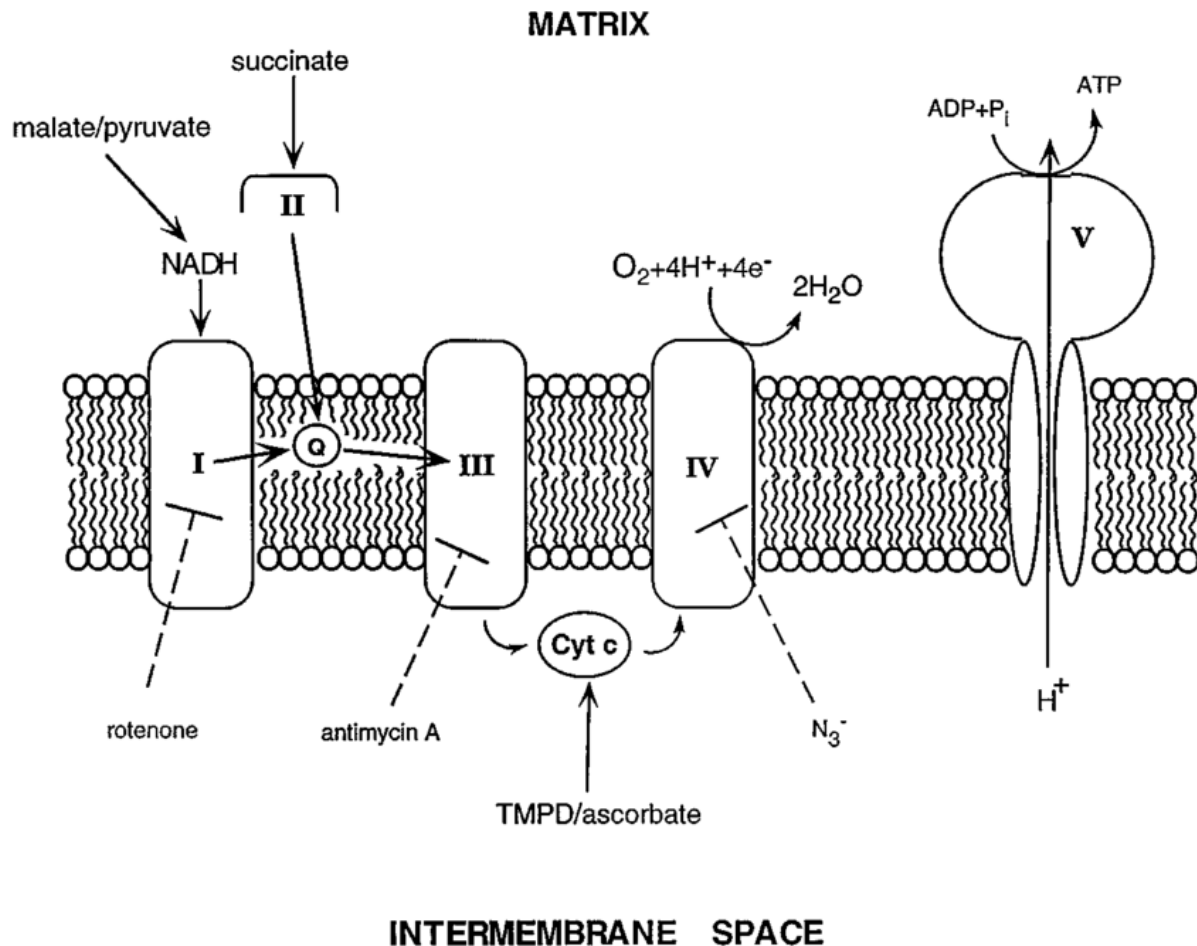


Figure 4: The electron transport chain is located within the mitochondria (Krippner et al., 1996)

As shown in Fig. 4, electrons are accepted by Complex I and II from NADH and FADH₂ respectively. However, electrons are accepted by complex I in an energy-dependent reaction to allow the complex to pump protons into the mitochondrial matrix to produce a proton gradient as well as oxidising the NADH to form NAD⁺ (Sharma et al., 2016). Unlike the complex I, complex II is unable to pump protons into the intermembrane space (Chaban et al., 2014).

These electrons gained by complexes I and II are then passed to complex III through a ubiquinone intermediate and are used to reduce the cytochrome c present in the complex (Krippner et al., 1996). The reduced cytochrome c carries over the electrons to Complex IV where they are used to completely reduce an oxygen molecule which is then able to form water as it needs to absorb a H⁺ present in the mitochondrial membrane as part of a redox reaction (Kadenbach, 2021).

Along with complex I, complexes III and IV work together to form an electrochemical gradient across the membrane through the pumping of protons into the mitochondrial matrix (Shultz & Chan, 2001). This allows complex V to produce ATP as when the proton's influx back into the matrix through the

ATP synthesising channel, they provide the force required for the rotation of ATP synthase for ATP synthesis (Nakamoto et al., 2008).

1.3.1 Mitochondrial Biogenesis

Mitochondria are constantly going through biogenesis, a process where they undergo fission and fusion, which control and regulate the morphology, and therefore function of the mitochondria, and are responsive to several stressors such as cigarette smoke and ethanol (Fig.5) (Fu et al., 2019; Chen et al., 2005; van Jaarsveld et al., 1992; Cederbaum et al., 2001). However, excess stress upon the mitochondria has been linked to many chronic diseases such as Parkinson's disease, as well as cancer and diabetes (Okarmus et al., 2020). During the process known as fission, the mitochondrion increases its volume through elongation before it separates into two smaller mitochondria. Fusion could be considered the reverse of fission, in this process, two mitochondria fuse together, combining their membranes and compartments to produce a single, larger mitochondrion (McInnes, 2013). These two processes are regulated by multiple cellular pathways, which include ubiquitylation, phosphorylation and dephosphorylation (Westermann, 2010).

Mitochondrial biogenesis is also regulated by several mitochondrial membrane lipids including cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidic acid (PA), and diacylglycerol (DAG) (Osman et al., 2011). CL is a negatively charged lipid found uniquely in the mitochondrial membrane and is primarily found within the inner and outer membrane (Ardail et al., 1990; Schlattner et al., 2014). CL is crucial for fusion via its interactions with the dynamin-related protein Optic Atrophy Protein 1 (Opa1), a GTPase which moderates inner membrane fusion (DeVay et al., 2009; Ban et al., 2010; Mishra et al., 2014b). CL can also play a role in mitochondrial fission when it is transferred from the inner to outer membrane (Montessuit et al., 2010; Macdonald et al., 2014; Schlattner et al., 2013). It plays a similar role as in fusion but targets Dynamin-Related Protein 1 (Drp1) in this case, where it can mediate its recruitment to the outer membrane and activate its GTPase activity (Montessuit et al., 2010; Macdonald et al., 2014; Ugarte-Urbe et al., 2014). PE is another lipid found in the inner and outer mitochondrial membrane and, like CL, facilitates mitochondrial fusion through similar mechanisms (Sperka-Gottlieb et al., 1998; Tasseva et al., 2013; Chan et al., 2012; Xu et al., 2005).

While CL has role within the regulation of mitochondrial dynamics, it also acts as a substrate for another lipid, PA. PA is thought to promotes mitochondrial fusion through its functional interactions with Mfn1/2; it has suggested this interaction to promote fusion is through providing support and pulling the outer membrane closer than Mfn is able to on its own (Choi et al., 2006; Chen et al., 2003). PA also is thought to play a role in the promotion of mitochondrial and membrane fission through recruitment of proteins that carry out membrane cleavage or through synthesis of lipids like DAG from PA which stimulate fission through reduction of fusion (Huang et al., 2011; Yang et al., 2008). DAG is thought to facilitate mitochondrial fission through the Golgi complex via the recruitment of Protein Kinase D (PKD), which is activated by phosphorylation and following a chain reaction, results in the generation of more DAG where then drives mitochondrial fission through creating membrane curvature (Bossard et al., 2007).

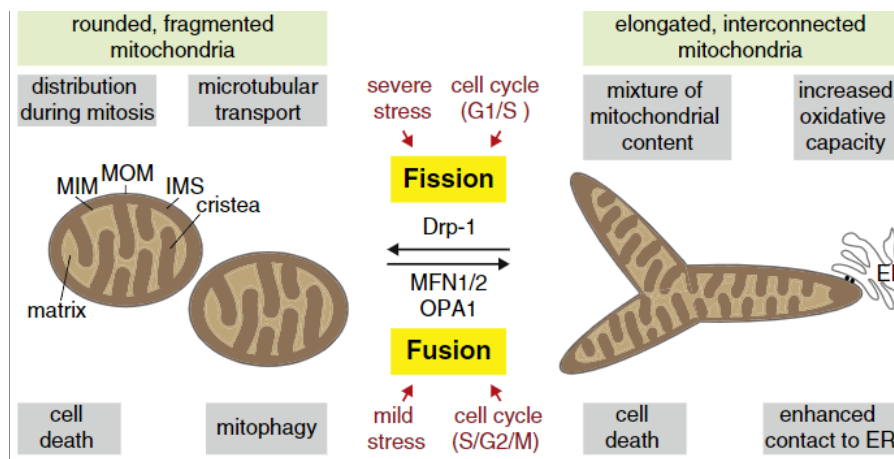


Figure 5: The morphological effects of fusion and fission on the mitochondria, which processes the mitochondria is most likely experience based on which process is occurring and possible external factors including mild stresses (e.g. nutrient starvation) and severe stress (e.g. apoptotic insults and hypoxia) which can influence the mitochondria to shift towards a certain process (Senft & Ronai, 2016). Abbreviations: Mitochondrial inner membrane (MIM); Mitochondrial Outer Membrane (MOM); Intermembrane Space (IMS); Dynamin-Related Protein 1 (Drp-1); Mitofusins 1/2 (MFN1/2); Optic Atrophy Protein 1 (OPA1).

During biogenesis, there are a family of peroxisome proliferator-activated receptor gamma coactivators (PGC-1 α , PGC-1 β) and PGC-1 related coactivator (PRC) which also play an essential role in this and processes including adaptive thermogenesis and fatty acid β -oxidation (Scarpulla, 2011; Finck and Kelly, 2006). Expression of each is usually dependent on function and tissue-specificity; PRC expression occurs through ubiquitination whereas PGC-1 α and PGC-1 β are preferably expressed within tissues such as skeletal muscle, where there is a high oxidative capacity, to regulate function and energy metabolism within the mitochondria (Puigserver et al., 1998; Andersson and Scarpulla, 2001; Lin et al., 2002; Kamei et al., 2003).

PGC-1 α is a coactivator that activates nuclear transcription factors involved in the regulation of oxidative phosphorylation and mitochondrial function; it is also responsible for the stimulation of mitochondrial biogenesis and metabolism of different substrates including glucose and fatty acids (Doughan & Dikalov, 2007; Wu et al., 1999; Liang & Ward, 2006). PGC-1 α does not bind directly to DNA, but instead works through the induction of the transcription of nuclear respiratory factor (NRF) 1 and NRF2, which in turn causes an increase in the expression of mitochondrial transcription factor A (mtTFA) and other subunits of the mitochondria such as complexes of the electron transport chain (Wu et al., 2016; Wu et al., 1999, Scarpulla, 2002a; Scarpulla, 2002b). By regulating these transcription factors, PGC-1 α can act as a molecular switch for multiple cellular processes, determining the ability of mtTFA to stimulate mitochondrial DNA replication and gene expression (Larsson et al., 1998; Garesse & Vallejo, 2001; Corona & Duchon 2015).

PGC-1 β , also plays a role in the regulation of oxidative phosphorylation while also being established as a regulator of β -oxidation of fatty acids (Shao et al., 2010). PGC-1 β , appears to interact with NRF1 identically to PGC-1 α , while also preferentially promoting the ligand-dependent activity of estrogen receptor- α (ER α) (Kressler et al., 2002; Lin et al., 2002). ER α is known to function as nuclear receptors and transcription factors within the nucleus, however it has also been shown to interact with the protein trifunctional protein β -subunit (HADHB) in the mitochondria and may play an important role estrogen-mediated lipid metabolism (Yasar et al., 2017; Zhou et al., 2012). While both PGC-1 α and PGC-1 β have similarities in their functions, PGC-1 β appears to promote a higher level of

coupled respiration compared to PGC-1 α because of differences in proton leaking, this could suggest there are differences in the metabolic efficiency between the two (St Pierre et al., 2003).

PRC is a growth-regulated transcriptional co-factor which, like PGC-1 α and PGC-1 β , interacts directly with transcription factors such as NRF-1, CREB and ERR α while also trans-activating multiple nuclear genes such as cytochrome c and mitochondrial transcription factor B isoforms TFB1M and TFB2M, which are required for the respiratory function of the mitochondria (Gleyzer & Scarpulla, 2011; Vercauteren et al., 2006; Vercauteren et al., 2008; Andersson & Scarpulla, 2001; Gleyzer et al., 2005). During the activation of cytochrome c, PRC can utilise CREB and NRF-1 while activation of all essential genes, the NRF-1 recognition site is required for PRC to reach maximal activation (Andersson & Scarpulla, 2001; Gleyzer et al., 2005). Unlike PGC-1 α and PGC-1 β , PRC differs in that its expression pattern is characteristic to regulators of the growth program within a cell such that its mRNA and protein levels within proliferating cells are higher compared to those in growth-arrested cells (Anderson & Scarpulla, 2001; Vercauteren, 2006). One potential role it may play in regulation of biogenesis is that it has been found to be a direct target for cellular-*Myc* (c-*Myc*), which plays a role in several functions of the cell including cell growth and metabolism (Li et al., 2005). In this study it was found that PRC levels correlated with those of c-*Myc* in *Myc*-expressing human B cells; high expression of c-*Myc* was also found to increase mitochondrial mass and oxygen consumption rate irrelevant of proliferation rates.

It has been suggested that maintaining function and repair of mitochondria is dependent on the remodelling of mitochondria (Wikstrom et al., 2009). The structure and genome of the mitochondria allow them to have a complex role in cell fate and energy metabolism (Wallace, 2012; van Vliet et al., 2014). Previous studies have found that mitochondrial dynamics can be linked to aspects of cancer cell characteristics including tumour growth and invasion and metastasis (Desai et al., 2013; Zhao et al., 2013). It is thought that it is through the upregulation of mitochondrial fission and increased Drp1 expression that the mitochondria play a role in cancer metastasis (Denisenko et al., 2019). Studies such as those in breast cancer and hepatocellular carcinoma (HCC) metastatic cells have found there to be an over-expression of DRP1 within tumour cells compared to normal tissue (Zhao et al., 2013; Hernández-Alvarez & Zorzano, 2021). Furthermore, the study using breast cancer metastatic cells also demonstrated that silencing of DRP1 or expression of Mfn1 (which is associated to mitochondrial fusion) was found to increase elongation of the mitochondria and significantly suppress metastatic properties of breast cancer cells (Zhao et al., 2013).

This suggests that mitochondrial fission is required for cancer cell migration and to support metastatic potential of cancer cells (Denisenko et al., 2019). However, the mechanism by which DRP1 regulation is involved in the process of cancer metastasis remains not fully understood. Studies have suggested fission is required for efficient redistribution of mitochondria, and that DRP1 upregulation/activation is associated with the migration of cancer cells (Zhao et al., 2013; Senft and Ronai, 2016).

1.3.2 The Mitochondrial Genome

During the evolution of the symbiotic relationship between bacterium, which had unique energy-producing capabilities, and a pro-eukaryotic cell, it is thought around 1500 genes were transferred from the bacterium. This transfer of genes resulted in the creation of mtDNA which retained 37 genes essential for producing energy (Wallace, 2005a; Holt et al., 2007). Each mitochondrion contains between 2 and 10 supercoiled circular copies of mtDNA found in the inner membrane (Rolo

& Palmeira, 2006). Within the mitochondrial genome, there are none or only a small number of non-coding bases between the genes, and in several cases, the termination codons are not coded within the DNA but post-transcriptionally produced through the polyadenylation of the mRNAs (Anderson et al., 1981). While the mitochondria's main role within the cell is the production of ATP, the mitochondrial genome is also responsible for controlling several mitochondrial functions also involved in energy production. These functions include oxidative phosphorylation, the production of ROS and the activation of the mitochondrial permeability transmission pores for the regulation of apoptosis (Wallace, 2005b).

Mutations within mtDNA accumulate within the mitochondria both due age and disease in both mitotic and post-mitotic tissue, with more than 250 mtDNA pathogenic mutations being identified so far (Tuppen et al., 2010; Lawless et al., 2020). Since the mitochondria is the main source of ROS within the cell, it is thought that mtDNA is under roughly 100 times more mutagenic stress than that experienced by nDNA; this is due to the lack of protection present in the form of histones as well as a deficient mismatch repair system (Schapira, 2006; Chistiakov et al., 2012). Since it is outside the nucleus and inherited maternally only, the mtDNA can also experience change through the accumulation of mutations along the maternal lineages (Schapira, 2006). The main region within the mtDNA to experience this damage is the displacement-loop (D-loop), the region with transcription/replication elements (Mishra & Kowluru, 2014a). This increased mutation rate within mtDNA results in heteroplasmy (co-existence of wild-type and mutant mtDNA within the same mitochondrion) which can reduce mtDNA copy number and protein production required for assembly of the respiratory chain when heteroplasmy exceeds 60%; this further results in mitochondrial dysfunction and mitophagy disruption (Chistiakov et al., 2012; Elorza et al., 2021)

As well as mutations within the mtDNA, the susceptibility of mtDNA to damage due to increased ROS exposure can also result in alterations to the copy number (the measure of the quantity of mitochondrial genomes within a cell) (Lee et al., 2000; Longchamps et al., 2020). One study looking at melanoma found that within the population that fell into the highest mtDNA content quartile were at a higher risk of developing the disease (Hu et al., 2016). However, it's not only as a result or cause of pathological conditions that mtDNA copy number changes are seen between tissues, but it can also change as a response in tissues to energetic and metabolic demands (D'Erchia et al., 2015). For example studies have found in oocytes the copy number is $\sim 4-6 \times 10^3$ in heart samples to $0.5-2 \times 10^3$ in kidney, liver, and lung samples (Chen et al., 1995; D'Erchia et al., 2015)

1.3.3 Mitochondrial fusion

In mammalian cells, fusion at the mitochondrial outer membrane (MOM) is mediated by mitofusin proteins MFN1 and MFN2, which are members of the membrane-anchored dynamin family that contain a conserved catalytic GTP-binding domain (Youle & Blik, 2012; Wai & Langer, 2016). MFNs can be found on the MOM and at other locations such as the endoplasmic reticulum membrane (Ngoh, et al., 2012). Fusion at the MIM is mediated by OPA1, a final dynamin-like GTPase structure found in the intermembrane space or anchored to the MIM by an N-terminal transmembrane domain (Delettre et al., 2000; Olichon et al., 2002). MFN 1 & 2 and OPA1, using energy from guanosine triphosphate (GTP) hydrolysis of the GTPase domains present on each, form an antiparallel intermolecular coil and induce a conformational change that generates the force required for fusion of two mitochondria (Ishihara et al., 2004; Koshiba, 2004). Whilst fusion occurs, the electrochemical membrane potential at the inner membrane is maintained (Meeusen et al., 2004). The fusion process results in a formation and elongation of mitochondrial tubules present in

the cytoplasm, causing an increase in the mitochondrial mass which supports some of the mitochondrion's roles such as oxidative phosphorylation (Yao et al., 2019).

Fusion is also able to mitigate the effects of stress on the mitochondria through mixing of the partially damaged mitochondrial contents as a form of complementation (Youle & van der Bliek, 2012). It has been shown that in the absence of fusion, there are pathological abnormalities similar to that seen in mitochondrial myopathies, especially those that have a role in mtDNA depletion conditions (Copeland, 2008). Mitochondria that contain mutated mtDNA are still able to fuse to other mitochondria within the same cell, as the DNA content within the healthy mitochondria are able to compensate for defects within the mutated mtDNA, if the mutation load is below 80-90% (Yoneda & Attardi, 1994; Nakada, 2001). It has been suggested that protection against mutations within the mtDNA may be a result of mitochondrial fusion and the content mixing ability, thus also reducing the effects of environmental damage (Nakada et al., 2001; Nakada et al., 2009). Fusion is therefore able to increase oxidative capacity in response to toxic stress below a critical threshold, even if there is no physical interaction of the genomes within the mitochondrion (Gilkerson et al., 2008).

It has been found that a reduction or complete loss of mitochondrial fusion can result in mtDNA point mutations and deletions. Mutations within the mitochondrial gene *Polg*^{D257A} in mice have shown a 2–3 order-of-magnitude increase in mtDNA point mutations. Another study looked at *Mfn1* mutant mice, where it was discovered that the mtDNA deletions found may be a cause for the premature aging seen within the mutator mice (Vermulst et al., 2007; Vermulst et al., 2008). Accumulation of mtDNA deletions has been seen in some cases where there is a dysfunction of the *OPA1* gene such as in a study by Amati-Bonneau and colleagues which looked at mutations of *OPA1* in non-syndromic optic neuropathy; it was found that those with the autosomal-dominant trait all had multiple deletions of mtDNA in their skeletal muscle (Amati-Bonneau et al., 2008). This suggests that *OPA1* may have a role in the stability of mtDNA, however, little is known of the mechanism resulting in multiple mtDNA deletions.

1.3.4 Mitochondrial fission

Fission, the splitting of one mitochondrion into two or more mitochondria, is mediated by a dynamin protein known as dynamin-related protein 1 (DRP1) (Smirnova, 2001). This protein is thought to be recruited to the mitochondrial outer membrane (MOM) by human fission protein 1 (FIS1) to form large complexes known as oligomeric rings which surrounds the MOM in a spiral structure and constrict until energy-dependent fission of the membrane occurs (Detmer and Chan, 2007; McBride and Soubannier, 2010; Scott & Youle, 2010). When fission is high, biogenesis tends to be low which helps in maintaining a lower mitochondrial mass (Fu et al., 2019). In excess, fission has been implicated in several human conditions such as neurological diseases and cancer (Serasinghe and Chipuk, 2017). An imbalance in mitochondrial biogenesis can result in mitochondrial fragmentation, which in turn can result in mitochondrial and cellular dysfunction (Wu et al., 2011).

Mutations in the *DRP1* gene block fission and cause an imbalance between fission and fusion (Otsuga et al., 1998). It has been suggested that fission is also associated to the early stages of apoptosis, as inhibiting mitochondrial fragmentation, a key aspect of the process, has been shown to reduce or prevent apoptotic factors being released (Frank et al., 2001; Lee et al., 2004). A study looking at the differences between mitochondrial fragmentation in various diseases found that in neurological diseases such as Huntington's disease, as well as cancer and diabetes, fragmentation

occurred as a result of a favouring of mitochondrial fission due to lateral interactions between mitochondria (Shah et al., 2019). Another study investigating a model of Ras-driven tumour growth found that phosphorylation of the DRP1 protein resulted in an increase of mitochondrial fragmentation, while knock down of DRP1 resulted in an inhibition of tumour growth (Kashatus et al., 2015). However, mitochondrial fragmentation has also been found to occur as a result of exposure to toxic chemicals. A study looking at the effect of hydrogen peroxide exposure found that there was a 41% increase in fragmentation within mitochondrial fibroblasts within 3 hours of exposure, suggesting that fragmentation occurs due to the oxidative stress inflicted on the mitochondria (Iqbal & Hood, 2014).

1.3.5 Mitochondria and Diabetes

It has been found that that mitochondrial biogenesis is dysregulated within human pancreatic β -cells of individuals with diabetes (Hong et al., 2020). In a study comparing diabetes and healthy mice, an increase in mitochondrial fragmentation and a significant decrease in OPA1 levels associated to mitochondria fusion were seen, whereas DRP1, which is associated with fission, was significantly increased in mice with Type 1 diabetes (Makino et al., 2010). While errors within mitochondrial biogenesis can result in disease, hyperglycaemia seen within individuals with diabetes could potentially have a genotoxic impact on the mitochondria, this could be a possible mechanism for the increased risk of cancer development, as cancer can be induced via both genotoxic and non-genotoxic mechanisms (Hananhan & Weinburg, 2011).

Hyperglycaemia was found to cause DNA strand breaks back in the 1980s, which can produce several mutations within DNA (Lorenzi, et al, 1986; Jena, 2012). A study done by Zhang and colleagues in 2007 confirmed that the DNA damage and mutations resulting from this could in fact result in mutagenesis (Zhang et al., 2007). However, hyperglycaemia has also been shown to increase 8-hydroxydeoxyguanosine (8-OHdG) levels due to an increase in the production of advanced glycation end products (AGEs) which are able to initiate DNA base oxidation as well as strand breaks (Mizutani, et al, 2000; Stopper, et al, 2003).

Metabolically, it has been found that diabetes could potentially inhibit mitochondrial metabolism. It has been found that several genes associated to both oxidative phosphorylation and ATP synthesis are downregulated in people with diabetes (Segerstolpe et al., 2016). A further study concluded this could be due to hyperglycaemia (Haythorne et al., 2019). They observed *in vivo* diabetes islet cell models showed a reduction in glycolysis, as well as a substantial reduction in mitochondrial metabolism, with similar changes also being noted in *in vitro* model INS-1 cells. They concluded that hyperglycaemia alone was responsible rather than mutations or polymorphisms, as they observed no elevation of fatty acid levels, as seen in in a study of cardiac metabolism where gene induction led to a shift towards lactate production as well as increased fatty acid metabolism (Rohm et al., 2018). Studies have also been done to investigate how PGC-1 α is affected by diabetes. One study looking at PGC-1 α polymorphisms in people with diabetes found that there was a variation which was associated with the development of insulin resistance often seen in diabetes (Hara et al., 2002), while another study identified another polymorphism which was associated with the switch from prediabetes to the development of Type 2 diabetes (Andrulionytė, et al, 2004).

1.3.6 Mitochondria and Cancer

It has been shown that there is an increase in mitochondrial fission present within cancer cells, resulting in an increase in fragmentation (Arismendi-Morillo, 2009; Inoue-Yamauchi & Oda, 2012; Rehman et al., 2012). In ovarian cancer, it was found that the morphology of the mitochondria changed as malignancy increased from filamentous network to single, enlarged organelles (Grieco et al., 2021). While in that study, there was induced fragmentation present, it was not due to an increase in the expression of fission proteins, but due to an imbalance of dynamic proteins as both fission and fusion proteins were both decreased. The role of fission in cancer progression has been further supported during a study which found that the inhibition of mitochondrial fission caused a decrease in cancer cell proliferation as well as increasing apoptosis within lung cancer models (Rehman et al., 2012) and colon cancer (Inoue-Yamauchi and Oda, 2012). As represented in Fig. 6 metabolic changes can also be observed in cancer cells. Otto Warburg first described this in the 1920s, in what is known as 'Warburg Effect' where even in the presence of oxygen, cells undergo 'aerobic glycolysis' thus increasing the glucose consumption and lactate production of the cell (Warburg, 1925).

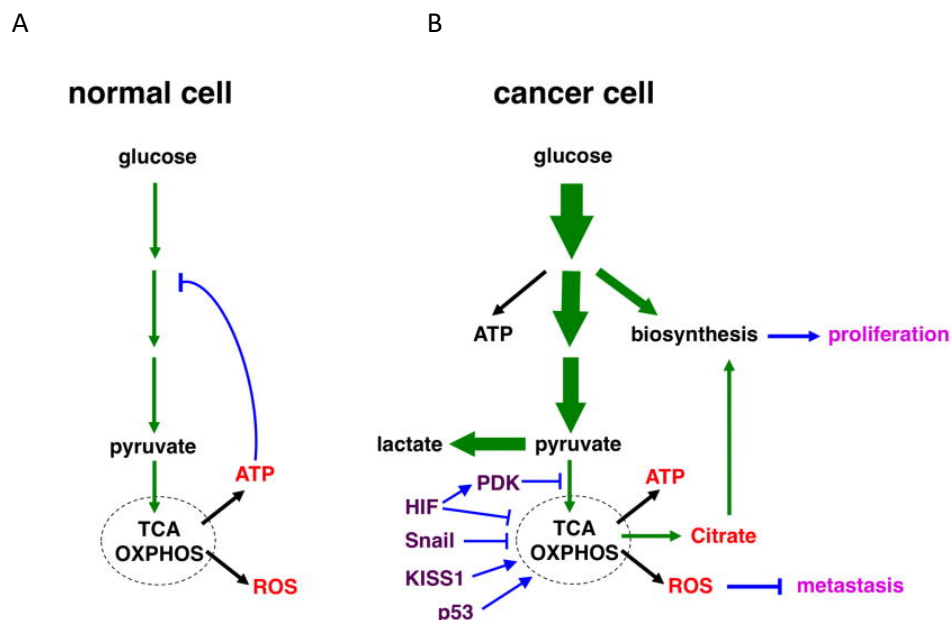


Figure 6: A) Glucose metabolism within a healthy cell, where pyruvate enters the mitochondria and undergoes oxidative phosphorylation. **B)** Glucose metabolism within a cancerous cell, where only small amount of the pyruvate enters the mitochondria but is instead converted to lactate, reducing the ATP produced thus drastically increasing in the glucose consumption (Lu, Tan & Cai, 2014).

The Warburg Effect and this dysregulated metabolism has been listed as one of the hallmarks of cancer; it is where glycolysis occurs even in the presence of oxygen (Warburg et al., 1927; Hanahan & Weinberg, 2011). Unlike in healthy cells, there is a reduction of ATP production but an increase in the consumption of glucose and preferential production of lactate. It is thought, due to cancer cells' highly proliferative nature, the reprogramming of cell metabolism to favour aerobic glycolysis supports the biosynthetic requirements of uncontrolled proliferation and growth (Liberti & Locasale, 2018). It is speculated that the glucose acts as a carbon source for anabolism which is needed to support cell proliferation (Vander Heiden et al., 2009; Levine & Puzio-Kuter, 2010; Boroughs & DeBerardinis, 2015).

However, while most cancer cells rely on glycolysis for ATP production, inhibiting this process has been shown to be insufficient for the suppression of cancer cells as they have the potential to adapt their metabolism to their environmental conditions (Zheng, 2012; Grasmann et al., 2021). One study found that multiple types of tumour cells achieved this via reprogramming energy metabolism oxidative phosphorylation in an autophagy-dependent manner (Shiratori et al., 2019).

Interestingly, it was discovered by Liu and colleagues in 2013 that pancreatic cancer patients often show hyperglycaemia and that this excess glucose increases levels of ATP present and stimulates migration of the cells (Liu et al., 2013). The function of the mitochondria remains intact, instead they reprogramme the mitochondria and alter its bioenergetic state to meet their requirements (Schiliro & Firestein, 2021). PGC-1 α also plays a role in the development of cancer. A study investigating PGC-1 α expression in breast cancer found levels altered depending on where the metastasis site was, with PGC-1 α levels being higher in sites such as lung and bone. It was also found that PGC-1 α enhanced the bioenergetic modulation of these cells which would make metabolic interventions less effective (Andrzejewski et al., 2017)

1.3.7 Metformin

According to Diabetes UK, metformin is the most common treatment for type 2 diabetes, with other uses during gestational diabetes (Diabetes UK). Metformin works by increasing insulin sensitivity through helping to restore how the body responds to insulin and decreases the quantity of blood sugar that the is produced by the liver and therefore the quantity the stomach or intestine can absorb (Kirpichnikov et al. 2002). Metformin has been found to specifically suppress hepatic gluconeogenesis without there being an increased burden on pancreatic β -cells to enhance insulin secretion or promoting adipocyte differentiation (Inzucchi et al., 1998). However, the exact mechanism by which metformin lowers blood sugar levels is not fully understood; although, it is believed that the drug acts via in various mechanisms within multiple tissues rather than in singular singular mode of action within a single organ (Foretz et al., 2019).

It has also been demonstrated that metformin acts directly on the mitochondria as a non-competitive inhibitor of Complex 1 within the electron transport chain (Andrzejewski et al., 2014; Feng et al., 2022). Other modes of action include inhibition of the mammalian target of rapamycin (mTOR) pathway; which has been to prevent protein synthesis, cell growth and ultimately the cell cycle (Saraei et al., 2019; Bridges et al., 2014). The mTORC1 pathway promotes protein synthesis by activating downstream targets within the mTORC1 pathway which directly promote the initiation and elongation phases of translation; this pathway is activated by insulin, such as shown in Fig. 8 (Graber et al., 2013; Norwitz & Querfurth, 2020). Within tumour cells, this pathway has been found to promote tumour growth (Deldicque et al., 2005; Zou et al., 2020). This pathway is inhibited by AMP-activated protein kinase (AMPK) direct and indirect mechanisms such as phosphorylation and therefore activation of transcription factors TSC1/2 as well as regulatory components of the mTORC1 pathway (Inoki et al., 2003; Gwinn et al., 2008)

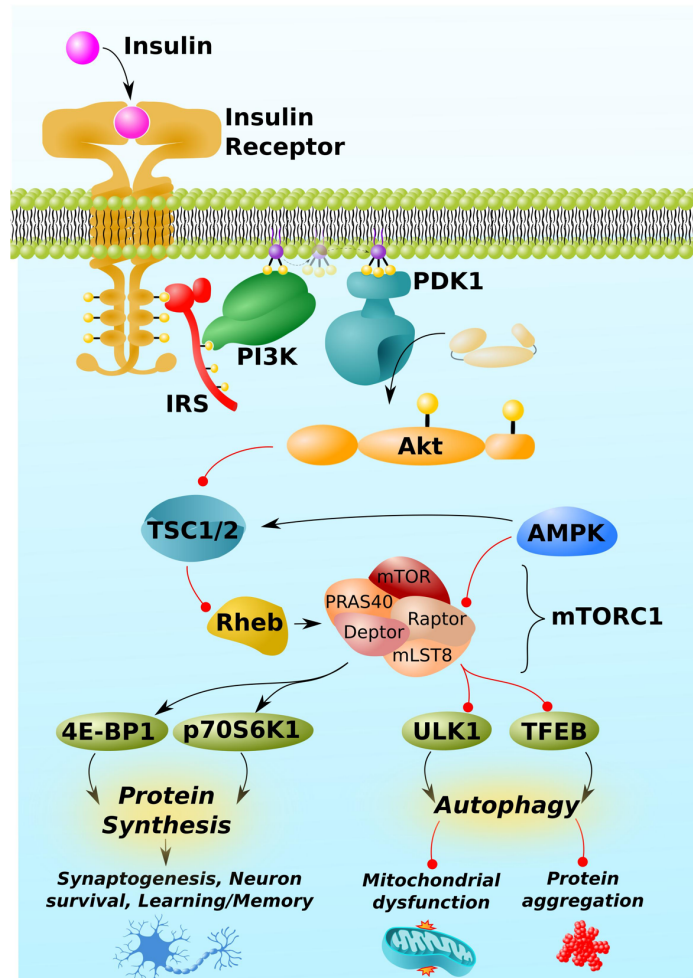


Figure 7: The (mTOR) pathway including insulin activation mechanism and both direct and indirect mechanisms of AMPK inhibition. Black and Red arrows represent positive and negative regulation. (Norwitz & Querfurth, 2020).

Due to its role within the mitochondria, metformin has been studied as a potential cancer therapeutic and has been shown to reduce proliferation of cancer cells and therefore possibility of malignancies in various types of cancer, including pancreatic, medullary thyroid and uterine cancer (Rêgo et al., 2015). It has also been demonstrated that alone or in combination with radiotherapy, metformin has reduced tumour growth in ovarian, prostate and breast cancer in animal models (Fortunato Rêgo et al., 2015; Zaharenko 2015). It is believed metformin acts by inhibiting the mTOR pathways through activation of AMPK (Sarai et al., 2019).

Furthermore, in tobacco carcinogen-induced lung tumourigenesis, it has been found that by administering metformin through injection in mice models, the mTOR pathway was inhibited through decreased activation of insulin-like growth factor-I receptor/insulin receptor (IGF-1R/IR), (Mommott et al., 2010). Within carcinogen-induced premalignant lesions, metformin again is believed to inhibit the mTOR pathway through both AMP-activated protein kinase (AMPK)-dependent and -independent mechanisms to prevent the development of Head and neck squamous cell carcinoma (HNSCC) (Vitale-Cross et al, 2012). As outlined above, the mTOR pathway is activated by insulin and within tumour cells, it promotes tumour growth (Deldicque et al., 2005; Zou et al., 2020).

1.4 Environmental toxins

Not only is hyperglycaemia able to promote cancer development, but environmental toxicants may also be a source of carcinogenicity, with The World Health Organization (WHO) estimating that 20% of cancers worldwide are attributable to environmental risks, predominantly air pollution; additionally, environmental toxins have also been found to be a contributing factor to the increased rate of Type 2 diabetes (Gatto, 2021; Lang et al., 2008). One study found that Dioxins, which are a group of endocrine disrupting chemicals that are persistent environmental pollutants, may have a relationship with increased risk of diabetes and breast cancer in both males and females (Villeneuve et al., 2010; Frentzel-Beyme, 2018; Natarajan et al., 2020; VoPham et al., 2020). Alongside environmental pollutants, other environmental toxins associated with diabetes, arsenic (which can be found in fruit juices, seafood and drinking-water) interferes with glucose sensing mechanisms and insulin secretion regulation. It is also linked to a range of cancers such as bladder and lung. (Bonini & Sargis, 2018; Khan et al., 2022). Within this project, the environmental toxins that will be tested are four xenobiotic carcinogens that have been found to promote cancer initiation, (i) acetaldehyde, (ii) hydrogen peroxide, (iii) 1-nitropyrene and (iv) Di(ethylhexyl) phthalate.

There is a growing range of literature that is suggesting that mitochondria, particularly mtDNA, are a target for a range of toxins including environmental pollutants (Meyer et al., 2013). It has been shown that within DNA, one of the types of ROS induced DNA damage caused by pollutant exposure is the oxidation of guanine to form 8-OHdG; this is also likely to occur within the mitochondria as reflected by data showing an increase in mitochondrial 8-OHdG levels (Cheng et al., 1992; Grevendonk et al., 2016). This accumulation of mitochondrial 8-OHdG and resulting mutations within mtDNA have been linked to diseases such as diabetes and cancer (Kakimoto et al., 2002; Lin et al., 2008). There are a large number of environmental chemicals such as pesticides including rotenone, 1-methyl-4-phenyl-1, 2, 3, 6-tetra-hydropyridine (MPTP) and xenobiotic chemicals such as acetaldehyde that are considered mitochondrial toxins (Harmon & Sanborn, 1982; Nicklas et al., 1987; Caito & Achner, 2015).

1.4.1 Acetaldehyde

Acetaldehyde is usually found in alcoholic beverages and tobacco smoke but can also be produced by the body during ethanol metabolism (Lachenmeier et al., 2009). Chronic alcohol consumption has been found to be a strong risk factor for the development of various cancers such as aerodigestive tract, liver, and breast (Seitz & Stickel, 2010). One of the first metabolites of alcohol oxidation is acetaldehyde, which has a carcinogenic effect using several mechanisms such as formation of DNA adducts, interference of DNA replication and induction of DNA damage (Seitz & Becker, 2007). It has also been found that acetaldehyde can bind to cellular proteins and DNA which in turn results in morphological changes as well as functional impairment of the cells (Fang & Vaca, 1995). The interaction acetaldehyde with nucleobases such as deoxyguanosine present in the DNA is one potential mechanism by which replication errors and/or mutations in oncogenes or tumour suppressor genes can be triggered (Wang et al., 2000; Fang & Vaca, 1995). The most stable DNA adduct to form, N2-ethylidene-2'-deoxyguanosine, has been found to inhibit DNA synthesis, induce mutations and both single and double strand breaks (Terashima et al., 2001; Mizumoto, et al., 2017).

While this is known of the interactions of acetaldehyde with nDNA, it has been noted in reviews such as Brooks and Zakhari, 2014, that there is a lack of information regarding the specific effect acetaldehyde has within the mitochondria. It can be expected though that the impact acetaldehyde

has on the mitochondrial genome may differ to that of the nuclear genome due to the presence of the primary acetaldehyde metabolism enzyme, acetaldehyde dehydrogenase 2 (ALDH2) in the matrix of the mitochondria (Endo et al., 2009; Wang et al., 2020). The location of the ALDH2 may result in the protection of mtDNA as its located at the inner membrane, meaning the ALDH2 is able to metabolise the acetaldehyde before interaction can occur (Goedde et al., 1983; Chinnery & Hudson, 2013; Zhang et al., 2020). However, the depletion of this ALDH2 enzyme has also been linked to cancer development (Zhu et al., 2022). Studies have found that the depletion of ALDH2, there was an increase in oxidative stress products which lead to mtDNA damage through mitochondrial dysfunction; there was also a promotion of cancer development through mtDNA damage in hepatocellular carcinoma (Gosselin et al., 2009; Seo et al., 2019).

1.4.2 Hydrogen peroxide

Hydrogen peroxide is used as an antiseptic but can also be produced naturally within the body due to impairments within the electron transport chain and is a reactive oxygen species (ROS) (Burgos-Morón et al., 2019). Superoxide molecules, which are a source of ROS can be reduced to hydrogen peroxide by mitochondrial superoxide dismutase 2 (SOD2) (Balaban et al., 2005). In the presence of free iron or copper ions, hydrogen peroxide can be transformed to a reactive hydroxyl radical, which is considered a highly reactive oxygen intermediate and results in DNA damage as its been found to interact with the DNA through the addition to the double bonds between DNA bases and the removal of a hydrogen atom from the methyl group of the thymine base as well as each of the C-H bonds found in 2'-deoxyribose (Collin, 2019). Oxidative stress has also been found to cause DNA base modifications, single- and double-strand breaks within the DNA and formation of potentially toxic and/or mutagenic apurinic/apyrimidinic lesions on the DNA (Raha & Robinson, 2000; Reuter, et al, 2010; Girard & Boiteux, 1997). Within the mitochondria, a study looking at hydrogen peroxide treatment in retinal pigment epithelial cells found there to be a significant increase in mtDNA compared to the control group which was not repaired completely following a 3-hour post treatment check (Ballinger et al., 1999). Interestingly, another study by Ballinger and colleagues, investigating the hypothesis that reactive species produced in the vascular environment causes mitochondrial damage; it was found that within both umbilical vein endothelial and aortic smooth muscle cells exposed to chemicals including hydrogen peroxide, mtDNA was preferentially damaged (Ballinger et al., 2000).

1.4.3 1-nitropyrene (1-NP)

1-nitropyrene (1-NP) is an aromatic hydrocarbon that is predominantly found in diesel and exhaust gasses (Riley et al., 2018). 1-NP is classed as a possible human carcinogen (Class 2B) by the International Agency for Research on Cancer (Möller, 1994). It has been found that 1-NP and mainly its metabolites such as 1-aminopyrene (1-AP) which can have a genotoxic effect (Hatanaka et al., 2001). 1-NP has been found to be metabolised mainly via two mechanisms, P450-mediated C-hydroxylation and nitroreduction (Oda et al., 1993; Chae et al., 1999). A study found that AP-1 transcription factor was highly activated to a N-hydroxylated intermediate by human P450 1B1, O-acetylation has also been found to also occur after N-hydroxylation to yield the reactive metabolites (Anderson et al., 1997; Hatanaka et al., 2001; Rendic & Guengerich, 2013). 1-NP itself was activated by the same P450 system to form a genotoxic compound via nitroreduction or O-acetylation at low 1-NP levels while at both low and/or high levels, activation occurs through epoxidation (Hatanaka et al., 2001). It has been shown that adducts are produced as a result of 1-NP metabolites binding to

guanine bases within DNA to form mainly N-[deoxyguanosine-8-yl]-1-aminopyrene adducts (Sherrer et al., 2009). These 1-NP- derived DNA adduct lesions generate DNA mutations, the most common of which are G to T transversions, a signature mutation associated with lung cancer (Kirouac et al., 2013). Within the mitochondria specifically, a study looking at many polycyclic aromatic hydrocarbon's components present in tobacco smoke including 1-NP was shown to increase mitochondrial genes and induce mitochondrial gene deregulation (Kim et al., 2014).

1.4.4 Di(ethylhexyl) phthalate (DEHP)

Di(ethylhexyl) phthalate (DEHP) is a distinctive endocrine-disrupting chemical commonly found in plastics (Caldwell, 2012; Rajagopal., 2019). Both human and rodent data suggest that cancer induction by DEHP occurs through several molecular signals such as oxidative stress and DNA damage, however, the mechanisms involved in its toxicity are poorly understood (Rowdhwil and Chen, 2018). A study conducted in breast cancer cells suggested one of the possible mechanisms involved in DEHP toxicity could be through increased cell proliferation without induction apoptosis (Rusyn et al., 2006; Crobeddu, et al, 2019). Increased proliferation plays an important role in the development of cancer as, through deregulating signalling pathways that control proliferation within normal cells, they are able to replicate uncontrollably resulting in alterations to expression/activity of cell cycle related proteins, as well as properties such as energy metabolism and cell survival which allow cancer cells to thrive (Hanahan & Weinberg, 2011; Feitelson et al., 2015). The altered cell survival results in an avoidance or resistance to apoptosis which reduces the cell's ability to eliminate cells that have potentially transformed into malignant ones (Kerr et al., 1972; Hanahan & Weinberg, 2000). A study looking at a condition DEHP has been linked to, skeletal muscle (SKM), where exposure to chemicals including DEHP has been linked to the development of metabolic myopathy, it was found that DEHP exposure resulted in impaired function of the mitochondria, further backed up by the reduction of mtDNA levels as well as increased ROS (Chen et al., 2020).

1.5 Reactive oxygen species (ROS)

The mutations found in many chronic diseases and cancer are believed to result from exposure to ROS produced during metabolism (Chatterjee, et al 2006). Mitochondria are the primary site of ROS production, with an estimated 2% of all O₂ consumed by mitochondria being used for ROS generation (Goossens, et al, 1995). Low levels of ROS and therefore these oxygen intermediates are essential in maintaining physiological functions as they are used as signal transduction messengers, which are important in the regulation of cellular function (Dröge, 2002; Reuter, et al, 2010). Under normal conditions, cells can maintain a homeostasis of the formation and removal through enzymatic pathways or via antioxidants (Lombard et al., 2005). An imbalance between the generation and removal of ROS in favour of the formation of oxidants results in the mitochondria and therefore the cell experiencing oxidative stress (Burgos-Morón et al., 2019). This excess generation of ROS can disrupt the function of the cell due to oxidation of lipids or DNA (Goossens et al., 1999). There are several electron transfers sites that have been identified as potential sources of mitochondrial ROS (mtROS) (illustrated in Fig.8), mainly complexes I and III; however, it remains unknown exactly how much is produced at each site (Chen et al., 2003).

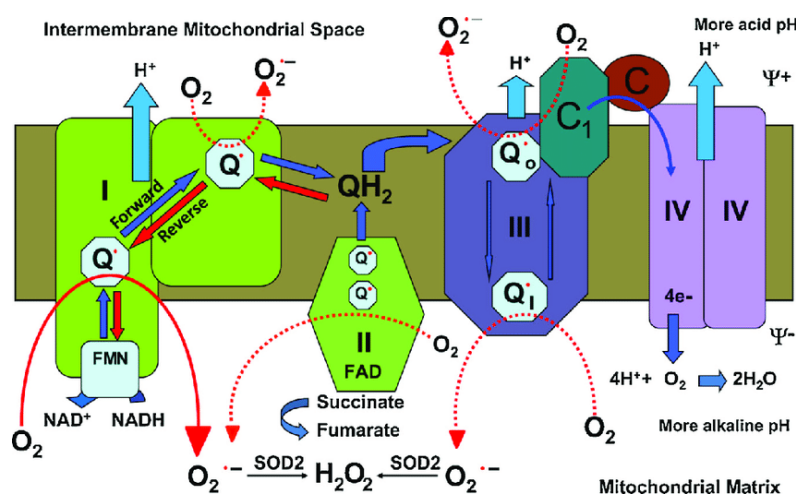


Figure 8: All the potential sites within the electron transport chain at the mitochondrial membrane for ROS production which contribute to oxidative stress. Complex I, II & III all contribute to the conversion of oxygen to a radical, which can go on to be converted to a superoxide and further to hydrogen peroxide (Kojouharova et al., 2019).

Superoxide is the main source of most ROS and is generated through a single electron reduction in an O_2 molecule (Balaban et al., 2005). The electron transport chain generates superoxide radicals through an electron leak at complexes I and III (Dröge, 2002; Brand, 2010) however, flavin-dependent enzymes present at the mitochondrial matrix may produce ROS at a higher rate than complex I (Quinlan et al., 2014). Upon reduction, superoxide can be transformed to a hydroxyl radical which is a highly reactive ROS that is formed in the presence of metal ions during a redox reaction with hydrogen peroxide (Winterbourn 2008). The imbalance between the ROS production and ability of the cells' defence mechanisms to eliminate the cellular stress results in a vicious circle, as the oxidative stress results in an increase in ROS production (Cui et al., 2012; Finkel, 2011).

It was believed that ROS production was solely an upstream step in oxidative damage as the main target for mitochondrial ROS (mtROS), mtDNA, is present within the mitochondrial matrix (Andreyev et al., 2005; Brandon et al., 2006). Each cell contains a significant number of mitochondria, which all contain multiple copies of mtDNA, therefore each cell can carry hundreds of mtDNA copies. However, mtDNA has also been shown to be more sensitive to oxidative damage, compared to nDNA with damaged more likely to persist in the mtDNA (Yakes & Van Houten, 1997). It has since been found that mtROS can impair ATP synthesis as well as other metabolic functions such as the citric acid cycle and amino acid metabolism (Zorov et al., 2014). Pathological effects of this increase in ROS production include formation of mutations or deletions to mtDNA, oxidative damage to the respiratory chain and overall dysfunction of the mitochondria (Massaad & Klann, 2011).

1.5.1 ROS and mtDNA damage

Under normal growth conditions, there is thought to be low levels of mtDNA damage as a result of ROS exposure; however, under high exposure to ROS mtDNA experiences extensive damage (Yakes & Van Houten, 1997). The main type of DNA damage is base modifications, with around 100 base lesions and 2-deoxyribose modifications identified due to oxidative stress (Claudia et al., 2018; Cadet et al. 2010, 2012b). One of the most prevalent base lesions seen within mtDNA is 8-OH-dG, which is formed due to the oxidation of guanine as a result of a hydroxyl radical being introduced to the C8

position, with A:T to C:C or G:C to T:A transversion mutations occurring (Kohen & Nyska, 2002; Altieri, 2008). The ability of 8-OHdG to pair incorrectly with adenine instead of cytosine also increases the mutational load and results in further oxidation and other DNA lesions (Kasai and Nishimura, 1984; Cheng et al., 1992; Cadet et al., 1999).

Damage to the DNA bases as a result of ROS can also lead to both single strand breaks (SSB) and double strand breaks (DSB) within the DNA, either directly or due to DNA repair intermediates (Hemnani & Parihar, 1998; Gonzalez-Hunt et al., 2018). SSBs commonly occur within DNA, and it is estimated that around >10,000 occur per cell per day compared to the less common type, DSBs, which are thought to occur ~50 DSBs per cell cycle (Vilenchik & Knudson, 2003; Mehta & Haber, 2014; Tubbs & Nussenzweig, 2017). The damage caused by ROS can lead to a failure of the mitochondria to produce energy due to damage of electron transport complexes, such as NADH dehydrogenase and ATP synthase, as well as other aspects required for mitochondrial function (Guo et al., 2014). Increased ROS has been linked to neurodegenerative diseases Huntington's disease as well as in spermatozoa with mutated mitochondria and lower levels of ATP as seen in men with asthenospermia (Feng et al., 2008; Siddiqui et al., 2021). However, despite our knowledge of the effects of ROS on disease and the mitochondria, little is known as to how ROS and the oxidative stress it causes affect mtDNA and its replication (Stojković et al., 2016).

1.5.2 Oxidative stress and diabetes

Oxidative stress is thought to be a contributory cause of hyperglycaemia and insulin resistance characteristically seen in Type 2 diabetes, it has been reported that compared to individuals without diabetes of normal weight, those with Type 2 diabetes and obesity had increased mitochondrial ROS (Burgos-Morón et al., 2019; Pinti et al., 2019). This is due to a calorie intake excess which is commonly associated with Type 2 diabetes and causes an impairment in the electron transport chain, increasing the chance of ROS production (Kwak, et al, 2010). The hyperglycaemia associated with diabetes and resulting ROS production cause a decrease in the expression of the insulin gene and subsequent production of insulin, and results in eventual apoptosis in a process known β -cell toxicity, which is frequently observed in those with diabetes (Moran et al., 1997; Evans et al., 2003; Kaneto et al., 2010) The increased oxidative stress is also known to cause an increased production of 8-OHdG from deoxyguanosine, resulting in more mtDNA deletions and induction of G-C and T-A transformation mutations (Richter, 1995). One study found that in kidneys of streptozotocin-induced diabetes rats that the levels of 8-OHdG, which can be used as a marker for oxidative DNA damage, was increased within the mtDNA only suggesting the damage predominantly occurred within the mtDNA (Kakimoto et al., 2002). Furthermore, it has been found that levels of 8-OHdG in muscle DNA from individuals with Type 2 diabetes was proportional to the severity of diabetic complications within patients such as diabetic nephropathy (Suzuki et al., 1999).

ROS production also occurs because of the dysfunction of the mitochondria due to diabetes. This dysfunction may be due to an excess of fission as diabetes has been associated with mitochondrial morphology (Barazzoni, 2012). Enhanced mitochondrial fission and fragmentation have been shown to be involved in the overproduction of ROS in the presence of hyperglycaemia in liver and muscle cells, implying there is a link between the altered dynamics of the mitochondria and oxidative stress seen in diabetes (Yu et al., 2006). A study investigating the role of fission in glucose induced cardiovascular cell injury found that mitochondrial fission which is necessary for fragmentation leads to an increase of ROS levels and ultimately cell death. From the data obtained, fragmentation is

thought to be an upstream event of hyperglycaemia-induced ROS overproduction and cell injury (Yu et al., 2008).

1.5.3 Oxidative stress and Cancer

ROS has been shown to have a role in various cancers via the promotion of several aspects of tumour growth and progression through mtDNA mutations (Brandon et al., 2006; Sosa et al., 2013; Trachootham et al., 2009). At moderate levels, ROS has been shown to upregulate the expression of several proteins that have been linked to the progression and survival of cancer cells through activation of proteins such as mitogen-activated protein kinase/extracellular signal-regulated protein kinases 1/2 (MAPK/ERK1/2) and phosphoinositide-3-kinase/ protein kinase B (PI3K/Akt) (Ranjan et al., 2006; Aggarwal et al., 2019). The MAPK/ERK1/2 pathway is activated by ROS through several mechanisms. One method is ROS activation of upstream products such as kinases, for example p90^{RSK}, within the pathway (Chan et al., 2008). It has been shown that in multiple cancers such as breast and ovarian, that ROS-activated Erk1/2 is able to increase cell survival (Roberts & Der, 2007). The P13K/Akt pathway has been shown to mediate survival of cancer cells through alterations of genes within the pathway that result in carcinogenic consequences on the cells (Yang et al., 2019; Jiang et al., 2020). In breast cancer, it was shown that ROS production through oestrogen metabolism activated the P13K/Akt pathway, while inhibition of ROS production in a study using pancreatic cancer found the same pathway to be inhibited, suggesting that the cells require constant activation of this pathway through the production of ROS (Burdick et al., 2003; Liu et al., 2006; Park et al., 2009).

At higher levels, ROS are also able to cause cancer cell apoptosis, this has been utilised in some anti-cancer therapeutics (Perillo et al., 2020). ROS is able to cause disruption to the mitochondrial membrane through opening of its permeability transition pore (PTP), together with the increase in the outer mitochondrial membrane permeabilization, this results in the dysregulation of the electron transport chain and causes the release of cytochrome c from complex III (Devun et al., 2010; Bernardi, 2013; Orrenius et al., 2015). Once in the cytosol, cytochrome c is able to form 'apoptosomes' which eventually result on the cleavage of proteins and cell death (Garrido et al., 2006). A paper investigating how ROS levels may influence response to ROS-stimulating treatments in cancer cells found that this mitochondrion specific method may be a more effective treatment method compared to conventional alternatives and that baseline levels of ROS levels within these cancer cells could be used as a predictive measure of drug efficiency (Zaidieh et al., 2019).

1.6 Aims & Objectives

The aim of this project is to establish how cellular reactions to carcinogens found in the environment affect high blood glucose concentrations associated with type 1 and type 2 diabetes, and impact the mitochondrial genome and function. These will be analysed by investigating mtDNA damage to see how this affects the overall genotype in both TK6 and MCL-5 cells. Mitochondrial health will also be investigated through changes to the mitochondrial phenotype, including their morphology and activity. This will provide knowledge of the effects of such chemicals on mtDNA and will help inform whether the increased susceptibility individuals with diabetes have to developing certain types of cancers is due to effects on the mtDNA (Biel et al., 2020).

Chapter 2- Methodology

2.1 Reagents

Unless otherwise stated, materials used were purchased from Merck (Dorset, UK).

2.2 Cell types

It has been shown that lymphoblastoid cells are promising for a range of studies including genotype-phenotype correlation studies (Sugimoto et al., 2004; Bernacki et al., 2005; Sie et al., 2009; Tan et al., 2011). In this study, lymphoblastoid cells TK6 and MCL-5 were used; TK6 cells were chosen as they are OECD-approved for applications in genotoxicity assays such as the micronucleus assay and are therefore well-characterised and genetically stable enough to be suitable for genotoxicity assessment (Zhang et al., 1995; OECD, 2014). MCL-5 cells were used in the case of 1-nitropyrene only within this study, as 1-nitropyrene requires metabolic activation which can occur in the MCL-5 line as they have the metabolic capacity via plasmids containing genes such as CYP450s to convert 1-nitropyrene to its genotoxic form (Busby et al., 1994; Hukkanen et al. 2000).

Previous studies have found success using lymphoblastoid cells as a diabetes and cancer model separately (Kassauei et al., 2006; Zhang et al., 2007; Mishima et al., 2008). One study found that exposure of lymphoblastoid cell lines to high glucose resulted in changes in gene expression, molecular, cellular, and functional levels and that recapitulated features found in the diabetic state (Grassi et al., 2016). Additionally, TK6 and MCL-5 cell lines also contain the wild-type human p53 tumour suppressor gene, which is advantageous for increasing specificity of genotoxicity testing such as in this study (Morris et al., 1996; Fowler et al., 2012; Babamohamadi et al., 2022).

2.2.1 Sub-culturing TK6 cells

All procedures were conducted in a Class II sterile laminar flow cell culture hood, with appropriate PPE always worn (lab coat, gloves and due to the ongoing pandemic, facemask). For all cell culture procedures, items were cleaned with 70% ethanol before being placed in the hood. Cell medium was produced from a mixture of RPMI 1640 medium with 10% horse serum and 1% glutamine (Invitrogen, Massachusetts, United States). A 1ml aliquot of spleen lymphoblastoid TK6 cell suspension (American Type Cell Collection, Virginia, USA) was removed from liquid nitrogen storage and added to 25 ml of the fresh medium in a T75 cell culture flask (Sarstedt, Germany). The cells were left to proliferate for 2-3 days in a 5.00% CO₂ incubator set at 37°C.

2.2.2 Sub-culturing MCL-5 cells

All procedures were conducted in a Class II sterile laminar flow cell culture hood and appropriate PPE work (listed in section 2.2.1). All items were cleaned with 70% ethanol before they were added to the hood. Cell medium was produced from a mixture of RPMI 1640 medium with 10% horse serum and 1% Glutamine and 0.44% Hygromycin B. A 1ml aliquot of MCL-5 cell suspension (American Type Cell Collection, Virginia, USA) was removed from liquid nitrogen storage and added to 50 ml of the fresh medium in a T175 flask. The cells were left to proliferate for 3-4 days in a 5.00% CO₂ incubator set at 37°C.

2.3 Treatment with glucose

10mM was selected for our normal glucose conditions as while within human blood, the normal blood glucose concentration ranges between 3.6-5mM; due to the high proliferation rate of these cells, a higher “normal” was required for sustained growth of cells (Ren et al., 2011). Therefore, such as in previous studies such as Zhang et al., (2007) and Czajka & Malik, (2016), where a glucose concentration for the high glucose group was more than two-fold that of the normal glucose group, 29mM was chosen as the high glucose concentration to mimic a comparative hyperglycaemic condition within the high glucose group.

2.3.1 Cell seeding and glucose treatment day one.

Medium was placed in a water bath set at 37°C to warm up 30 minutes before the experimental setup. The cells were removed from the incubator and examined under a microscope at 40x magnification to survey the viability, morphology, and density of the cells as well as absence of microbial contamination. In a sterile cell culture hood, the contents of the flask were split equally between two 50mL falcon tubes and centrifuged at room temperature for 5 minutes at 145 xg to remove from the original medium. Following centrifugation, the supernatant was then removed and discarded, and the cell pellet resuspended in 20mL of fresh medium and cell counts were taken using a standard haemocytometer (Hawksley, UK), counting two of the four 4x4 squares and producing an average cell count per ml. In a new sterile T175 flask, the TK6 cells were diluted to 100,000 cells/ml with medium. 10ml of the diluted cell solution was each added to ten labelled T25 flasks (Sarstedt, Germany). The same process was conducted for the MCL-5 cells also; however, the MCL-5 cells were diluted to 150,000 cells/ml due to the slower proliferating nature of the cells.

To treat the high glucose cells, a 1900mM glucose stock solution (calculations shown in Appendix 6.2.1) was made up each week dissolving 1.71g of glucose in 5ml of water. This solution was then filter-sterilised using a 0.45µm filter into a fresh sterile container; 5ml of water was also sterilised using a 0.45µm filter into a sterile container before treatment of cells. Half of the T25 flasks then had 100µl of the glucose stock solution added to each of them while the remaining half had 100µl of water added to them (as a control group due to glucose being dissolved in water). Finally, the cells were viewed under the microscope to ensure there was no contamination and transferred to the incubator for 24 hours set at the same conditions as described in section 2.2/2.3.

2.3.2 Glucose treatment day two

The following day a second glucose treatment was performed. Cell counts were taken using a haemocytometer as described previously to check for growth, and the contents of each flask poured into corresponding 15ml Falcon tubes and centrifuged at room temperature for 5 minutes at 145 xg. Once removed from the centrifuge, the supernatant was removed within a sterile cell culture hood and the pellet resuspended. 10ml of medium was added to the tube and the entire contents transferred into a labelled sterile T25 flask. The cells received the same glucose treatments as the previous day using the sterilised glucose stock or water solutions prepared during the previous treatments before being treated with the appropriate carcinogen solutions (see section 2.5 for further details).

2.4 Carcinogen Treatment

Relative population doubling (RPD) was calculated throughout using cell counts before and after chemical dosing to ensure that excessive cellular toxicity did not occur; according to official guidelines, an RPD of >50% has been recommended as a cytotoxicity limit (Greenwood et al., 2004). RPD data related to this study can be found in Appendix (6.1) while calculations relating to the composition of stock required can be found in Appendix (6.2.2-6.2.5)

The concentrations of each of the chemicals were chosen from this previous work, such as (Wilde et al., 2018), to reduce non-specific chemical effects due to the environmental carcinogens chosen to allow only the effect of the high glucose level in conjunction with these carcinogens to be seen; this would allow us to fully investigate the effects of high glucose in this pilot study.

DMSO was used as the vehicle of two of the environmental carcinogens used, and therefore a DMSO control was also used where the same volume of treatment of the carcinogens and water controls were used, meaning the cells received a treatment of 1% (as shown in section 2.5.1). A study using TK6 cells to investigate genotoxicity testing used a DMSO control, stating the concentration of this control never exceeded 1% while other studies with varying cell types also used 1% DMSO as a control group (Li et al., 2020; Kreuzer et al., 2022; Chen et al., 2022).

There have been studies showing DMSO has a genotoxic effect at higher doses of DMSO, however, there is conflicting data at DMSO concentrations $\geq 1\%$ (Valencia- Quintana et al., 2012; Anwar et al., 1989). While DMSO is generally accepted as non-toxic below 10% (v/v) and therefore the results thought to be negligible, at lower doses some studies have shown otherwise (Verheijen et al., 2019). One study using 3T3-L1 cells found that doses $\geq 1\%$ resulted in an increase in oxidative stress-associated damage and apoptosis as well as alterations to mitochondrial membrane potential, however doses as low as 0.1% were also found to have similar findings (Dludla et al., 2014). However, two studies looking at the effects of 1% DMSO on cell survival rates found varying results with one suggesting 1% DMSO did significantly decrease the survival rate to 72.6% while the other suggested doses as high as 1.4% did not cause any significant changes (Chen & Thibeault, 2014; Schmidt et al., 1989). One study using the same dose and exposure time as within this study found that 1% DMSO did not cause significant changes to astrocyte survival rates but did result in impairments in mitochondria integrity and cell viability (Yuan et al., 2014). Therefore, while lower doses of DMSO are recommended, 1% has been shown to have varying effects in various cell types. While these changes have been seen in some studies, the changes are thought too only be mild and non-genotoxic. (Aye et al., 2010; Kollerup Madsen et al. 2018).

2.4.1 TK6 Carcinogen Treatment

Medium was placed in a water bath set at 37°C to warm up 30 minutes before the experimental setup. On the same day as the second glucose treatment (described in section 2.4.2), the carcinogen dosing solutions used to treat cells were made up in a sterile cell culture hood using the dilutions in Table 1 to produce the desired concentrations.

Table 1: The final dosing concentration of the carcinogens used to treat TK6 cells, acetaldehyde, DEHP and hydrogen peroxide, and dilution of stock solution.

Carcinogen	Master stock concentrations (as sold)	Master Stock concentration (g/L)	Final concentration needed in cell flask (μM)	Composition of stock required
Acetaldehyde	40%	400	1000	3 μ l Master Stock + 297 μ l H ₂ O
DEHP	0.98 g/mL	985	2000	24 μ l Master Stock + 276 μ l DMSO
Hydrogen peroxide	30%	300	20	Stock A: 5 μ l Master Stock + 495 μ l H ₂ O (88 mM) (Use sterile 15 ml centrifuge tube to make above 1:100 dilution) Stock B (for dosing into flasks): 7 μ l Stock A + 293 μ l H ₂ O

100 μ L of each carcinogen solution was added to separate flasks of cells cultured in 10mM and 29mM glucose (prepared as described in 2.4.2); additionally, further control groups were treated with 100 μ L of water or DMSO, as appropriate; a 1% (v/v) was chosen for the controls as it kept the volume of treatment solutions consistent between groups. The flasks were then returned to the incubator for a further 24 hours set at the same conditions as described in section 2.2.

2.4.2 MCL-5 Carcinogen Treatment

Medium containing 0.44% Hygromycin B was placed in a water bath set at 37°C to warm up 30 minutes before the experimental setup. On the same day as the second glucose treatment (described in section 2.4.2), the carcinogen dosing solution used to treat cells were made up in a sterile cell culture hood using the dilutions in Table 2 to produce the desired concentration.

Table 2: The final dosing concentration of the carcinogen used to treat MCL-5 cells, 1-nitropyrene and dilution of stock solution required.

Carcinogen	Master Stock concentration (µg/ml)	Master Stock concentration (g/L)	Final concentration needed in cell flask (µM)	Composition of stock required
1-Nitropyrene	20	0.02	80.9	Stock A: 100mg Master Stock + 500 µl DMSO (809 mM) (Use sterile weighing pot for this dilution) Stock B (for dosing into flasks): 3 µl Stock A + 297 µl DMSO <i>* The volume of Stock A added to Stock B varied depending on the weight of 1-nitropyrene measured and therefore DMSO required to dissolve the 1-Nitopyrene powder, however the ratio of 1-nitopyrene and DMSO used and therefore concentrations remained the same.</i>

100µL of 1-nitropyrene dosing solution was added to separate flasks of cell cultured in 10mM and 29mM glucose (prepared as described in 2.4.2); additionally, a further control group was treated with 100µL DMSO. The flasks were then returned to the incubator for a further 24 hours set at the same conditions as described in section 2.3.

2.5 Recovery of cells

Medium was placed in a water bath set at 37°C to warm up 30 minutes before the experimental setup. In a sterile cell culture hood, the contents of each flask were added to separate, labelled 15ml centrifuge tubes and centrifuged at room temperature for 5 minutes at 145 xg. The supernatant is then removed into a toxic waste pot and the cells resuspended. 5ml PBS is then added to each tube and centrifuged again to wash the cells and remove any remaining medium. The supernatant is again removed and the cells resuspended. 10ml of fresh medium is then added to each tube, invert the tubes before transferring the cells into a sterile, labelled T25 flask. The flasks were then returned to the incubator for a further 24 hours set at the same conditions as described in section 2.2/2.3. All toxic waste was disposed of appropriately.

2.6 DNA extraction

All procedures were carried out on the benchtop. DNA extraction from TK6 and MCL-5 cells was conducted using a DNeasy Blood and Tissue kit (Qiagen, Manchester, UK), according to the manufacturer's protocol provided with the kit. The cells used were treated as described previously in sections 2.2-2.6. The cells were removed from the incubator and cell counts were carried out using a haemocytometer, as described previously, to ensure cell growth has occurred. The contents of each flask were poured into a labelled centrifuge tube and centrifuged at room temperature for 5 minutes at 145 xg. The supernatant was then removed and 200µl PBS was added to each tube, and the pellet resuspended.

The contents of each tube were transferred into a labelled DNeasy mini spin column. 20µl proteinase K and 200µl solution AL was added to each of the spin columns, and vortexed approximately 5 seconds after each solution added. The tubes were incubated at 56°C for 10 minutes. Once incubated, 200µl 90% ethanol was added, and the spin columns centrifuged in a microcentrifuge (STAR lab, Belgium) for 1 minute at 5724xg. Once centrifuged, the collection tubes containing the flow through were discarded and replaced. 500µl solution AW1 was added to each of the spin columns and centrifuged again under the same conditions. The collection tubes containing the flow through were discarded and replaced and 500µl solution AW2 was added to each. Tubes were centrifuged for 3 minutes at 15584xg. Once centrifuged, spin columns were placed in labelled microcentrifuge tubes and collection tubes discarded. 200µl Buffer AE was added to each of the spin columns and centrifuged for 1 minute at 5724xg. The spin columns were then discarded. DNA concentrations were measured using a Nanodrop NP-100 spectrophotometer (ThermoFisher, Massachusetts, United States) or NanoPhotometer NP80/N60/N50/C40 (Implen, Massachusetts, United States). Each sample was then divided into 20µl aliquots and stored at -20°C until use.

2.7 Analysis of mtDNA fold change using qRT-PCR

Within several mitochondrial diseases, there has been seen to be aberrant mtDNA replication (Stumpf & Copeland, 2011). Therefore, in this study, we have used qRT-PCR to measure the abundance of mtDNA within the samples. The genetic region chosen to measure was within the *MT-ND1* gene (shown in Fig.9) as it has little overlap with the nDNA in terms of sequence; additionally, NADH dehydrogenase 1 is transcribed from this gene, it plays a role within the electron transport chain and alterations to the copy number have been associated with both diabetes and various cancers such as breast and colorectal (Yusnita et al., 2010; Al-Ghamdi et al., 2022; Lemnrau et al., 2015; Tseng et al., 2006; Cui et al., 2013).

The house keeping gene, β -actin, was chosen as it is encoded within the nDNA. It is considered to have a uniform pattern abundance whereas mtDNA replicates at different rates due to several factors (Bustin, 2002; Korumutla et al., 2016; Yasukawa & Kang, 2018). It is known that TK6 and MCL5 have wild type ACTB gene sequence and therefore would be compatible with the primers used within this study (Ellinger-Ziegelbauer et al., 2009; David et al., 2016)

Studies such as those by Pieters et al. (2013) and Clemente et al., (2016) have been seen to use both *MT-ND1* and β -actin within their experiments to measure the relative mtDNA content such as in this study; additionally other studies looking at mtDNA content in varying cancers and diabetes complications also were found to use this same house keeping gene as a nDNA to control to other varying mtDNA targets (Mambo et al., 2005; Yu et al., 2010; Tewari et al., 2012).

ACACCCTCACCCTACAATCTTCCTAGGAACAACATATGACGCACTCTCCCCTGAACTCTA
 CACAACATATTTTGTCAACCAAGACCCTACTTCTAACCTCCCTGTTCTTATGAATTCGAACA
 GCATACCCCGATT

Figure 9: The sequence that was amplified (5'-3') during qRT-PCR that encodes for the genetic region of interest in this study, which falls within the *MT-ND1* gene at base location 4010-4145 within the mitochondrial genome; mitochondrially encoded NADH dehydrogenase 1 (NIH, 2023).

Before all experiments, a plate layout was created, such as the one illustrated in Fig.10, plotting the positions of the serial dilutions and a maximum of 11 samples investigating both the mitochondrial genetic region of interest (GOI) copy number and housekeeping gene, β -actin, copy number. The DNA samples and reagents were removed from the freezer for both the gene of interest and β -actin (SYBR Green, nuclease- free water and forward and reverse primers) and placed on ice to defrost. The PCR hood was cleaned with 70% ethanol and UV sterilised. All equipment was cleaned with 70% ethanol and UV sterilized before use.

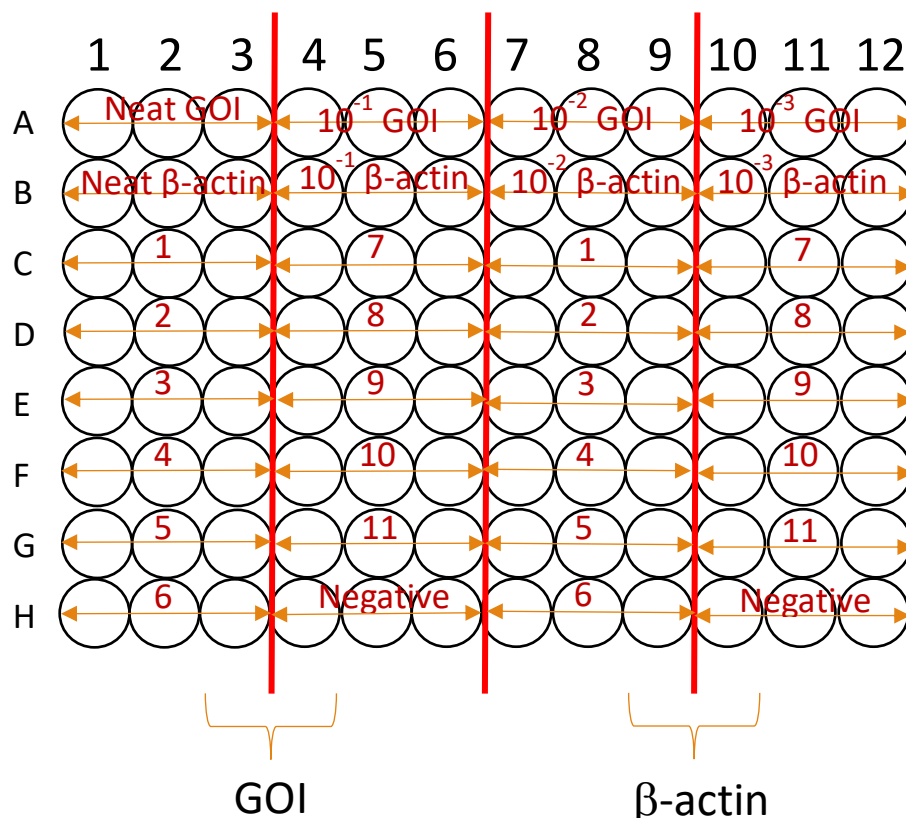


Figure 10: An example of the layout of a plate and the position of each triplet for samples and serial dilutions for the genetic region of interest (GOI) and β -actin genes

A normal glucose sample which had no additional chemical treatment was chosen to produce a serial dilution of neat DNA, 10^{-1} , 10^{-2} and 10^{-3} as illustrated in Fig. 11 to produce a standard curve, to be used later to analyse results.

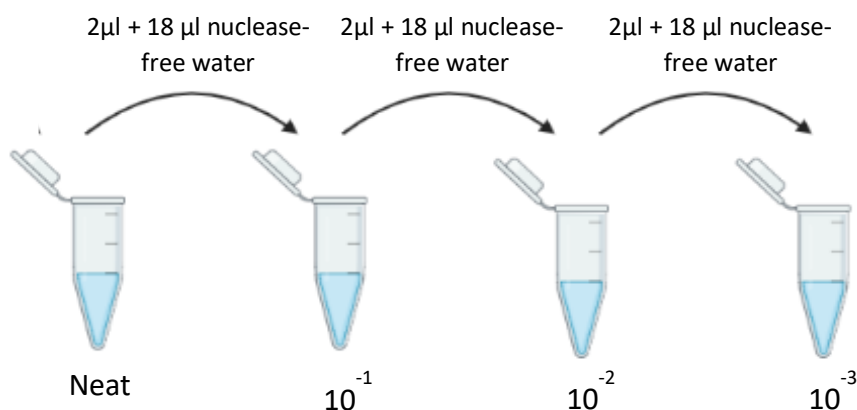


Figure 11: The serial dilution was done to create a standard curve for the analysis of RT-PCR results. 18μl nuclease-free H₂O was pipetted into each tube, then 2μl neat DNA was pipetted into the 10⁻¹ tube, then agitated and centrifuged to mix the sample. 2μl of 10⁻¹ was pipetted into 10⁻² and again agitated and centrifuged and finally 2μl of 10⁻² was pipetted into 10⁻³ and agitated and centrifuged.

The DNA samples were diluted to 15ng/μl in a 20μl solution using nuclease-free H₂O in separate tubes. Following this, 2 master mixes were made using the reagents listed in Table. 3, one containing the GOI primers and one containing the β-actin primers.

Table 3: The reagents and volumes used to produce the master mixes for GOI and b-actin. SYBR Green mix from the Quantifast PCR kit, the sequences of the forward and reverse primers for both the GOI and β-actin are shown in Table. 4.

Reagents	Volume (μl)
SYBR Green	676
Nuclease Free water	574
Forward Primer	13
Reverse Primer	13

Following the mixing of the master stock, 4.5μl of each of the diluted DNA solution was added to either 75μl the GOI or β-actin master stock.

Table 4: Primer sequence for the mitochondrial genome of interest and the housekeeping β-actin gene, and their position on the genome.

Primer name	Primer sequence (5'-3')
GOI Forward primer	ACACCCTCACCCTACAATCT
GOI Reverse primer	AATCGGGGGTATGCTGTTCG
β-actin Forward primer	AGATTGGGACAAAGGAAGCC
β-actin Reverse primer	GACGCCTCCGACCAGTGTGTTT

In a 96-well plate, following the plate layout plan (an example shown in Fig.9), 25µl of the DNA/master stock mix was pipetted into each well. Once completed, an adhesive plate film was placed over the plate and the edges smoothed down to ensure proper adherence. The plate was then centrifuged for 1 minute at 101xg before being placed in a Bio-Rad CFX connect qt-PCR machine (Bio-Rad, California, United States) following the protocol in Table. 5.

Table 5: The protocol used for the qt-PCR reaction as recommended by primer manufacturers Sigma Aldrich (Dorset, UK).

Stage	Cycles	Temperature	Time
Initial Denaturation	1	95°C	5 minutes
Denaturation	40	95°C	10 seconds
Annealing/ Extension		60°C	30 seconds
Final Extension	1	65°C	5 seconds
		95°C	50 seconds

Following the qRT-PCR run, the data was exported to a Microsoft Excel spreadsheet where the fold changes were calculated following the normalisation of the data.

2.8 Fluorescence Microscopy

2.8.1 Staining cells for image analysis of mitochondria

The cells used were treated as described previously in sections 2.2-2.6. Medium was placed in a water bath set at 37°C to warm up 30 minutes before the experimental setup. MitoTracker Green tube was protected from light using foil and all work was conducted in an unlit Class II sterile laminar flow cell culture hood as the stain is light sensitive. After the recovery stage as described in section 2.6, cell counts were taken using a haemocytometer as described above and the volume required to obtain 2,000,000 cells was calculated, which was then removed from the flask and placed in a 15ml centrifuge tube. All samples were centrifuged for 5 minutes at 145 xg, and the supernatant removed from the pellet using a pipette to ensure as much as possible is removed. A 20nM MitoTracker Green, (Invitrogen, Massachusetts, United States), staining solution was made up using the dilutions shown in Table 6.

Table 6: The steps and volumes required to dilute the MitoTracker Green staining solution to stain the mitochondria of the cells for imaging.

Step	Solution	Concentration of solution (mM)
1.	50µg dye sample in vial + 74µL DMSO	1
2.	3µL of solution from step 1 + 297µL DMSO	0.01
3.	10µL solution from step 2 + 4.99mL medium	0.00002

200µL of staining solution (final solution from step 3) was added to each pellet and resuspended. The centrifuge tubes were then wrapped in foil and placed in a dark cupboard to incubate at room temperature for 45 minutes.

2.8.2 Fixing Cells

After the 45-minute incubation period, the cells were centrifuged again at 145 xg for 5 minutes, the supernatant removed and 200µL 4% paraformaldehyde (PFA) added to each tube to fix cells and the pellet resuspended. The cells were left to incubate in the dark at room temperature for a further 15 minutes.

Following the second incubation, 3ml PBS was added, and the samples centrifuged at 145 xg for 5 minutes. The supernatant was removed, and the cells resuspended in 1ml PBS and then imaged.

2.8.3 Imaging cells

Images were taken using a Zeiss Axio Imager M1 microscope with the AxioCam 503 colour camera (Carl Zeiss NTS Ltd, Jena, Germany) and the Zen 3.1 program. Slides were prepared in the microscope room 3 samples at a time to allow enough time for each slide to dry to place a coverslip on while also reducing the time exposed to light and therefore prevent bleaching of the cells by the dye. 30µL sample was pipetted in a zig zag motion and spread over the slide before a cover slip was placed on top. Air bubbles were removed by pressing the slide gently to prevent squashing of cells. The ARF 888 filter was used with the R-L shutter open and set to MitoTracker Green. Images were taken with x40 magnification with an automatic camera shutter speed used. Eight greyscale images were taken per sample to obtain quantitative data related to the mitochondria using Cell Profiler 4.2.1 software with a pre-made pipeline by Dr Kate Chapman, measuring parameters relating to shape and size of mitochondria. One coloured image was taken per sample for illustrative purposes compare fluorescent signals visually.

2.9 Long-Range PCR for sequencing

2.9.1 Amplification of DNA samples

The PCR laminar flow hood was cleaned with 70% ethanol and UV sterilised. All equipment was cleaned with 70% ethanol and UV sterilized before use. The samples used were prepared as described previously in sections 2.2-2.6. DNA samples and reagents were placed on ice and left to defrost. All samples were resuspended through agitation of tubes followed by a brief pulse centrifugation. Two tubes for each sample were labelled, one for amplicon 1 (9kb) and the second for amplicon 2 (11kb).

75ng of each DNA sample was added to a 0.5ml Eppendorf tube along with the volumes of reagents shown in Table.7, the nuclease-free water volume is adjusted to maintain the overall total volume of 25 μ l. Three separate kits were used due to the large number of samples and continuous optimisation as described in 2.10.5.

Table 7: A) Shows the volumes of each reagent from (Thermo Fisher, Massachusetts, United States) required for the negative control B) Shows the volumes of each reagent from (Promega, Wisconsin, United States) required for the negative control C) Shows the volumes of each reagent required for the negative control for the Platinum Superfi II Master PCR Mix kit. All samples with an asterisk were added last. the sequences of the forward and reverse primers are shown in Table. 8.

A

Reagent	Volume (μ l)
Ultra-Buffer	5
Forward primer	1
Reverse primer	1
Template DNA	0
Polymerase	0.5
Nuclease-free water	17.5

B

Reagent	Volume (μ l)
Buffer	5
Forward primer	0.5
Reverse primer	0.5
dNTP mix	1
Template DNA	0
Polymerase	0.5
Nuclease-free water	17.5

C

Reagent	Volume (μ l)
Platinum PCR mix	12.5
Forward primer	0.5
Reverse primer	0.5
Template DNA	0
Nuclease-free water	17.5

Table 8: The primer sequence of each of the primers used for amplicon 1 (9kb) and amplicon 2 (11kb) regions of the mitochondrial genome.

Primer name	Primer sequence (5'-3')
F1	AAAGCACATACCAAGGCCAC
R1	TTGGCTCTCCTTGCAAAGTT
F2	TATCCGCCATCCCATACATT
R2	AATGTTGAGCCGTAGATGCC

The samples were then placed in a Bio-Rad T100 (Thermocycler, California, United States) with a protocol recommended by the reagent manufacturer set out in Table. 9.

Table 9: A. Shows the protocol used for the Long-Range PCR reaction in the thermal cycler as recommended by (Promega, Wisconsin, United States). B. Shows the protocol used for the Long-Range PCR reaction in the thermal cycler as recommended by (Sigma-Aldrich, Dorset, UK)

A

Stage	Cycles	Temperature	Time
Initial Denaturation	1	95°C	1 minute
Denaturation	35	95°C	15 seconds
Annealing		55°C	15 seconds
Extension		72°C	45-60 seconds per 1kb 11 minutes
Final Extension	1	72°C	5 minutes
		4°C	hold

B

Stage	Cycles	Temperature	Time
Initial Denaturation	1	98°C	30 seconds
Denaturation	35	98°C	10 seconds
Annealing		60°C	10 seconds
Extension		72°C	15-30 seconds per 1kb 6 minutes
Final Extension	1	72°C	5 minutes
		4	hold

2.9.2 Gel Electrophoresis (pre-run staining)

0.5g of agarose and 25ml of 0.5x TBE buffer were mixed in a glass conical flask and microwaved for roughly 1 minute. The mixture was then left to cool until the conical flask could be touched. 5 µl SYBR Gold Nucleic Acid Gel Stain, (Invitrogen, Massachusetts, United States), was added to the conical flask and swirled until fully combined, then poured into a gel holder after tape was placed on the exposed sides. A comb was placed at one end to form sample wells and air bubbles removed with a disposable pipette tip. The gel was then left to polymerise for 20 minutes at room temperature.

Once the gel had solidified, the comb and tape were removed. The gel electrophoresis apparatus was set up and the gel holder inserted. The gel was submerged in 0.5x TBE buffer and electrophoresis tank filled to the maximum point. 5µl DNA ladder solution was mixed with 2µl loading buffer, and 10µl of each PCR product mixed with 2µl loading buffer separately. 7µl of the DNA ladder solution and 12µl of each PCR product solution were loaded into separate wells. The lid was fixed to the tank, with the black (negative) plug jack paired with the black lead and the red (positive) plug jack paired with the red lead. The leads were then connected to a power supply and run at 80V for 70 minutes. During running, the progress of the sample migration, indicated by the dye front, was tracked by eye. After running was completed, the gel holder was removed from the tank, the gel separated from the holder and an image captured using a Bio-Rad ChemiDoc Imaging System (Bio-Rad, California, USA).

2.9.3 Gel Electrophoresis (post-run staining)

0.5g of agarose and 25ml of 0.5x TBE buffer were mixed in a glass conical flask and microwaved for roughly 1 minute. The mixture was then left to cool until the conical flask could be touched. The mixture was poured into a gel holder after tape placed on exposed sides. A comb was placed at one end to form sample wells and air bubbles removed with a disposable pipette. The gel was then left to polymerise for 20 minutes at room temperature.

Once the gel had solidified the comb and tape were removed. The gel electrophoresis apparatus was set up and the gel holder inserted. The gel was submerged in 0.5x TBE buffer and electrophoresis tank filled to the maximum line. 5µl DNA ladder solution was mixed with 2µl loading buffer, and 10µl of each PCR product mixed with 2µl loading buffer separately. 7µl of the DNA ladder solution and 12µl of each PCR product solution were loaded into separate wells. The lid was fixed to the tank, with the black (negative) plug jack paired with the black lead and the red (positive) plug jack paired with the red lead. The leads were then connected to a power supply and left to run at 80V for 70 minutes. After running, the gel holder is removed from the tank, the gel separated from the holder and placed in a staining tray. A staining solution made up of 50ml 0.5x TBE and 5 µl SYBR Gold (Invitrogen, Massachusetts, USA) was combined in a conical flask and swirled until fully combined before submerging the gel. The gel was left to incubate for 40 minutes at room temperature with a foil cove to protect from light. After the incubation period, the gel was removed from the solution and the image captured using a Bio-Rad ChemiDoc (Bio-Rad, California, USA).

2.9.4 PCR product purification

Purification of the long-range PCR products was done using the QIAquick PCR purification kit (Qiagen, Manchester, UK). 5 volumes of Buffer PB, containing pH indicator (1:250 pH indicator to Buffer PB) was mixed with 1 volume of PCR product and added to a QIAquick column, then centrifuged for 1 minute at 15115 xg. The flow through was discarded from the collection tube and the column placed back into the tube. 750µl Buffer PE was added and spun again under the same conditions to wash the DNA. The flow through was again discarded from the collection tube and the column placed back into the tube, which were spin again for 1 minute at 15115 xg. The collection tube was then discarded, and the column placed in a clean and labelled 1.5ml microcentrifuge tube. 50µl Buffer EB was placed at the centre of the QIAquick membrane and centrifuged again at the same conditions as previously stated. Samples are stored in a -20°C freezer until use.

2.9.5 Optimisation of amplicon 1

Due to issues highlighted during imaging of amplicon 1 samples, further optimisation was carried out on amplicon 1 samples to determine optimal conditions. The further optimisation process has been described in further detail in Appendix 8.1

2.10 Next Generation Sequencing

The samples used were prepared as described previously in sections 2.2-2.7 and amplified as described in section 2.10; the samples were then transferred to the Swansea University Medical School Sequencing Facility where the Next Generation Sequencing was to be carried out by Dr Matthew Hitchings who provided the following information of the rationale used.

“ Samples were purified using a standard AMPureXP approach using 1.8X bead mix volume to PCR sample volume. A total of 1 ng of purified DNA was taken as input to the Nextera XT library preparation procedure as per the standard Nextera XT library preparation protocol. Libraries were normalised using the XT bead approach and pooled before sequencing on a MiSeq system to obtain 250 bp paired end reads using a the V2 reagent chemistry.

To carry out the variant analysis reads were adapter trimmed using Scythe (<https://github.com/ucdavis-bioinformatics/scythe.git>) using the read1 and 2 sequences. Sickle (Joshi, *et al.* 2011) was used for quality filtering in default mode (threshold for trimming based on average quality in a window = Q20 and 20 bp minimum read length). The following shell script was used for batch processing.

```
#!/bin/bash
```

```
for sample in `cat samples.txt`  
do
```

```
R1=${sample}_*_L001_R1_001.fastq.gz  
R2=${sample}_*_L001_R2_001.fastq.gz
```

```
scythe -a adapter.fasta -q sanger -o ${sample}.scythe.R1.fastq $R1  
scythe -a adapter.fasta -q sanger -o ${sample}.scythe.R2.fastq $R2  
sickle pe -f ${sample}.scythe.R1.fastq -r ${sample}.scythe.R2.fastq  
-t sanger -o ${sample}.sickle.R1.fastq -p ${sample}.sickle.R2.fastq
```

```
-s  
  ${sample}.singles.fastq
```

```
done
```

Reads were aligned to the indexed mitochondrial genome sequence as part of the GRCh38.p13 genome assembly using the BWA mem algorithm (Li 2013). Alignments were processed using Samtools (Li, et al. 2009) to retain reads with mapping qualities above Q30 and position sorted prior to indexing.

```
#!/bin/bash
```

```
for sample in `cat samples.txt`  
do
```

```
R1=${sample}.sickle.R1.fastq  
R2=${sample}.sickle.R2.fastq
```

```
bwa mem -t 8 chrM ${sample}.sickle.R1.fastq ${sample}.sickle.R2.fastq | samtools view -@ 8 -b -q 30 - | samtools sort -@ 8 -O BAM -o ${sample}_mt.sorted.BAM - ;  
samtools index -@ 8 ${sample}_mt.sorted.BAM  
done
```

Read groups were amended using Picard's AddOrReplaceReadGroups function using the sample name as readgroup. Genotype likelihoods were generated using the bcftools mpileup option, recalibrating base qualities with minimum quality of Q20. Maximum depth was set to 1,000,000 and positions were annotated with the depth (DP) and allele depth (AD). Variants were called using the call function using two separate approaches, firstly using the multiallelic and rare-variant calling option (-m) keeping all alternative alleles (-A) and setting the ploidy to 1. Further, a strict consensus approach was employed keeping only variant positions.

```
#!/bin/bash
```

```
for sample in `cat samples.txt`  
do
```

```
bcftools mpileup --redo-BAQ --min-BQ 20 -d 1000000 -a DP,AD -f chrM.  
fasta ${sample}_mt.sorted.BAM | bcftools call --ploidy-file ploidy_  
file -A -m -Ov - | bcftools ann  
otate -x FORMAT/PL - > ${sample}_mt.vcf -;  
bcftools mpileup --redo-BAQ --min-BQ 20 -d 1000000 -a DP,AD -f chrM.  
fasta ${sample}_mt.sorted.BAM | bcftools call --ploidy-file ploidy_  
file -m -Ov -o ${sample}_strict_mt.vcf -
```

```
done
```

Within this use of Next Generation Sequencing, the Cambridge Reference gene was used for comparison against the sample genomes. Variant allele frequencies (VAF), that is the fraction of

reads aligning to a genomic coordinate that support a non-reference allele, were added to the VCF using the bcftools +fill-tags option. The VAF value was calculated from each variation that occurred within each of the samples in comparison to the reference gene and locations of these VAF sites were later mapped across the genome to help identify regions where variation was occurring. Within the genome, a VAF of 0 and 1 represent homoplastic sites, with 0 meaning 0% alternative alleles while 1 means 100% alternative alleles.

Graphical representation of variant positions and or VAF of each reference position was carried out using RStudio using a variety of packages. Statistical comparisons of VAF between treatment groups was carried out using paired samples Wilcoxon tests using Benjamini Hochberg adjustment for multiple tests."

2.11 Seahorse Bioanalyzer

2.11.1 Day 1- Plate preparation

The cells used were prepared as described previously in sections 2.2-2.6. Prior to loading cells on the plate, Cell Tak reagent (Corning) was added to each well to allow the cells to adhere to the base of the well. 29µl of Cell Tak was reagent was added to 221µl sterile water; within 10 minutes of making this Cell Tak solution, 10µl was added to every well of a 24 well Cell Culture Microplate along with 40µl of 0.1M sodium bicarbonate. This was left for 20 minutes before the 50µl solution was removed. 500µl of water was then used to wash the wells, taking care not to scratch the base of the well when removing the water. The plate was left to air dry in the cell culture hood and then stored at 4°C until use. Once the cell plate has been prepared, a Seahorse sensor cartridge was hydrated by removing the lid, hydro booster and cartridge and adding 1ml of MitoStress test calibrant into each well of the Utility plate; the lid, hydro booster and cartridge were then placed back on the plate and the plate stored overnight in a non-CO₂ incubator at 37°C.

2.11.2 Day 2- Seahorse Run

The cells used were treated as described previously in sections 2.2-2.6. Seahorse medium, sodium pyruvate, L-glutamine and glucose solutions was placed in a water bath set at 37°C to warm up 30 minutes before the experimental setup. In a sterile cell culture hood, the Mito Stress test medium in two 50ml falcon tubes, each had 48.5ml of Seahorse medium and 10 mM glucose, 2mM Pyruvate and 2mM L-glutamine; the plate containing Cell Tak that was prepared the day prior (described in 2.12.1) was also removed from the 4°C fridge and placed in the cell culture hood and allowed to warm to room temperature.

Cell counts were carried out for each flask as described previously using a haemocytometer before being centrifuged at room temperature for 5 minutes at 145 xg. Each pellet was resuspended before cells were diluted to 400,000 cells/ml using a volume of Seahorse Mito Stress Test medium made up earlier calculated from the cell counts. 100µl of cell solution was added to each well before the plate was spun at 20 xg for 1 minute with an acceleration of 4 and a brake of 1 to adhere the cells to the base of the wells. The wells were then checked to ensure a consistent number of cells were adhered to each. 400µl of Seahorse Mito Stress Test medium was added slowly to each well before the cell culture plate was then placed in a non-CO₂ incubator set at 37°C for 30 minutes.

The injection solutions that were loaded into each cartridge port during the Seahorse run were prepared during the 30-minute incubation from stock compounds using the dilutions described in Table. 10.

Table 10: The dilutions required to prepare the injection solutions used to load the cartridge ports which were used during the Seahorse run and their concentrations after dilutions.

Stock compound	Dilution
Oligomycin	Stock A: Oligomycin powder + 630µl Seahorse Mito Stress Test medium Stock B (2.5µM): 630µl Stock A + 1890µl Seahorse Mito Stress Test medium
FCCP	Stock A: FCCP powder + 720µl Seahorse Mito Stress Test medium Stock B (2.0µM): 600µl Stock A + 2400µl Seahorse Mito Stress Test medium
Rotenone/Antimycin A	Stock A: Rotenone/Antimycin A powder + 540µl Seahorse Mito Stress Test medium Stock B (0.5µM): 300µl Stock A + 2700µl Seahorse Mito Stress Test medium

After 30 minutes, the cell culture plate was removed from the incubator. The hydrated Seahorse plate prepared the day before was removed from the non-CO₂ incubator, the hydration booster was removed, and the cartridge ports were loaded with the appropriate solution.

A Oligomycin 56 µl	B FCCP 62 µl
C Rotenone/Antimycin 69 µl	Blank

Figure 12: The appropriate solution and the required volume loaded into each of the cartridge ports on the XFe24 plate.

On the XFe²⁴ Seahorse Bioanalyzer, the plate pan was inputted, the injections and protocol were checked before the cartridge and utility plate were placed into the machine to calibrate the machine.

Following the calibration, the utility plate was replaced with the cell culture plate and the Seahorse left to run. Following the run, the data was exported into a Microsoft Excel spreadsheet.

2.12 Statistical Analysis

At least three biological replicates were carried out (except where stated) independently of each other. Error bars were produced using standard deviation of the data. Normality and Statistical significance were calculated using SPSS Version 28 using a one-way ANOVA for normally distributed data or a Kruskal-Wallis for data that was not normally distributed; the p-values for the Next Generation Sequencing were calculated using a Pairwise Wilcoxon test. Conclusions were deemed statistically significant following an outcome of $p \leq 0.05$. Statistical significance was shown on figures using asterisks, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Chapter 3- The effect of environment chemical exposure and hyperglycaemia on the mitochondrial genome

3.1. Next Generation Sequencing

3.1.1. Comparison of all TK6 samples for significance

Each of the TK6 comparisons were done with a threshold of 1% applied, excluding all Variant Allele Frequency (VAF) points with a value of below 1% to remove errors while also accounting for low variation frequencies that may be significant. (Wessel et al., 2015; Shin et al., 2017) The comparison of all the VAF points were tested for significance and the results shown in Fig. 13 (full list of p-values found in table 3 in appendix 6.4.1.1).

Pairwise comparison of mitochondrial VAF > 1% in TK6 cell line

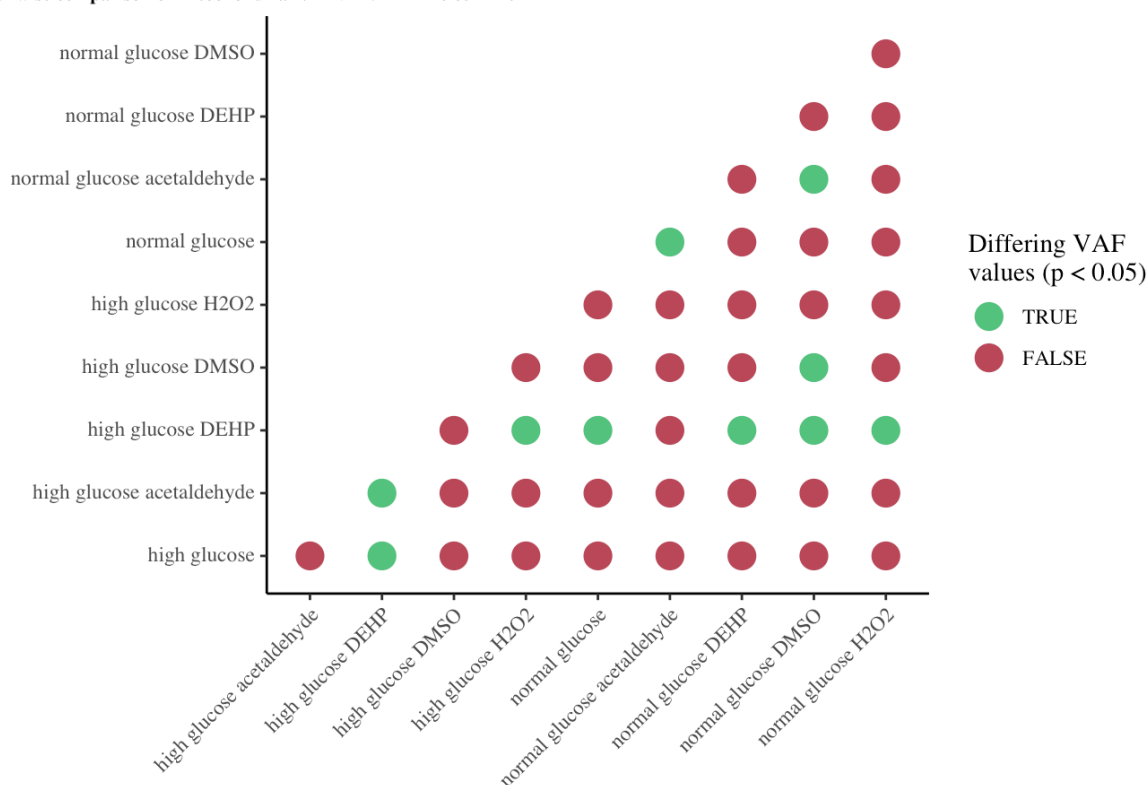


Figure 13: Comparisons of the average VAF value above 1% of TK6 sample groups grown under normal or high glucose concentration with a water (n=7) or DMSO control (1% (v/v) (n=7) or with one the test chemicals, acetaldehyde (1000μM) (n=7), di(ethylhexyl) phthalate (DEHP) (2000μM) (n=5) or hydrogen peroxide (H₂O₂) (20μM) (n=7). Each dot represents a comparison of all the data points above the 1% of two groups being compared for significance, with the significant or true results (p<0.05) are represented as green circles, while non-significant or false results are represented as red dots. N numbers represent biological replicates. Graphical representation of these comparisons was carried out using RStudio by Dr Matt Hitchings.

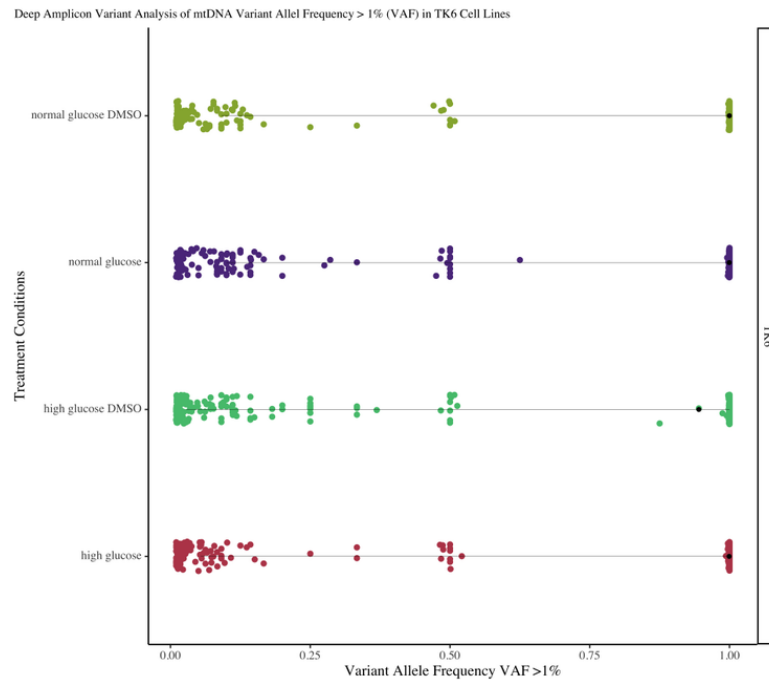
There were three comparisons of interest that were saw significant changes between the VAF points: normal glucose DMSO v high glucose DMSO, normal glucose v normal glucose + Ace and normal glucose DEHP v high glucose DEHP.

3.1.2. The effect of high glucose within the TK6 control groups

Within Fig.14.A, the values of the VAF sites in the normal and high glucose water control groups show there appears to be a higher proportion of VAF sites with a value in the range of 0.00 and 0.5 in the normal glucose group compared to be high glucose group; however, the average VAF value

within both sample groups appears to be similar. Within the regions of genome sequenced (as shown in Fig.14.B), it appears that within both the normal and high glucose groups, there is a higher proportion of VAF sites below a VAF of 0.25, particularly below 1000bp within the genome. Within the normal glucose control genome, the region of the genome sequenced below 10000bp has more VAF sites compared to high glucose, this is particularly noticeable below a 5000bp within the genome. Above a VAF of 0.5, there appears to be little difference between the two groups in the number of VAF sites, their values or location.

A



B

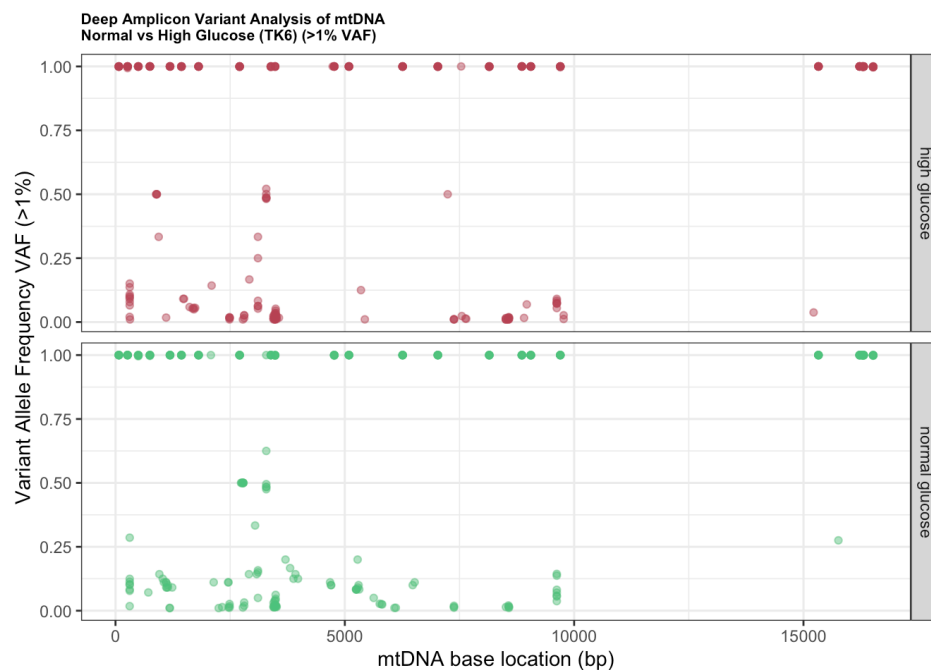


Figure 14: A) Comparison of the range of Variant Allele Frequency (VAF) values above 1% within the TK6 samples grown under a normal or high glucose concentration within the water control groups or DMSO control groups (n= 7). Each dot represents a variation site while the average VAF observed are represented as a singular black dot within the data while the standard deviation is represented by solid black lines. **B)** Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of TK6 water control samples grown under a high or normal glucose concentration (n=7). Each dot represents a variation site. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 1% (v/v) DMSO treatment. The DNA samples extracted from the cells were then amplified and purified before being transferred to the Swansea University Sequencing department where

were sequenced. The Cambridge Reference gene was used for comparison and VAF values were calculated. Graphical representation was carried out using RStudio by Dr Matt Hitchings.

When looking at the VAF sites values of the differing glucose treatments within the DMSO control groups in Fig.14.A, there appears to be a larger number of sites with a VAF value between 0.00 and 0.25 within the high glucose DMSO group compared to the normal glucose DMSO group; within Fig.15, many of these VAF sites can be seen within the 0bp and 5000bp region of the gene, particularly in the high glucose group. Additionally, when comparing the average VAF values of the variation sites, normal glucose DMSO appears to have an average value of 1.00% while high glucose DMSO appears to have a lower average of approximately 0.90%. In the final region of the gene sequenced (above 15000bp), there appears to be noticeably more VAF sites within the high glucose DMSO group across the VAF scale, however, the majority of them fall between 0.00% and 0.25%. This shows that it is at the low-frequency values that the main differences between the two samples occur. Furthermore, the reduction in the average VAF value within the high glucose DMSO group compared to the normal glucose DMSO group stated earlier was shown to be significant ($p = 0.031$).

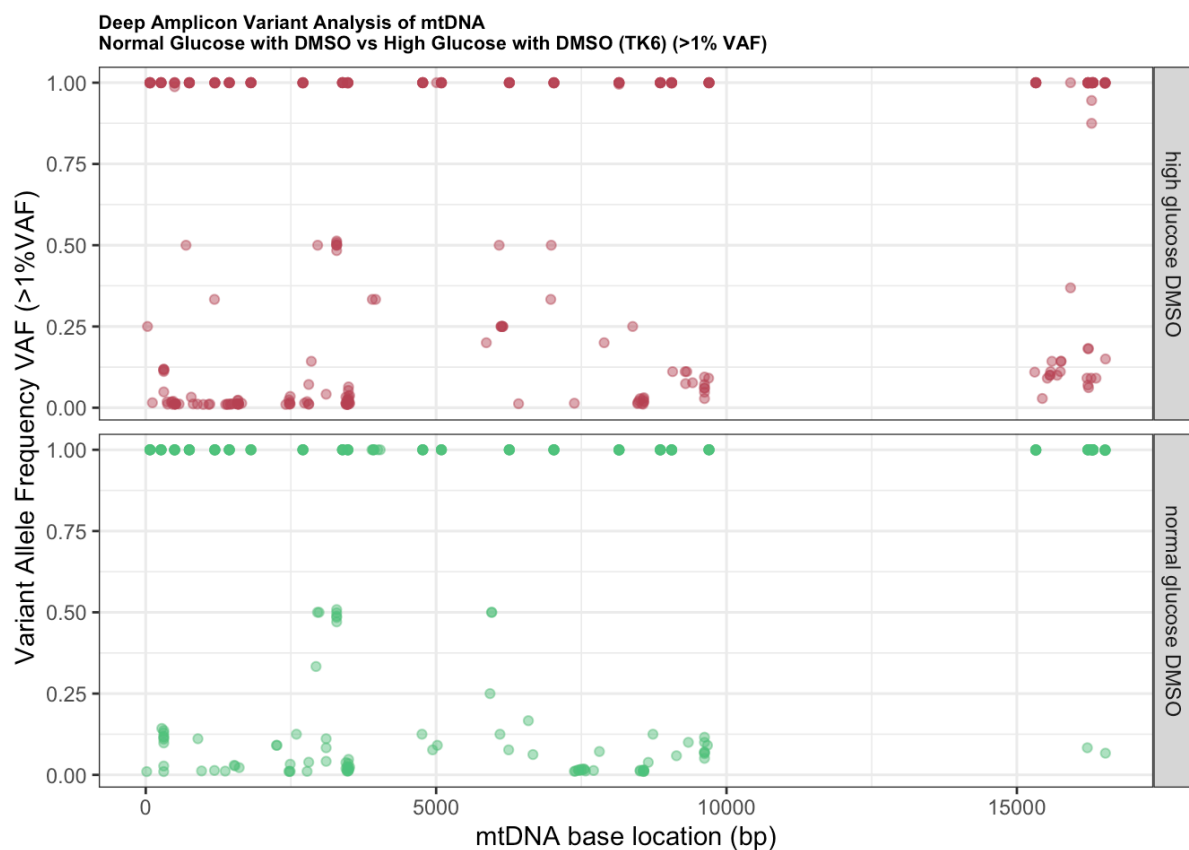
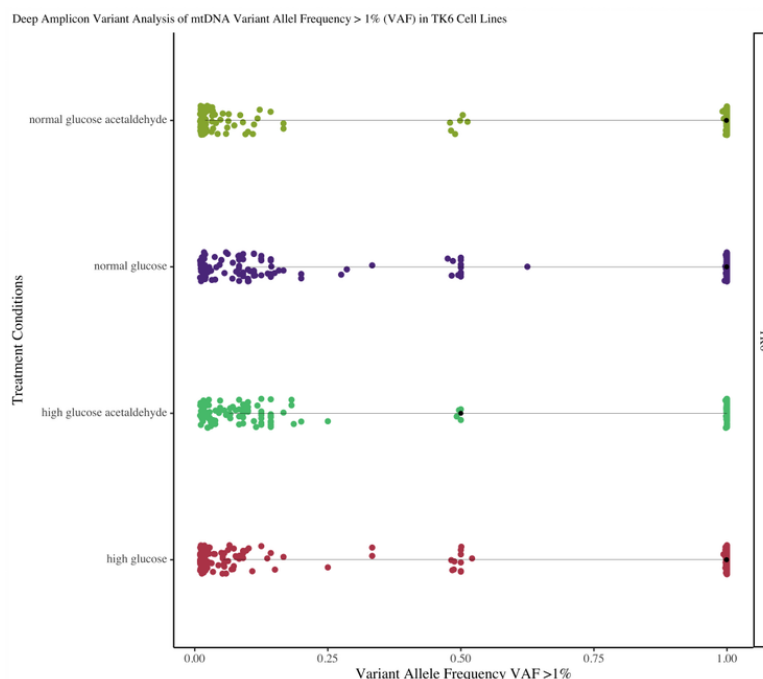


Figure 15: Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of TK6 DMSO control samples grown under a high or normal glucose concentration ($n=7$). Each dot represents a variation site. N number represents biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups received (1% (v/v) DMSO treatment. The DNA samples extracted from the cells were then amplified and purified before being transferred to the Swansea University Sequencing department where they were sequenced. The Cambridge Reference gene was used for comparison and VAF values were calculated. Graphical representation was carried out using RStudio by Dr Matt Hitchings.

3.1.3. The effect of high glucose in the presence of acetaldehyde

Looking at the effect of high glucose in the presence of acetaldehyde, there appears to be a larger proportion of VAF sites with a value of 0.00% and 0.25% within the high glucose group compared to that of the normal glucose (Fig.16.A), this increase can be seen across all regions of genome that was sequenced (Fig 16.B). Since the increase in variation sites below 0.25% is seen across the whole region of genome sequenced, this would suggest the decrease in the average VAF seen within high glucose acetaldehyde group may be due to an increase in the number of VAF sites at a low VAF value. Looking at variation sites of the higher VAF values, it can be seen at a VAF value above 0.05%, there is little difference between the two groups. Additionally, at 0.50%, there is only a slight variation of the number of VAF sites at similar base location within both samples at a base location between 2500bp and 5000bp; with the only noticeable difference is a slight increase in sites present in normal glucose acetaldehyde between 2500bp and 5000bp. The main difference seen between the two groups are within the sites with a VAF of below 0.25%. However, there is no significance within this comparison ($p=0.23$).

A



B

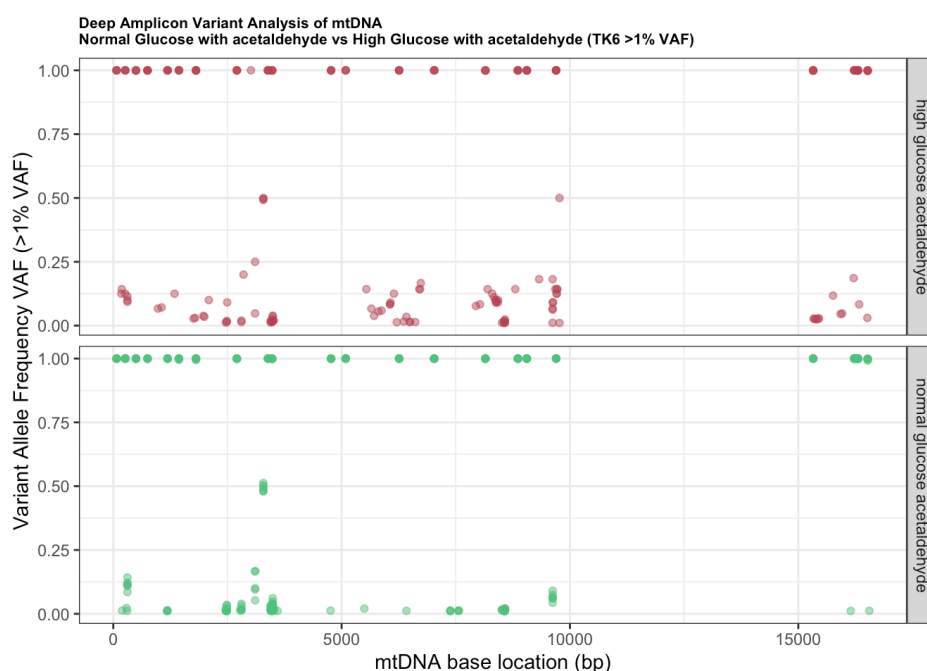


Figure 16: A) Comparison of Variant Allele Frequency (VAF) values above 1% within the TK6 cells grown under a normal or high glucose concentration within the water control groups (n=7) or under acetaldehyde (n=7). Each dot represents a variation site while the average VAF observed are represented as a singular black dot within the data while the standard deviation is represented by solid black lines. **B)** Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of acetaldehyde and either a high or normal glucose concentration (n=7). Each dot represents a variation site. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatment or a 1000µM acetaldehyde treatment. The DNA samples extracted from the cells were then amplified and purified

before being transferred to the Swansea University Sequencing department where were sequenced. The Cambridge Reference gene was used for comparison and VAF values was calculated. Graphical representation was carried out using RStudio by Dr Matt Hitchings.

As shown in Fig.17. within the normal glucose group treated with acetaldehyde, there appears to be fewer VAF sites across the genome when compared to the normal glucose water control; this is particularly true when looking at sites with a VAF below 0.5%. Above 0.5% there appears to be little difference in both the value and location of the VAF sites within the genome between groups. Additionally, while there appears to be little difference between the average VAF value (Fig.16.A), when comparing the two groups, there was significance seen when comparing the two groups in Fig.10. ($p=0.015$). All of this shows that rather than a shift in VAF values within the acetaldehyde group, there is a reduction in variation sites within the acetaldehyde group, however those that are present are of a higher VAF value than those in the normal glucose group.

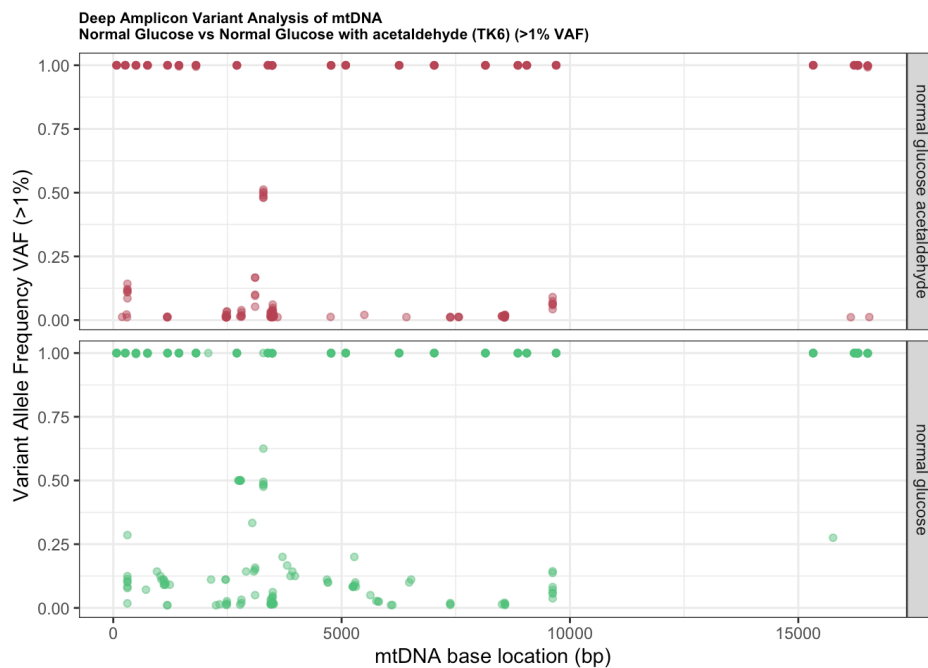
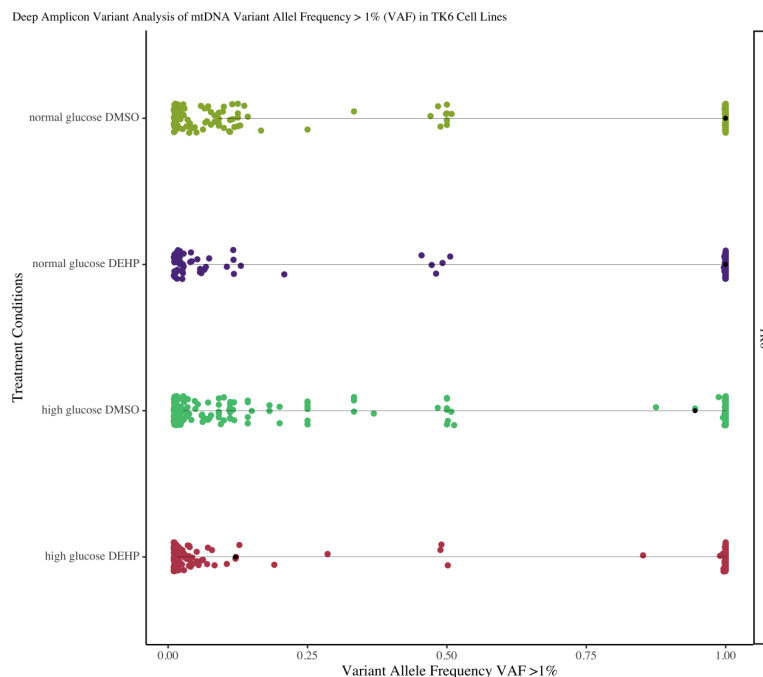


Figure 17: Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of TK6 cells grown under normal glucose conditions treated concentration with the water control groups ($n=7$) or acetaldehyde ($n=7$). Each dot represents a variation site. N number represents biological replicates. Normal glucose samples were cultured in 10mM of glucose and treated with either received 1% water treatments or a 1000 μ M acetaldehyde treatment. The DNA samples extracted from the cells were then amplified and purified before being transferred to the Swansea University Sequencing department where were sequenced. The Cambridge Reference gene was used for comparison and VAF values was calculated. Graphical representation was carried out using RStudio by Dr Matt Hitchings.

3.1.4. The effect of high glucose in the presence of DEHP

During the comparison normal and high glucose DEHP groups, as shown in Fig. 18.A, there is a higher proportion of VAF sites within the high glucose DEHP group compared to the normal glucose group DEHP; this is especially apparent below a VAF value of 0.25% and above 0.5%, with the opposite being true around a value of 0.5%. At base locations, as shown in Fig. 18.B, below 5000bp and above 15000bp is where many of the additional VAF sites within the high glucose DEHP genome appear to be. Above 15000bp, there is only VAF sites with a value below 1.00% in the high glucose DEHP group, with many of these additional sites falling below a VAF of 0.25. At a VAF of 1.00%, many of the sites within both the normal and high glucose DEHP groups appear to be in similar locations. Between 2500bp and 5000bp, many of the sites within the high glucose DEHP have a similar value and location to those in the normal glucose group; this could suggest that the DEHP is responsible for the variation at these sites opposed to the high glucose. Despite the increase in VAF sites within the high glucose DEHP group, there was also a significant decrease in the average VAF value in the high glucose DEHP group; this may be due to the increase in sites with a lower VAF, suggesting that there is an increase in low frequency variations within the genome of the high glucose DEHP compared to the low glucose DEHP group ($p=0.005$).

A



B

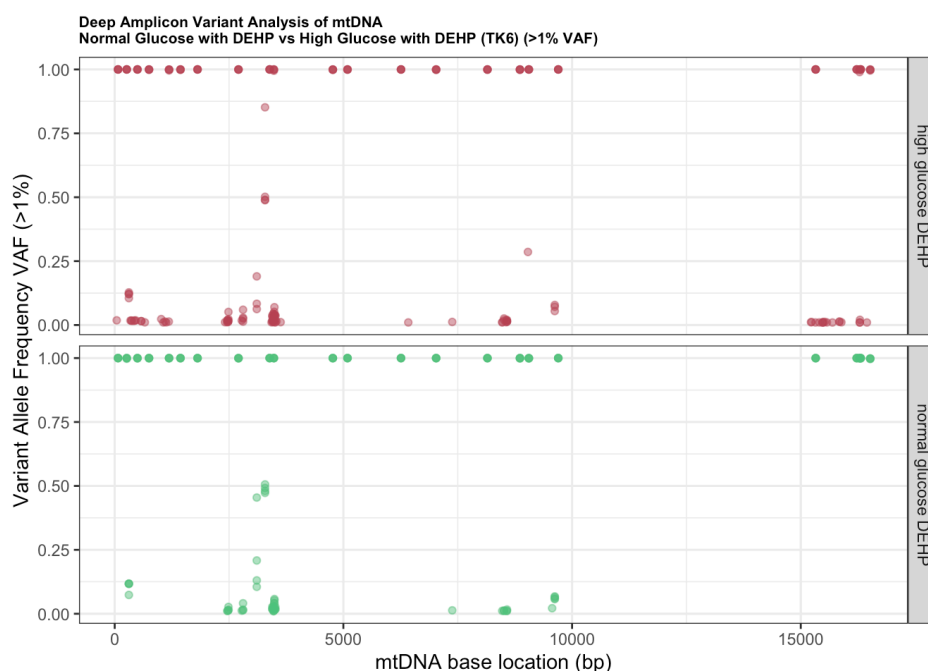


Figure 18: A) Comparison of Variant Allele Frequency (VAF) values above 1% within the TK6 samples grown under a normal or high glucose concentration within the DMSO control groups (n=7) or under di(ethylhexyl) phthalate (DEHP) (n=5). Each dot represents a variation site while average VAF observed are represented as a singular black dot within the data while the standard deviation is represented by solid black lines **B)** Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of di(ethylhexyl) phthalate (DEHP) samples grown under a high or normal glucose concentration (n=5). Each dot represents a variation site. N number represents biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received (1% (v/v) DMSO treatment or a 2000µM di(ethylhexyl) phthalate (DEHP) treatment. The DNA samples extracted from the cells were then amplified and purified before being

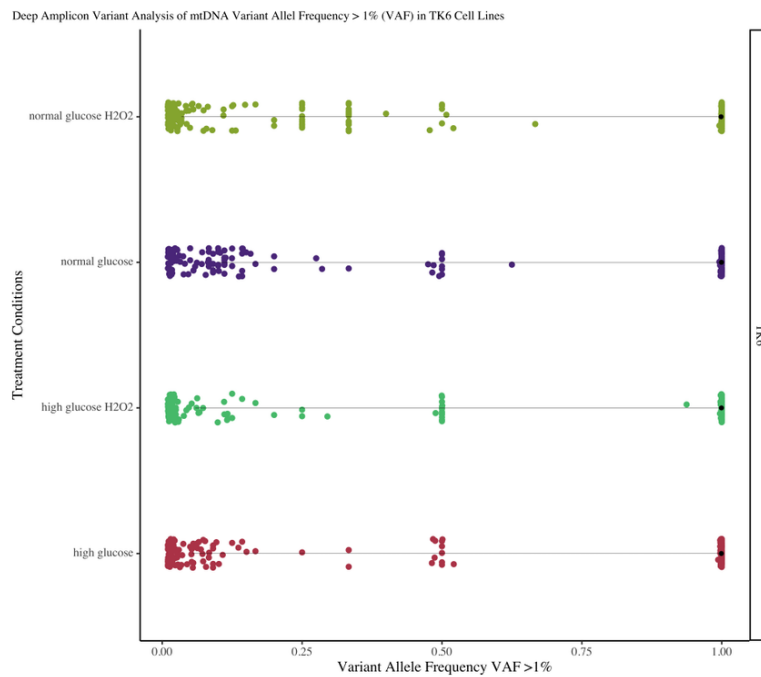
transferred to the Swansea University Sequencing department where were sequenced. The Cambridge Reference gene was used for comparison and VAF values was calculated. Graphical representation was carried out using RStudio by Dr Matt Hitchings.

Comparisons between both the normal and high glucose DMSO control groups and their respective DEHP counterparts yielded no significance as shown in Fig. 10, implying the effects seen within the previous comparison are a result of high glucose in the presence of DEHP only.

3.1.5. The effect of high glucose in the presence of Hydrogen Peroxide

During the comparison of high glucose hydrogen peroxide against normal glucose hydrogen peroxide, as represented in Fig.19 .A there appears to be a larger number of points below a VAF of 0.5 within the normal glucose hydrogen peroxide group, particular below a value of 0.25%. Looking at the mitochondrial genome (Fig.19.B), a region where this is particularly noticeable is above 15000bp, as there is only one site of variation in the high glucose group while there is considerably more in the normal glucose group. Unlike in the previous comparisons, at all values below 1.00%, many of the sites of variation appear to differ between samples, this is particularly true between 5000bp and 10000bp. At a VAF value of 1.00%, there are only one noticeable site in each that does not line up with the respective sample. When comparing the average VAF within the sample groups, there appears to be no significant difference between the normal and high glucose hydrogen peroxide ($p= 0.45$).

A



B

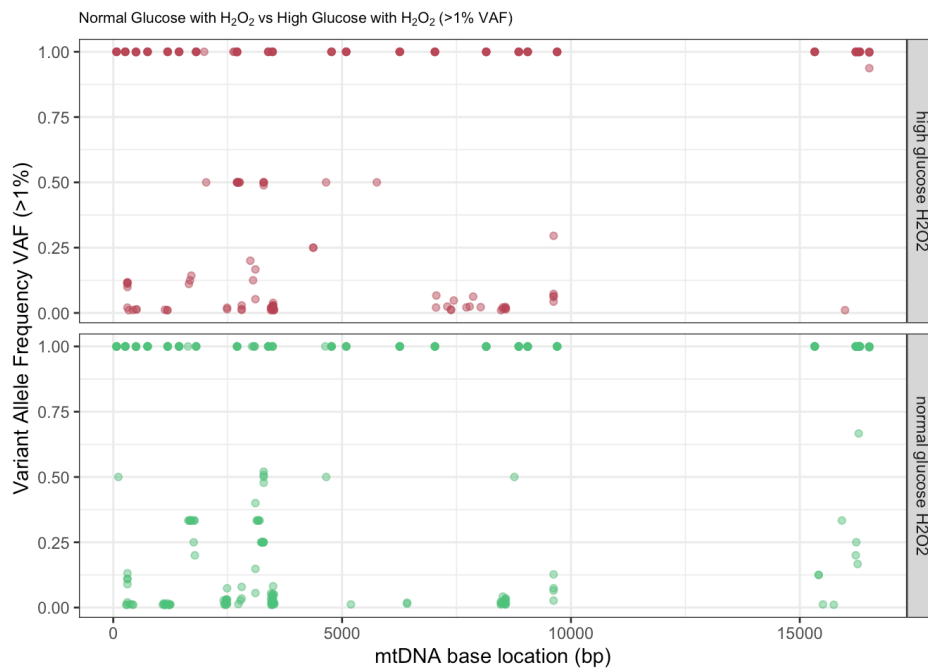


Figure 19: A) Comparison of Variant Allele Frequency (VAF) values above 1% within the TK6 samples grown under a normal or high glucose concentration within the water control groups (n=7) or under Hydrogen Peroxide (H₂O₂) (n=7). Each dot represents a variation site while the average VAF observed are represented as a singular black dot within the data while the standard deviation is represented by solid black lines. **B)** Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of TK6 Hydrogen Peroxide (H₂O₂) samples grown under a normal or high glucose concentration (n=7). Each dot represents a variation site. N number represents biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or a 20μM Hydrogen Peroxide (H₂O₂) treatment. The

DNA samples extracted from the cells were then amplified and purified before being transferred to the Swansea University Sequencing department where were sequenced. The Cambridge Reference gene was used for comparison and VAF values was calculated. Graphical representation was carried out using RStudio by Dr Matt Hitchings.

Comparisons between both the normal and high glucose water control groups and their respective hydrogen peroxide counterparts yielded no significance as shown in Fig. 10, implying no effects seen due to high glucose or the presence of hydrogen peroxide.

3.1.6. Comparison of all sites with a VAF of 1.00% in all MCL-5 samples

Such as in section 3.1.1, comparisons of each of the MCL-5 samples were done with a threshold of 1% applied. The results of these comparisons, as shown in Fig.20, there were no comparisons of interest that produced a significant change (full list of p-value found in Appendix 6.4.1)

Pairwise comparison of mitochondrial genome wide VAF above 1% in MCL-5 cell line

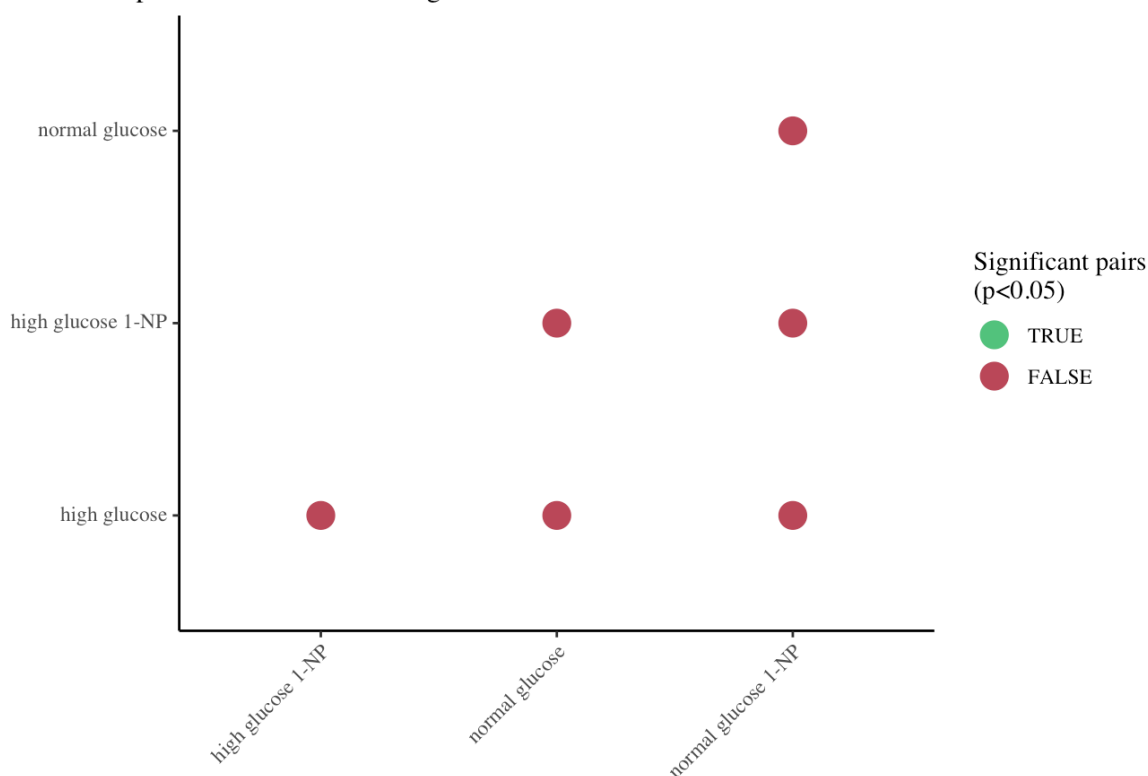
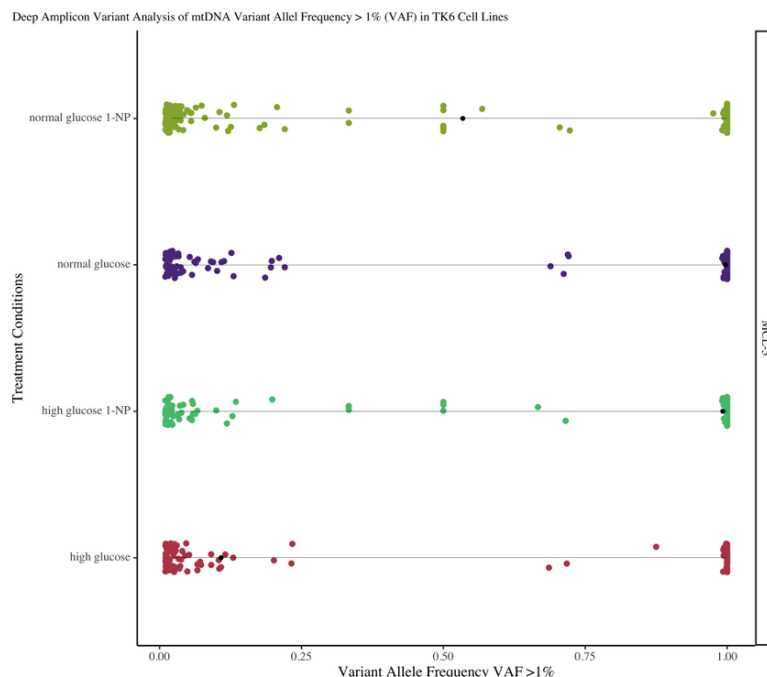


Figure 20: Comparisons of the average VAF value above 1% of MCL-5 sample groups grown under normal or high glucose concentration with (1% (v/v) DMSO control or with the test chemicals 1-nitropyrene (1-NP) (80.9µM). Each dot represents a comparison of all the data points above the 1% of two groups being compared for significance, with the significant or true results ($p<0.05$) are represented as green circles, while non-significant or false results are represented as red dots. N numbers represent biological replicates. Graphical representation of these comparisons was carried out using RStudio by Dr Matt Hitchings.

3.1.7 The effect of high glucose within the MCL-5 control groups.

Comparison of the values of the VAF sites between normal glucose control group and high glucose DMSO control group, as shown in Fig. 21. A, show there is a slight increase VAF sites below a VAF of 0.25% in the high glucose control; this can be further seen within Fig. 21.B, particular below a base location of 10000bp within the genome. Looking at the base locations of each site of variation within the genome within both samples, they appear very similar in both samples, with there being a few additional points within the high glucose group. Above a VAF of 0.5%, there does not appear to be much difference between both number of VAF sites or their base locations within the genome. Finally, above a base location of 15000bp within both normal and high glucose control groups, there appears to be no variations in location of sites at a VAF of 1.00%. Finally, comparing the average VAF values for each group of samples, there appears to be a noticeable decrease in the high glucose DMSO control group compared to the normal glucose DMSO control, however it was non-significant ($p=0.205$).

A



B

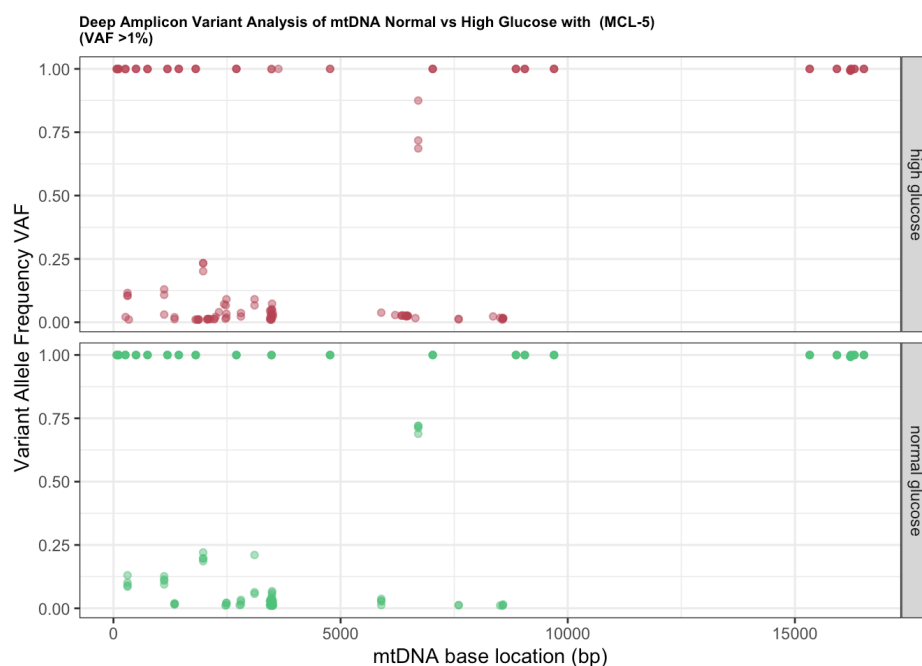


Figure 21: A) Comparison of Variant Allele Frequency (VAF) values above 1% within the MCL-5 samples grown under a normal or high glucose concentration within the DMSO control groups (n=4) or under 1-nitropyrene (1-NP) (n=4). Each dot represents a variation site while average VAF observed are represented as a singular black dot within the data while the standard deviation is represented by solid black lines. **B)** Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of MCL-5 DMSO control samples grown under a normal or high glucose concentration (n=4). Each dot represents a variation site. N number represents biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received (1% (v/v) DMSO treatment or an 80.9µM 1-nitropyrene (1-NP) treatment. The DNA samples extracted from the cells were then amplified and purified before being transferred to the Swansea University Sequencing department where were

sequenced. The Cambridge Reference gene was used for comparison and VAF values was calculated. Graphical representation was carried out using RStudio by Dr Matt Hitchings.

3.1.8. The effect of high glucose in the presence of 1-NP

Comparison of the normal and high glucose 1-NP groups there appears to be more sites of variation with a VAF of below 0.50% within the normal glucose group (Fig.22. A). As shown in Fig.22.B, the increased number of VAF sites within in the normal glucose 1-NP sample (particularly below a VAF value of 0.25%) appear to be increased, particularly at a base location of between 5000bp and 7500bp. Furthermore, above a base location of 15000bp, there appears to be variations with a VAF of below 1.00% present in the normal glucose group only. Above a VAF of 0.50%, the number of sites within both samples and their base locations appear to be very similar while also appearing at similar VAF values. While there are more sites within the normal glucose 1-NP group, the average VAF for each sample group is a larger within the high glucose 1-NP group; there was no significant difference found within this comparison (p-values= 0.738).

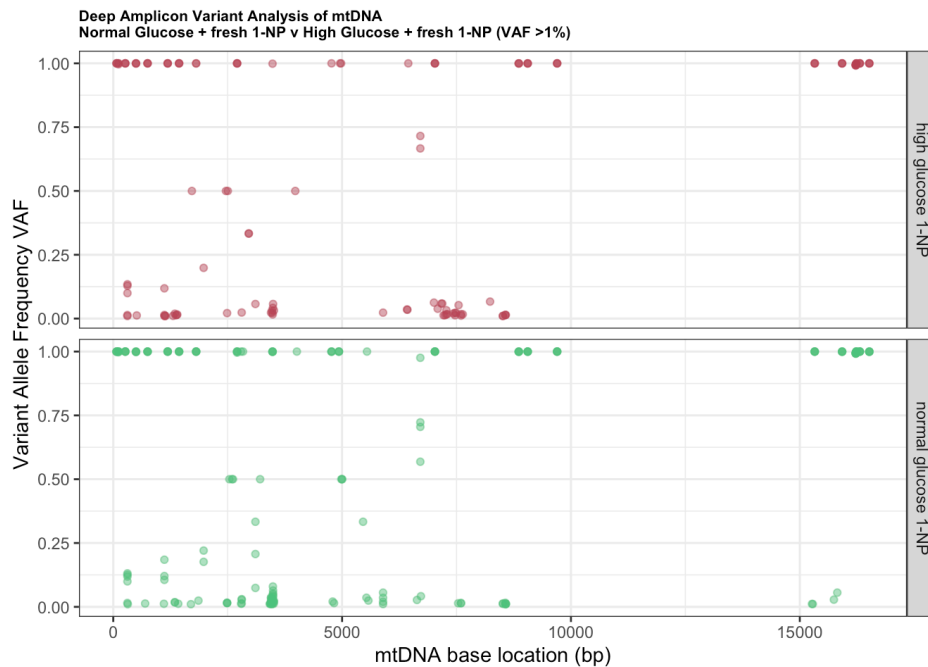


Figure 22: Comparison of the base location (bp) of Variant Allele Frequency (VAF) points above 1% within the mitochondrial genome of MCL-5 Nitrotyrene (1-NP) samples grown under a normal or high glucose concentration (n=4). Each dot represents a variation site. N number represents biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups received 80.9µM 1-nitrotyrene (1-NP) treatment. The DNA samples extracted from the cells were then amplified and purified before being transferred to the Swansea University Sequencing department where were sequenced. The Cambridge Reference gene was used for comparison and VAF values was calculated. Graphical representation was carried out using RStudio by Dr Matt Hitchings.

3.1.9. Types of mutations within the sample groups

3.1.9.1 Absolute variant counts within samples

There are a total of 1,912 variant sites within the mitochondrial genome across all samples and treatment groups. VAF values have been compared between 3.1.1-3.1.9, while in this section the absolute variant counts (Fig.23), which represents the number of total variants within the samples, are being compared to investigate the number of mutations present.

Within both the comparison of high glucose DMSO against normal glucose DMSO and high glucose DEHP against normal glucose DEHP, which both produced a significant result in the comparison of VAF sites, there appears to be an increase in the average absolute variant counts within the high glucose groups compared to the normal glucose groups. Within the DMSO comparison there a small difference between the average absolute variant count, compared to the difference seen between the DEHPs which is larger. This in conjunction with observations in sections 3.1.2 and 3.1.4 which show that within the DMSO and DEHP comparison, there is a decrease in average VAF but appeared to be an increased number of sites within the lower VAF range, further suggests an increase in low variation frequencies present within both high glucose groups; the similarities within the findings of differing glucose concentrations under DMSO and DEHP implies high glucose may have a similar effect with both chemicals.

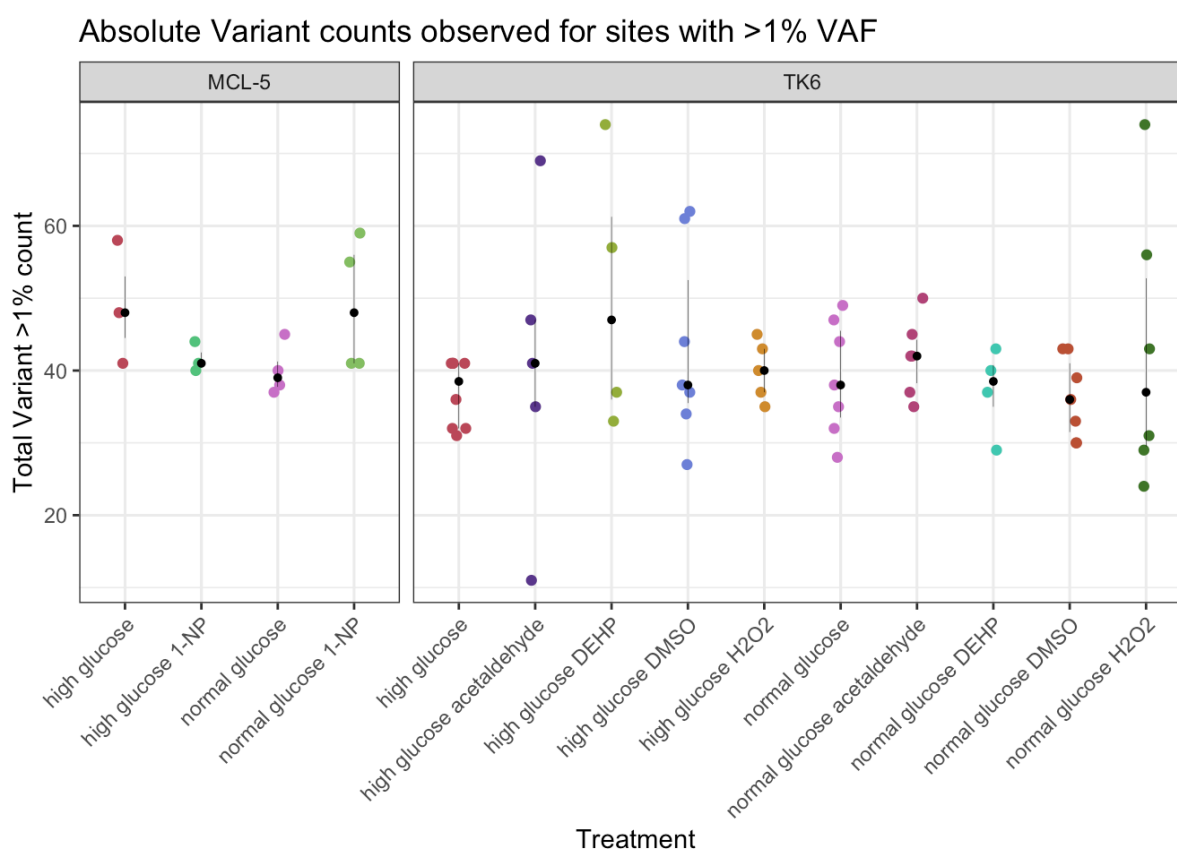


Figure 23: Comparison of the absolute variant counts within samples with a VAF value above 1% of TK6 sample groups grown under normal (10mM) or high glucose (29mM) concentration with a water (n=7) or DMSO control (n=7) ((1% (v/v)) or with one the test chemicals, acetaldehyde (1000µM) (n=7), di(ethylhexyl) phthalate (DEHP) (2000µM) (n=5) or hydrogen peroxide (H₂O₂) (20µM) (n=7) and of MCL-5 sample groups grown under normal or high glucose concentration with DMSO control (1% (v/v)) (n=4) or with the test chemicals 1-nitropyrene (1-NP) (80.9µM) (n=4). Each variant count for each sample is represented by a coloured dot while

the average total variant counts observed are represented as a singular black dot within the data while the standard deviation is represented by solid black lines.

While in DEHP and DMSO samples there was an increase in absolute variant counts within the genome, and therefore an increase in the number of VAF sites present; within the other significant comparison, normal glucose acetaldehyde against normal glucose (section 3.1.3), where there was a decrease in VAF sites in the presence of acetaldehyde, the average absolute variant within normal glucose acetaldehyde appears to be larger at approximately 41 compared to the approximate count of 39 within the seen in the normal glucose group. This slight increase in average absolute variant count alongside the observations described in 3.2.5 would suggest that suggest there is a small amount of variation due to the presence of acetaldehyde.

3.1.9.2 The type of variants present

As well as the absolute variant counts, Single Nucleotide Polymorphisms (SNPs) and Insertion and Deletion (INDEL) counts were taken for each sample tested within each group, as shown within Fig.24 to investigate the types of variations within the samples; particularly in the comparisons that produced significance during the comparison of VAF. Within the comparison of high glucose DMSO against normal glucose DMSO while there appeared to be an increase in VAF sites and absolute variant counts within the high glucose group; there appears to be no noticeable difference between the SNP and INDEL counts with the averages within the normal and high glucose also appearing to be very similar (Fig,24). All of this would suggest that within the DMSO comparison, there is little difference between the presence of SNPs and INDELs present within the samples and that the variation present is not because of these mutations.

During the comparison of normal and high glucose concentration DEHP groups, there was an increase in VAF sites and absolute variant counts; in the normal glucose DEHP group, SNP count per sample and the average SNP count appear to be equal and slightly lower than that of the high glucose DEHP group (Fig. 24.A). Comparison of the INDEL counts (Fig. 24.B) show that within in the normal glucose DEHP group, all samples appears to have an INDEL count of zero; this is mostly true in the high glucose DEHP group apart from one sample with a count of 1. Furthermore, the average count in this figure between the same two samples appear to be very similar. All of this would suggest that in the presence of high glucose and DEHP, there appears to be a SNPs mainly present due to the presence of high glucose.

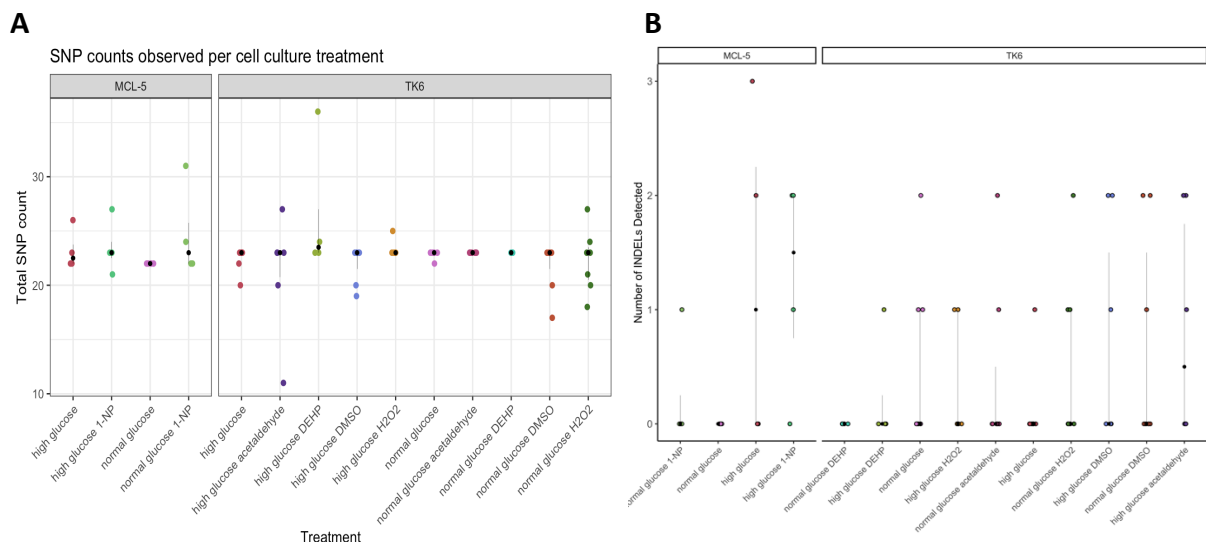


Figure 24: A) Comparison of the Single nucleotide polymorphisms (SNPs) count within samples with a VAF value above 1% of TK6 sample groups grown under normal or high glucose concentration with a water (n=7) or DMSO control (n=7) or with one the test chemicals, acetaldehyde (1000 μ M) (n=7), di(ethylhexyl) phthalate (DEHP) (2000 μ M) (n=5) or hydrogen peroxide (H₂O₂) (20 μ M) (n=7) and of MCL-5 sample groups grown under normal (10mM) or high glucose (29mM) concentration with DMSO control (n=4) or with the test chemicals 1-nitropyrene (1-NP) (80.9 μ M) (n=4). B) Comparison of the Insertion and Deletion (INDEL) counts within samples with a VAF value above 1% of TK6 sample groups grown under normal or high glucose concentration with a water or DMSO control or with one the test chemicals, acetaldehyde, di(ethylhexyl) phthalate (DEHP) or hydrogen peroxide (H₂O₂) and of MCL-5 sample groups grown under normal or high glucose concentration with DMSO control or with the test chemicals 1-nitropyrene (1-NP). Each SNP and INDEL for each sample is represented by a coloured dot while the average SNP and INDEL counts observed are represented as a singular black dot within the data while the standard deviation is represented by solid black lines.

Finally, the comparison of normal glucose acetaldehyde against normal glucose appears to show similar findings as the previous comparisons. Within Fig. 24.A, the average SNP counts for both samples again appear to be equal at between 23-24.. When comparing the INDEL counts within Fig. 24.B between these groups, within both the normal glucose and normal glucose acetaldehyde groups, many of the samples have an INDEL count of 0; the average of both groups being the same at 0. However, there are samples within each sample group with a count greater than 0 in both groups, with the normal glucose having a higher number of samples with a count above 1 than normal glucose acetaldehyde. All of this suggests that, as before, there are only small differences between the two groups which suggests that the variation in the presence of acetaldehyde is not due to these types of mutations.

3.1.10 Summary of NGS results discussed.

3.1.11.1 Summary of results discussed within the TK6 samples.

Table 10: Summary of the comparisons of interest illustrated within chapter 3 sections 3.3.1- 3.3.6 and 3.3.10 in all TK6 samples.

Samples of comparison	Observations
Normal Glucose v High Glucose	Within the normal glucose sample, there is a higher number of VAF sites within the genome compared to the high glucose group, with many of the additional points falling at the lower range values (>0.00%-0.25%). No significance was found within this comparison ($p=0.556$). Looking at the absolute variant counts within each sample, there appears to be little difference in the average counts in both types of mutations looked at (SNPs and INDELs).
Normal Glucose DMSO v High Glucose DMSO	Within the normal glucose DMSO group, there appears to be more VAF sites within the lower values between 5000bp-1000bp whereas between 0bp-5000bp the opposite can be seen within the high glucose DMSO group. Additionally, above 15000bp, there is a noticeable increase in the number of VAF sites in the high glucose DMSO group. There is also a slight decrease in the average VAF in the high glucose DMSO which was found to significant with the lower threshold of 1% ($p= 0.003$). Furthermore, there was only a small increase in the absolute variant counts within the high glucose DMSO group, with little difference between the SNP and INDEL counts between the two samples.
Normal Glucose Acetaldehyde v High Glucose Acetaldehyde	Comparison of both samples found there to be little difference in the VAF sites position and value above a VAF of 0.5%. However, within the high glucose acetaldehyde group, there was a noticeable increase in the number of VAF sites below 0.25% across the genome sections sequenced. Additionally, there was also a decrease in the average VAF value within the high glucose group compared to the normal glucose group; however, this observation was non-significant ($p= 0.227$).
Normal Glucose v Normal Glucose Acetaldehyde	In comparison to the normal glucose control, there is a decrease of VAF sites across all values within the normal glucose acetaldehyde group, particularly below a VAF value of 0.25%. Additionally, most of the variation sites within the normal glucose acetaldehyde group are also in very similar base locations as those in the normal glucose group. The average VAF value appears to be very similar between the two groups. Statistical analysis of this comparison found there to be significance with the lower VAF threshold of 1% ($p= 0.01$). Looking at the absolute variant counts between these groups, there appears to be a small increase in the normal acetaldehyde. Comparing the types of mutations, there appears to be little difference in the SNP and INDEL counts between the two samples.

Normal Glucose DEHP v High Glucose DEHP	Across the genome, there appears to be a higher number of variation sites within the high glucose DEHP compared to the normal glucose DEHP group, although many of those sites within the high glucose group, particularly between 2500bp and 5000bp appear to be in a similar position to those in the normal glucose group. Above a base location of 15000bp, there appears to be the most noticeable difference between normal and high glucose DEHP. Within this region of the genome, there appears to be variation sites with a VAF of below 1.00% in the high glucose DEHP group. Looking at the average VAF values for each of the sample groups, there appears to be a significant decrease in the high glucose DEHP group compared to the normal glucose DEHP group with the lower threshold of 1% ($p = 0.005$). Samples that meet this threshold have also shown that there is a noticeable increase in the absolute variant counts within the high glucose DEHP compared to the normal glucose DEHP, with the SNP count being increased/decreased while the INDEL count appears to be similar between samples.
Normal Glucose H ₂ O ₂ v High Glucose H ₂ O ₂	Comparing both normal and high glucose H₂O₂ groups there appears to be a higher proportion of VAF sites at the lower values (>0.00%-0.50%) within the normal glucose H₂O₂ group; particularly above 15000bp. There also appeared to be no change to the average VAF value between the two groups and no significance was found in the comparison of the two groups ($p = 0.45$).

3.1.10.2 Summary of results discussed within the MCL-5 samples.

Table 12: Summary of the comparisons of interest illustrated within chapter 3 sections 3.3.7- 3.3.10 in all MCL-5 samples.

Samples of comparison	Observations
Normal Glucose DMSO v High Glucose DMSO	Comparing the two DMSO control groups, the position of many of the variation sites are similar within both groups, with the high glucose group experiencing an increased number of variation sites. This increase in variation sites is particularly noticeable between 0bp and 5000bp. In addition, below a VAF of 1.00%, there are no variation sites above 15000bp. Comparison of the average VAF value show a noticeable but non-significant decrease in the high glucose DMSO control group compared to the normal glucose DMSO control group ($p=0.205$).
Normal Glucose 1-NP v High Glucose 1-NP	Within the high glucose 1-NP sample group, there is a reduced number of variation sites across the genome and the range of VAF values, this is particularly noticeable below a VAF value of 0.25% and base location of 10000bp. Within both groups, many of the VAF sites appear to be positioned at similar locations within the genome and VAF value, the main difference is the absence of any lower VAF value sites above a location of 15000bp within the high glucose 1-NP group. Comparison of the average VAF value show a noticeable but non-significant decrease in the normal glucose 1-NP group compared to the high glucose 1-NP group ($p=0.738$).

3.2. qRT-PCR

qRT-PCR was done to measure the fold change of the number of copies of a region of the *MT-ND1* due to the presence of high glucose under various chemical environments and the effects of each chemical under differing glucose conditions. As discussed in section 2.8, the housekeeping gene, ACTB (β -actin) was used to normalise the data collected from the mtDNA chosen as it is from nDNA as it is considered to have a uniform abundance whereas mtDNA replicates (Bustin, 2002; Koramutla et al., 2016; Yasukawa & Kang, 2018).

3.2.1. *MT-ND1* copy number change within TK6 control groups.

Comparing how the fold-change of the *MT-ND1* copy number within control groups of the TK6 cell line is affected by the presence of high glucose, as presented in Figure. 25.A; in the high glucose water control group there was a 1.65- fold increase in the copy number compared to the normal glucose group ($p = 1.000$). However, in the presence of DMSO, the effect of high glucose appeared to differ compared to the effect of high glucose within the water control as there was a 0.46-fold increase in the copy number when comparing HG + DMSO against NG + DMSO (Fig. 25.B) ($p = 0.996$). To investigate the effect of DMSO, each of the glucose concentrations were compared to their respective water controls, as shown in Fig. 25.C and Fig. 25. D. When comparing NG + DMSO against the NG water control, there was a 7.66-fold increase of the *MT-ND1* gene ($p = 1.000$). This increase in the presence of DMSO is also seen under high glucose, but of a smaller magnitude at 1.09-fold increase ($p = 0.970$). However, none of these observations were statistical significance.

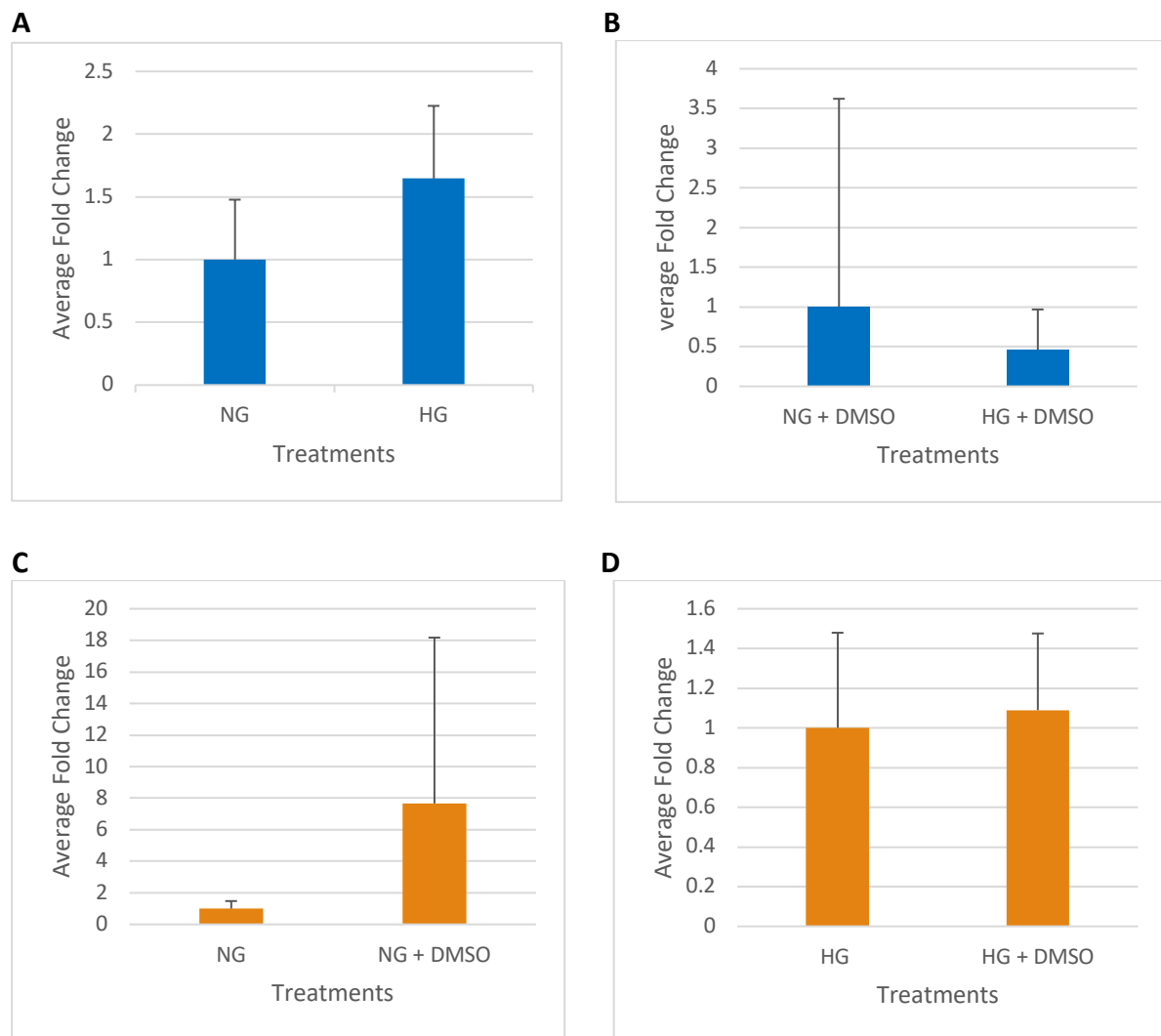


Figure 25- **A)** Representation of the fold change of the *MT-ND1* genetic region when normalised against β -*actin* within the high glucose water control (HG) samples when compared against normal glucose water control group (NG) (n=5). **B)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose DMSO control (HG + DMSO) samples when compared against normal glucose DMSO control group (NG + DMSO) (n=4). **C)** Representation of the fold change of the *MT-ND1* genetic region within the normal glucose DMSO (NG + DMSO) samples when compared against normal glucose (NG) (n=4). **D)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose DMSO control (HG + DMSO) samples when compared against high glucose water control (HG) samples respectively (n= 4). Error bars are Standard Deviation bars. Blue graphs represent glucose is being investigated whereas orange graphs represent DMSO is being investigated. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 1% (v/v) DMSO treatment. Fold-change was measured using qRT-PCR with the housekeeping gene, β -*actin*. The DNA samples extracted from the cells were diluted as appropriate and added to a master mix containing primers for either the *MT-ND1* genetic region or β -*actin* gene. The PCR plate was containing all samples alongside a serial dilution for both master mixes was run on a Bio-Rad CFX connect qt-PCR machine following a recommended protocol form the primer manufacturers.

3.2.2. *MT-ND1* copy number change within acetaldehyde.

When comparing HG + Ace against the NG + Ace group to investigate the effects of high glucose in the presence of acetaldehyde, as shown in Fig.26.A, there was a 3.96-fold increase ($p = 0.923$). When comparing this to the 1.65-fold increase seen within the glucose control groups, there is a greater fold-change increase in the presence of acetaldehyde. When investigating how the acetaldehyde itself is affecting the copy number of the *MT-ND1* gene, comparing the NG + Ace group against its NG water control, as represented in Fig. 26.B, there was a 11.08-fold increase seen ($p = 0.541$); while the comparison of HG + Ace group against the HG water control as shown in Fig. 26.C, produced a 10.54-fold increase ($p = 0.378$). This shows that in the presence of acetaldehyde, there is a greater increase in *MT-ND1* copy number due to high glucose than in the water controls. Additionally, while in the presence acetaldehyde there was also an increase in copy number, this increase was higher under normal glucose conditions. However, none of these observations yielded any statistical significance.

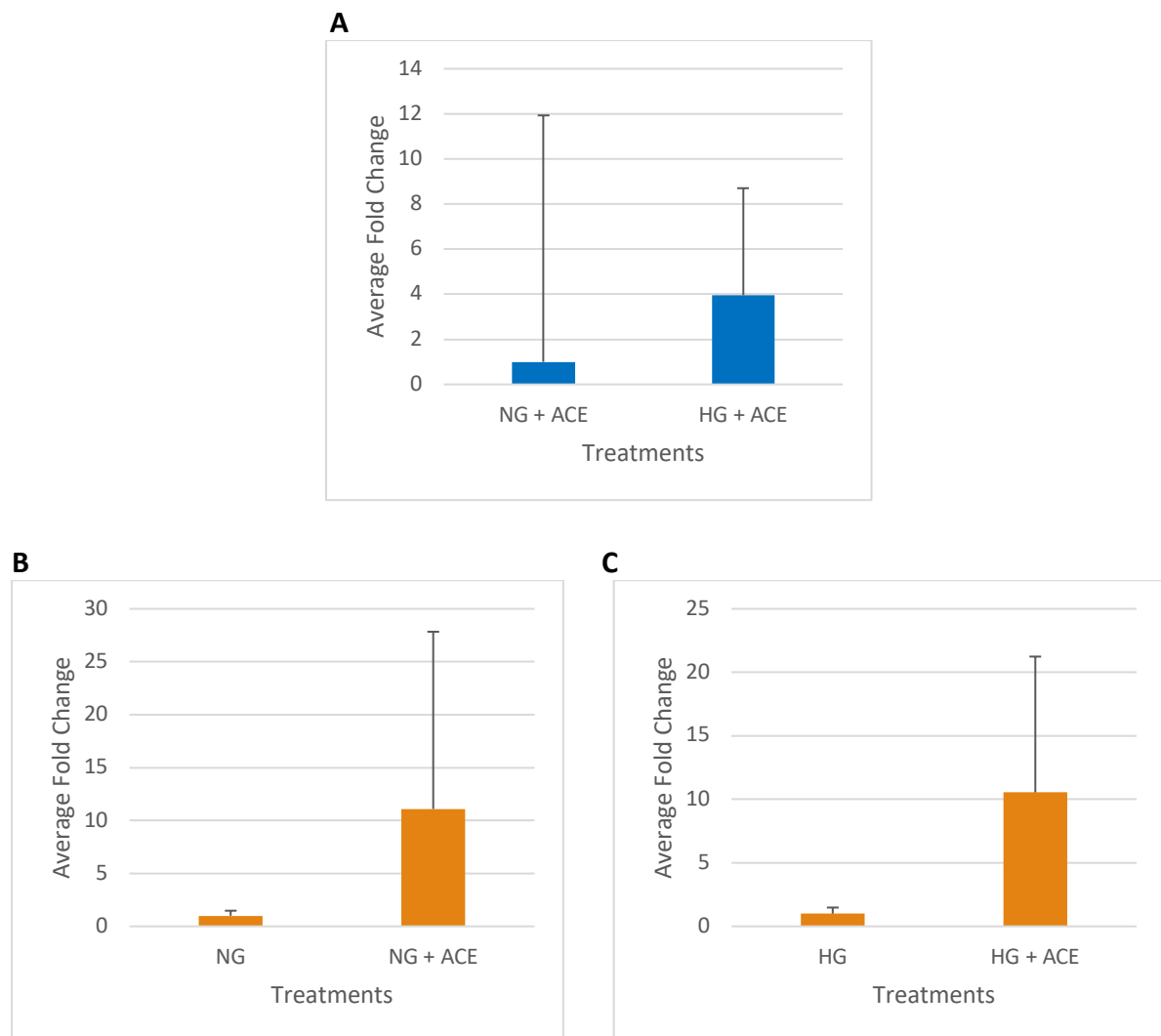


Figure 26: A) Representation of the fold change of the *MT-ND1* genetic region within the high glucose acetaldehyde (HG + Ace) samples when compared against normal glucose acetaldehyde (NG + Ace) samples (n=4). **B)** Representation of the *MT-ND1* genetic region within the the fold change of normal glucose acetaldehyde (NG + Ace) samples when compared against normal glucose water control (NG) samples (n=4) **C)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose acetaldehyde (HG + Ace) samples when compared against high glucose water control (HG) samples (n=4). Error bars are Standard Deviation bars. Blue graphs represent glucose is being investigated whereas orange graphs represent DMSO is being investigated. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 1000 μ M of acetaldehyde (Ace) treatment. Fold-change was measured using qRT-PCR with the housekeeping gene, *β -actin*. The DNA samples extracted from the cells were diluted as appropriate and added to a master mix containing primers for either the *MT-ND1* gene or *β -actin* gene. The PCR plate was containing all samples alongside a serial dilution for both master mixes was run on a Bio-Rad CFX connect qt-PCR machine following a recommended protocol form the primer manufacturers.

3.2.3. *MT-ND1* copy number change within DEHP.

When investigating how high glucose affected the copy number of the *MT-ND1* gene under DEHP conditions, as shown in Fig. 27.A, within the HG + DEHP group, there was a 2.12-fold increase compared to the NG + DEHP group ($p = 0.100$). This is the opposite of the effects seen within the DMSO control groups. This would suggest that there is a down regulation within the control group while in the presence of DEHP there is an upregulation of a similar magnitude. To see how DEHP influences the genetic region of *MT-ND1*, firstly the NG + DEHP group was compared to the NG + DMSO control (Fig. 27.B). In this comparison, there is only a slight 1.09-fold upregulation, whereas comparing the high glucose counterparts (as shown in Fig. 27.C), there is a 5.50-fold increase of the *MT-ND1* gene copy number ($p = 0.445$ and 0.480 respectively). However, none of these observations yielded any statistical significance.

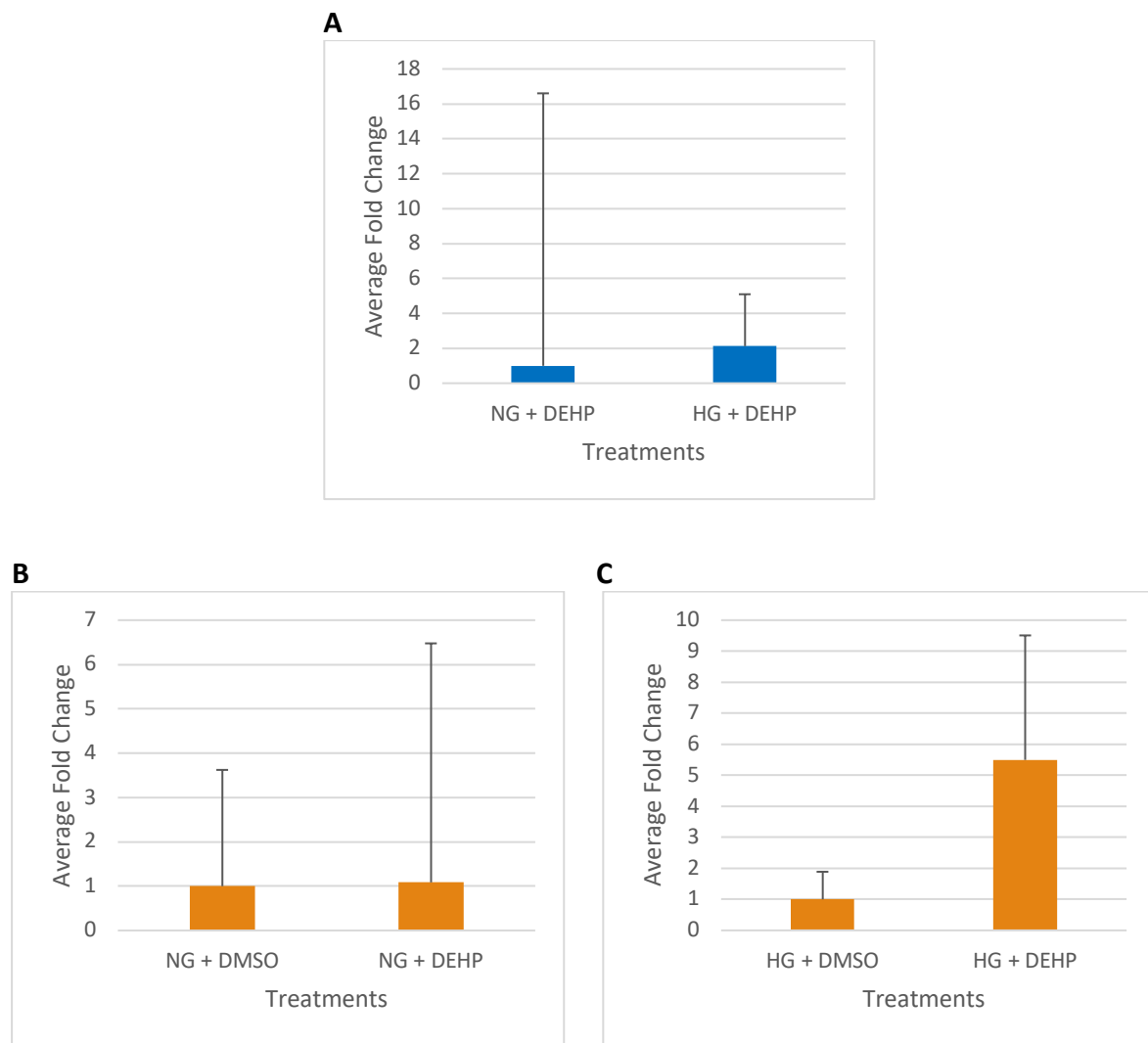


Figure 27: **A)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose di(ethylhexyl) phthalate (HG + DEHP) samples when compared against normal glucose di(ethylhexyl) phthalate (NG + DEHP) samples (n=4). **B)** Representation of the fold change of the *MT-ND1* genetic region within the normal glucose di(ethylhexyl) phthalate (NG + DEHP) samples when compared against normal glucose DMSO control (NG + DMSO) control (HG + DMSO) samples (n=4). **C)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose di(ethylhexyl) phthalate (HG + DEHP) samples when compared high glucose DMSO control (HG + DMSO) samples (n=4). Error bars are Standard Deviation bars. Blue graphs represent glucose is being investigated whereas orange graphs represent DMSO is being investigated. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received (1% (v/v) DMSO treatment or a 2000 μ M di(ethylhexyl) phthalate (DEHP) treatment. Fold-change was measured using qRT-PCR with the housekeeping gene, *β -actin*. The DNA samples extracted from the cells were diluted as appropriate and added to a master mix containing primers for either the *MT-ND1* gene or *β -actin* gene. The PCR plate was containing all samples alongside a serial dilution for both master mixes was run on a Bio-Rad CFX connect qt-PCR machine following a recommended protocol form the primer manufacturers.

3.2.4. *MT-ND1* copy number change within hydrogen peroxide.

To see the effects of high glucose under hydrogen peroxide, as represented in Fig. 28.A, when comparing the HG + H₂O₂ *MT-ND1* copy number against that of the NG + H₂O₂ group, there appears to be a 7.69-fold increase ($p = 0.990$). These are similar findings as seen within the water control groups, except the upregulation within the hydrogen peroxide group being noticeably higher in magnitude. Looking at Fig. 28.B when NG + H₂O₂ was compared against its NG water control, there was a 3.74-fold increase, which is over half that seen when comparing the HG + H₂O₂ group against the HG water control, as shown in Fig. 28. C, which had an 8.14-fold increase ($p = 0.668$ and 0.662 respectively). This shows that hydrogen peroxide under high glucose has a greater copy number than the high glucose in the presence of the hydrogen peroxide. However, none of these observations yielded any statistical significance.

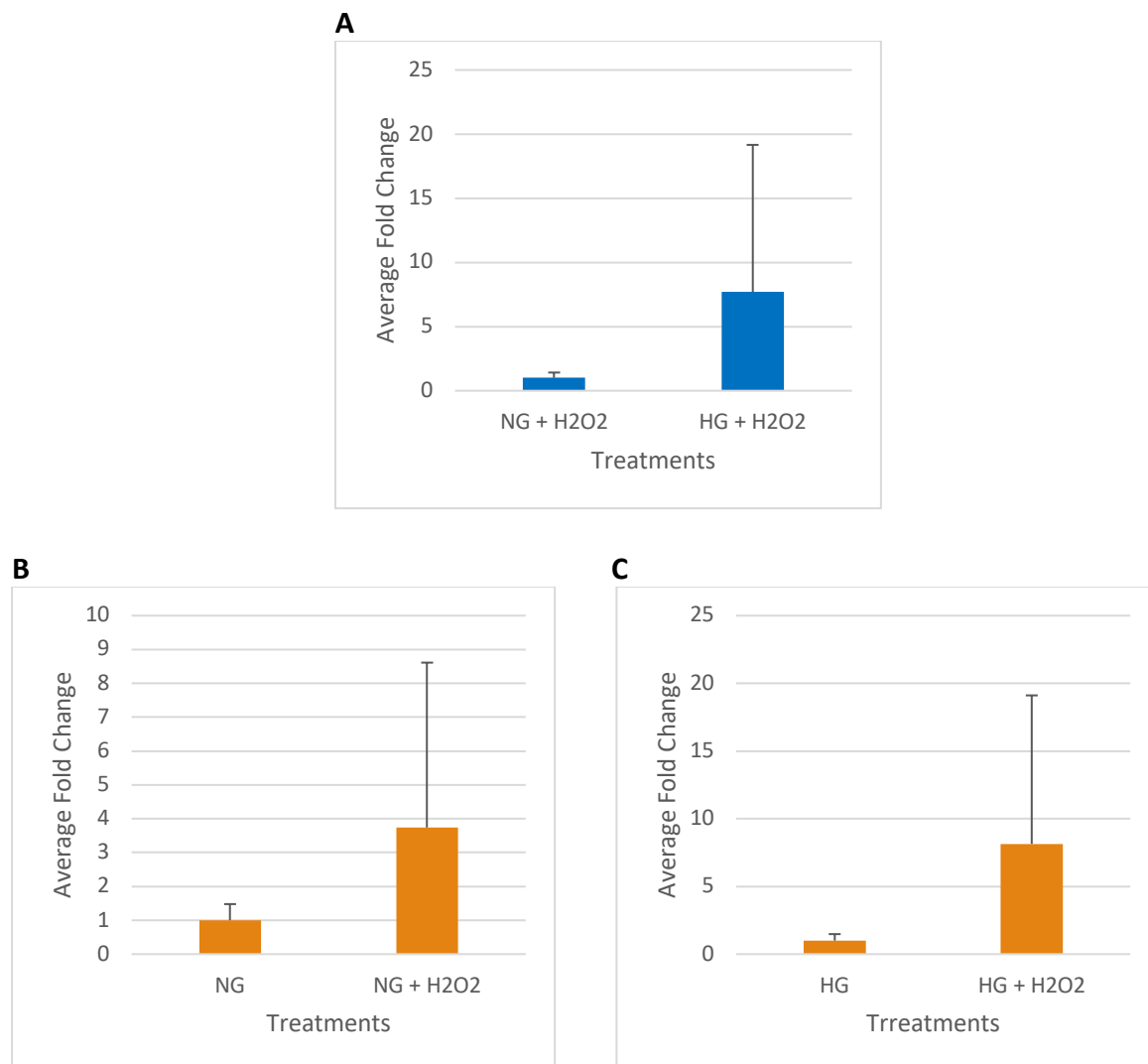


Figure 28: **A)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose hydrogen peroxide (HG + H₂O₂) samples when compared against normal glucose hydrogen peroxide (NG + H₂O₂) samples (n=5). **B)** Representation of the fold change of the *MT-ND1* genetic region within the normal glucose hydrogen peroxide (NG + H₂O₂) samples when compared against high glucose water control (HG) samples respectively (n=5). **C)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose hydrogen peroxide (HG + H₂O₂) samples when compared against high glucose water control (HG) samples. Error bars are Standard Deviation bars (n=5). Blue graphs represent glucose is being investigated whereas orange graphs represent DMSO is being investigated. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatment or a 20μM of Hydrogen Peroxide (H₂O₂) treatment. Fold-change was measured using qRT-PCR with the housekeeping gene, *β-actin*. The DNA samples extracted from the cells were diluted as appropriate and added to a master mix containing primers for either the *MT-ND1* gene or *β-actin* gene. The PCR plate was containing all samples alongside a serial dilution for both master mixes was run on a Bio-Rad CFX connect qt-PCR machine following a recommended protocol form the primer manufacturers.

3.2.5. *MT-ND1* copy number change within MCL-5 samples.

Looking at the *MT-ND1* copy number within the control DMSO groups of the MCL-5 cell line (Fig. 29. A), within the HG + DMSO group there appears to be a 2.87-fold increase when compared against the NG + DMSO group ($p = 0.987$). Within the *MT-ND1* gene in the 1-NP groups of Fig. 29.B, there also appears to be upregulation of 1.41-fold in the HG + 1-NP group compared to be NG + 1-NP ($p = 0.594$). This shows that in the presence of the DMSO and 1-NP, there is an increased copy number in the high glucose groups, however there is a larger increase in the DMSO control. To look at the effect of 1-NP in the presence of different glucose concentrations, each 1-NP was compared against their respective controls. When comparing HG + 1-NP group to its HG + DMSO control (shown in Fig.29.D), there appears to a 1.65-fold change in the *MT-ND1* copy number, which is nearly half the upregulation seen within the NG + 1-NP comparison which yielded a 3.09-fold increase compared to NG + DMSO (shown in Fig.29.C) ($p = 0.621$ and 0.976 respectively). This shows that in the presence of normal glucose and 1-NP, there is a greater upregulation than that of the high glucose and 1-NP. However, none of these observations yielded any statistical significance.

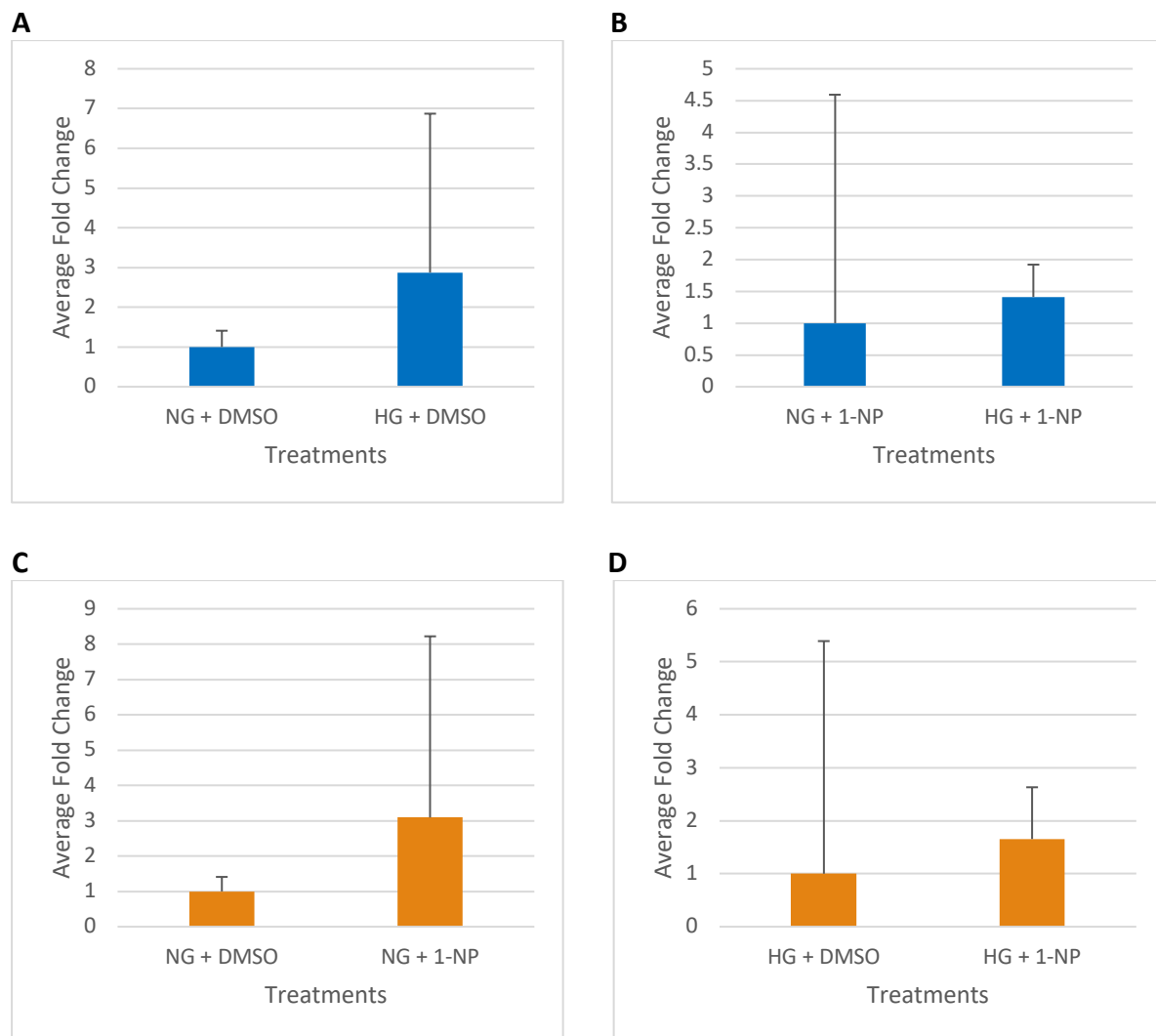


Figure 29: A) Representation of the fold change of the *MT-ND1* genetic region within the high glucose DMSO control (HG + DMSO) samples when compared against normal glucose DMSO control (NG + DMSO) (n=4). **B)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose 1-nitropyrene (HG + 1-NP) samples when compared against normal glucose 1-nitropyrene (NG + 1-NP) samples (n=4). **C)** Representation of the fold change of the *MT-ND1* genetic region within the normal glucose 1-nitropyrene (NG + 1-NP) samples when compared against normal glucose DMSO control (NG + DMSO) samples (n=4). **D)** Representation of the fold change of the *MT-ND1* genetic region within the high glucose 1-nitropyrene (HG + 1-NP) samples when compared high glucose DMSO control (HG + DMSO) samples (n=4). Error bars are Standard Deviation bars. Blue graphs represent glucose is being investigated whereas orange graphs represent DMSO is being investigated. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received (1% (v/v) DMSO treatment or an 80.9 μ M 1-nitropyrene (1-NP) treatment. Fold-change was measured using qRT-PCR with the housekeeping gene, *β -actin*. The DNA samples extracted from the cells were diluted as appropriate and added to a master mix containing primers for either the *MT-ND1* gene or *β -actin* gene. The PCR plate was containing all samples alongside a serial dilution for both master mixes was run on a Bio-Rad CFX connect qt-PCR machine following a recommended protocol form the primer manufacturers.

Chapter 4- The effect of environment chemical exposure and hyperglycaemia on the mitochondrial genome

4.1 Fluorescence Microscopy

Fluorescence microscopy was used to obtain images to allow the measurement of changes in mitochondrial mass. Fluorescence Microscopy was carried out on live cells stained with MitoTracker Green only, with between 24 and 32 images taken for each treatment over 3 or 4 replicates respectively. The mitochondria were imaged in this study using MitoTracker Green as stains such as DAPI do not work well on live cells due to its inability to efficiently pass through the membrane in live cells (Wallberg et al., 2016). Additionally, as DAPI can be toxic, there may be false positive changes that may occur that could be due to the presence of DAPI rather than the conditions we are measuring (Azqueta et al., 2022). Furthermore, DAPI can also be used to stain both nDNA and mtDNA while MitoTracker green is selectively accumulates in the mitochondrial matrix (Dellinger & Gèze, 2001; Presley et al., 2003). As this is a pilot study, only a mitochondrial stain was used as this was the main area of interest, and the only thing being measured within the CellProfiler pipeline.

4.1.1 Effect of high glucose on intensity changes within the mitochondria in TK6 control groups

Comparison of the differing glucose concentrations used in the water control groups (Fig.30) show the average intensity within the high glucose water control appeared to be 14.40% lower than that of the normal glucose water control ($p=0.984$). Within the second control group, DMSO, the same trend appears, however, the decrease between the NG + DMSO and HG + DMSO was of a higher magnitude at 29.66% ($p=0.689$). Examples of these comparisons can be found within Fig.31, where comparison between the normal and high glucose water control images (Fig.31.A and Fig.31.C) indicated brighter regions within the cells in Fig.31.A; additionally, the same can be seen in Fig.31.B and Fig.31.D for the DMSO controls.

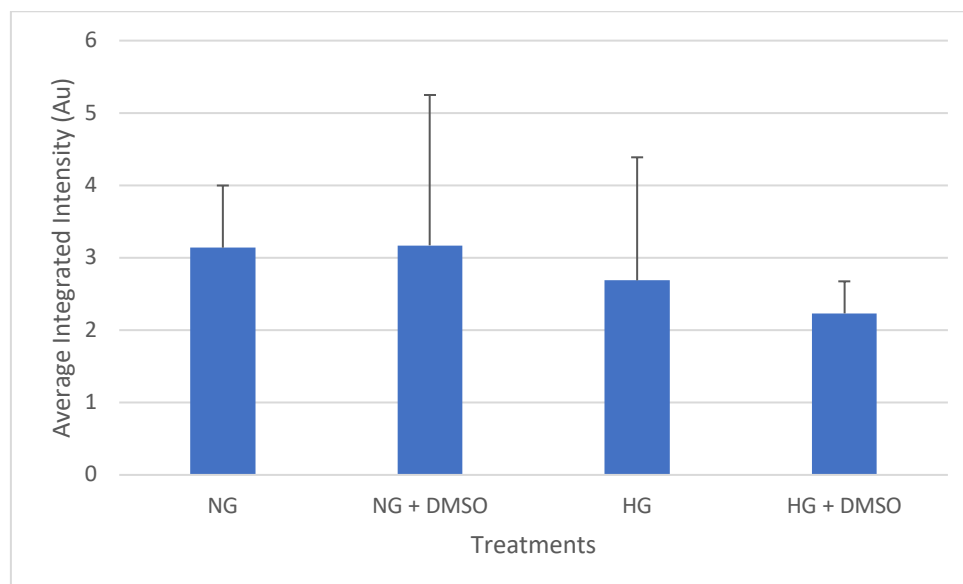


Figure 30: Average fluorescence intensity for normal glucose and high glucose water controls (NG and HG respectively) ($n=4$) and the normal glucose and high glucose DMSO control (NG + DMSO and HG + DMSO) ($n=4$). The intensities were measured using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. A total of 32 images were used for each of the samples. Error bars are Standard

Deviation bars. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 1% (v/v) DMSO treatment. 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

To investigate the effect of DMSO on mitochondrial mass, the water and DMSO control groups at the same glucose concentrations were compared. Comparing, NG against NG + DMSO average integrated intensities (Fig.30) there was a non-significant increase of 1.03% within the NG + DMSO group compared to the NG group ($p=1.00$). Comparison of two high glucose controls show HG + DMSO has an average integrated intensity 16.98% lower than that of the HG water control group ($p=0.991$). Examples can be seen in Fig. 31, within in the NG sample (Fig.31.A) have a dimmer appearance than in those of the NG + DMSO (Fig.31.B) groups whereas the opposite can be seen in the comparison of HG (Fig.31.C) and HG + DMSO (Fig.31.D). However, these changes were not statistically significant.

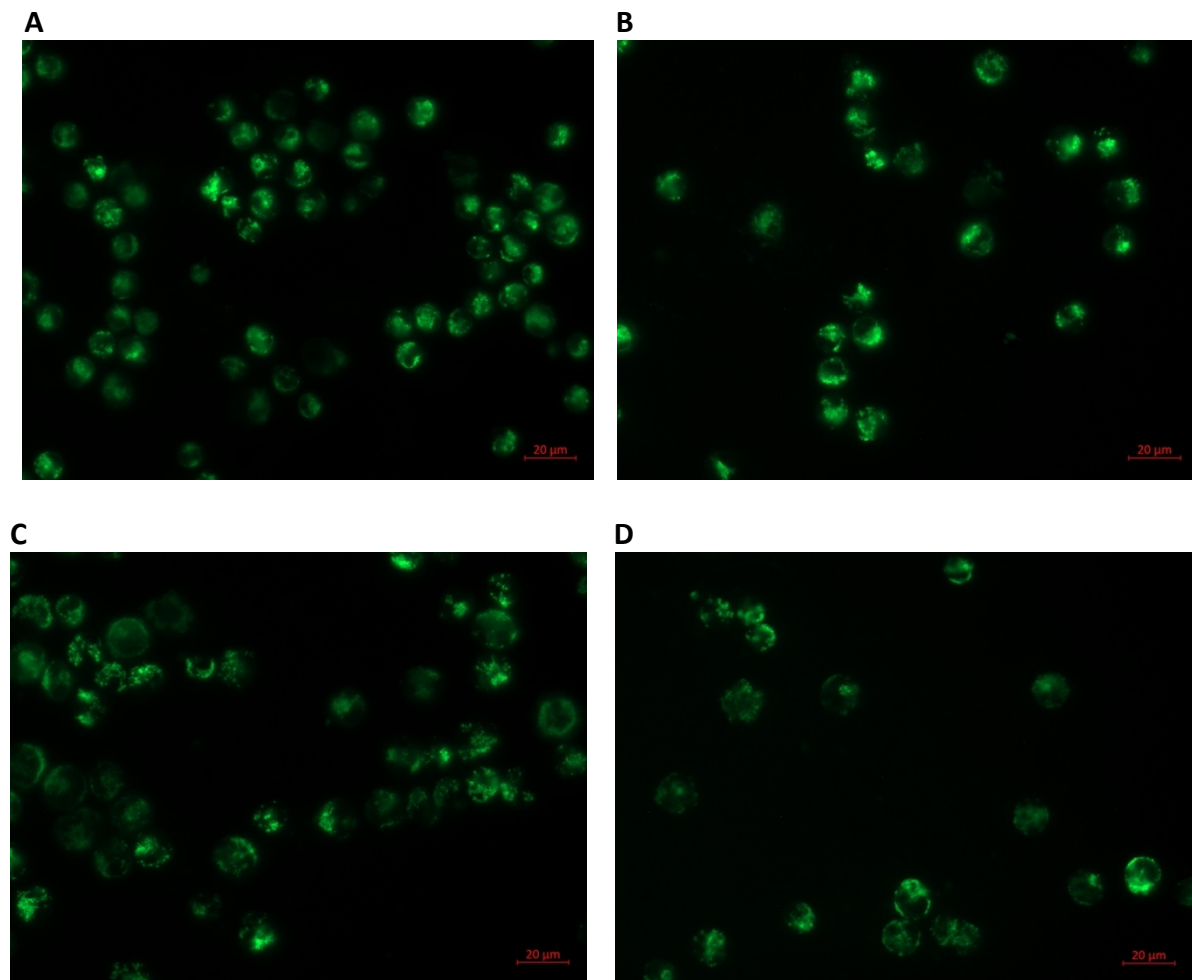


Figure 31: A) Fluorescence microscope image taken from experiments carried out on normal glucose water control (NG) within TK6 cells. B) Fluorescence microscope image taken from experiments carried out on normal glucose + DMSO (NG + DMSO) control within TK6 cells. C) Fluorescence microscope image taken from

experiments carried out on high glucose water control (HG) within TK6 cells. D) Fluorescence microscope image taken from experiments carried out on high glucose + DMSO (HG + DMSO) treatment within TK6 cells. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 1% (v/v) DMSO treatment. 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

The trends within the average intensity data were also investigated through quintiles created using various controls and compared against appropriate treatment groups. When comparing the trends within the data for the water and DMSO control as shown in Fig.32, there is a noticeable but non-significant increase of 54.53% in the frequency of mitochondria that fall within the lowest integrated intensity category when comparing the HG group against the NG group (p-value= 1.000). However, the same comparison of the DMSO controls indicated a decrease of 11.22% to the frequency of cells within those mitochondria with an integrated intensity that falls within the low category (p-value= 0.931). Comparisons of each glucose concentration DMSO group when compared to their respective glucose water control group also found no significant changes within the quintiles produced; further information can be found within appendix 6.6.1.

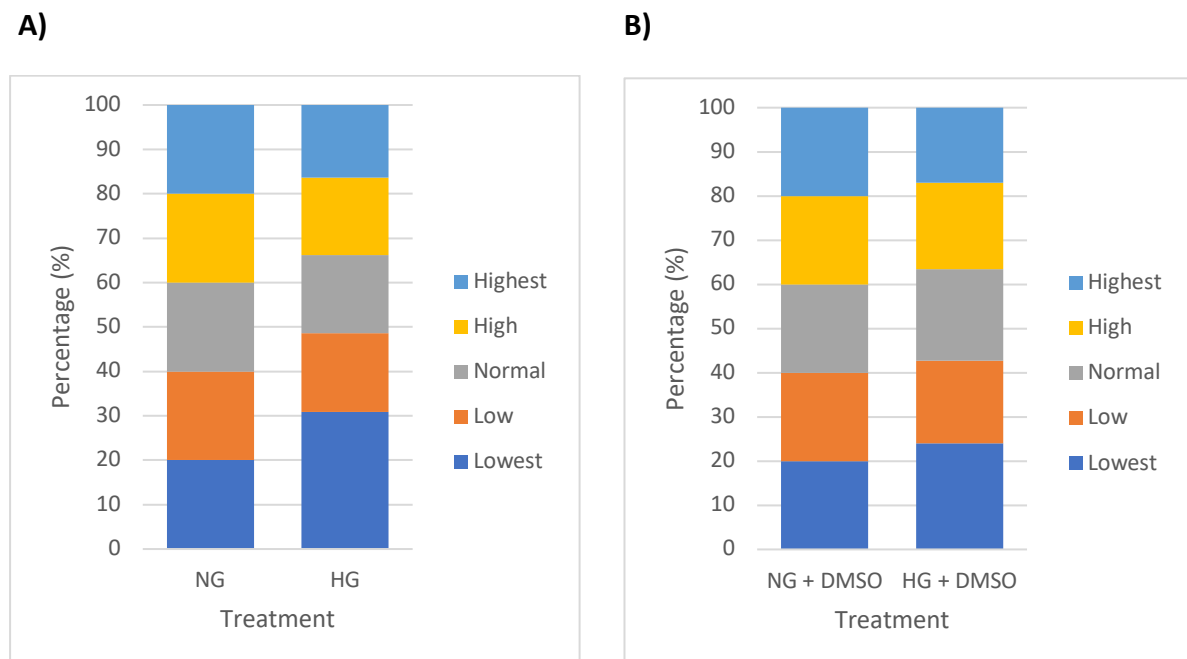


Figure 32: A) The intensity changes seen within the data obtained for normal glucose control in comparison to the high glucose controls (NG and HG respectively) (n=4) **B)** The intensity changes seen within the data obtained for normal glucose and DMSO control in comparison to the high glucose and DMSO control (NG + DMSO and HG + DMSO) (n=4) with the fluorescence microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 32 images were used for each of the samples. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 1% (v/v) DMSO treatment. 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed

with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

4.1.2 Effect of high glucose on intensity changes within the mitochondria in acetaldehyde groups

Comparing the average data of NG + Ace to the NG water control group, as shown in Fig.33, the average intensity appears to be 6.68% within the acetaldehyde group compared to the control; this can further be seen within Fig. 34, it can be seen in NG and NG + Ace (Fig.34.A and Fig.34.B respectively) that the mitochondria within the NG + Ace group are brighter than those within the NG cells. When comparing the average intensity of the HG water control against the HG + Ace group there appears to be a larger increase of 42.89% in the average integrated intensity (p-value= 0.946). Within Fig.39, the HG + Ace group (Fig.34. D) compared to the HG group (Fig. 34. C) as a higher proportion of cells within Fig.34.B have a brighter appearance than those in Fig.34.D.

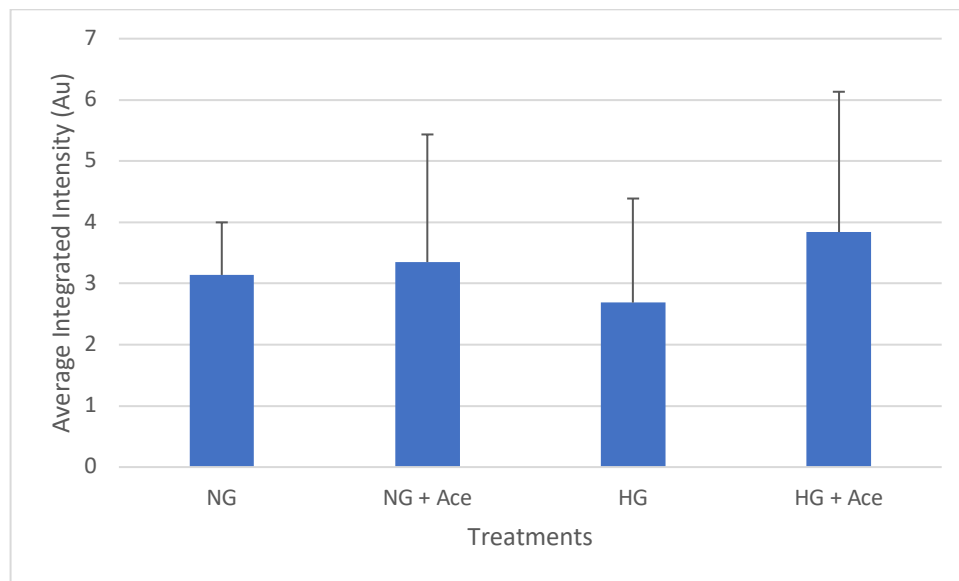


Figure 33: Average fluorescence intensity for normal glucose and high glucose water controls (NG and HG respectively) (n=4) and the normal glucose and high glucose treated with acetaldehyde (NG +Ace and HG +Ace respectively) (n=4).The intensities were measured using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells.A total of 32 images were used for each of the samples. Error bars are Standard Deviation bars. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or a 1000μM of acetaldehyde (Ace). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

To further investigate the effects of high glucose alone in the presence of acetaldehyde, HG + Ace was compared against NG + Ace; this comparison saw an increase of 14.64% in average integrated intensity within the mitochondria (p-values= 0.762).This can be seen within images shown in Fig. 31.C and Fig.31.D, as more of the cells within the Fig.31.D (HG + Ace) have a higher percentage of mitochondria with a brighter green appearance compared to the Fig.31.C (NG + Ace).

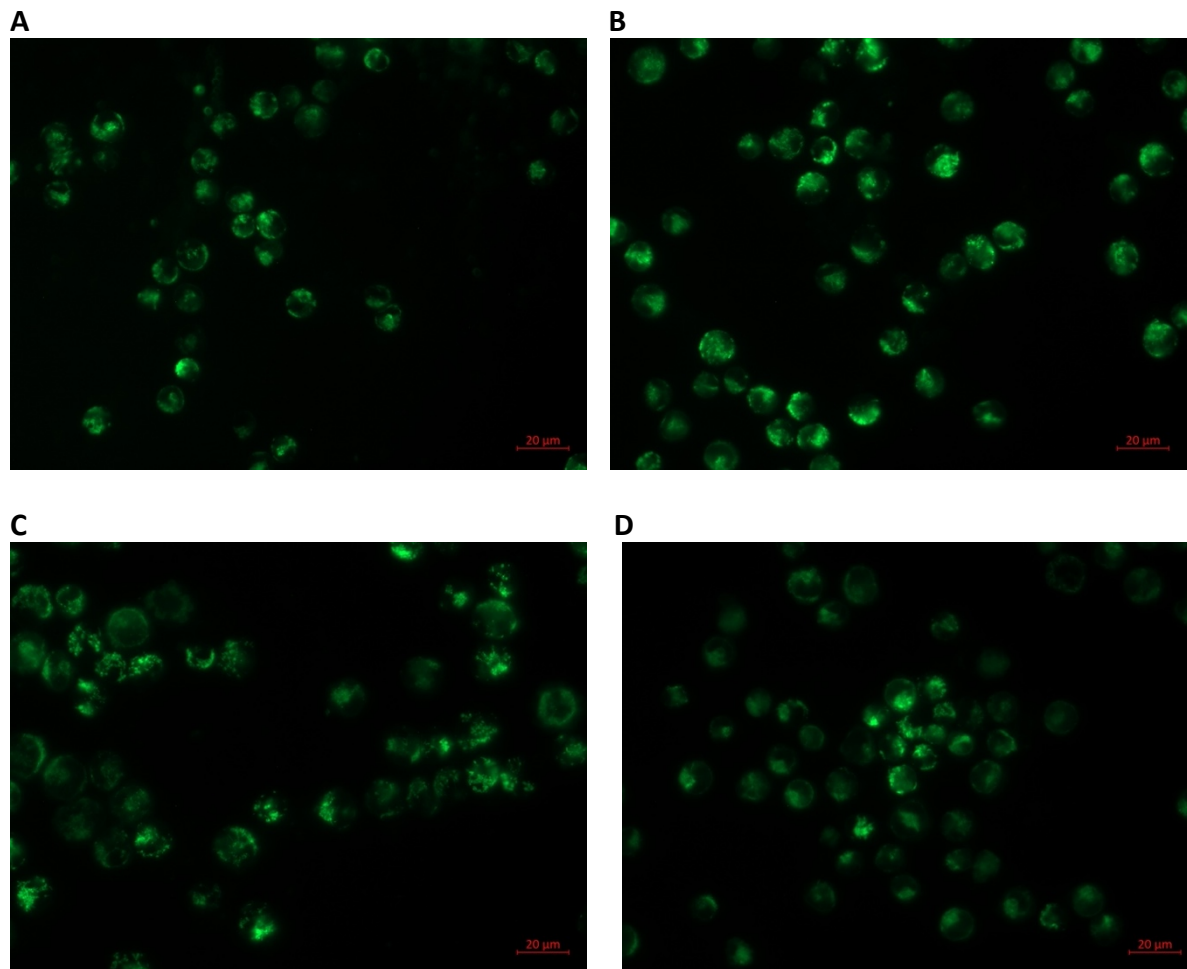


Figure 34: A) Fluorescence microscope image taken from experiments carried out on normal glucose and water control (NG) within TK6 cells. B) Fluorescence microscope image taken from experiments carried out on normal glucose treated with acetaldehyde (NG + Ace) within TK6 cells. C) Fluorescence microscope image taken from experiments carried out on high glucose and water (HG). D) Fluorescence microscope image taken from experiments carried out on high glucose + acetaldehyde (HG + Ace) within TK6 cells. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or a 1000µM of acetaldehyde (Ace). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the AxioCam 503 colour camera and the Zen 3.1 program.

Within the quintiles illustrated in Fig. 35, comparison of the HG + Ace sample group against the NG + Ace sample group showed that percentage of mitochondria that fall within the low quintile are significantly decreased by 12.94% ($p=0.023$). Additionally, there was a large but non-significant increase of mitochondria with integrated intensities that fall within the highest quintile (23.70%) ($p=0.122$). This would suggest that there is a shift in the data within the HG + Ace group to higher average integrated intensities and therefore mitochondrial mass. Comparisons of each glucose concentration Ace group when compared to their respective glucose water control group found no significant changes within the quintiles produced; further information can be found within appendix 6.6.1.

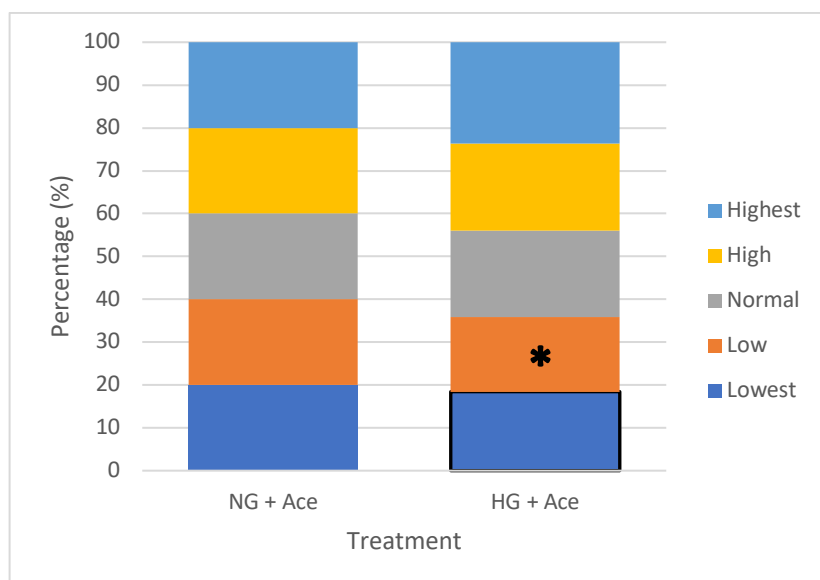


Figure 35: The intensity changes seen within the data obtained for normal glucose treated with acetaldehyde (NG + Ace) (n=4) in comparison to high glucose and acetaldehyde (HG + Ace) (n=4) with the fluorescence microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 32 images were used for each of the samples. Significant results shown through p-values, where * $p < 0.05$. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or a 1000 μ M of acetaldehyde (Ace). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the AxioCam 503 colour camera and the Zen 3.1 program.

4.1.3 Effect of high glucose on intensity changes within the mitochondria in DEHP groups

Comparison of NG + DMSO control to the NG + DEHP (Fig. 36) shows there is an increase of 9.38% in the average integrated intensity within the DEHP group such as that seen within the normal glucose acetaldehyde group (p -value= 0.986); this can be seen in Fig.37.A (NG + DMSO) and Fig.37.C (NG + DEHP) where the cells within Fig.37.C appear to have a brighter, more intense staining compared to those in Fig.37.A. Comparing HG + DEHP against its HG + DMSO control, the same observations were seen with the HG + DEHP having an average integrated intensity 36.10% higher ($p=0.629$); this can further be seen within Fig.37.B (HG + DMSO) and Fig.37.D (HG + DEHP) as the mitochondria appear to have a higher intensity than those in Fig.37. B.

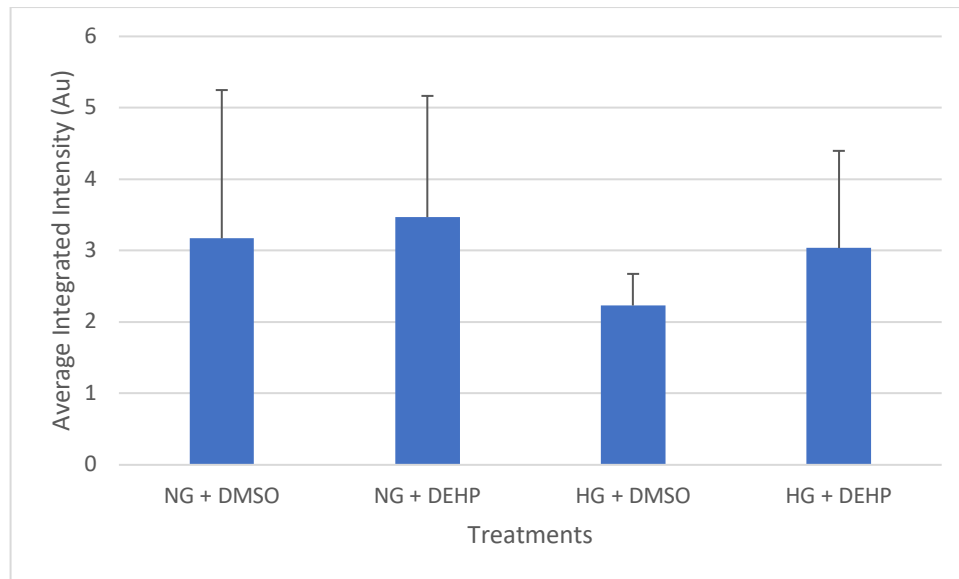


Figure 36: The intensities were measured using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. Shows the average intensity for normal glucose treated with diethyl hexyl phthalate (NG + DEHP) (n=4) and high glucose and diethyl hexyl phthalate (HG + DEHP) (n=4) in comparison to the normal and high glucose and DMSO control (NG + DMSO and HG + DMSO respectively) (n=4). A total of 32 images were used for each of the control samples while 24 were used for each of the DEHP samples. Error bars are Standard Deviation bars. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or a 2000 μ M of di(ethylhexyl) phthalate (DEHP). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

Comparison of NG + DEHP and HG + DEHP showed the opposite as the previous comparisons as the HG + DEHP has a decreased average integrated intensity by 12.50% compared to that of the NG + DEHP group found ($p=0.748$). A further example of this can be seen within images Fig.37.C (NG + DEHP) and Fig.37.D (HG + DEHP) as while some of the cells within Fig.37.C have a high intensity, a greater proportion of those within the Fig.37.D have a higher intensity of staining in the mitochondria. Such as in previous comparisons, there was no statistical significance.

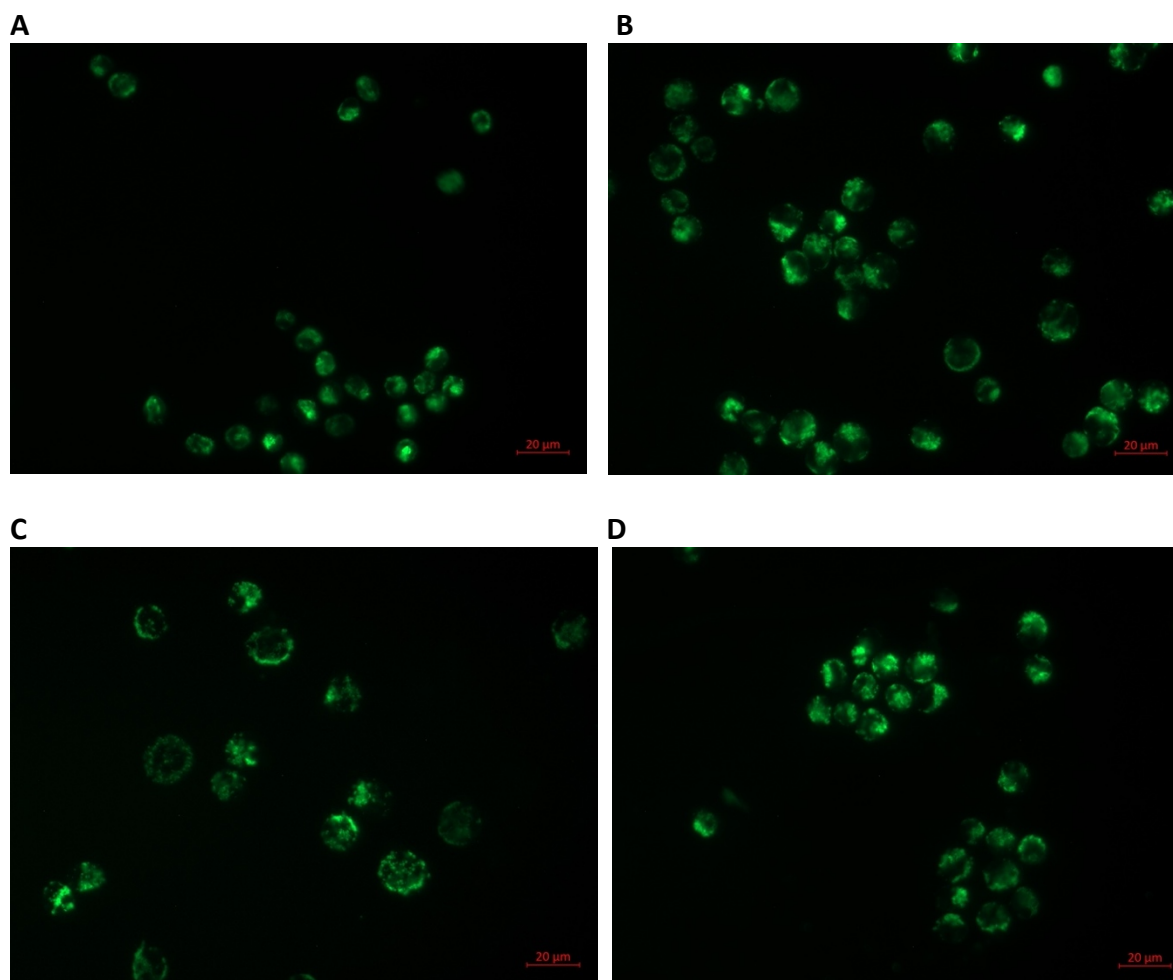


Figure 37: A) Fluorescence microscope image taken from experiments carried out on normal glucose and DMSO control (NG + DMSO) within TK6 cells. B) Fluorescence microscope image taken from experiments carried out on Normal glucose + diethyl hexyl phthalate (NG + DEHP) control within TK6 cells. C) Fluorescence microscope image taken from experiments carried out on high glucose and DMSO control (HG + DMSO) within TK6 cells. D) Fluorescence microscope image taken from experiments carried out on high glucose + diethyl hexyl phthalate (HG + DEHP) treatment within TK6 cells. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or a 2000µM of di(ethylhexyl) phthalate (DEHP). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde (and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

To further study the effect of the high glucose in the presence of DEHP, NG + DEHP was additionally used as a control to compare quintiles, as shown in Fig.38. During this comparison there is a noticeable but non-significant increase of 20.85% and 4.52% in the frequency of mitochondria within the lowest and normal categories respectively (p-value=0.598 and 0.557). The remaining two categories, high and highest, indicate a decrease in the number of mitochondria within these groups, with the high group experiencing the largest decrease of 13.93% (p-values= 0.285). Comparisons of each glucose concentration DEHP group when compared to their respective glucose water control

group also found no significant changes within the quintiles produced; further information can be found within appendix 6.6.1.

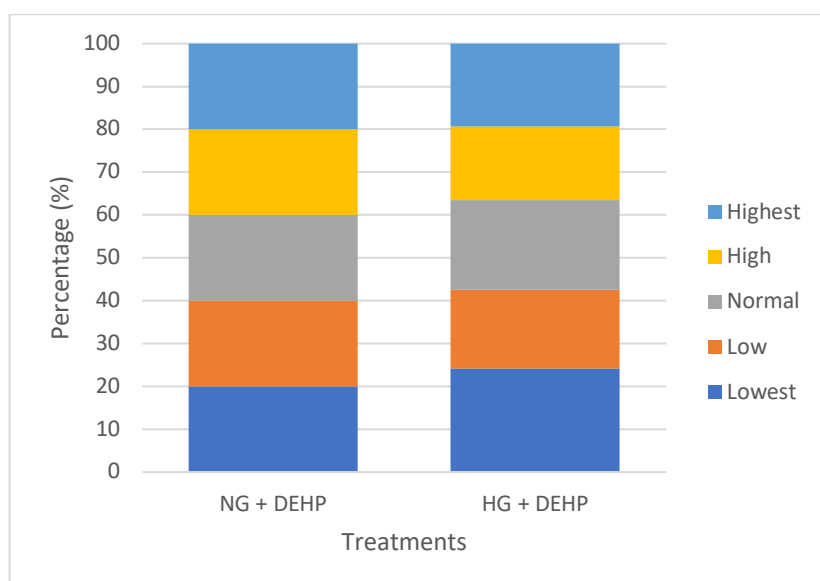


Figure 38: The intensity changes seen within the data obtained for normal glucose treated with diethyl hexyl phthalate (NG + DEHP) (n=4) in comparison to high glucose and diethyl hexyl phthalate (HG + DEHP) (n=4) with the fluorescence microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 32 images were used for each of the control samples while 24 were used for each sample. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or a 2000µM of di(ethylhexyl) phthalate (DEHP). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

4.1.4 Effect of high glucose on intensity changes within the mitochondria in hydrogen peroxide groups

Within the comparison of the NG + H₂O₂ against its NG water control, the average integrated average (shown in Fig.39) is 17.30% lower within the hydrogen peroxide group compared to its glucose control (p = 0.970). An example of this is shown in Fig.40.A and Fig.40. C, as many of the cells in Fig. 40.C (NG + H₂O₂) are of a duller appearance than those in Fig.40.A (NG). During the comparison of the average intensity of HG + H₂O₂ against its HG water control, the same conclusion as the previous H₂O₂ can be drawn, as there was a decrease of 16.01% of the average integrated intensity within the HG + H₂O₂ group (p= 0.995). This is illustrated in Fig.40.B and Fig.40.C, where such as the previous comparison, the mitochondria within Fig.40.D have a duller appearance, with very few having regions of higher intensity than the cells within Fig.40. B.

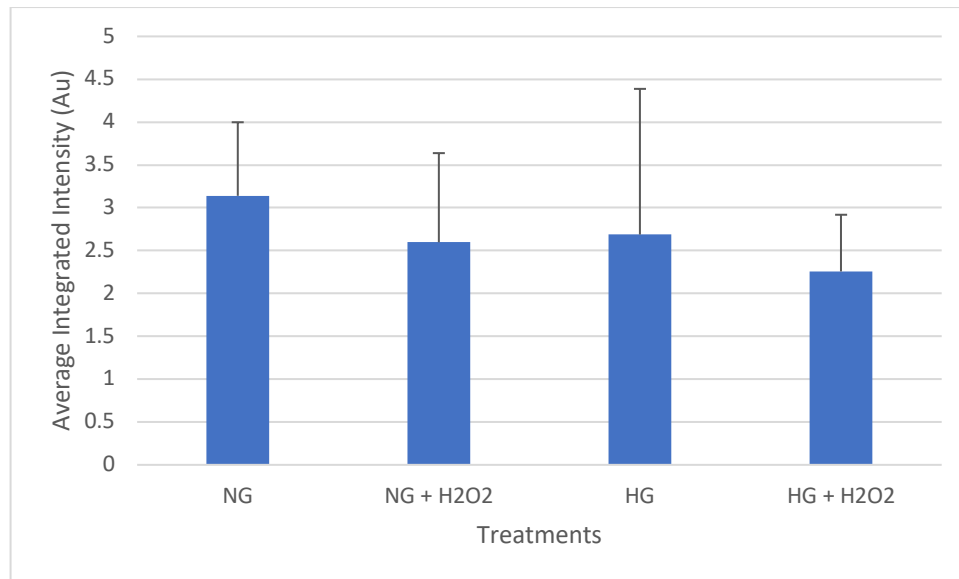


Figure 39: Average fluorescence intensity for normal glucose and high glucose water controls (NG and HG respectively) (n=4) and the normal glucose and high glucose treated with hydrogen peroxide (NG + H₂O₂ and HG + H₂O₂ respectively) (n=4). The intensities were measured using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. A total of 32 images were used for each of the samples. Error bars are Standard Deviation bars. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 20μM of Hydrogen Peroxide (H₂O₂). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

The comparison of the HG + H₂O₂ against its NG + H₂O₂ (Fig.39) also showed that there is a 13.10% decrease in average integrated intensity within the HG + H₂O₂ (p-value= 0.601). This can be seen within the comparison of Fig.40.C (NG + H₂O₂) and Fig.40.D (HG + H₂O₂), while in many of the mitochondria within the cells appear to have similar of intensities, there also appears to be a few cells within Fig.40.C that have regions of higher intensity than those in Fig.40. D.

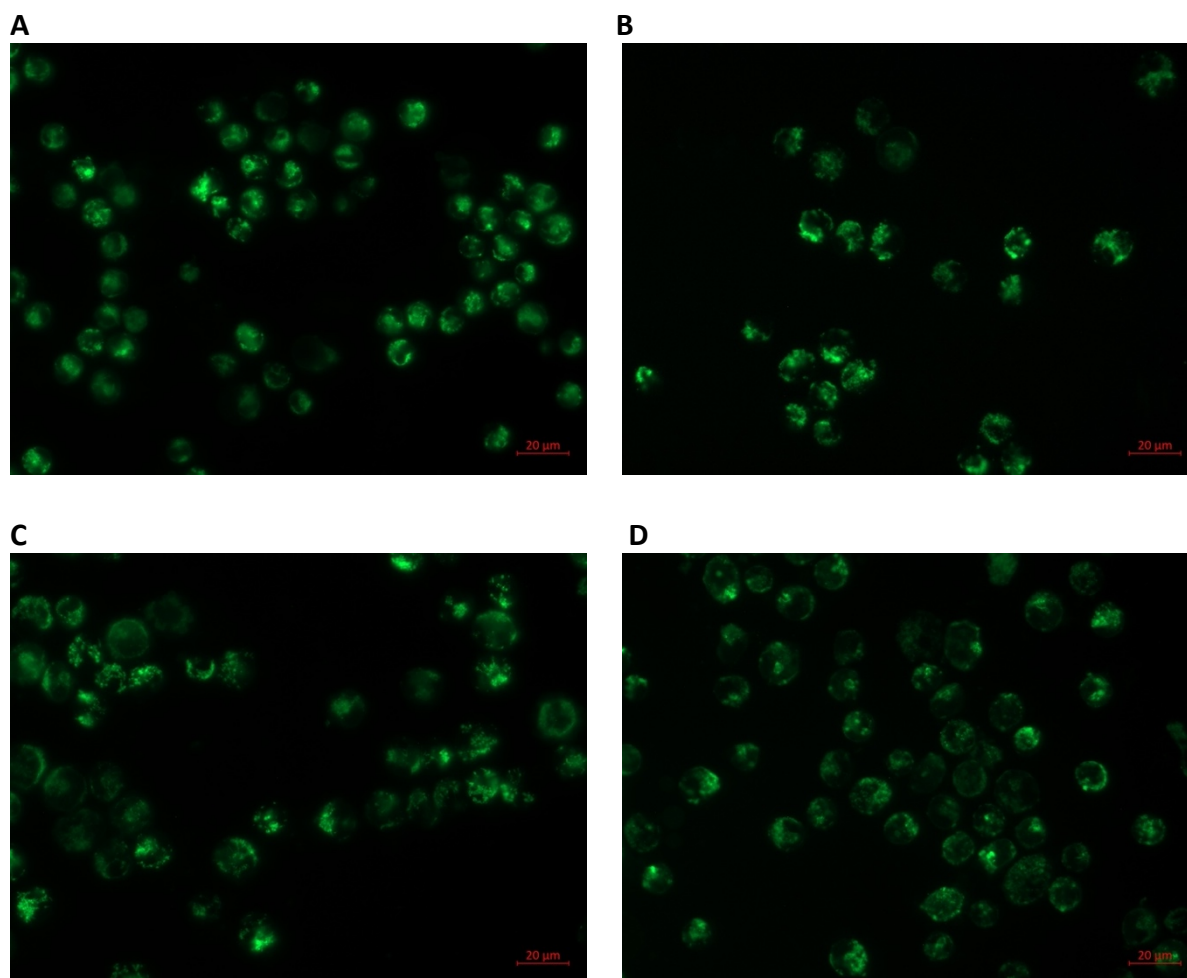


Figure 40- A) Fluorescence microscope image taken from experiments carried out on normal glucose and water control (NG) within TK6 cells. B) Fluorescence microscope image taken from experiments carried out on normal glucose with treated with hydrogen peroxide (NG + H₂O₂) within TK6 cells. C) Fluorescence microscope image taken from experiments carried out on high glucose and water (HG). D) Fluorescence microscope image taken from experiments carried out on high glucose with treated with hydrogen peroxide (HG + H₂O₂) within TK6 cells. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 20µM of Hydrogen Peroxide (H₂O₂). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

Within the quintiles of HG + H₂O₂ sample group when compared against those of the NG + H₂O₂ (Fig. 41) there was a significant change within this comparison is the decrease of 10.06% in the number of mitochondria with an average integrated intensity that falls within the normal quintile ($p < 0.001$). Additionally, there was a non-significant increase of 13.24% in the number of mitochondria within the lowest category ($p = 0.462$) while the frequency of those that fall within the highest categories are decreased by 7.03% ($p = 0.687$). Comparisons of each glucose concentration H₂O₂ group when compared to their respective glucose water control group found no significant changes within the quintiles produced; further information can be found within appendix 6.6.1.

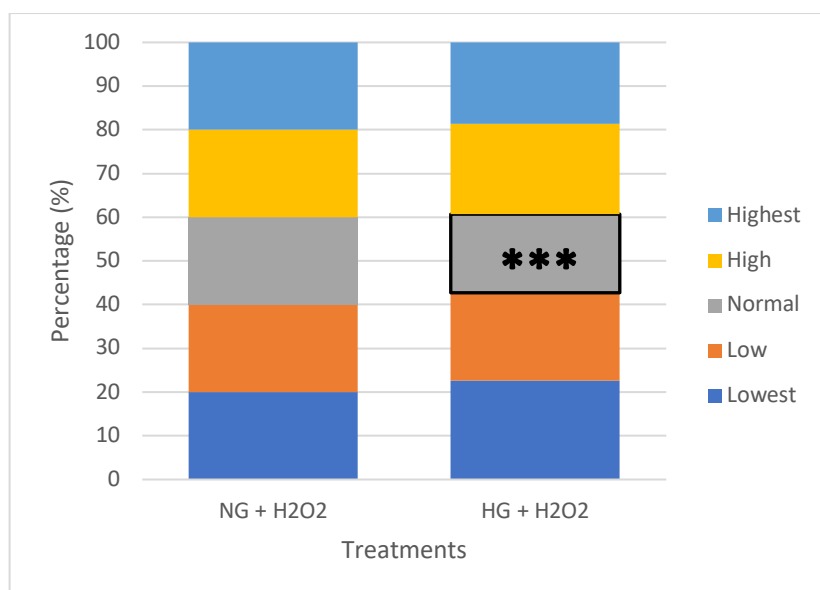


Figure 41: Shows the intensity changes seen within the data obtained on normal glucose treated with hydrogen peroxide (NG + H₂O₂) (n=4) in comparison to the high glucose and hydrogen peroxide (HG + H₂O₂) (n=4) with the fluorescence microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 32 images were used for each of the samples. Significant results shown through p-values, where ***p<0.001. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 20μM of Hydrogen Peroxide (H₂O₂). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the AxioCam 503 colour camera and the Zen 3.1 program.

4.1.5 Effect of high glucose on intensity changes within the mitochondria in 1-nitropyrene groups

Comparing the average integrated intensity within the mitochondria in the DMSO controls used in the MCL-5 cell line (Fig.42), the average integrated intensity appears to be an increase of 6.16% in the HG + DMSO group compared to the NG + DMSO group (p= 1.000). This can be seen within Fig. 43.A (NG + DMSO) and Fig.43.C (HG + DMSO), the intensities in each image are similar but within Fig.43.C there is a slightly larger proportion of mitochondria treated with HG + DMSO that have a higher intensity compared to Fig.43.A that are treated with NG + DMSO.

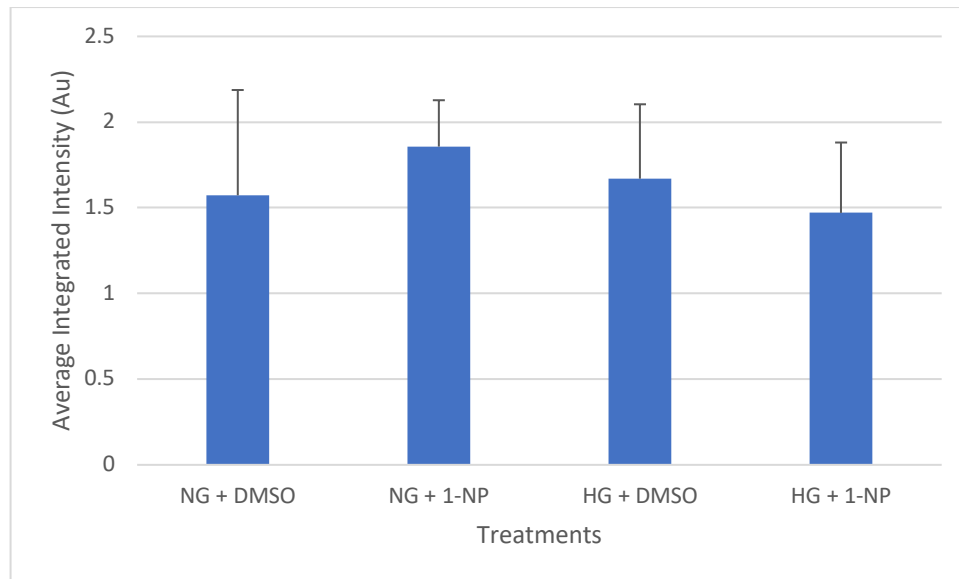


Figure 42: Average fluorescence intensity for normal glucose and high glucose DMSO controls (NG + DMSO and HG + DMSO respectively) (n=3) and the normal glucose and high glucose treated with 1-nitropyrene (NG + NG + 1-NP and HG + 1-NP respectively) (n=3). The intensities were measured using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. A total of 24 images were used for each of the samples. Error bars are Standard Deviation bars. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mMM, both glucose groups either received 1% (v/v) DMSO treatment or 80.9 μ M of 1-nitropyrene (1-NP). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

When comparing the average integrated intensities of the NG + 1-NP with the HG + 1-NP sample groups shown in Fig.42, there also appears to be a 20.83% decrease in the intensity ($p= 0.594$). This trend can be seen within Fig.43, when comparing Fig.43.C (NG + 1-NP) and Fig.43.D (HG + 1-NP), overall, the cells appear to have a duller appearance in Fig.43.D than in Fig.43.C, with the proportion of mitochondria with the brightest appearance being considerably lower within the HG + 1-NP (Fig.43.D) image.

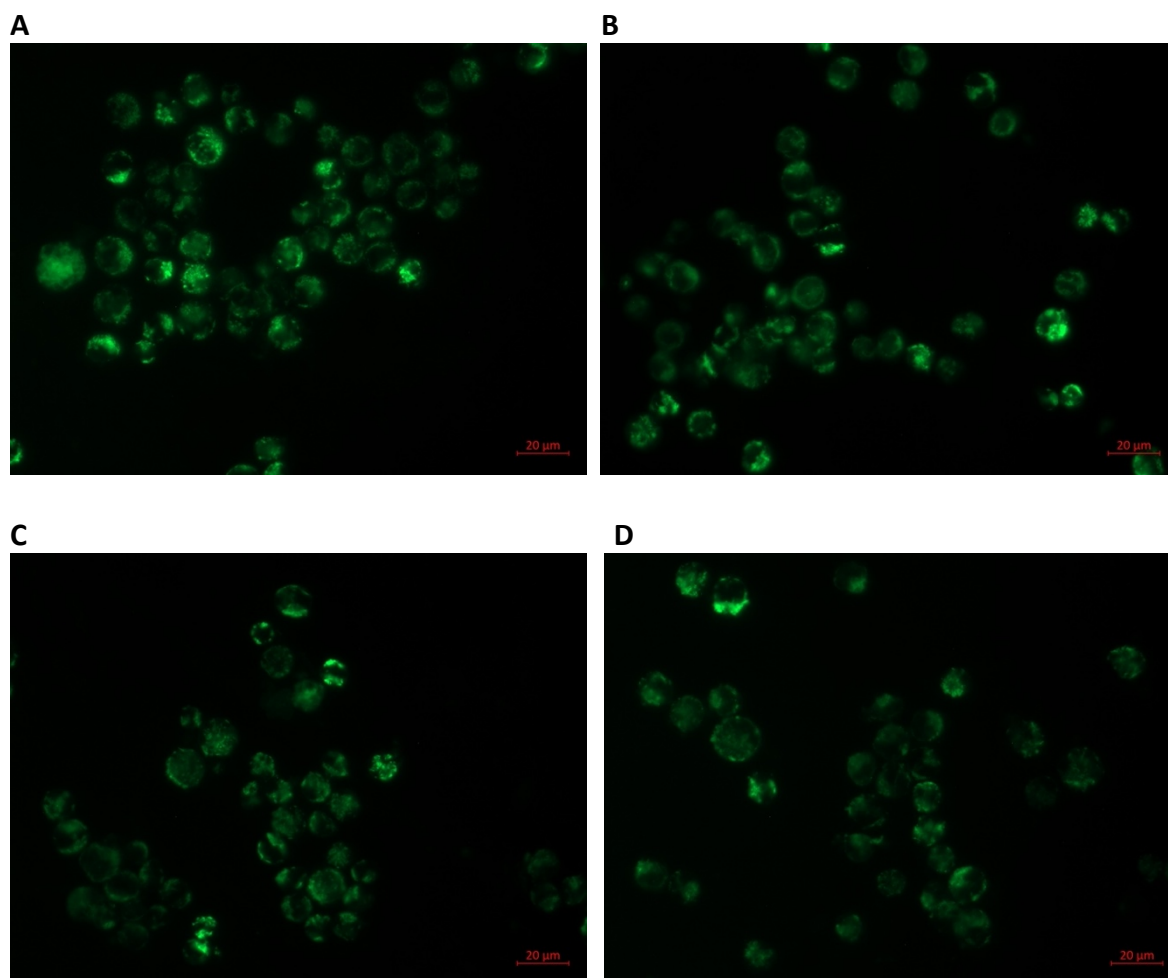


Figure 43: A) Fluorescence microscope image taken from experiments carried out on normal glucose and DMSO control (NG + DMSO) within MCL-5 cells. B) Fluorescence microscope image taken from experiments carried out on normal glucose with treated with hydrogen 1-nitropyrene (NG + 1-NP) within MCL-5 cells. C) Fluorescence microscope image taken from experiments carried out on high glucose and DMSO (HG + DMSO). D) Fluorescence microscope image taken from experiments carried out on high glucose with treated with 1-nitropyrene (HG + 1-NP) within MCL-5 cells. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or 80.9µM of 1-nitropyrene (1-NP). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the AxioCam 503 colour camera and the Zen 3.1 program.

Using quintiles to see further changes within the data; as seen within Fig.44.A, comparison of NG + DMSO to HG + DMSO shows an increase of 5.92% and 16.68% in the frequency of mitochondria that fall within the high or highest categories ($p=0.737$ and 0.314); the remaining categories (lowest, low and normal) appear to have a slightly decreased frequency in each (p -values= $0.941, 0.128$ and 0.671 respectively). Shown in Fig.44B, comparison of HG + 1-NP against NG + 1-NP showed there is an increase in the frequency of mitochondria with integrated intensities that fall in lowest, low, and high categories ($p=0.507, 0.251$ and 0.153 respectively) in the 1-np group. There were also decreases of 5.92% and 15.08% in the frequency in the mitochondria found within the normal and highest categories (p -values= 0.700 and 0.124 respectively).

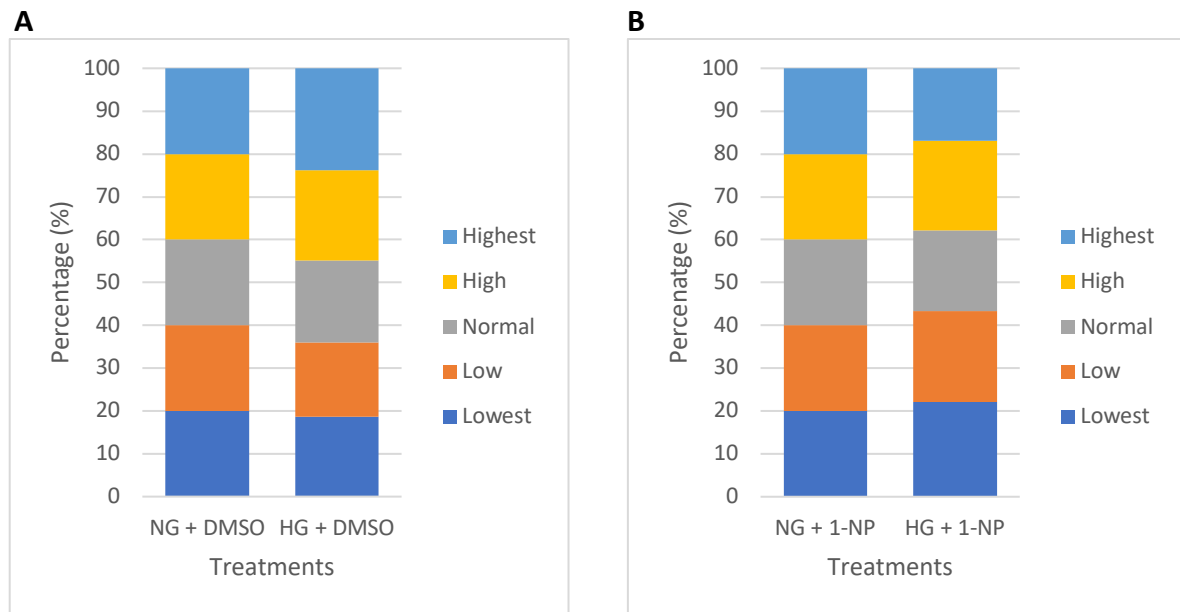


Figure 44: A) The fluorescence intensity changes for MCL-5 cells grown under normal glucose and DMSO control in comparison those under high glucose and DMSO control (NG = DMSO and HG + DMSO respectively) (n=3). **B)** The intensity changes seen within the data obtained for MCL-5 cells grown under normal glucose and treated with 1-nitropyrene (NG + 1-NP) (n=3) in comparison to the high glucose and treated with 1-nitropyrene (HG + 1-NP) (n=3) with the fluorescence microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 32 images were used for each of the samples. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or 80.9µM of 1-nitropyrene (1-NP). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS. Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

During the comparison of the trends within mitochondrial integrated intensity data shown in Fig.45.A, comparison of the NG + DMSO and NG + 1-NP groups showed there was a decrease of 18.32% and 4.98% in the frequency of mitochondria within the lowest and low categories (p= 0.649 and 0.496 respectively) while there is an increase in the high, and highest categories, with the highest increase of 11.42% seen within the high quintile (p= 0.381 and 0.909 respectively). The opposite was seen during the comparison of HG + DMSO and HG + 1-NP (Fig.45.B), as there was a significant increase of 19.16% in the low category and a significant decrease of 27.47% in the highest category (p-value= 0.011 and 0.006), suggesting a shift towards the lower average integrated intensities and therefore mitochondrial masses within the HG + 1-NP group.

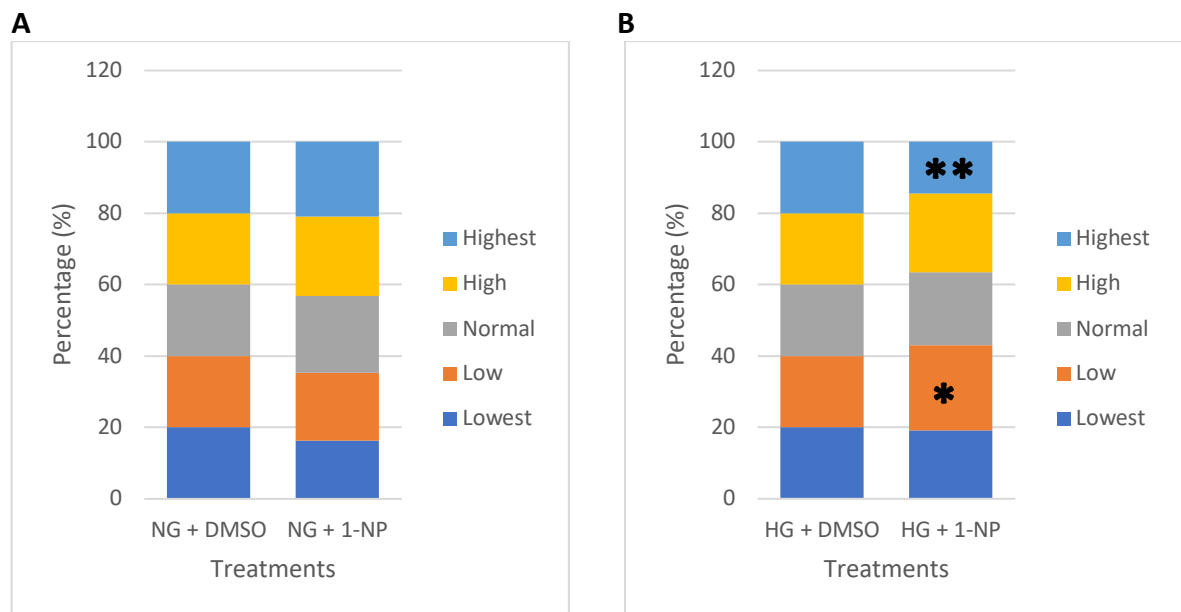


Figure 45: A) The intensity changes seen within the data obtained for MCL-5 cells grown under normal glucose and treated with 1-nitropyrene (NG + 1-NP) (n=3) in comparison to the normal glucose DMSO controls (NG + DMSO) (n=3). **B)** The intensity changes seen within the data obtained for MCL-5 cells grown under high glucose and treated with 1-nitropyrene (HG + 1-NP) (n=3) in comparison to the high glucose and DMSO controls (HG + DMSO) (n=3) with the fluorescence microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 24 images were used for each of the samples. Significant results shown through p-values, where * $p < 0.05$ and ** $p < 0.01$. N numbers represent biological replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or 80.9 μ M of 1-nitropyrene (1-NP). 2,000,000 cells were obtained and treated with 20nM MitoTracker Green staining solution before being fixed with 4% paraformaldehyde and suspended in PBS Cells were Imaged using a Zeiss Axio Imager M1 microscope with the Axiocam 503 colour camera and the Zen 3.1 program.

4.2 Seahorse Bioanalyzer

4.2.1 Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in TK6 control groups

Before any injections were added, comparison of the oxygen consumption rate (OCR) profiles between the water and DMSO control groups for cells grown under normal and high glucose concentrations (Fig.46. A) show HG had the highest OCR values, with an average value of 809.73 pmol/min, showing HG had the highest basal respiration at this stage. Cells within the NG + DMSO and NG groups had similar OCR values at each of the time points with averages of 784.85 pmol/min and 809.56 pmol/min respectively; HG + DMSO had the lowest OCR values at each time points and average of 671.51 pmol/min. Following the oligomycin injection, all four samples appear to have lower OCR values due to the decrease in the electron transport chain because of complex V inhibition. Following the second injection, FCCP, all samples saw an increase in OCR values and therefore maximal respiration rate due to complex IV increased oxygen consumption. After the FCCP injection, NG + DMSO had the lowest in OCR values with an average of 903.63 while HG remained the highest OCR value of 1364.74 pmol/min. Following the Rotenone/Antimycin A, all the samples decreased to the similar values between 65.07-84.61 pmol/min, showing there was complex I and complex III inhibition and therefore a stopping of mitochondrial respiration has occurred. Further information relating to the effect of the same injections on the ECAR values within the samples can be found within Appendix 6.6.2.

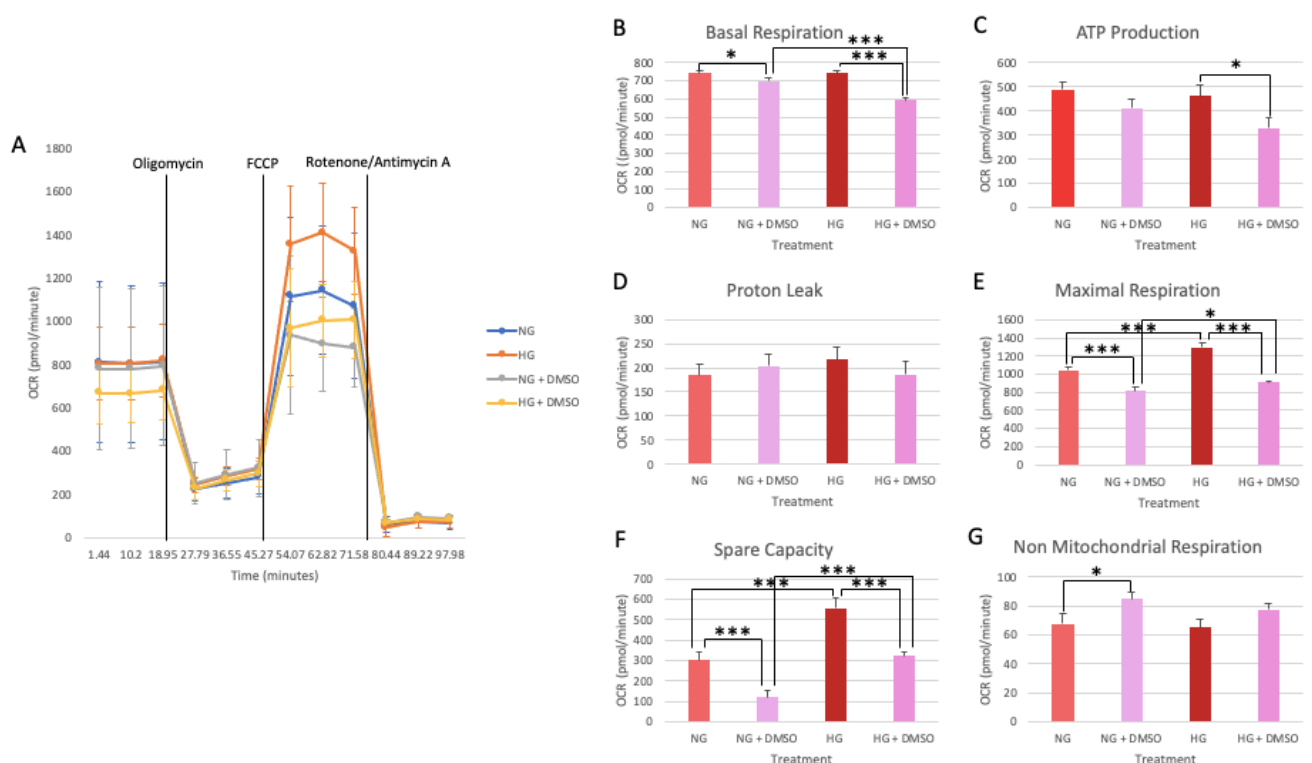


Figure 46: A) Oxygen consumption rate (OCR) profile for cells grown under normal glucose control (NG) (n= 4) in comparison to the high glucose controls (HG) (n= 4) and the normal glucose and DMSO control in comparison to the high glucose and DMSO control (NG + DMSO and HG + DMSO) (n= 4). B) Basal respiration rate, calculated from the subtraction of the non-mitochondrial respiration C) ATP production within the cells, calculated following the oligomycin injection. D) Proton leak within the cells, calculated following the oligomycin injection in conjugation within the non-mitochondrial respiration. E) Maximal respiration within the

cell calculated following the FCCP injection. F) Spare capacity calculated from the difference between maximal respiration and basal respiration. G) Non-mitochondrial respiration measured following the rotenone/antimycin A injection. Error bars are Standard Deviation bars. Significant results shown through p-values, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N numbers represent technical replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 1% (v/v) DMSO treatment. Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF²⁴ Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Comparison of the NG and HG water control groups found there was statistically significant increases of 24.62% and 84.33% in both the maximal respiration and spare capacity seen in Fig.46.E and Fig.46.F ($p = 0.001$ and 0.003 respectively). This shows that in the presence of high glucose there is an increase in the energy demand, which the cells have the capability to respond to and have the fitness to work towards their “theoretical” maximum. The increase in maximal respiration would suggest that there would also be an increase in proton leak and ATP production, however, the ATP production shows a decrease of 5.74% while proton leak was increased by 18.34%; however, neither of these observations were statistically significant (p -values= 0.808 and 0.422 respectively).

Within the DMSO controls, there is a significant decrease of 15.14% seen within the basal respiration (Fig.46.B) ($p < 0.001$). There were also further increases within the maximal respiration (Fig.46.E) and spare capacity (Fig.46.F) of 11.92% and 171.49% within the HG + DMSO ($p = 0.006$ and < 0.001 respectively). The decrease in basal respiration shows that the oxygen consumption required to meet the ATP or energetic demand has decreased under baseline conditions; the increase in maximal respiration and spare capacity is due to the increase in the maximum rate of respiration rate the mitochondria can achieve as increased cell fitness or flexibility.

Comparison of the NG water controls to the NG+ DMSO controls found there was a significant increase in the non-mitochondrial respiration by 24.86% (Fig.46.G) ($p = 0.003$) while there were significant decreases in spare capacity by 60.55% (Fig.46.F), maximal respiration by 21.47% (Fig.46.E) and the basal respiration by 5.60% (Fig.46.A) ($p < 0.001$, < 0.001 and 0.012 respectively). Comparison of the HG water control group to the HG+ DMSO control group yielded similar observations to NG and NG + DMSO with decreases in spare capacity (Fig.46.F) by 41.90%, ATP production (Fig.46.C) by 28.42%, basal respiration (Fig.46.A) by 20.21% and maximal respiration by 29.47% (Fig.46.E) also seen (p -values= < 0.001 , 0.01 , < 0.001 and < 0.001 respectively). The decreased basal and maximal respiration suggests that the energetic demand at baseline and the maximal rate of mitochondrial respiration within the DMSO group is lower; this may be due to decreased fitness of the cells as suggested by the decreased spare. Within the NG + DMSO comparison, the increase in non-mitochondrial respiration may be the cell trying to compensate for the reduced ATP production within the mitochondria, which the HG + DMSO cells are unable to do and would account for the decreased ATP production also seen in the high glucose comparison.

4.2.2 Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in acetaldehyde groups

Comparison between water control and the acetaldehyde groups OCR Profiles (seen in Fig.47.A) show, at the start of the experiment, the baseline the OCR values appears to be highest in the HG sample group, with an average OCR of 809.56 pmol/min, showing HG had the highest basal respiration such as before; HG + Ace had the lowest OCR and therefore basal respiration, with an average of 738.71 pmol/min. The oligomycin injection appears to have a similar effect of decreasing OCR in all groups, with NG samples now having the lowest OCR values, with a decrease in OCR value of 68.92%; this shows that within complex V in the NG sample group, inhibition has occurred at the highest rate due to the largest decrease in OCR value. The FCCP injection appears to increase OCR in all samples, to a higher value than the baseline values seen before the first injection; HG remains the highest with an average of 1364.74 pmol/min, suggesting HG had the highest maximal respiration rate due to increased oxygen consumption by complex IV; NG remains the lowest. Both NG + Ace and HG + Ace appear to have OCR values of 1189.07pmol/min and 1235.98 pmol/min respectively. Following the final Rotenone/Antimycin A injection, all samples see a noticeable decrease in their OCR values, with the largest decrease seen in the HG group, with a decrease of 95.23% showing mitochondrial respiration has been shut down. Further information relating to the effect of the same injections on the ECAR values within the samples can be found within Appendix 6.6.2.

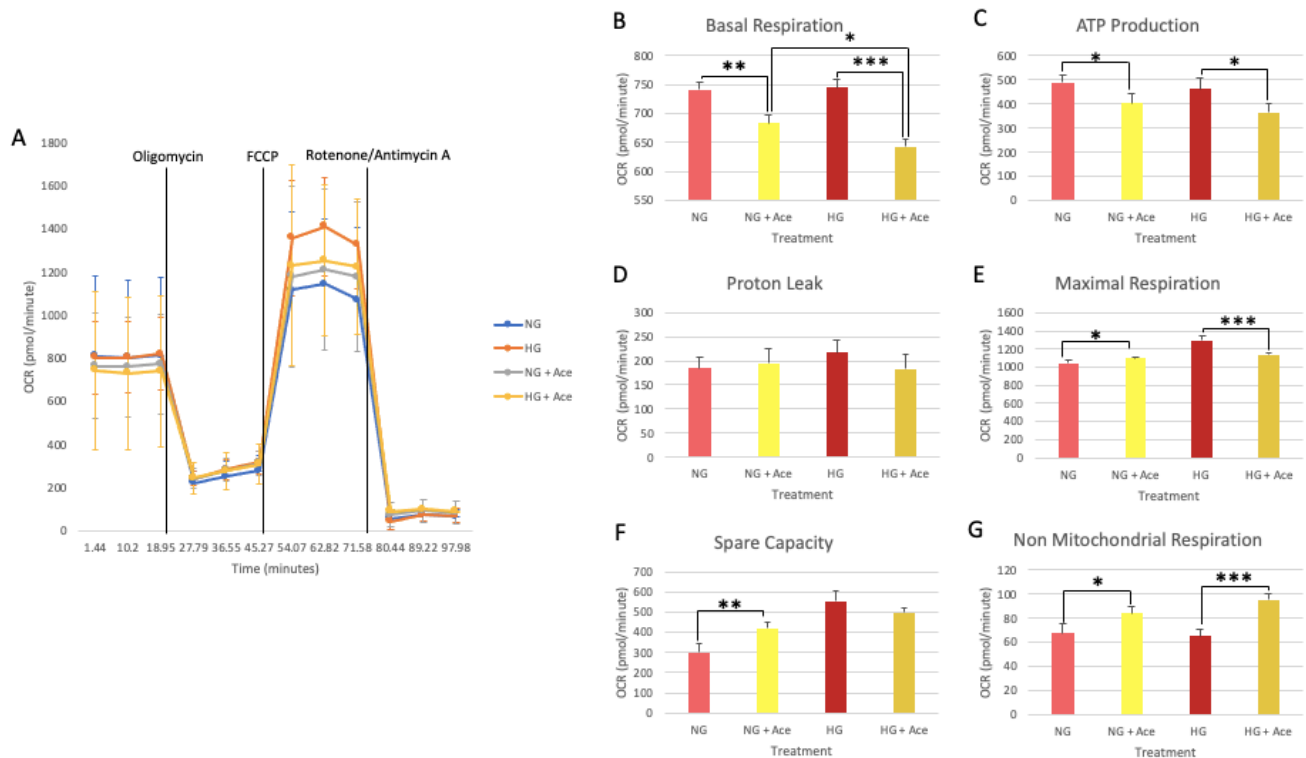


Figure 47: A) Oxygen consumption rate (OCR) profile for cells grown under normal glucose treated with acetaldehyde (NG + Ace) (n= 4) and high glucose and acetaldehyde (HG + Ace) (n= 4) in comparison to the normal glucose (NG) (n= 4) and high glucose controls (HG) (n= 4). B) Shows the basal respiration rate, calculated from the subtraction of the non-mitochondrial respiration C) Shows the ATP production within the cells, calculated following the oligomycin injection. D) Shows the proton leak within the cells, calculated following the oligomycin injection in conjugation within the non-mitochondrial respiration. E) Shows the maximal respiration within the cell calculated following the FCCP injection. F) Shows the spare capacity

calculated from the difference between maximal respiration and basal respiration. G) Shows the non-mitochondrial respiration measured following the rotenone/antimycin A injection. Error bars are Standard Deviation bars. Significant results shown through p-values, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. N numbers represent technical replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 1000 μ M of acetaldehyde (Ace). Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF[®]24 Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Upon comparison of NG + Ace with its NG control group, as shown in Fig. 47.B, the basal respiration has significantly decreased by 7.886% ($p = 0.001$) while the spare capacity (Fig.47.F) has significantly increased by 39.94% ($p = 0.007$). These results show that there is a lower oxygen consumption to meet the energetic demand, while also being able to better respond to the energetic demand and indicates a greater fitness, which is further supported by the significant decrease in ATP production (Fig.47.C) by 17.59% ($p\text{-value} = 0.049$). However, the results also show there to be increases in maximal respiration (Fig.47.E) by 5.94% and non-mitochondrial respiration (Fig.47.F) by 24.29% ($p = 0.026$ and 0.011 respectively), which suggests that the maximum respiration and non-mitochondrial respiration pathways are increased within the mitochondria.

Comparison of HG + Ace against its HG control also appears to show decreases in basal respiration (Fig.47.B) by 13.57% and ATP production (Fig.47.C) by 20.77% ($p = < 0.001$ and 0.045 respectively); showing that in the high glucose groups, there is also a decrease in oxygen consumption to meet the lower energetic demand and therefore energy production, such as illustrated in the NG and NG + Ace comparisons. There were also an increased non-mitochondrial respiration (Fig.47.G) by 46.10% within HG + Ace while the maximal respiration (Fig.47.E) has decreased by 12.21% ($p\text{-values} = 0.003$ and 0.011). This shows that the maximum respiration rate within the mitochondria has decreased, and the cells are using an increase rate of non-mitochondrial respiration pathway.

Such as the previous two comparisons, when comparing the results from the HG + Ace group against the NG + Ace group, there appears to be a significant decrease by 5.83% in basal respiration (Fig.47.B) ($p = 0.016$). This could suggest that the high glucose in conjunction in the presence acetaldehyde also results in a decrease in the energetic demand at baseline conditions suggesting a decrease in mitochondrial respiration.

4.2.3 Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in DEHP groups

As shown in Fig.48.A, it appears that before any injections are applied, the basal respiration within HG + DEHP is the highest due to its average OCR value of 1122.17 pmol/min. HG + DMSO had the lowest average OCR values with an average of 671.51 pmol/min. After the oligomycin injection, the OCR values in all samples decreased at varying rates across a larger range, suggesting varying inhibition of complex V and therefore ETC inhibition; while HG + DEHP still remains the sample with the highest OCR values, it also saw the biggest decrease in the OCR value by 68.61%. Following the addition of FCCP, all samples had an increase in OCR suggesting oxygen consumption and therefore maximal respiration has been reached. However, NG + DEHP now appears to have the highest OCR value with an average of 1332.75 pmol/min while NG + DMSO appears to have the lowest with an average of 993.99 pmol/min. In addition, both the HG + DEHP group and HG + DMSO have very similar OCR values. Finally, following the Rotenone/Antimycin A injection, all samples demonstrated

a decrease in OCR values, all samples also appear to have a similar OCR value after this showing a stop in mitochondrial respiration. Further information relating to the effect of the same injections on the ECAR values within the samples can be found within Appendix 6.6.2.

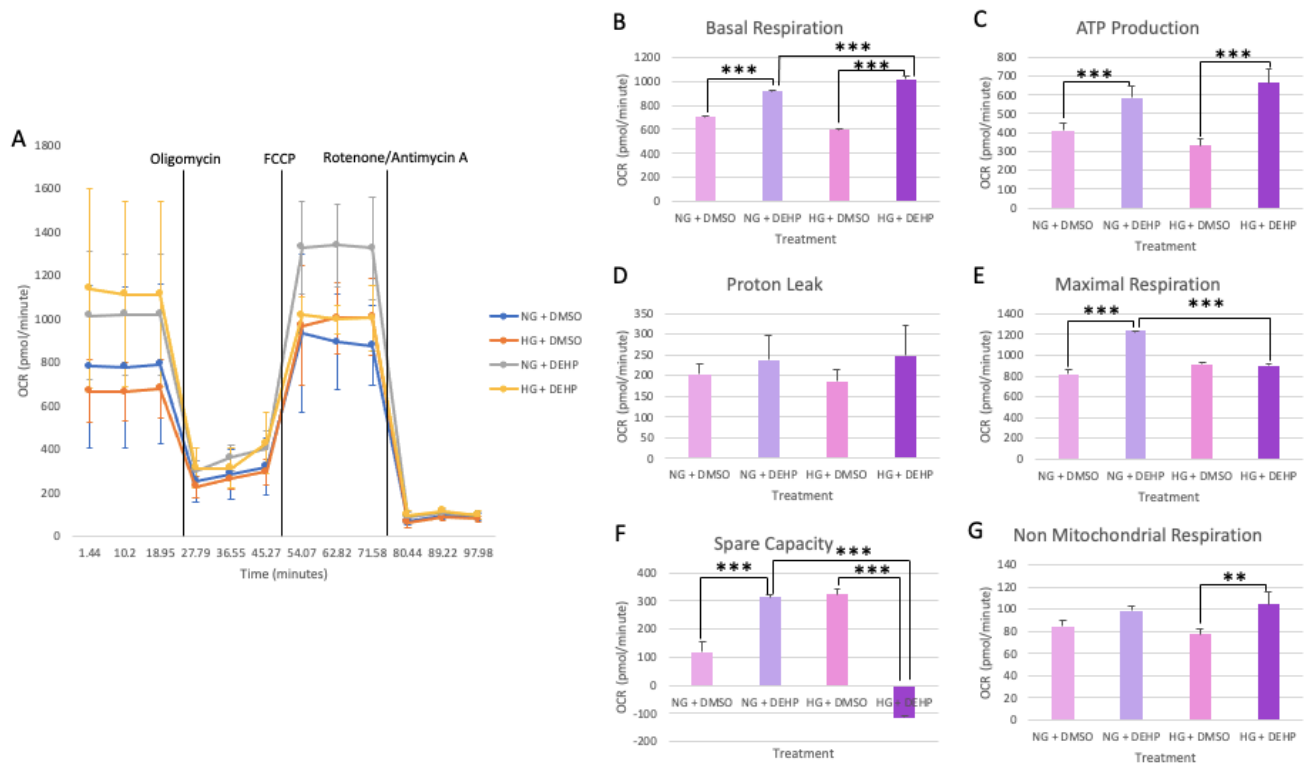


Figure 48: A) Oxygen consumption rate (OCR) profile for cells for cells grown under normal glucose treated with diethyl hexyl phthalate (NG + DEHP) (n= 4) and high glucose and diethyl hexyl phthalate (HG + DEHP) (n= 4) in comparison to the normal glucose and DMSO (NG + DMSO) (n= 4) and high glucose and DMSO control (HG + DMSO) (n= 4). B) Shows the basal respiration rate, calculated from the subtraction of the non-mitochondrial respiration C) Shows the ATP production within the cells, calculated following the oligomycin injection. D) Shows the proton leak within the cells, calculated following the oligomycin injection in conjugation within the non-mitochondrial respiration. E) Shows the maximal respiration within the cell calculated following the FCCP injection. F) Shows the spare capacity calculated from the difference between maximal respiration and basal respiration. G) Shows the non-mitochondrial respiration measured following the rotenone/antimycin A injection. Error bars are Standard Deviation bars. Significant results shown through p-values, where *p<0.05, **p<0.01, ***p<0.001. N numbers represent technical replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or 2000µM of di(ethylhexyl) phthalate (DEHP). Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF²⁴ Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Comparing NG + DEHP against the NG + DMSO control, it appears there is a significant increase in basal respiration by 31.50% (Fig.48.B) (p= <0.001). This shows that there is an increase in oxygen consumed to meet the cells energetic demand, and therefore mitochondrial respiration. These results can further be supported by the increase in maximal respiration (Fig.48.E) and ATP

production (Fig.48.C) by 50.71% and 42.03% respectively, as this shows there is in fact there is an increased maximal rate of respiration within the mitochondria as well as increased energy production by the mitochondria ($p = <0.001$ and 0.02).

Non-mitochondrial respiration (Fig.48.D) and ATP production (Fig.48.C) were significantly increased by 35.36% and 101.16% respectively in the HG + DEHP sample group when compared to the HG + DMSO control ($p = 0.009$ and <0.001). This suggests that there is an increase in non-mitochondrial respiration pathways such as glycolysis to meet the try meet the ATP demand within the cells. The decreased spare capacity (Fig.48.F) by 135.49% ($p\text{-value} = <0.001$) shows that the mitochondria within the HG + DEHP group have a lower capacity compared to the HG + DMSO control to respond to the increased energy demand of the cells suggested by the increased basal respiration rate and is unable to work to its maximum rate ($p\text{-value} =$ and <0.001). The decrease in maximal respiration (Fig.48.E) would suggests that respiration within the mitochondria has decreased,, reducing the amount of ATP produced within the mitochondria, however upon analysis the decrease in maximal respiration was not statistically significant ($p\text{-value}=0.78$).

Finally, comparing NG + DEHP and HG + DEHP, there is an increase of 10.50% in the basal respiration rate (Fig.48.B) and a decrease of 136.50% in the spare capacity (Fig.48.F) ($p = <0.001$ and <0.001 respectively). Such as the comparison of HG + DEHP and HG + DMSO control, there is also significant decrease in the maximal respiration (Fig.48.E) while unlike in the previous comparison the increase in the non-mitochondrial respiration within the cells was not statistically significant ($p = <0.001$ and 0.827 respectively). These results suggest that the mitochondria within the cell have a reduced respiration capacity under high glucose and DEHP meaning they are unable to respond to the energetic demand suggested by the significant increase in basal respiration, potentially due to the decrease in the maximal respiration within the mitochondria.

4.2.4. Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in hydrogen peroxide groups

Within Fig.49.A, NG+ H_2O_2 appears to have the higher OCR values before injection of oligomycin, with an average value of 954.52 pmol/min across the three time-points, while both NG and HG controls and HG + H_2O_2 all appear to have similar OCR values between 806.03 pmol/min-809.72 pmol/min. This shows a similar level of baseline respiration in all samples. After the administration of oligomycin, all samples see a decrease in OCR values, suggesting a complex V has been inhibited and ETC stopped. NG + H_2O_2 remained the sample group with the highest OCR values, although this group also saw the largest decrease by 66.99%. Following the addition of FCCP, all the samples saw their OCR values increase, showing an increase in oxygen consumption and therefore maximal respiration. NG + H_2O_2 appear remain as the highest OCR values at 1377.33 pmol/min; NG has the lowest OCR at 1110.65 pmol/min. This would show that while the FCCP was successful in accelerating mitochondrial respiration, there is varying degrees of effect. Following the final Rotenone/Antimycin A injection, all samples saw a decrease in OCR values all to similar values between 67.24 pmol/min-86.16 pmol/min, showing a stop in mitochondrial respiration. Further information relating to the effect of the same injections on the ECAR values within the samples can be found within Appendix 6.6.2.

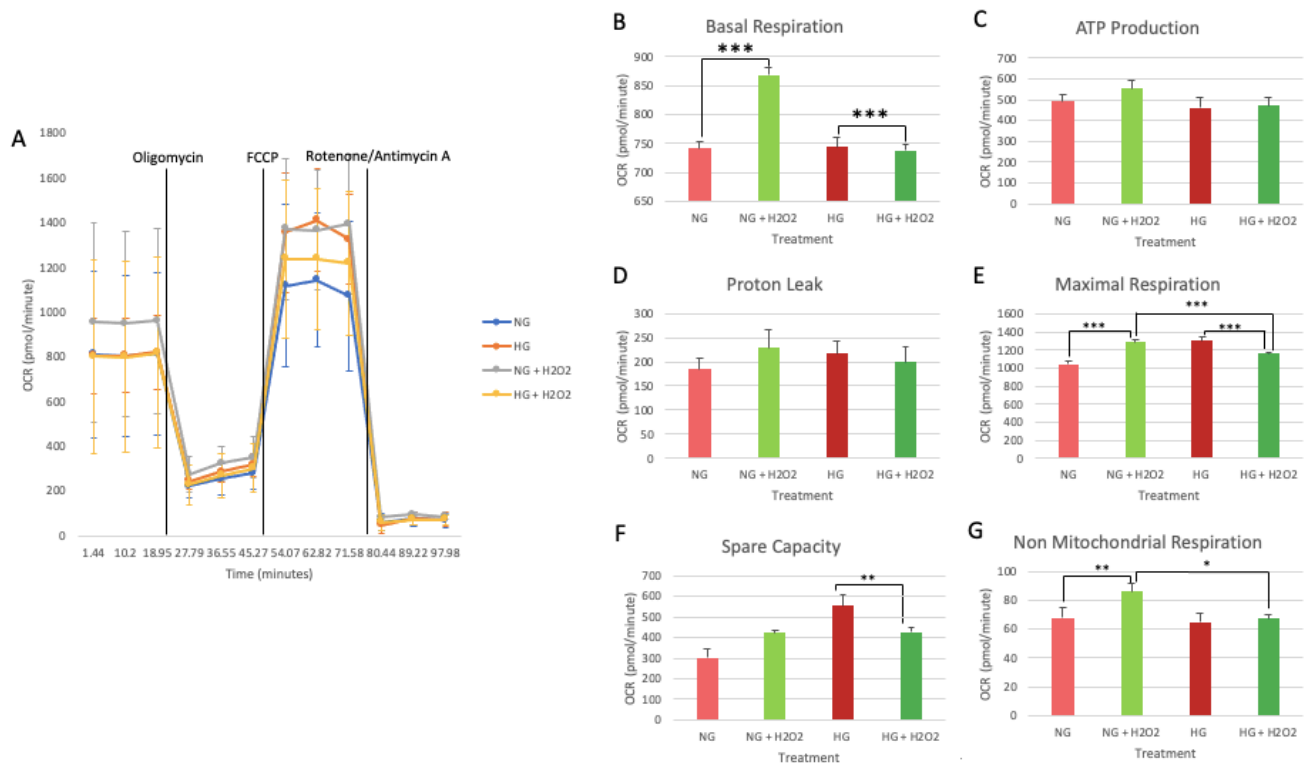


Figure 49: A) Oxygen consumption rate (OCR) profile for cells grown under normal glucose treated with hydrogen peroxide (NG + H₂O₂) (n= 4) and high glucose and hydrogen peroxide (HG + H₂O₂) (n= 4) in comparison to the normal glucose (NG) (n= 4) and high glucose controls (HG) (n= 4). B) Shows the basal respiration rate, calculated from the subtraction of the non-mitochondrial respiration C) Shows the ATP production within the cells, calculated following the oligomycin injection. D) Shows the proton leak within the cells, calculated following the oligomycin injection in conjugation within the non-mitochondrial respiration. E) Shows the maximal respiration within the cell calculated following the FCCP injection. F) Shows the spare capacity calculated from the difference between maximal respiration and basal respiration. G) Shows the non-mitochondrial respiration measured following the rotenone/antimycin A injection. Error bars are Standard Deviation bars. Significant results shown through p-values, where *p<0.05, **p<0.01, ***p<0.001. N numbers represent technical replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 20μM of Hydrogen Peroxide (H₂O₂). Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF[®]24 Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Comparing NG + H₂O₂ treated cells against their NG control, there appears to be a significant increase of 23.81% maximal respiration (Fig.49.E) (p= <0.001), suggesting that the mitochondrial respiration has increased, potentially due to a higher demand of energy, as suggested by the increase in basal respiration by 17.06 (Fig.57.B) (p= <0.001). Alongside the increase in basal and maximal respiration, there is also an increase of non-mitochondrial respiration (Fig.49.F) by 27.17% (p=0.007). This data suggests that in the presence of DEHP, the cell is undergoing more respiration because of an increase in energy demand of the cell.

HG + H₂O₂ treated cells compared to the HG control group have significant decreases in their spare capacity (Fig.49.F) by 23.43% and maximal respiration (Fig.49.E) by 10.46% (p= 0.002 and <0.001 respectively). This suggests that the cells within the HG + H₂O₂ treated cells have a lower capability to respond to the energetic demand within the cell potentially due to having a lower fitness than those in the control group. This decreased capability appears to come from the decreased in the maximum respiration within the mitochondria, it would therefore be expected that there would also be a decrease in ATP production (Fig.49.C), which is not seen within the data; however, the slight increase of 2.23% seen within this data was shown not to be significant (p-value=0.987).

When comparing the HG + H₂O₂ treatment group against NG + H₂O₂, there were significant decreases in basal respiration (Fig.49.B) and maximal respiration (Fig.49.E) by 14.92% and 9.87% (p= <0.001 and 0.001 respectively), while the non-mitochondrial respiration (Fig.49.G) within the HG + H₂O₂ group also appears to be decreased by 21.96% (p= 0.014). This suggests that within the mitochondria, respiration rates have decreased both at a baseline as well as at maximum rates within the mitochondria while other non-mitochondrial respiration pathways are also decreased within the cells.

4.2.5 Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in MCL-5 control groups.

Comparing the OCR profiles (Fig.50.A) for both DMSO control and 1-NP groups, the average OCR value before the first injection appears to be highest in NG + DMSO at 981.79 pmol/min, suggesting it has the highest baseline respiration; HG + 1-NP had the lowest OCR average of 657.00 pmol/min. After the oligomycin injection, the OCR values for all samples drop, suggesting a reduced respiration rate because of complex V inhibition. While the lowest and highest remained the same as before, their average OCR values dropped by 73.35% and 73.83%. Following the addition of FCCP, NG + DMSO remained the highest OCR values, averaging at 1457.74 pmol/min, while HG + DMSO samples had the lowest OCR values with an average of 876.69 pmol/min. The increase in all sample's OCR values show that the FCCP has been successful in accelerating the last stage of the electron transport chain and maximal respiration reached. After the final injection, all samples fall to a similar OCR value between 89.95 pmol/min-135.27 pmol/min suggesting a marked shut down of mitochondrial respiration. Further information relating to the effect of the same injections on the ECAR values within the samples can be found within Appendix 6.6.2.

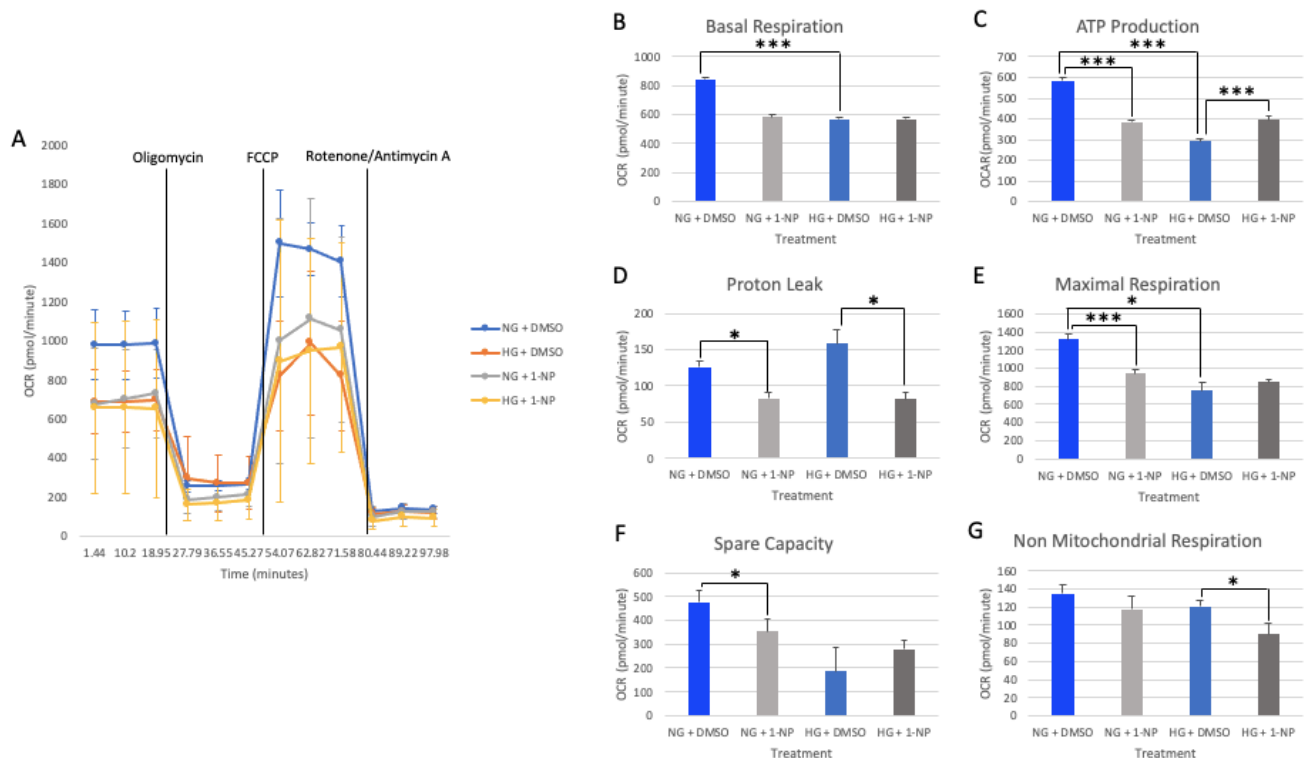


Figure 50: A) Oxygen consumption rate (OCR) profile for cells grown under normal glucose treated with 1-nitrotyrene (NG + 1-NP) (n=6) and high glucose and 1-nitrotyrene (HG + 1-NP) (n=6) in comparison to the normal and DMSO (NG + DMSO) (n=6) and high glucose and DMSO controls (HG + DMSO) (n=5). B) Shows the basal respiration rate, calculated from the subtraction of the non-mitochondrial respiration C) Shows the ATP production within the cells, calculated following the oligomycin injection. D) Shows the proton leak within the cells, calculated following the oligomycin injection in conjugation within the non-mitochondrial respiration. E) Shows the maximal respiration within the cell calculated following the FCCP injection. F) Shows the spare capacity calculated from the difference between maximal respiration and basal respiration. G) Shows the non-mitochondrial respiration measured following the rotenone/antimycin A injection. Error bars are Standard Deviation bars. Significant results shown through p-values, where *p<0.05, **p<0.01, ***p<0.001. N numbers represent technical replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or 80.9µM of 1-nitrotyrene (1-NP). Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF[®]24 Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Comparing NG + DMSO to HG + DMSO, there appears to be significant decreases in basal respiration (Fig.50.B) by 29.86%, ATP production (Fig.50.C) by 35.15% and maximal respiration (Fig.50.E) 39.41% (p<0.001, <0.001 and <0.001). This shows that the mitochondria have a reduced fitness and capability to respond to energetic demand, and thus respiration within the mitochondria at baseline and at maximum rates are decreased due to reduced fitness. Under these conditions, it may be expected that there would be an increase in non-mitochondrial respiration to as a source of ATP, however, there appears to be a non-significant decrease in non-mitochondrial respiration (Fig.50.G) by 11.54% within the HG + DMSO group (p=0.151).

4.2.6 Effect of high glucose on mitochondrial and non-mitochondrial respiration within the mitochondria in 1-nitropyrene groups

Comparing cells grown under 1-NP and normal glucose with its NG + DMSO control group, there appears to be a decrease in the maximal respiration (Fig.50. E) of 29.01% in the 1-NP cells ($p < 0.001$). This suggests that the maximum respiration rate of the mitochondria has decrease, potentially due to a decrease in the energetic demand of the cell suggested by the decreased ATP production (Fig.50.C) of 34.27% ($p < 0.001$). Consequently, there also appears to be decreases in the proton leak (Fig.50.D) of 34.65, and spare capacity (Fig.50.F) of 25.62% ($p = 0.012$ and 0.034 respectively). This further supports the idea that there is a decrease in mitochondrial respiration as proton leaking is an essential part of the later stage of the electron transport chain which allows the production of ATP. The decrease in spare capacity suggests that the reduction of the mitochondrial respiration could be due to a decrease in the capability of the cell to meet the energetic demand due to a decrease in the fitness of the cell.

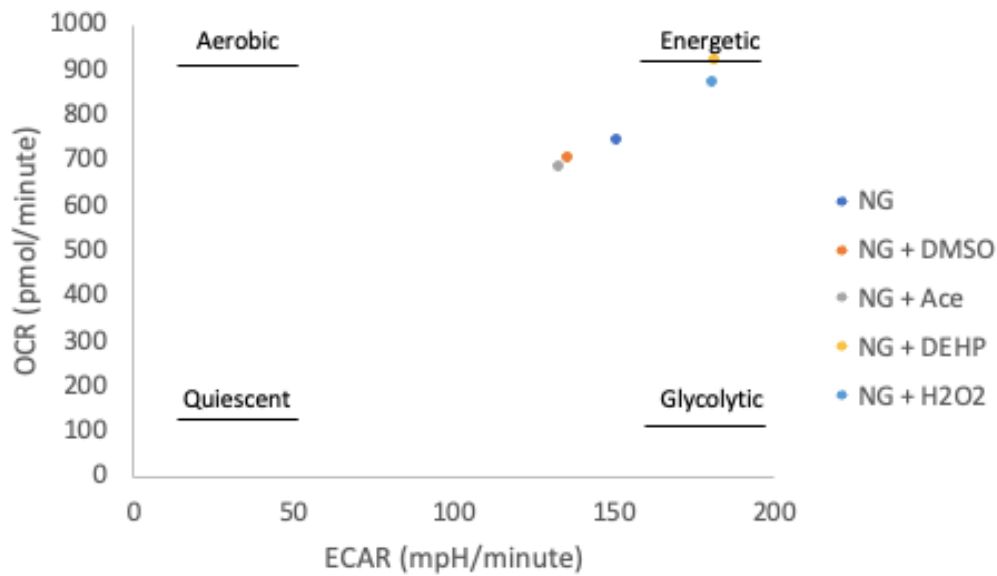
Comparison of HG + 1-NP against its HG + DMSO control, showed there were significant decreases in the basal respiration by 4.50% (Fig.50.B) and non-mitochondrial respiration by 25.74% (Fig.50.G) ($p = 0.033$ and 0.015 respectively). This would suggest that within the cells, there is a reduced energetic demand within the cell under baseline conditions while also experiencing a decreased rate of respiration both in and outside the cell. This would be further supported by a decrease in ATP production, however in this data there is a slight 4.15% increase, although this was found not to be significant ($p = 0.374$).

While the previous comparisons yielded significant differences, the comparison of NG + 1-NP and HG + 1-NP shows produced no significant differences within any of the parameters measured. However, the decrease in non-mitochondrial respiration (Fig.50.G) and maximal respiration (Fig.50.E) produced p -values near significance (p -values= 0.051 and 0.071) This could suggest that upon further work including more replicates, these results could produce significant differences.

4.2.7. Effect of varying glucose concentrations on energy state within TK6 cell line groups

Within Fig.51.A, the energy map for normal glucose samples shows within this group, NG + DEHP is the most energetic with an average OCR value of 920.83 pmol/min and an average ECAR value of 181.85 mpH/min; this shows that within this group, there is a highest rate of both mitochondrial and glycolytic respiration within the cells while NG + Ace is the most quiescent of this group with OCAR and ECAR values of 683.50 pmol/min and 133.00 mph/min respectively. Both the NG and NG + DMSO control groups appear to have similar ECAR values at 151.04 mpH/min and 135.69 mpH/min, showing similar rates of glycolysis while the rate of mitochondrial respiration appears to be slightly higher within the NG group, as shown by the higher OCR value in the NG group of 741.79 pmol/min compared to the OCR value of the NG + DMSO group of 700.23 pmol/min.

A



B

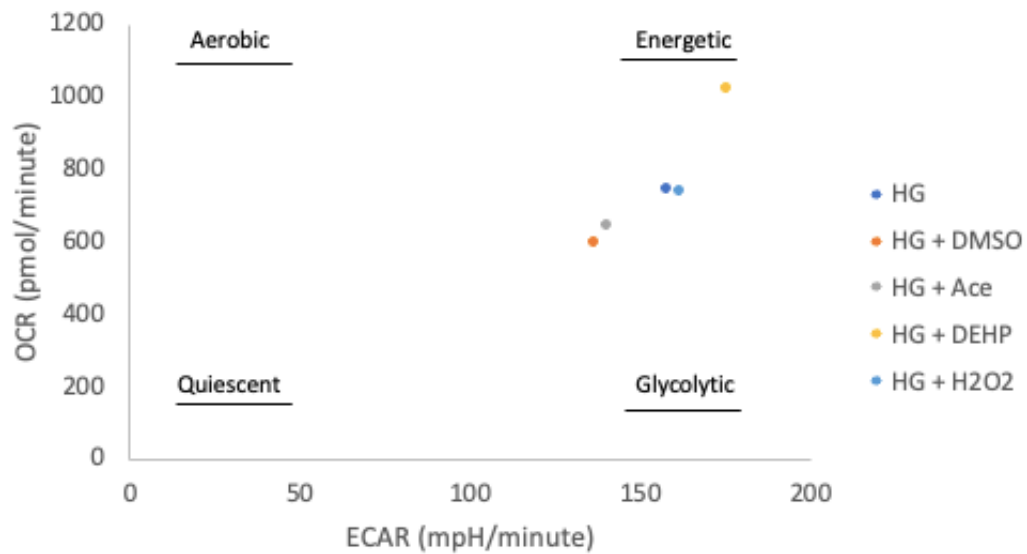


Figure 51: Shows the Energy Map produced using the average Oxygen Consumption Rate (OCR) values and the average Extracellular Acidification Rate (ECAR) values for the same time points as the OCR values for TK6 cells. A) Energy map produced from the samples grown under normal glucose (NG) treated with DMSO (1%), acetaldehyde (Ace) (1000 μ M), diethyl hexyl phthalate (DEHP) (2000 μ M) and hydrogen peroxide (H_2O_2) (20 μ M). B) Energy map produced from the samples grown under high glucose (HG) treated with DMSO (1%), acetaldehyde (Ace) (1000 μ M), diethyl hexyl phthalate (DEHP) (2000 μ M) and hydrogen peroxide (H_2O_2) (20 μ M) (n=4 for all groups). Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatment or 1% (v/v) DMSO treatment as a control group and or either an acetaldehyde, di(ethylhexyl) phthalate (DEHP) or Hydrogen Peroxide (H_2O_2)

treatment. N numbers represent technical replicates. Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF[®]24 Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

The energy maps for samples under high glucose conditions (Fig.51.B) show that within this group HG + DEHP is the most energetic group with an OCR value of 1017.51 pmol/min and an ECAR value of 175.75 mpH/min while the most quiescent is the HG + DMSO group with an OCR value of 594.1904378 pmol/min and an ECAR value of 136.76 mpH/min; as both values are lower in the HG + DMSO group, both mitochondrial and glycolytic respiration pathways are happening at a lower rate in this sample. With HG having an average OCR value of 744.66 pmol/min and an average ECAR value of 158.18 mpH/min and HG + H₂O₂ having an average OCR value at 738.79 pmol/min and an average ECAR value at 161.44 mpH/min, there are similar rates of both mitochondrial respiration and glycolysis as the OCAR and ECAR values are similar in both.

Within both glucose concentration energy maps, the DEHP samples appear to be the most energetic; comparing the OCAR and ECAR values of both groups, the OCR value appears higher within the HG + DEHP while the ECAR value is slightly higher within the NG + DEHP. This shows that while mitochondrial respiration is higher within the HG + DEHP sample group, the rate of glycolysis is slightly increased within the NG + DEHP.

4.2.8. Effect of varying glucose concentrations on energy state within MCL-5 cell line groups

When comparing the MCL-5 samples shown in Fig.52, the NG + DMSO is the most energetic of the samples as it has the highest average OCR value of 846.52 pmol/min and highest average ECAR value of 168.19 mpH/min, whereas HG + 1-NP appears to be least with average OCR value of 567.04 pmol/min and an average ECAR value of 115.10 mpH/min. Within the NG + 1-NP sample group, the average OCR value is 584.85 pmol/min and the average ECAR value is 132.12 mpH/min; the OCR and ECAR values are very similar to that seen within the HG + DMSO, which has an average OCAR value 570.09 pmol/min and an average ECAR value of 133.73 mpH/min. This shows that within NG + 1-NP and HG + DMSO, there are similar rates of both mitochondrial respiration and glycolysis.

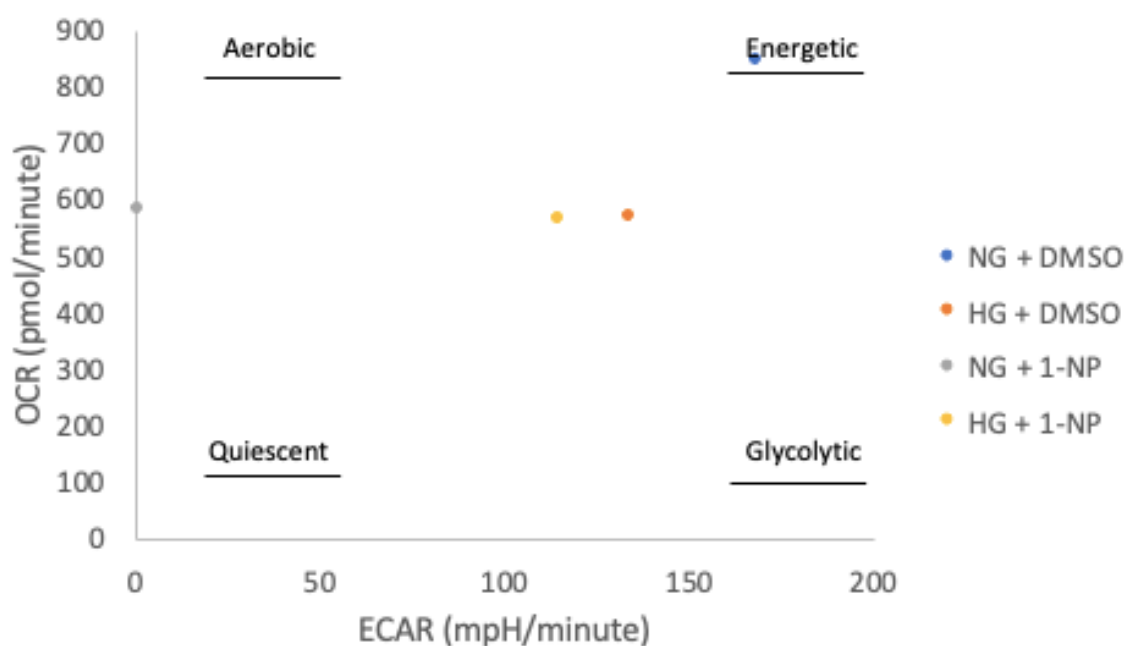


Figure 52: Shows the Energy Map produced using the average Oxygen Consumption Rate (OCR) values and the average Extracellular Acidification Rate (ECAR) values for the same time points as the OCR values for MCL-5 cells. Samples grown under normal glucose (NG) treated with DMSO (1%) or 1-nitropyrene (1-NP) (80.9 μ M) and samples grown under high glucose (HG) treated with DMSO (1%) or 1-nitropyrene (1-NP) (80.9 μ M). Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or 80.9 μ M of 1-nitropyrene (1-NP) (n=6 for all but HG + DMSO where n=5). N numbers represent technical replicates Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF[®]24 Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Chapter 5- Discussion

This study was performed to investigate how high glucose exposure impacts the mitochondrial genome and function and whether this impact is altered in the presence of four carcinogens, acetaldehyde, DEHP, hydrogen peroxide and 1-nitropyrene. By studying various mitochondrial genomic changes such as mutation rates and mtDNA copy number variations as well as changes to mitochondrial dynamics and function (mainly metabolism as that is the primary function of the mitochondria), this study was designed to gather a full view of the effects of high glucose in the presence of environmental carcinogens. While *in vivo* hyperglycemia occurs over a longer period of time than that used in the present study, one of the reasons mtDNA was chosen as the target was its greater sensitivity to damage which allowed study of acute treatments within a shorter timeframe. Additionally, the increased sensitivity of mtDNA could assist with detection of low-level or early-stage damage and allow more targeted approach. This was done to establish whether the effects of high glucose associated with diabetes is influenced by these four common carcinogens; furthermore, there is evidence mtDNA is more sensitive to damage and could assist with detection of low-level or early stage damage. This was done to establish if exposure to these everyday chemicals plays a role in the established link between those with type 1 and type 2 diabetes and their increased risk to develop many forms of cancer.

5.1. The effect of high glucose on the mitochondria within the TK6 groups

5.1.1. The effect of high glucose on the genome

Within the comparison of the NG and HG water control groups in the TK6 cell line there were no statistically significant changes seen within the next generation sequencing data; there was however significance within the data obtained for NG + DMSO and HG + DMSO control groups. Within the VAF values obtained for DMSO control groups, there was a significant decrease found within HG + DMSO ($p = 0.03$). This in conjunction to figures shown in section 3.1.2, which illustrated, above 15000bp, there was a visible increase in the number of low frequency variations ($<0.01-0.25\%$) within HG + DMSO. This suggests that the decrease in average VAF may be due to increased VAF sites of lower values. This may suggest that while there is an increased mutation rate in the presence of high glucose above 15000bp, there is a significant increase in variation sites present.

It has been illustrated in multiple studies that the stress caused by hyperglycaemia seen in diabetes results in damage and destabilisation of the mtDNA, such as an increase in mtDNA copy number (Gilkerson, 2016, Al-Kafaji & Golbahar, 2013; Malik et al., 2009). While no significance was found within our qt-PCR data, a study also using real-time PCR but in human mesangial cells cultured at glucose concentrations of 5 mM and 25 mM, found there to be a significant increase in the mtDNA copy number within the cells cultured at 25 mM compared to those cultured at 5 mM (Al-Kafaji & Golbahar, 2013). Furthermore, these increased copy numbers have been proposed to occur due to high glucose induced oxidative stress (Al-Kafaji & Golbahar, 2013; Santos et al., 2003; Bohr, 2002). This may cause oxidative damage to the mtDNA, such as those seen within this current study (Ide et al., 2001). Additionally, studies on the effects of diabetes have also found there to be a significant increase in mtDNA mutations because of hyperglycaemia (Chistiakov et al., 2012, Li et al., 2018).

Additionally, the comparison of HG + DEHP group to the NG + DEHP group also produced significant changes within the next generation sequencing data. Such as seen within the DMSO controls, there appears to be a significant decrease within the VAF values including the average data in HG + DEHP ($p\text{-value} = 0.005$). When looking at the regions of VAF sites, there appears to be a noticeably

increased number of VAF sites within the HG + DEHP, with many of them falling points between >0.01% and 0.25% between 0bp and 5000bp. Looking at the types of mutations, there appeared to be an increased number of SNPs within the HG + DEHP group, while there appeared to be little difference in the INDEL counts between the two samples. This suggests that some of the variation sites may be due to SNPs mutations. It has been found that exposure to DEHP, as well as related compounds such as Mono-2-ethylhexyl phthalate (MEHP), promote ROS production, which has been linked to increased mtDNA mutations (Rosado-Berrios et al., 2011; Andreyev et al., 2005). This ROS production, or oxidative stress, has been found to result in DEHP-induced DNA damage in both human and rat cells (Cho et al., 2015; Zhang et al., 2012). One study looking at the mechanisms of DEHPs testicular toxicity found that DEHP was able to induce testicular toxicity through DNA damage because of inhibited DNA replication caused by DEHP exposure with additional induction of cellular oxidative stress (Li et al., 2014). This same mechanism of damage to DNA via increased oxidative stress has also been found to occur in the presence of DMSO within 1-cell zygotes and may therefore be the source for DNA damage within the DMSO group (Kange et al., 2017).

5.1.2 The effect of high glucose on the phenotype

Within the metabolic data, there appears to be different effects within both controls and the carcinogen groups used. Within the comparison of NG and HG water controls in the TK6 cell line, it was shown there was a significant increase to the maximum respiration rate and spare capacity in the HG group ($p=0.001$ and 0.003). These results would suggest that there is an increase in maximal mitochondrial respiration and cell fitness due to the presence of high glucose. However, other studies have found the opposite to be true (Czajka & Malik, 2016; Rabøl et al., 2009). One study looking at the development of diabetic kidney disease in podocytes found that due to high glucose exposure, the same significant parameters in this study, maximal respiration and spare capacity were significantly decreased while ATP production, and basal respiration were also significantly decreased (Audzeyenka et al., 2021).

Such as within the water control, within the DMSO control group, there were significant increases seen within the maximal respiration and spare capacity of HG + DMSO compared to NG + DMSO ($p=0.006$ and <0.001 respectively). However, unlike the water control groups, within the DMSO control groups in the TK6 cell line, there was a significant decrease of the basal respiration rate within the HG + DMSO group ($p=<0.001$). This difference could be attributed to the significant VAF site variations, as mutations both homoplasmic and heteroplasmic, are seen within the mtDNA data which may have an influential effect on the mitochondrial OXPHOS capacity within diabetes patients (Chistiakov et al., 2012).

Similarly, within the MCL-5 cell line, as with the TK6 cell line, there was a significant decrease in mitochondrial basal respiration of HG + DMSO compared to NG + DMSO ($p=<0.001$). However, while there were significantly increased within in the TK6 HG + DMSO control group to the maximal respiration rate, within the MCL-5 cell line the same is significantly decreased HG + DMSO ($p=<0.001$). Furthermore, ATP production was significantly decreased within the HG + DMSO group ($p=<0.001$). These observations agree with previous studies which found that mitochondrial metabolism is dysregulated with reduced levels of mitochondrial glucose utilization (Kim et al., 2008; Stump et al., 2003; Zhang et al., 2013). One study investigating the effect of high glucose on mitochondrial metabolic changes has shown that high glucose decreases metabolic capacity and exhibited a reduced oxygen consumption in retinol endothelial cells (Trudeau et al., 2011), while another conducted with pancreatic β - cells, showed inhibition of mitochondrial metabolism (Haythorne et al., 2019). Results may differ between various models due to methodology

differences. Within the Trudeau study (2011), cells were culture for 7 days under normal (5 mM) or high glucose (30 mM) medium, while the models in this current study were grown for 4 days under 10mM (normal) and 29mM (high) glucose conditions.

Under acetaldehyde, unlike within the water control, there was a small but significant decrease in the mitochondrial basal respiration rate within the HG + Ace group compared to NG + Ace ($p=0.016$). Furthermore, HG + Ace when compared against NG + Ace, appeared to cause a significant decrease in the number of mitochondria with an integrated intensity that falls within the low quintile. This implied increase in integrated intensity of MitoTracker green dye suggests an increase in mitochondrial mass, which is consistent with a study observing breast cancer cells which found that within breast cancer cells, there was an increase in mitochondrial mass (De Luca et al., 2015).

Such as under acetaldehyde, under hydrogen peroxide respiration rates are decreased as there was a significant reduction in maximal respiration and non-mitochondrial respiration within the HG + H_2O_2 group ($p=0.001$ and 0.014 respectively). This decrease in metabolic capacity may be due to increased fragmentation suggested by the change in mitochondrial mass seen within HG + H_2O_2 as there is a significant decrease in the number of mitochondria with a mitochondrial mass that is considered normal compared to NG + H_2O_2 ($p<0.001$). While no further significance is found within the data, the trends within the quintile and intensity would suggest that this change would be a reduction in the integrated intensity and therefore mass; this would suggest an increase in the fission process (Trudeau et al., 2010; Serasinghe & Chipuk, 2019). It has been shown that fission has been linked to mitochondrial dysfunction while the reduction of fusion has been linked to decreased respiration (Jheng et al., 2012; Chen & Chan, 2005). Further studies have found that there has been a decrease in mitochondrial mass and therefore suggest a decrease in mass within both diabetes and cancer cell lines (Makino, Scott & Dillmann, 2010; Choo et al., 2006; Kashatus et al., 2015).

Similarly, to acetaldehyde and hydrogen peroxide, within the comparison of HG + DEHP against NG + DEHP, there were significant decreases in the maximal respiration rate and spare capacity ($p<0.001$ and <0.001). However, unlike within acetaldehyde and hydrogen peroxide TK6 carcinogen groups, within the DEHP comparison, there is a significant increase in the basal respiration rate within the HG + DEHP group ($p<0.001$). While under these three chemical conditions, alterations have been seen to respiration rates, it has been shown that the mitochondrial function within many cancer cells is not impaired (Weinhouse, 1976; Fantin et al., 2006; Moreno-Sánchez et al., 2007). However, the increase seen in basal respiration within the HG + DEHP may be showing that there is an increase in energetic demand within the cell under baseline conditions, which would suggest that mitochondrial respiration rates at these conditions are increased. Interestingly, a study investigating radio sensitising effects of glycolytic and mitochondrial function inhibitors found that inhibition of both glycolysis and mitochondrial respiration increased the radio sensitisation of patients with neuroblastoma, suggesting that increased rates of both play a role in the resistance of patients to treatments such as radiotherapy (Nile et al., 2021).

While the observations of this study differ, it has been suggested that there may be mitochondrial respiration defects present due to mtDNA deletions which result in glycolysis dependency, suggesting an increase in non-mitochondrial respiration or aerobic glycolysis as described within the “Warburg effect” (Pelicano et al., 2006; Warburg, 1925; Warburg & Negelein, 1927). One study investigating the effects of differing glucose concentrations on cell proliferation in various endometrial cell lines found that in two of these cell lines there was increased cell growth with high glucose, increasing the glucose uptake and glycolytic activity within those cells (Han et al., 2016).

5.2 The effect of DMSO on the mitochondria

It has been shown DMSO has a range of adverse effects, from inducing metabolic stress to having cytotoxic effects depending on the dose, where doses $\geq 1\%$ were shown to result in oxidative stress (Dludla et al., 2018). Within this current study, the effects of DMSO only appear to occur within the metabolic data. Under normal glucose conditions, NG + DMSO caused a significant increase in non-mitochondrial respiration ($p = 0.003$), while there were significant decreases in spare capacity, maximal respiration, and basal respiration ($p = <0.001$, <0.001 and 0.012 respectively). Under high glucose conditions there also appeared to be significant decreases in spare capacity, maximal respiration, basal respiration, and ATP production within the HG + DMSO group ($p = <0.001$, <0.001 , <0.001 and 0.001 respectively). All of this shows that in the presence of DMSO, there is a decrease in mitochondrial respiration as both the baseline and the maximal respiration rate have decreased, potentially due to a decrease in the fitness/flexibility of the mitochondria. Additionally, the energetic demand of the cell has also decreased. The increase to non-mitochondrial respiration in normal glucose alone suggests that the cells is attempting to try and maintain energetic demand outside the mitochondria through processes such as glycolysis.

Within the water control, where only high glucose was interacting with the mitochondria, there were increases in mitochondrial respiration which is not seen during the comparisons of NG and HG to NG + DMSO and HG + DMSO respectively. DMSO is a radical scavenger, which may provide protection against higher levels of ROS, however, at normal or reduced levels of ROS, DMSO may scavenge for electrons required for ATP production and therefore reduce basal respiration rates, as seen in both normal and high glucose, and is backed up by studies looking at hepatic and cardiac models (Eberhardt & Colina, 1988; Zorov et al., 2014; Lienhard et al., 2019).

5.3 The effect of acetaldehyde on the mitochondria

5.3.1 The effect of acetaldehyde glucose on the genome

Within the next generation sequencing data, under normal glucose, NG + Ace produced a significant decrease in the VAF values of the sites present, with a larger proportion of these variation sites appearing to be within the range of >0.01 and 0.25% compared to NG ($p = 0.015$). However, overall, there is a larger number of the lower frequency range (>0.01 and 0.25%) within the NG control group in general compared to the NG + Ace group. This would suggest that within the NG + Ace group, there are less variation sites in total while many of them fall within a low frequency range. This protective effect of acetaldehyde may be due to the presence of the ALDH2 enzyme; studies have shown that the presence of the ALDH2 enzyme in the inner mitochondrial membrane produces a protective effect over mtDNA against the genotoxic consequence of the acetaldehyde (Goedde et al., 1983; Chinner & Hudson, 2013; Zhang et al., 2020). Studies have shown that ALDH2 variants have been found to have a significant indirect protective role on cancers of the digestive track for moderate alcohol consumers (Koyanagi et al., 2020; Yin et al., 2007). Additionally, while within this current study there was no significance found for the 11.08-fold increase of *MT-ND1* ($p = 0.541$), other studies have shown that within young adults with glioblastoma, there is a higher mtDNA copy number which may result in decreased tumour aggressiveness due to its link with oxidative metabolism (Dardaud et al., 2019; Liberti & Locasale, 2016). The increase in oxidative metabolism has been found to decrease tumour aggressiveness potential through the increased synthesis of citrate, which when reduced, diminishes intracellular acidity and reduces acetyl-CoA production. This results in in favoured production of deacetylation state of proteins which favours apoptosis resistance and epigenetic changes, both of which contribute to tumour aggressiveness (Zhang et al., 2009; Cai et al., 2011; Philippe & Hubert, 2016).

While there is a lack of significance within the comparison of HG and HG + Ace, further work may show the same effect as within both normal and high glucose acetaldehyde groups there is a non-significant increase in mtDNA copy number by 11.08-fold increase and 10.54-fold increase respectively ($p=0.541$ and 0.378 respectively). This is further supported by a study in human mesangial cells where an increase in mtDNA copy number was seen due to high glucose exposure, with the conclusion that this increase may be a compensatory response to mtDNA damage and a decrease in the electron transport chain due to oxidative stress (Al-Kafaji & Golbahar, 2013).

5.3.2 The effect of acetaldehyde on the phenotype

Within NG + Ace, the basal respiration and ATP production had significantly decreased ($p= 0.003$ and 0.049) compared to NG. The same measurements also showed decreases within HG + Ace compared to HG ($p= <0.001$ and 0.045 respectively). Additionally, both NG + Ace and HG + Ace, there were significant increases in the non-mitochondrial respiration rate ($p=0.011$ and 0.003). This demonstrates that acetaldehyde causes a decrease in baseline respiration rates and ATP production from the cell, suggesting the increased non-mitochondrial pathways are not able to compensate for the decrease in the basal respiration rates. Furthermore, within the high glucose group there was an additional decrease in maximal respiration rate ($p=<0.001$), which suggests that in the presence high glucose conditions, the acetaldehyde results in a decrease in mitochondrial respiration rates. Studies have also shown that acetaldehyde inhibits mitochondrial energy production (Cederbaum et al., 1974), with a recent study investigating the effects of acetaldehyde on T-cell hepatitis demonstrating that T-cells treated with acetaldehyde showed inhibited glucose metabolism (Gao et al., 2018). This observation differs from that seen in NG + Ace when compared to NG, there was an increase in maximal respiration ($p= 0.026$). This increase in maximal respiration could be attributed to the protective effect of acetaldehyde implied in 5.3.1. It was found that it is through increased mitochondrial respiration that oxygen associated-DNA damage can be protected against (Sung et al., 2010). Furthermore, mtDNA damage within both mouse and human atherosclerosis has been linked with reduced mitochondrial respiration (Yu et al., 2017).

5.4 The effect of DEHP on the mitochondria

The DEHP exposure resulted in significant changes in the metabolic data. Comparison of NG + DEHP against NG + DMSO produced an increase in the basal respiration ($p= <0.001$). HG + DEHP compared to HG + DMSO produced similar findings, the high glucose only affected the magnitude of increases within the basal respiration and non-mitochondrial respiration ($p= <0.001$ and 0.009 respectively). This shows that DEHP exposure resulted in increased basal respiration rates under both normal and high glucose conditions while there is also an increased non-mitochondrial respiration pathway under high glucose conditions to maintain the enhanced energetic demands. However, it has been observed in adult male rats following DEHP exposure that energy intake and glucose intolerance are increased, which may suggest that this may be a potential reason for the increased respiration rates seen within the mitochondria (Strakovsky et al., 2015). Additionally, under the high glucose conditions the only other change present was a decreased spare capacity by 135.49% ($p\text{-value}= <0.001$). This in conjunction with the previous study mentioned by Strakovsky *et al* further suggests that the mitochondria are not able to respond to the increased glucose present.

Within the comparison of NG + DEHP against NG + DMSO, the maximal respiration and ATP production were also increased ($p= <0.001$ and 0.02). This would suggest DEHP caused an increase in both mitochondrial and non-mitochondrial respiration to increase ATP production to meet the increased energetic demand. Previous studies looking at urinary metabonomic changes following a

30-day dietary DEHP exposure found that exposure to DEHP can affect energy-related metabolism, indicated by decreases of citric acid and nonate; thus excess energy is converted to fat and retained which can result in obesity related diseases such as diabetes (Dong et al., 2017; Martinelli et al., 2010). However, this observation differs to that seen in other studies where it has been shown that metabolites of DEHP decrease ATP production while 8-OHdG levels are increased (Lee et al., 2020; Yang et al., 2015).

5.5 The effect of hydrogen peroxide on the mitochondria

Hydrogen peroxide resulted in significant changes within the metabolic data under both normal and high glucose conditions. Comparison of NG + H₂O₂ against NG produced significant increases were seen in both the maximal and basal respiration respectively ($p < 0.001$ and < 0.001). There was also an additional significant increase in non-mitochondrial respiration capacity ($p = 0.007$). This is in line with previous results where it has been shown that hydrogen peroxide may play a role in several cellular functions including coordinating oxidative metabolism through its role as a secondary messenger (Veal et al., 2007; Mailloux et al., 2018). It does this through the activation of downstream proteins in a cascade event which results in a metabolic response from the cell (Di Marzo et al., 2018). A study back in 2015 found that it was through the Syk pathway that the regulation of metabolism as well as other processes including transcription and translation that hydrogen peroxide can act as a messenger as it is activated by ROS (Patterson et al., 2015).

These same observations were not seen during the comparison of HG + H₂O₂ against HG as the hydrogen peroxide resulted in significant decreases in spare capacity and maximal respiration ($p = 0.002$ and < 0.001 respectively). Hydrogen peroxide can also be produced from superoxide anion ($O_2^{\cdot-}$) within normal cellular metabolism and can oxidatively alter cellular components, through inhibition of enzymes from the Krebs cycle, particularly α -ketoglutarate dehydrogenase, and succinate dehydrogenase (Nulton & Szveda, 2001). This in conjunction with previous studies which found that high glucose also produced these superoxide anions may suggest that within the high glucose hydrogen peroxide condition there is more oxidative stress present resulting in both a decrease in mitochondrial respiration and fitness of the cell (Piwkowska et al., 2011; Cosentino et al., 1997; Yano et al., 2013).

5.6 The effect of 1-nitropyrene on the mitochondria

1-NP exposure also produced significant changes within metabolic data both under normal and high glucose conditions. Comparison of NG and NG + 1-NP produced a decrease in the maximum respiration rate and a near significant decrease in basal respiration ($p = < 0.001$ and 0.05 respectively). Furthermore, there were also significant decreases in the proton leak, ATP production and spare capacity ($p = 0.012$, < 0.001 and 0.034 respectively). This would suggest that under normal glucose, 1-NP causes a decrease in the total mitochondrial respiration rate due to a reduction in cell fitness/flexibility. Furthermore, it was found in a study of mouse oocytes that there was a decrease in ATP production, while also disturbing mitochondrial function, due to 1-NP exposure and consequently increased ROS production (Yu et al., 2022b). This can also be seen within this current study in the comparison of HG + DMSO and HG + 1-NP where basal respiration decreased due to 1-NP exposure ($p = 0.033$). Additionally, mitochondrial mass within HG + 1-NP compared to HG + DMSO was significantly reduced within the quintiles in Fig. 45 (p -value = 0.011 and 0.006). This would further suggest activity is reduced. It has been found that decreased mitochondrial mass may be due to oxidative stress through the over production of ROS (Lee, Yin, Chi & Wei, 2002; De Luca et al., 2015); this in conjunction with other studies which have shown that 1-NP causes extreme ROS production suggests these changes are due to excess ROS production and therefore oxidative stress

(Kim et al., 2005; Liang et al., 2021; Su et al., 2021). This decrease in mitochondrial mass may be due to an increase in the fission process, which has been found to increase in the presence of increased generation of ROS (Zhao et al., 2017; Brillo et al., 2021). It has also been found that inhibition of mitochondrial fission prevented ROS production during high glucose exposure (Yu et al., 2006).

However, there was also a decrease in non-mitochondrial respiration because of 1-NP exposure ($p=0.015$). This would suggest that within the high glucose group, non-mitochondrial respiration rate is decreased, suggesting damage to the cell. It has also been found that 1-NP may increase apoptosis in part due to oxidative damage and ROS production (Asare et al., 2008; Asare et al., 2009; Su et al., 2021). This in conjunction with the observations in normal glucose may suggest that the addition of both high glucose and 1-NP increases ROS production and oxidative damage further to induce apoptosis.

5.7 Limitations

Within this study, there were several limitations to the starting and continued progression of this work, such as increased work to obtain a permit for the laboratory delaying the start date of experimental work and delays in delivery times of supplies due to the ongoing COVID-19 pandemic. Additionally, various issues such as failed optimisation of long-range PCR for amplicon 1 as well as primer issues within qt-PCR delayed gathering of results and subsequently reduced time and replicates done for these samples.

5.8 Future work

Within this study should further work continue, I would make alterations to the current methodology. These include changing the imaging technique used from a fluorescence microscope to an InCell bioanalyzer, due to its ability to image at various timepoints. Additionally, increasing the recovery period following the carcinogen treatment to 48 hours to allow further time for recovery, to see the full impact of the treatments.

To extend the work from this study, I would include additional techniques to further our understanding of our endpoints. To investigate the effect of the genome, duplex sequencing could be used to detect rarer mutations that may have an impact, as suggested by the significant number of low frequency variations. Additionally, I would sequence other nuclear genes that have an influence on mitochondrial activity such as *ALDH2*. To investigate the effects on the phenotype, I would use western blotting to monitor protein levels involved in mitochondrial processes such as DRP-1 and OLA1 to conclude whether morphological changes are occurring due to alterations in mitochondrial activity. Additionally, the use of flow cytometry to assess ROS level changes between treatment groups could be included, as these changes may account for many of the observations seen within this current study. Furthermore, the use of mass spectrometry to measure the amounts of metabolites produced both within and outside of the mitochondria.

5.9 Overall Conclusions and findings

Under high glucose within each of the control groups, there appears to be differing effects on mitochondrial respiration. During the comparison of HG against NG, there was an increase in maximal mitochondrial respiration and cell fitness while in the comparison of HG + DMSO against NG+ DMSO, there was a decrease in basal respiration rates both, within the TK6 and MCL-5 cell line. However, within the MCL-5 cell line, there was also decreases in cell fitness, maximal mitochondrial respiration and therefore ATP production. Therefore, it can be said that DMSO is having differing effects within cell lines compared to the water controls and therefore could be influencing the

mitochondrial genome itself. This is further supported by significant changes seen within the mitochondrial genome in DMSO conditions where there is an increase in low frequency variations above 15,000bp under high glucose conditions.

Looking at the various effects of high glucose exposure in the presence of all four environmental carcinogens tested, as shown in Fig. 53, DEHP was the only environmental chemical that produced changes within the sequence of the mitochondrial genome due to high glucose as there was increased variation sites of lower frequencies seen between 0bp and 5000bp; this was potentially due to increased SNP levels. Additionally, while DEHP showed no morphological changes to suggest a change in mass/activity, the significant decrease in maximum respiration rate suggests there was a decrease in mitochondrial activity.

While due to high glucose exposure, there were changes to both the mitochondrial mass and activity following acetaldehyde and hydrogen peroxide treatments; the changes seen were not similar between the two groups. Under hydrogen peroxide treatment, the mitochondrial mass decrease; this would suggest there was a decrease in mitochondrial activity. Within the metabolic data there was reduced activity seen, as implied by the decreased basal and maximum respiration rates. Under acetaldehyde treatment, there was an increase in mitochondrial mass/activity, potentially a recovery mechanism for ROS damage associated with high glucose exposure. The metabolic activity within the mitochondria under acetaldehyde conditions did not show increased activity as suggested by the increased mitochondrial mass, but a significant reduction in basal respiration. The only similarity between the four environmental carcinogens is non-significant increases were seen within the mtDNA copy number of varying magnitudes.

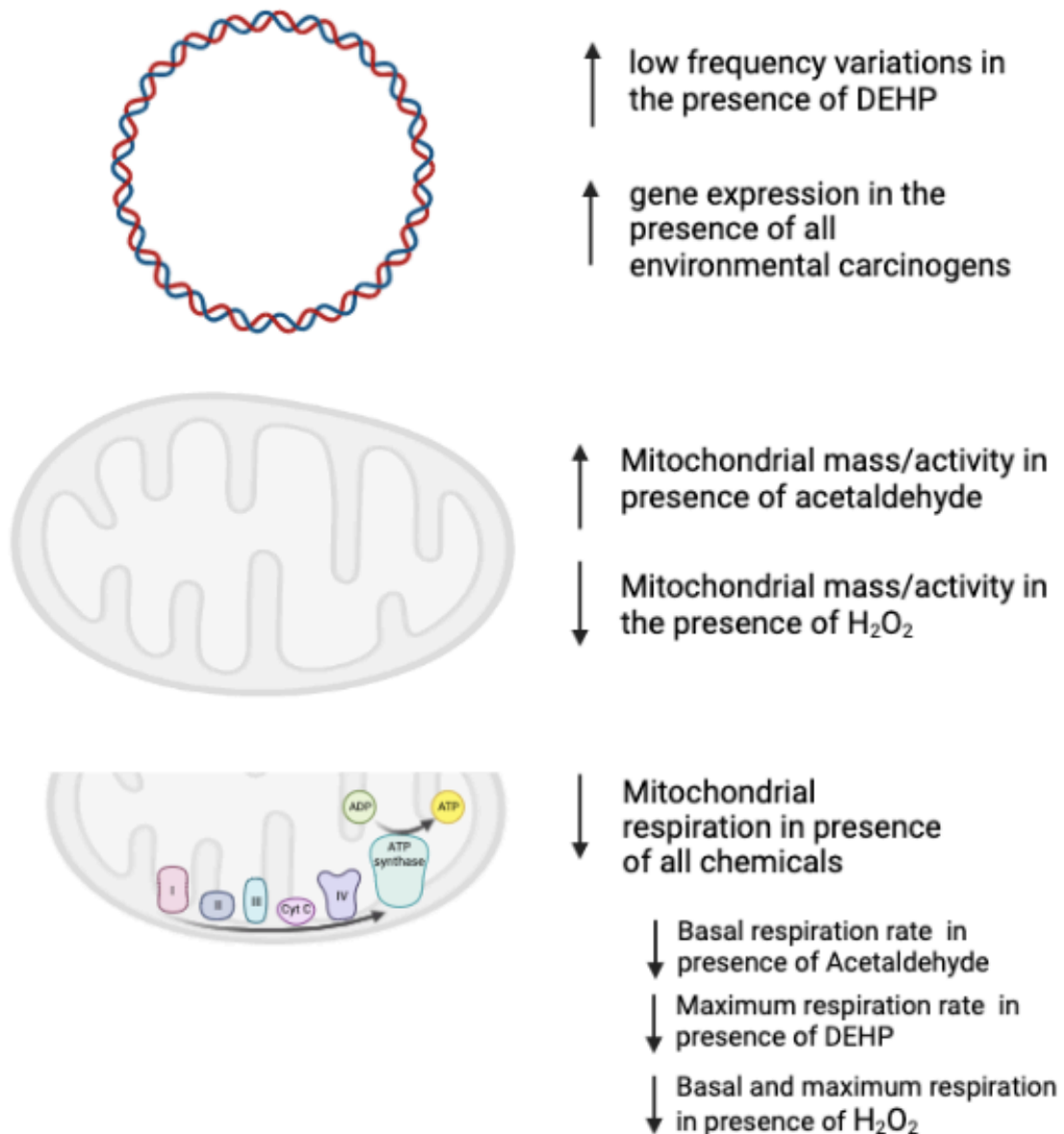


Figure 53: The effects of high glucose in the presence of Acetaldehyde, diethyl hexyl phthalate (DEHP) and Hydrogen Peroxide (H₂O₂) Decreases are represented with downwards arrows while increases are represented with upwards arrows. Image was made using Biorender.

While the presence of the majority of the environmental carcinogens tested appears to be affecting how the high glucose impacts the mitochondria, the effects of the chemicals produced few changes when comparing acetaldehyde and DEHP exposure under normal and high glucose conditions. Under normal glucose conditions, there was an increase in cell fitness, mitochondrial and non-mitochondrial respiration rates and therefore ATP production due to DEHP under normal glucose conditions. Under high glucose, DEHP has similar observations apart from within the maximum respiration rate, where there was a non-significant decrease, while the cell fitness was also found to be significantly decreased.

Under both normal and high glucose conditions, acetaldehyde exposure, unlike DEHP, produced significant decreases in mitochondrial basal respiration rates and therefore ATP production. Additionally, non-mitochondrial respiration was significantly increased such as within the comparison. The one differing effect within the cells exposed to acetaldehyde within the maximal respiration rate was under normal glucose conditions, there was a significant increase while under high glucose conditions there was a significant decrease. This suggests the acetaldehyde is having similar, damaging effects on mitochondrial respiration. Furthermore, acetaldehyde under normal glucose conditions only produce significant genomic changes within the sequencing data; there was a decrease in variations within the gene due to acetaldehyde exposure, potentially due to a protective effect ALDH2 has against genotoxic damage caused by acetaldehyde exposure.

Unlike the previous two chemicals, the effects of hydrogen peroxide exposure appeared to differ because of high glucose exposure compared to under normal glucose exposure, particularly within the metabolic data. Under normal glucose conditions, hydrogen peroxide exposure appears to result in an increase in both mitochondrial and non-mitochondrial respiration processes, while under high glucose, the opposite is seen within the mitochondrial respiration rate, potentially due to a decrease in cell fitness.

1-NP was the only one of the four environmental chemicals that resulted in significant changes to mitochondrial mass, shown through an increase in mitochondria with a mass that fall in the low quintile and a decrease in those that fall in the highest quintile under high glucose conditions only. Furthermore, under high glucose there was also a significant decrease in non-mitochondrial respiration due to 1-NP exposure, while under normal glucose, both basal and non-mitochondrial respiration was significantly decreased. Under normal glucose, 1-NP exposure also results in a decrease in basal respiration as well as decreases in maximal respiration, proton leaking, ATP production and cell fitness. All of this would suggest that 1-NP is having similar effects within the cell regardless of glucose concentration exposed to. In the groups HG + 1-NP and NG + 1-NP when compared against HG + DMSO and NG + DMSO, there was a decrease in mitochondrial respiration, while within HG + 1-NP, both mitochondrial and non-mitochondrial processes are affected.

While DMSO itself was used as a solvent and therefore an additional control group, it too was shown to cause significant changes to respiration within the cell. Under both normal and high glucose, there appears to be a decrease in basal and maximal respiration rates, while the fitness of the cell is also decreased due to DMSO exposure. The only differing effect between the two is an increase in non-mitochondrial respiration within the normal glucose group, suggesting an attempt to compensate ATP loss due to a decrease in mitochondrial respiration under normal glucose only.

This study has not only produced an overall snapshot of the effects of high glucose alone and in conjunction with four common environmental carcinogens on the mitochondria, but it has allowed us to further our knowledge of how those with type 1 and type 2 diabetes are more susceptible to cancer development through exposure to these everyday chemicals. This information will allow improvements of chemical safety assessment, to account for those individuals with increased susceptibility for cancer development especially as prevalence of diabetes within the population is increasing. Furthermore, these findings can provide those living with diabetes further advice to reduce potential risk of cancer development through avoidance of these chemicals as well as providing further information to improve treatment plans for those at a higher risk of cancer, such as the use of metformin. Within this study, there are increases in mtDNA variation and decreases to mitochondrial respiration, as expected with cancer development, as mentioned in section 1.3.7,

further studies have shown that metformin prevents tobacco carcinogen induced lung tumourgenesis (Memmott et al., 2010). It has also been shown reduced development of head and neck squamous cell carcinoma within oral-specific carcinogenesis mouse model with oral premalignant lesions following carcinogen exposure (Vitale-Cross et al., 2012).

In the future, more chemicals can be investigated and compared to results seen here to provide further insight into other environmental carcinogens mechanism within diabetic individuals to improve safety assessment and public knowledge further. Finally, following future work, these results can be used potentially as a monitoring strategy within diabetic individuals where i.e., blood can be taken to monitor cells within the blood for these changes to catch early onset cancer or prevent cancer development through detection of pre-cancerous changes.

Within this study, it has been found that high glucose associated with type 1 and type 2 diabetes alone and under differing environmental chemical exposure had different effects within the mitochondrial genome in the case of DEHP and DMSO while all, but 1-NP treatment, showed varying changes to the metabolic profile of the mitochondria due to high glucose. Additionally, all four environmental chemicals in the presence of high glucose have slightly differing effects, mainly metabolic changes within the mitochondria. However, as stated previously, there have been studies suggesting effects not found to be significant in this study and therefore future work is required to develop our understanding to the effect of hyperglycaemia and its role in increase susceptibility to cancer development.

Chapter 6. Appendix

6.1 RPD data

In accordance with guidelines set or cytotoxicity limits which require reductions in cell growth to be greater than 50% after the removal of anomalous results, the data used in this study meets these guidelines due to all Relative Population Doubling (RPD) values calculated from cell counts taken before and after chemical treatment being above 50%, as shown in Fig. 54 (Greenwood et al.,2004).

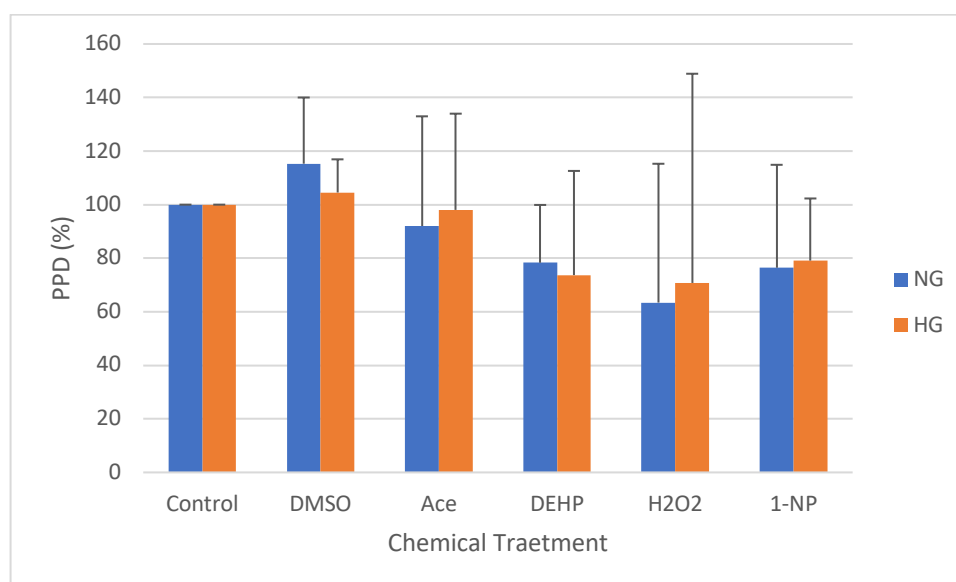


Figure 54: The average Relative Population Doubling (RPD) values for all chemical treatments used, Acetaldehyde (Ace), Di-2-ethylhexyl Phthalate (DEHP), Hydrogen Peroxide (H₂O₂), 1-nitropyrene (1-NP) and controls (TK6- water controls, MCL-5- DMSO controls)

6.2 Stock Calculations

6.2.1 19mM glucose dosing solution calculations

Molarity wanted in cells= 0.000019M

Molarity needed for dilution glucose solution = 1.9M (as 100μL of solution used as dosing solution)

Total volume of dilution Stock A= 5mL

$$\text{Grams used of 1-NP powder} = \frac{1.9\text{M} \times 180.156\text{g/mol} \times 5\text{mL}}{1000} = 1.71\text{g}$$

6.2.2 Carcinogens stock concentrations

6.2.2.1 Acetaldehyde

Concentration of stock= 40%/ 400 g/L

Molarity of stock= 9.1M

Molarity wanted in cells= 1000 μ M/ 0.001M

Molarity needed for dosing= 0.001M x 100 (as 100 μ L of carcinogen placed in 10mL solution)
= 0.1M

M1=9.1M

M2= 0.1M

V1= ?

V2= 300 μ L

M1V1= M2V2

$$\frac{9.1\text{M} \times V1}{9.1\text{M}} = \frac{0.1\text{M} \times 300\mu\text{L}}{9.1\text{M}}$$

$$V1 = 3 \mu\text{L}$$

Therefore, 3 μ L of acetaldehyde stock is added to 297 μ L of water.

6.2.2.2 DEHP

Concentration of stock= 0.985g/mL or 985g/L

Molarity of stock= 2.52M

Molarity wanted in cells= 2000 μ M/ 0.002M

Molarity needed for dosing= 0.002M x 100 (as 100 μ L of carcinogen placed in 10mL solution)
= 0.2M

M1=2.52mM

M2= 0.2M

V1= ?

V2= 300 μ L

M1V1= M2V2

$$\frac{2.52\text{M} \times V1}{2.52\text{M}} = \frac{0.2\text{M} \times 300\mu\text{L}}{2.52\text{M}}$$

$$V1 = 24\mu\text{L}$$

Therefore, 24 μ L of DEHP stock is added to 276 μ L of DMSO.

6.2.2.3. Hydrogen Peroxide

Stock A

Concentration of stock= 30%/ 300 g/L

Molarity of stock= 8.8M

Molarity needed for Stock A= 0.088M

M1=8.8M

V1= ?

M2= 0.088M

V2= 500μL

$$M1V1= M2V2$$

$$\frac{8.8M \times V1}{8.8M} = \frac{0.088M \times 500\mu L}{8.8M}$$

$$V1 = 5 \mu L$$

Stock B

Molarity of stock= 0.088M

Molarity wanted in cells= 20μM/ 0.00002M

Molarity needed for dosing= 0.002M

M1=0.088M

V1= ?

M2= 0.002M

V2= 300μL

$$M1V1= M2V2$$

$$\frac{0.088M \times V1}{0.088M} = \frac{0.002M \times 300\mu L}{0.088M}$$

$$V1 = 7 \mu L$$

Therefore, 7μL of H₂O₂ is added to 293μL of water.

6.2.2.4 1-nitropyrene

Stock A (dilution)

Concentration of stock= 0.02g/L

$$\text{Molarity of stock} = \frac{0.02\text{g/L}}{247.25\text{g/mol}} \\ = 0.000081\text{M}$$

Molarity wanted in cells= 0.0081M

Molarity needed for dilution solution 1= 0.81M

Total volume of dilution Stock A= 500μL/0.5mL (as same used in Hydrogen Peroxide)

$$\text{Grams used of 1-NP powder} = \frac{0.81\text{M} \times 247.5\text{g/mol} \times 0.5\text{mL}}{1000} \\ = 0.1\text{g}$$

Therefore 0.1g of 1-nitropyrene stock is added to 500μL of DMSO.

Stock B (dosing solution)

M1=0.81M

M2= 0.0081M

V1= ?

V2= 300μL

$$M1V1= M2V2$$

$$V1 = \frac{0.0081 \times 300}{0.81}$$

$$V1 = 3 \mu\text{L}$$

Therefore, 3μL of 1-NP stock is added to 297μL of DMSO.

6.3 Amplicon 1 optimisation

6.3.1. Amplicon 1 optimisation methodology

Due to issues highlighted during imaging of amplicon 1 samples, further optimisation was carried out on amplicon 1 samples to determine optimal conditions. In Table. 13, conditions described in A were carried out first and used to determine the conditions used in B.

Table 13: A) The protocol used for the Long-Range PCR reaction in the thermal cycler at a larger gradient of annealing temperatures to determine optimal annealing temperature B) Shows the protocol used for the Long-Range PCR reaction in the thermal cycler at a smaller gradient of annealing temperatures to determine optimal annealing temperature.

A

Stage	Cycles	Temperature	Time
Initial Denaturation	1	98°C	30 seconds
Denaturation	35	98°C	10 seconds
Annealing		59-65°C	10 seconds
Extension		72°C	15-30 seconds per 1kb 6 minutes
Final Extension	1	72°C	5 minutes
		4	hold

B

Stage	Cycles	Temperature	Time
Initial Denaturation	1	98°C	30 seconds
Denaturation	35	98°C	10 seconds
Annealing		59-60°C	10 seconds
Extension		72°C	15-30 seconds per 1kb 6 minutes
Final Extension	1	72°C	5 minutes
		4	hold

Further optimisation was required following the results of the first two gradients, as shown in Table. 14. The conditions described in A were used to test the original concentration of DNA (75ng) and double the concentration of DNA (150ng).

Table 14: A) The protocol used for the Long-Range PCR reaction in the thermal cycler with a larger gradient of annealing temperatures to determine optimal annealing temperature B) Shows the protocol used for the Long-Range PCR reaction in the thermal cycler at a smaller gradient of annealing temperatures at lower temperatures to determine optimal annealing temperature.

A

Stage	Cycles	Temperature	Time
Initial Denaturation	1	98°C	30 seconds
Denaturation	35	98°C	10 seconds
Annealing		59.2-62°C	10 seconds
Extension		72°C	15-30 seconds per 1kb 6 minutes
Final Extension	1	72°C	5 minutes
		4 °C	hold

B

Stage	Cycles	Temperature	Time
Initial Denaturation	1	98°C	30 seconds
Denaturation	35	98°C	10 seconds
Annealing		57-60°C	10 seconds
Extension		72°C	15-30 seconds per 1kb 6 minutes
Final Extension	1	72°C	5 minutes
		4 °C	hold

Following the previous runs, 59.2°C. appeared to be the optimal temperature. A further run was done at this temperature to confirm this (Table. 15).

Table 15: A. Shows the protocol used for the Long-Range PCR reaction in the thermal cycler at an annealing temperature of 59.2°C.

Stage	Cycles	Temperature	Time
Initial Denaturation	1	98°C	30 seconds
Denaturation	35	98°C	10 seconds
Annealing		59.2°C	10 seconds
Extension		72°C	15-30 seconds per 1kb 6 minutes
Final Extension	1	72°C	5 minutes
		4	hold

The final optimisation was done by altering the annealing time from 10 seconds to 20 seconds under the conditions described in Table 16.

Table 16: A. Shows the protocol used for the Long-Range PCR reaction in the thermal cycler at an annealing temperature of 59.2°C and altered annealing time from 10 seconds to 20 seconds.

Stage	Cycles	Temperature	Time
Initial Denaturation	1	98°C	30 seconds
Denaturation	35	98°C	10 seconds
Annealing		59.2°C	20 seconds
Extension		72°C	15-30 seconds per 1kb 6 minutes
Final Extension	1	72°C	5 minutes
		4	hold

6.3.2. Amplicon 1 optimisation results

Initially, a gradient was set up as described in table 1.A to try determining the optimal temperature region for the primers; as seen in Fig. 54, this appeared to be in the range of 59°C and 59.4°C due to the presence of bands in region which was expected of the fragment size.

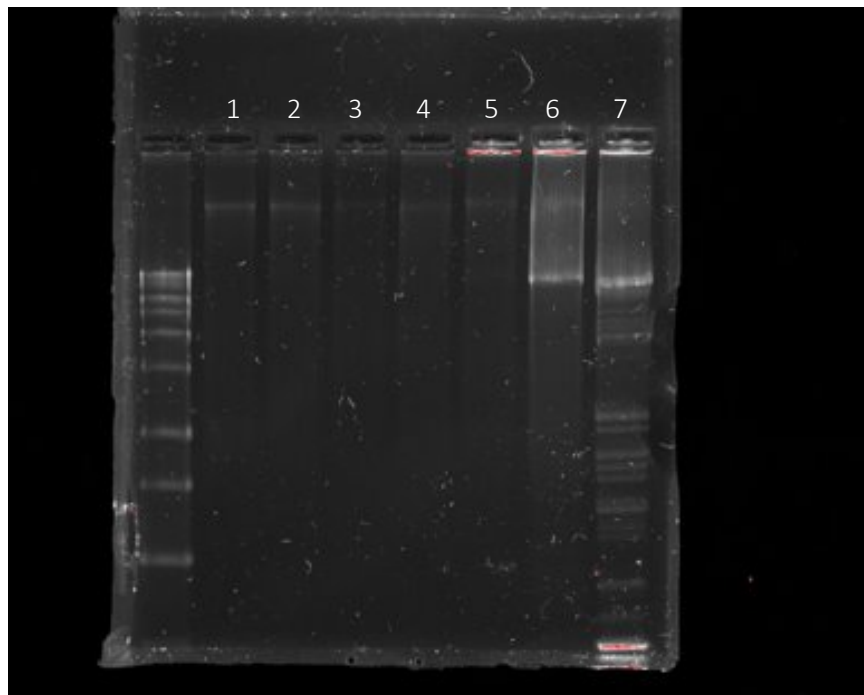


Figure 54: Gel run for normal glucose water control TK6 sample extracted 2nd June following the protocol described in Table 13.A where 1= 64.6°C, 2= 63.8°C, 3= 62.7°C, 4= 61.3°C, 5= 60.2°C, 6= 59.4°C, AND 7= 59°C

As the amplicon primer showed it was able to work around 59°C, a second gradient was set up as described in table 13.B to further optimise the temperature accounting for temperatures slightly below 59°C. From the results shown in Fig. 55, a temperature of 59.2°C appeared to give the best results in the MCL-5 sample (Fig.55.B) while the bands in TK6 were still too high up on the gel compared to what was expected (Fig. 55. A).

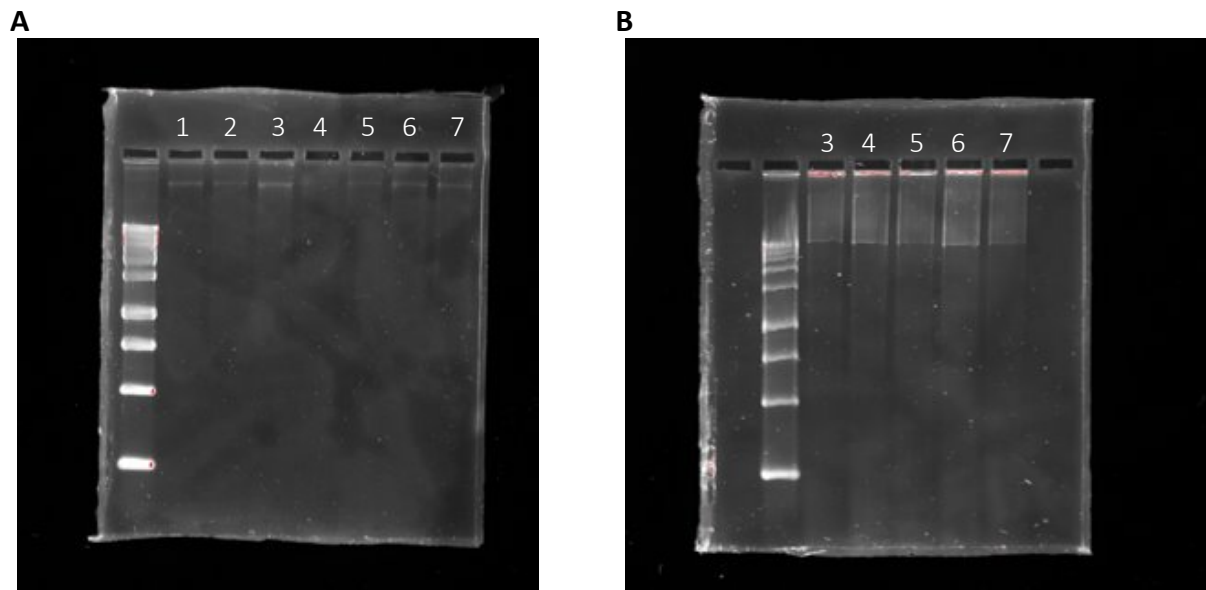


Figure 55: A) Gel run for normal glucose water control TK6 sample extracted 2nd June 2021 following the protocol described in Table 13.B) Gel run for normal glucose MCL-5 sample extracted 29th January 2021 following the protocol described in Table 12.B. 1= 60°C, 2= 59.9°C, 3= 59.8°C, 4= 59.6°C, 5= 59.4°C, 6= 59.2°C, 7= 59.1°C

Following these results, while within Fig.56.B there appears to be a band for amplicon 1 at 59.2°C, within the TK6 sample and remaining temperatures within the MCL-5 sample used, there were no bands present. Therefore, further optimisation looking at DNA concentration was done (Fig.56). Following this, as there were bands present at the original concentration used (75ng), shown in Fig. 56. A, it was decided this concentration would be used in further optimisation experiments.

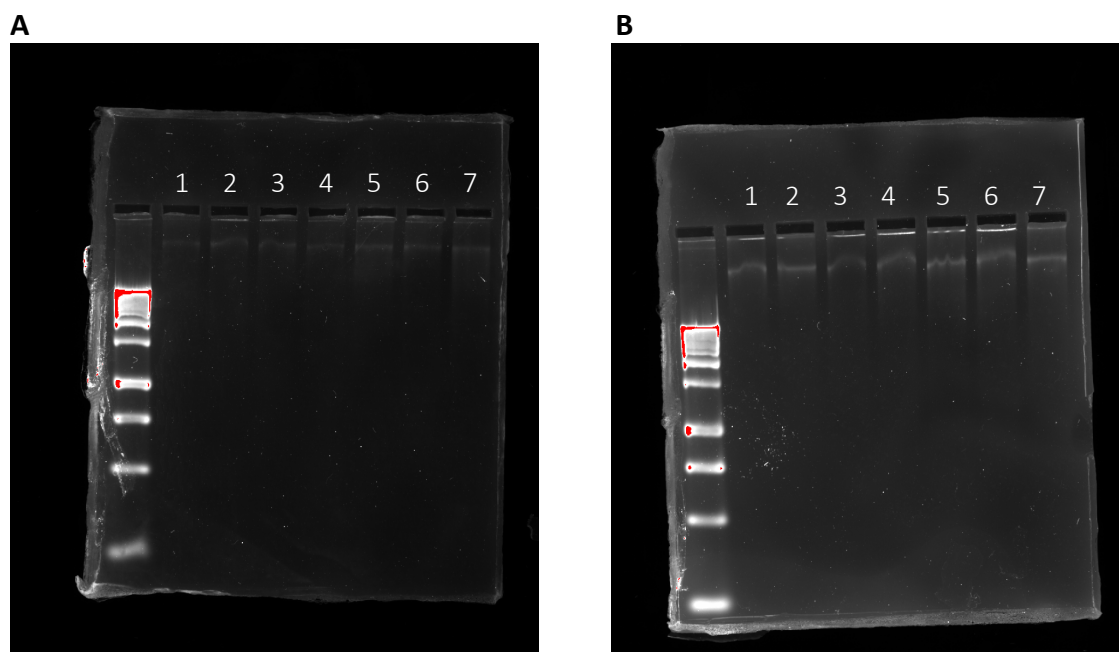


Figure 56: A) Gradient described in Table 13.A tried on normal glucose water control TK6 sample extracted 2nd June 2021 to determine amplicon 1 optimal temperature in TK6 samples at a DNA concentration of 75ng B) Gradient described in Table 13.A tried on normal glucose water control TK6 sample extracted 2nd June 2021 to determine amplicon 1 optimal temperature in TK6 samples at a DNA concentration of 150ng. 1= 62°C, 2= 61.8°C, 3= 61.4°C, 4= 60.9°C. 5= 60.2°C, 6= 59.6°C, 7= 59.2°C.

As there was no consistency within these results, a slightly larger gradient including temperatures below 59°C were tested if the optimal temperature was just out of the previous gradients used range while also including a few higher temperatures that showed bands present previously, even though they were not in the correct position. As seen in Fig. 57, this gradient did not yield any bands within the MCL-5 sample while there were only bands present with 60°C and 58.2°C bands, which again were in the wrong position and very faint.

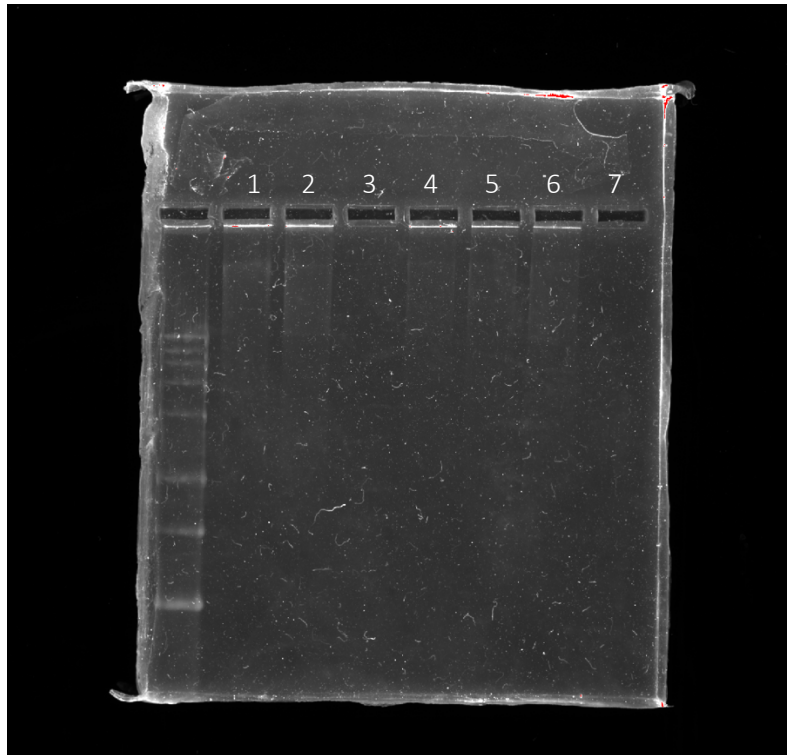


Figure 57: A) Gradient described in Table 13.B tried on normal glucose water control TK6 and MCL-5 sample extracted 4TH February 2021 to determine amplicon 1 optimal temperature in TK6 samples. 1= 60°C (TK6), 2= 58.2°C (TK6), 3= 57.0°C (TK6), 4= 60°C (MCL-5), 5= 58.2°C (MCL-5), 6= 57.0°C (MCL-5), 7= Negative control

Following the positive results at 59.2°C within the MCL-5 in Fig. 55.B, multiple samples were tested using this optimal temperature as described in Table 3, as shown in Fig. 58. However, such as within the TK6 samples, in Fig. 58.A there was no bands present in the region of 11kb as would be if amplicon 1 has successfully amplified amplicon 1.

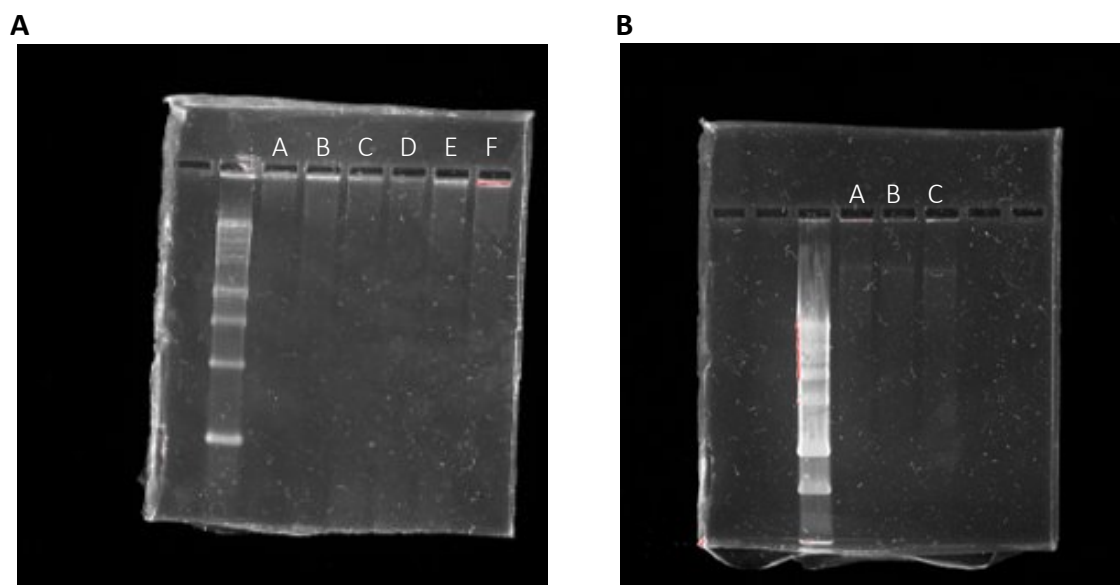


Figure 58: A) Gel run for a full replicate of MCL-5 samples extracted 29th January 2021 following the protocol of Table 14. A= Normal Glucose + DMSO, B=High Glucose +DMSO, C=Normal Glucose + 1-nitropyrene, Old D=High Glucose + 1-nitropyrene Old, E=Normal Glucose + 1-nitropyrene New, F= High Glucose + 1-nitropyrene New, B) Gel run for MCL-5 samples following the protocol of Table 14. A= Normal Glucose + DMSO extracted 28th January 2021, B= High Glucose extracted 28th January 2021, C= Normal Glucose + DMSO extracted 4th February 2021.

A larger gradient was used again within the TK6 normal glucose sample to further try obtaining an optimal temperature. However, as within previous runs, many of the bands were too high to be our fragment size. While within Fig. 59.A, lane 7, there appeared to be a second smaller band present roughly where we would expect; the presence of the additional larger band and the absence of the band of the smaller fragment within lane 7 of Fig. 59. B further suggests this temperature is not the amplicon optimal annealing temperature.

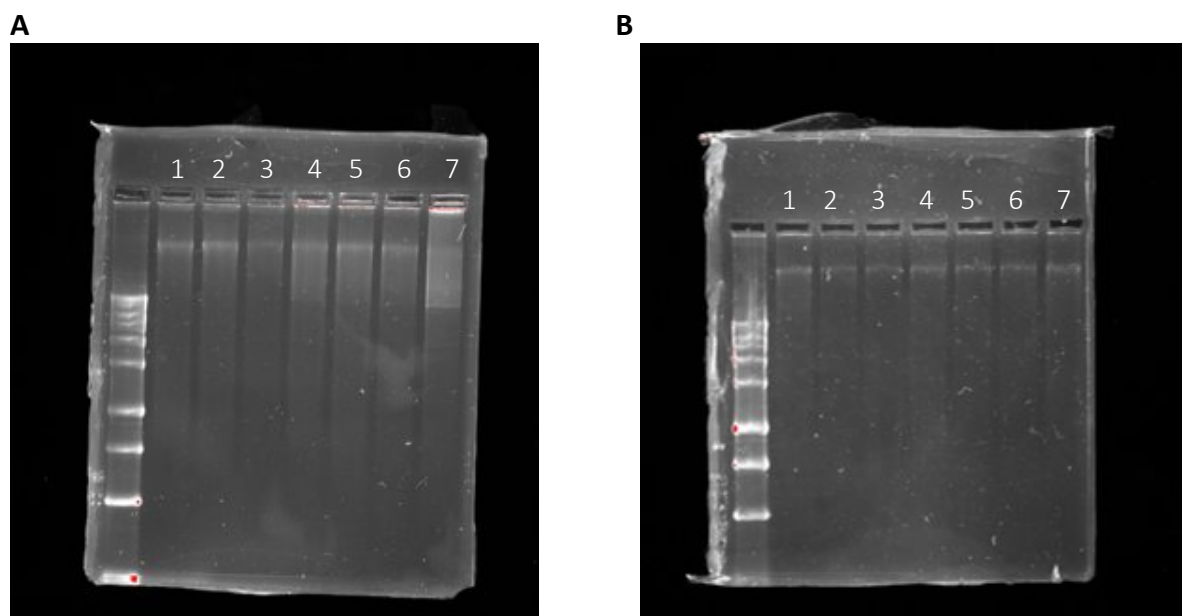


Figure 59: Gradient described in Table 15 tried on different normal glucose water control TK6 samples to determine amplicon 1 optimal temperature in TK6 samples A) sample extracted from pellet stored from 4th February 2021. B) sample extracted 3rd December 2020. 1= 62°C, 2= 61.8°C, 3= 61.4°C, 4= 60.9°C. 5= 60.2°C, 6= 59.6°C, 7= 59.2°C.

Due to time restraints, no further optimisation could occur and therefore only amplicon 2 was taken forward to sequencing.

6.4 SPSS p-values obtained.

6.4.1 Next Generation Sequencing Results

6.4.1.1 No Threshold applied.

6.4.1.1.1 TK6 comparisons

Table 17: All results found within the Next Generation Sequencing data with no threshold during the comparison of TK6 samples.

	high glucose	high glucose acetaldehyde	high glucose DEHP	high glucose DMSO	high glucose H2O2	normal glucose	normal glucose acetaldehyde	normal glucose DEHP	normal glucose DMSO
high glucose acetaldehyde	3.34E-223	NA	NA	NA	NA	NA	NA	NA	NA
high glucose DEHP	2.79E-186	0	NA	NA	NA	NA	NA	NA	NA
high glucose DMSO	1.25E-173	2.21E-12	0	NA	NA	NA	NA	NA	NA
high glucose H2O2	0.439	1.84E-186	7.18E-160	1.62E-125	NA	NA	NA	NA	NA
normal glucose	0.541	2.34E-217	3.14E-182	1.19E-170	0.841	NA	NA	NA	NA
normal glucose acetaldehyde	1.36E-281	0	0.294	0	1.36E-217	8.14E-280	NA	NA	NA
normal glucose DEHP	0	0	2.51E-83	0	0	0	5.11E-104	NA	NA
normal glucose DMSO	1.30E-29	2.07E-104	0	1.53E-62	9.28E-21	3.06E-32	0	0	NA
normal glucose H2O2	4.25E-07	1.39E-263	9.87E-114	1.10E-201	7.57E-08	1.44E-07	2.20E-162	0	1.03E-54

6.4.1.1.2 MCL-5 comparisons

Table 18: All results found within the Next Generation Sequencing data with no threshold during the comparison of MCL-5 samples.

	high glucose	high glucose 1- NP	normal glucose
high glucose 1- NP	1.24E-61	NA	NA
normal glucose	1.04E-24	2.27E-191	NA
normal glucose 1- NP	0.864	3.06E-70	5.09E- 30

6.4.1.2 1% Threshold

6.4.1.2.1 TK6 comparisons

Table 19: All results found within the Next Generation Sequencing data with a 1% threshold during the comparison of TK6 samples.

	high glucose	high glucose acetaldehyde	high glucose DEHP	high glucose DMSO	high glucose H2O2	normal glucose	normal glucose acetaldehyde	normal glucose DEHP	normal glucose DMSO
high glucose acetaldehyde	0.707	NA	NA	NA	NA	NA	NA	NA	NA
high glucose DEHP	0.003	0.005	NA	NA	NA	NA	NA	NA	NA
high glucose DMSO	0.252	0.561	0.075	NA	NA	NA	NA	NA	NA
high glucose H2O2	0.707	0.519	0.002	0.171	NA	NA	NA	NA	NA
normal glucose	0.556	0.236	6.38E-05	0.061	0.841	NA	NA	NA	NA
normal glucose acetaldehyde	0.138	0.227	0.208	0.707	0.075	0.015	NA	NA	NA
normal glucose DEHP	0.760	0.638	0.00541135	0.236	0.971	0.745	0.138	NA	NA
normal glucose DMSO	0.276	0.208	6.38E-05	0.031	0.618	0.707	0.007	0.561	NA
normal glucose H2O2	0.618	0.971	0.015	0.561	0.450	0.251	0.326	0.556	0.162

6.4.1.2.2 MCL-5 comparisons

Table 20: All results found within the Next Generation Sequencing data with a 1% threshold during the comparison of MCL-5 samples.

	high glucose	high glucose 1-NP	normal glucose
high glucose 1-NP	0.498	NA	NA
normal glucose	0.205	0.499	NA
normal glucose 1- NP	0.499	0.738	0.498

6.4.2 qt-PCR

6.4.2.1 TK6 comparisons

Table 21: All results found within the qt-PCR data during the comparison of TK6 samples.

Sample 1	Sample 2	p-values
NG	HG	1.000
NG + DMSO	HG + DMSO	0.996
NG	NG + DMSO	1.00
HG	HG + DMSO	0.970
NG	NG + Ace	0.541
HG	HG + Ace	0.378
NG + Ace	HG + Ace	0.923
NG + DMSO	NG + DEHP	0.445
HG + DMSO	HG + DEHP	0.480
NG + DEHP	HG + DEHP	1.000
NG	NG + H2O2	0.668
HG	HG + H2O2	0.662
NG + H2O2	HG + H2O2	0.990

6.4.2.2 MCL-5 comparisons

Table 22: All results found within the qt-PCR data during the comparison of MCL-5 samples.

Sample 1	Sample 2	p-values
NG + DMSO	HG + DMSO	0.987
NG + DMSO	NG + 1-NP	0.976
HG + DMSO	HG + 1-NP	0.621
NG + 1-NP	HG + 1-NP	0.594

6.4.3 Morphology Results

6.4.3.1 Average Integrated Intensity results

6.4.3.1.1 TK6 Comparisons

Table 23: All results found within the Fluorescence Microscopy average integrated intensity data during the comparison of TK6 samples.

Sample 1	Sample 2	p-values
NG	HG	0.984
NG + DMSO	HG + DMSO	0.689
NG	NG + DMSO	1.00
HG	HG + DMSO	0.991
NG	NG + Ace	
HG	HG + Ace	0.946
NG + Ace	HG + Ace	0.762
NG + DMSO	NG + DEHP	0.986
HG + DMSO	HG + DEHP	0.629
NG + DEHP	HG + DEHP	0.748
NG	NG + H2O2	0.970
HG	HG + H2O2	0.995
NG + H2O2	HG + H2O2	0.601

6.4.3.1.2 MCL-5 Comparisons

Table 24: All results found within the Fluorescence Microscopy average integrated intensity data during the comparison of MCL-5 samples.

Sample 1	Sample 2	p-values
NG + DMSO	HG + DMSO	1.000
NG + DMSO	NG + 1-NP	1.000
HG + DMSO	HG + 1-NP	0.400
NG + 1-NP	HG + 1-NP	0.594

6.4.3.1 Quintile results

6.4.3.2.1 TK6 Comparisons

Table 25: All results found within the Fluorescence Microscopy quintile data during the comparison of TK6 samples.

Sample 1	Sample 2	Lowest	Low	Normal	High	Highest
NG	HG	1.000	0.931	0.787	0.979	0.873
NG	NG + DMSO	0.343	1.000	1.000	0.808	1.000
HG	HG + DMSO	0.997	0.745	0.981	0.777	0.998
NG + DMSO	HG + DMSO	0.893	0.808	1.000	0.971	0.688
NG	NG + Ace	0.343	0.343	0.957	0.972	1.000
HG	HG + Ace	0.851	0.520	0.786	0.804	0.518
NG + Ace	HG + Ace	0.627	0.023	0.871	0.774	0.122
NG + DMSO	NG + DEHP	0.327	0.996	0.819	0.443	0.999
HG + DMSO	HG + DEHP	0.752	0.931	0.629	0.652	0.722
NG + DEHP	HG + DEHP	0.598	0.400	0.557	0.285	0.859
NG	NG + H2O2	0.343	0.343	1.000	0.969	0.528
HG	HG + H2O2	1.000	0.659	0.818	0.892	1.000
NG + H2O2	HG + H2O2	0.462	0.943	<0.001	0.732	0.687

6.4.3.2.2 MCL-5 Comparisons

Table 26: All results found within the Fluorescence Microscopy quintile data during the comparison of MCL-5 samples.

Sample 1	Sample 2	Lowest	Low	Normal	High	Highest
NG + DMSO	HG + DMSO	0.941	0.128	0.671	0.737	0.314
NG + DMSO	NG + 1-NP	0.649	0.496	0.424	0.381	0.909
HG + DMSO	HG + 1-NP	0.890	0.011	0.897	0.445	0.006
NG + 1-NP	HG + 1-NP	0.941	0.128	0.671	0.737	0.314

6.4.4 Seahorse Data

6.4.4.1 TK6 comparisons

Table 27: All results found within the Seahorse Bioanalyzer data during the comparison of TK6 samples.

Sample 1	Sample 2	Basal Respiration	ATP Production	Proton Leak	Maximal Respiration	Spare Capacity	Non-Mitochondrial Respiration
NG	HG	0.996	0.808	0.442	<0.001	<0.001	0.852
NG	NG + DMSO	0.012	0.115	0.808	<0.001	<0.001	0.003
HG	HG + DMSO	<0.001	0.01	0.437	<0.001	<0.001	0.072
NG + DMSO	HG + DMSO	<0.001	0.133	0.825	0.006	<0.001	0.127
NG	NG + Ace	0.003	0.049	0.847	0.026	0.006	0.011
HG	HG + Ace	<0.001	0.045	0.359	<0.001	0.124	<0.001
NG + Ace	HG + Ace	0.016	0.398	0.815	0.162	0.44	0.198
NG + DMSO	NG + DEHP	<0.001	0.02	0.704	<0.001	<0.001	0.05
HG + DMSO	HG + DEHP	<0.001	<0.001	0.266	0.78	<0.001	0.009
NG + DEHP	HG + DEHP	<0.001	0.242	0.965	<0.001	<0.001	0.827
NG	NG + H2O2	<0.001	0.143	0.204	<0.001	0.05	0.007
HG	HG + H2O2	0.903	0.977	0.774	<0.001	0.002	0.987
NG + H2O2	HG + H2O2	<0.001	0.066	0.44	0.001	0.513	0.014

6.4.4.2 MCL-5 comparisons

Table 28: All results found within the Seahorse Bioanalyzer data during the comparison of MCL-5 samples.

Sample 1	Sample 2	Basal Respiration	ATP Production	Proton Leak	Maximal Respiration	Spare Capacity	Non-Mitochondrial Respiration
NG + DMSO	HG + DMSO	<0.001	<0.001	0.05	<0.001	0.05	0.151
NG + DMSO	NG + 1-NP	0.05	<0.001	0.012	<0.001	0.034	0.208
HG + DMSO	HG + 1-NP	0.033	0.374	0.127	0.631	0.513	0.015
NG + 1-NP	HG + 1-NP	0.275	0.601	0.827	0.071	0.173	0.051

6.6 Additional Data for Chapter 4

6.6.1 Effects of chemicals under differing glucose concentrations in TK6 cells on the shift of integrated intensity data.

As shown in Fig. 60. A there is a marked but non-significant increase of 22.44% in the number of mitochondria that fall within the lowest category for integrated intensity when comparing NG + DMSO against NG (p-value= 0.343) while the remaining categories are slightly decreased (p-value= 1.00, 1.00, 0.808 and 1.000 respectively); the opposite is seen when comparing the HG group with the HG + DMSO group (Fig. 60. B), where there is a 30.25% increase in mitochondria with an integrated intensity that is considered in the high category, however this was not found to be significant (p-value= 0.777). The data shown in Fig.37.B suggests a contradictory trend to that seen within the data collected for the average integrated intensity as the increase in mitochondria within the high category would suggest that there is an increase in intensity in those treated with DMSO under high glucose conditions.

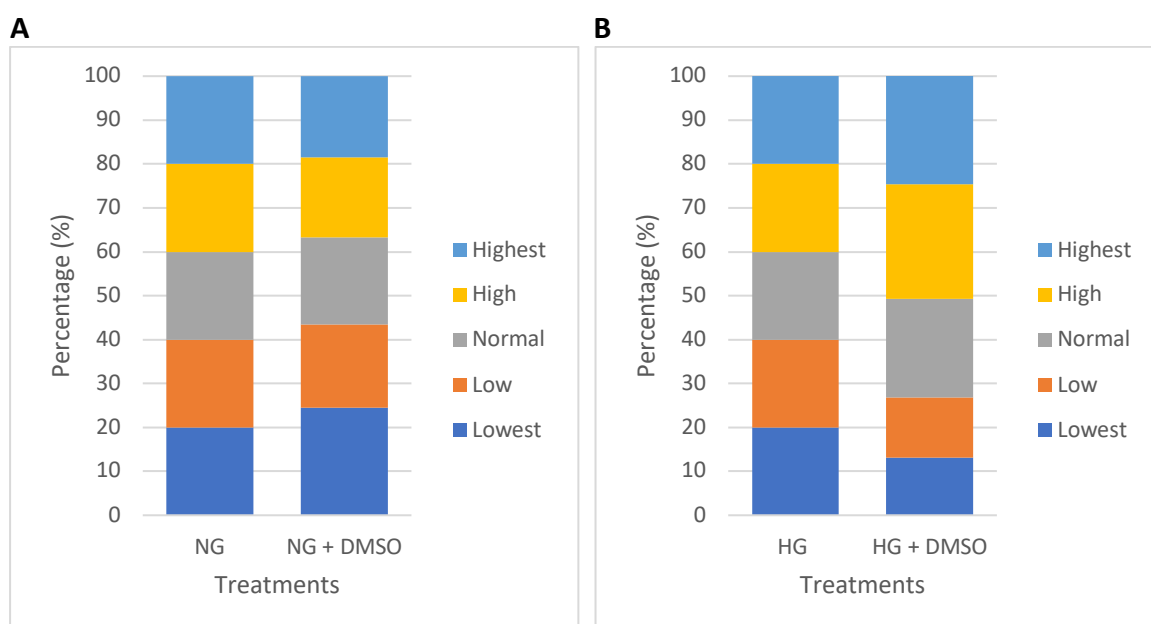


Figure 60: A) The intensity changes seen within the data obtained for normal glucose control in comparison to the microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 32 images were used for each of the samples. B) The intensity changes seen within the data obtained for high glucose controls in comparison to the high glucose and DMSO control (HG and HG + DMSO) with the fluorescence microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 32 images were used for each of the samples. Normal glucose and DMSO (NG and NG + DMSO respectively) with the fluorescence

Comparing the shift in the average integrated intensity data shown in Fig.57 within the NG + Ace sample group against the NG water control, the only noticeable changes are the percentage of mitochondria with the integrated intensities that fall within the low and normal quintiles, with both groups increasing by 3.05% and 7.23% respectively. However, none of these observations were significant (p-value= 0.343 and 0.957). Comparison of HG + Ace against the HG water control to see the effect of acetaldehyde under high glucose conditions show the opposite effect as the previous

normal glucose comparison as the number of mitochondria with an integrated intensity within the lowest, low, and normal categories all decrease (with the biggest decrease of 26.37% seen within the low group) (p-values= 0.851, 0.520, and 0.786 respectively) whereas the number of mitochondria with an integrated intensity seen within the high and highest groups are increased by 15.21% (p= 0.804) and 45.80% respectively (p=0.518).

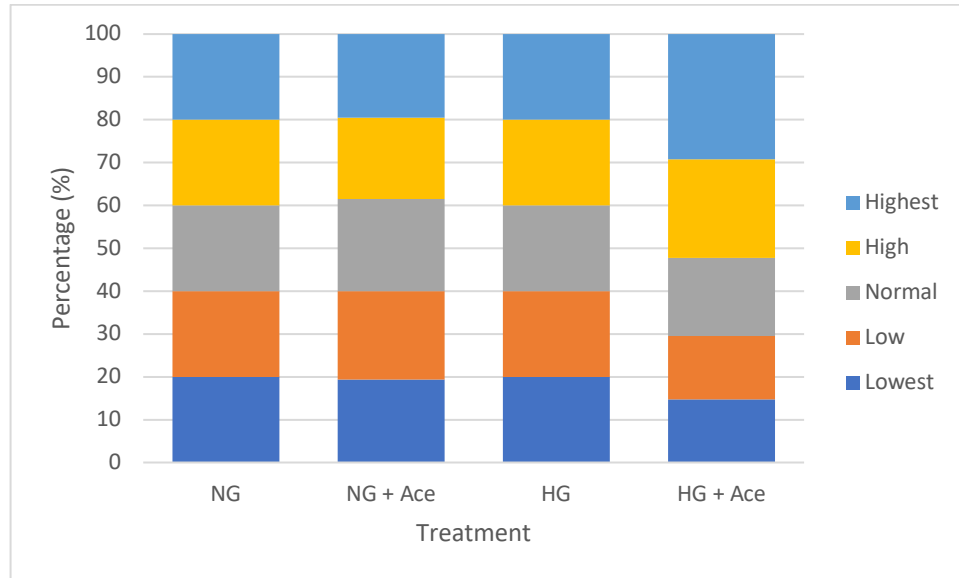


Figure 57: Shows the intensity changes seen within the data obtained for normal glucose treated with acetaldehyde (NG + Ace) and high glucose and acetaldehyde (HG + Ace) in comparison to the normal and high glucose controls (NG and HG respectively) with the fluorescence microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 32 images were used for each of the samples.

Comparing the effect of the DEHP on the integrated intensity data and its trends, it can be seen in Fig.58 that in comparison of NG + DEHP against the NG + DMSO control, there is a decrease of 32.87% and 6.04% in the number of mitochondria that have an integrated intensity that fall within the lowest (p= 0.327) and low category (p= 0.996) respectively. The remaining categories, normal, high and highest saw increases, with the largest being in the high category of 26.70% (p=0.443). Furthermore, comparing HG + DEHP against HG + DMSO yielded similar results, with the only difference being a slight increase of 0.39% in the frequency of mitochondria that fall within the low category (p= 0.931); there was also a decrease of 10.08% in the frequency in the normal category (p=0.629).

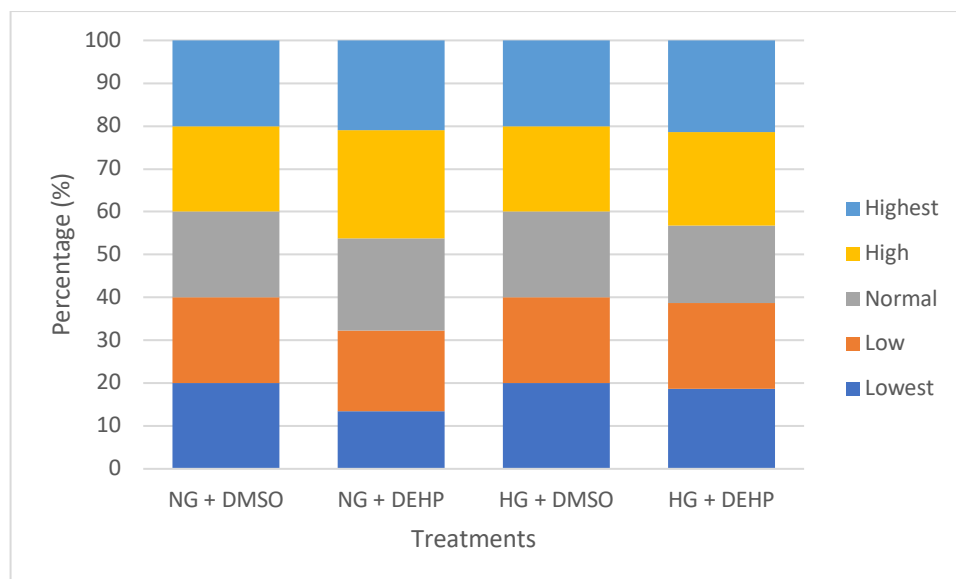


Figure 58: The intensity changes seen within the data obtained for normal glucose treated with diethyl hexyl phthalate (NG + DEHP) and high glucose and diethyl hexyl phthalate (HG + DEHP) in comparison to the normal and high glucose and DMSO control (NG + DMSO and HG + DMSO respectively) with the fluorescence microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 32 images were used for each of the control samples while 24 were used for each of the DEHP samples.

As shown in Fig. 59, the trends within the data when comparing NG + H₂O₂ to the NG water control shows that there is an increase in the frequency of mitochondria that fall within the lowest category within the NG + H₂O₂ data of 16.16% ($p = 0.343$). Additionally, there is also a smaller increase in those that fall within the low and normal categories as well as decreases to the frequency of mitochondria that fall in the high and highest categories, with the largest being 19.59% in the highest category ($p = 0.343, 1.000, 0.969$ and 0.528 respectively). Unlike the comparison of the normal glucose groups, comparing HG + H₂O₂ to the HG water control results in a slight increase of 8.04% and 11.81% in the frequency of mitochondria within the normal and high categories respectively while the frequency that fall within the low category decreases by 21.52% (p -values = $0.818, 0.892$ and 0.659 respectively). This observation differs to that seen within the average intensity data, where it shows a reduction between the two groups.

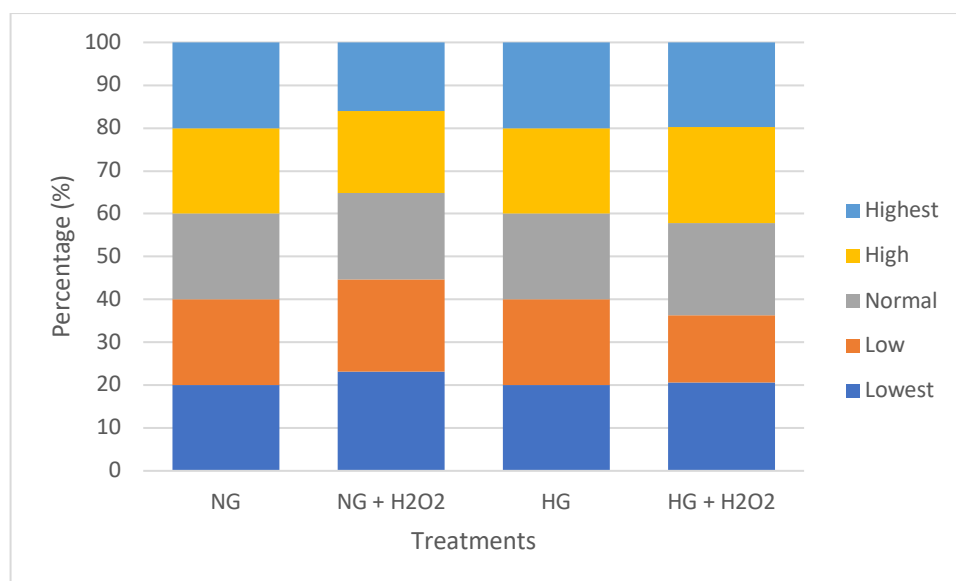


Figure 59: The intensity changes seen within the data obtained for normal glucose treated with hydrogen peroxide (NG + H₂O₂) and high glucose and hydrogen peroxide (HG + H₂O₂) in comparison to the normal and high glucose controls (NG and HG respectively) with the fluorescence microscope images using CellProfiler with a pipeline set to detect the intensity of the mitochondria with the cells. The percentage frequency of mitochondria within each of the quintiles is plotted. A total of 32 images were used for each of the samples.

6.6.2 Effects of chemicals under differing glucose concentrations on the ECAR traces following Seahorse injections.

As shown in Fig. 60, before any injections, there appears to be the highest ECAR and therefore glycolysis within the HG water control group while both glucose DMSO groups appear to be similar and the lowest of the four. Following the Oligomycin injection, which inhibits complex V, there is an increase within the ECAR values within all samples. This shows that the decrease in mitochondrial respiration due to complex V inhibition is causing an increase in glycolysis within all samples, with HG remaining the highest while NG + DMSO is now the lowest. Following the second injection, FCCP, which accelerates the last stage of the electron transport chain and inhibits complex V, there is a decrease in all samples of ECAR values; this would suggest that the cell is no longer trying to compensate and is preferentially producing energy through mitochondrial respiration. Following the last injection, which shuts down the mitochondria, there is little change in ECAR values.

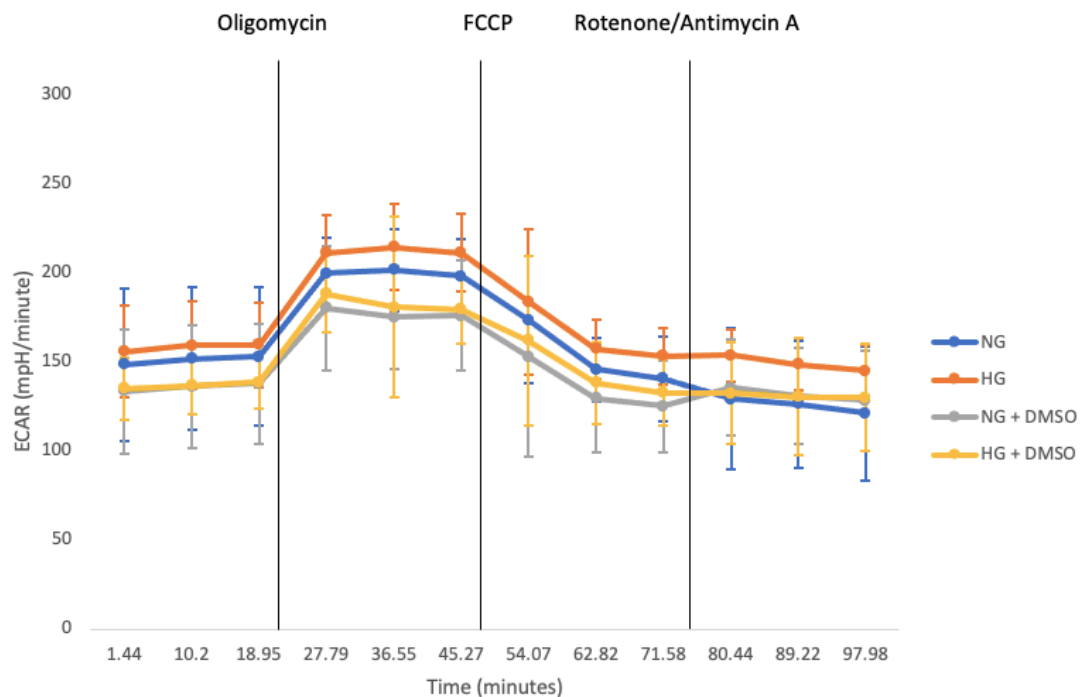


Figure 60: Extracellular acidification rate (ECAR) profile for cells grown under normal glucose control (NG) (n= 4) in comparison to the high glucose controls (HG) (n= 4) and the normal glucose and DMSO control in comparison to the high glucose and DMSO control (NG + DMSO and HG + DMSO) (n= 4). N numbers represent technical replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 1% (v/v) DMSO treatment. Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF[®]24 Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Looking at the ECAR trace produced within the Acetaldehyde groups and their respective controls (Fig. 61), before any injection was administered, HG had the highest ECAR value while NG + Ace had the lowest. Following the first injection, there is an increase seen in the ECAR values in all samples due to the inhibition of complex V and therefore reduction of mitochondrial respiration, thus increasing the need to produced energy through glycolysis; on average, the samples remained in the same order as before the injections. Following the second injection, FCCP, there was a decrease in ECAR values seen within all samples due to an increase in mitochondrial respiration as a result of acceleration of the last stages. HG and HG + Ace at this stage appear to have similar ECAR values while NG + Ace remains the lowest. Following the final injection, Rotenone/Antimycin A, there appears to be a slight increase of ECAR values within the HG + Ace and NG + Ace samples while within the NG and HG water control samples, there appear to be slight decreases.

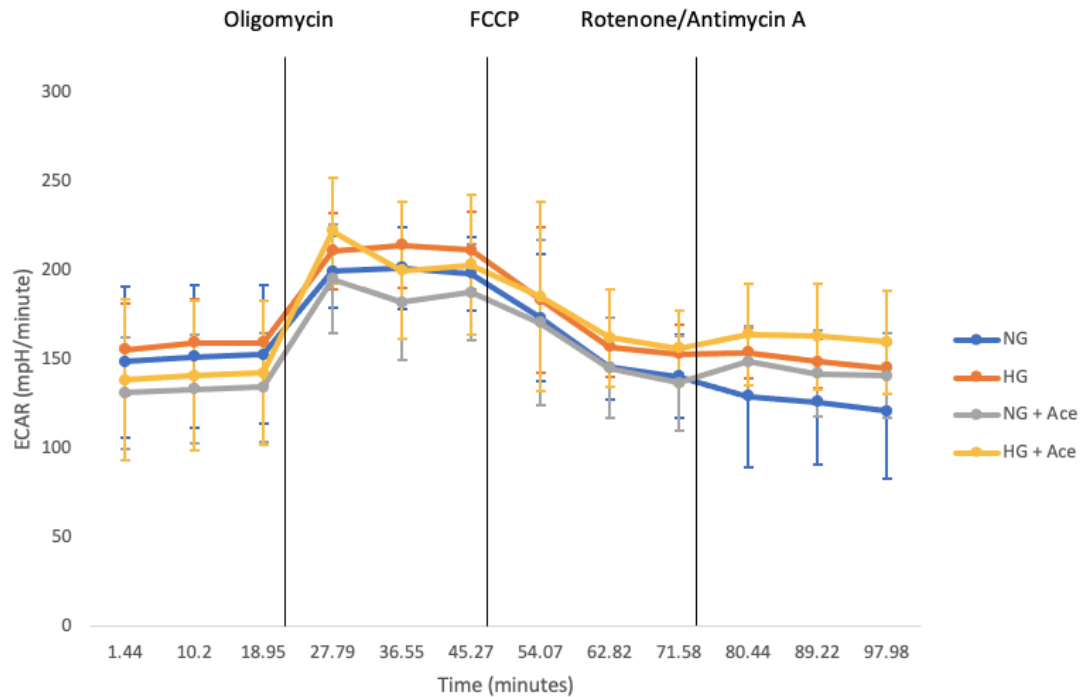


Figure 61: Extracellular acidification rate (ECAR) profile for cells grown under normal glucose treated with acetaldehyde (NG + Ace) (n= 4) and high glucose and acetaldehyde (HG + Ace) (n= 4) in comparison to the normal glucose (NG) (n= 4) and high glucose controls (HG) (n= 4). N numbers represent technical replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 1000 μ M of acetaldehyde (Ace). Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF^e24 Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Within Fig. 62, the effect of each injection on the ECAR rates within the cells can be seen within the DEHP and DMSO groups. Before any injections, NG + DEHP had the highest ECAR rate implying a higher glycolysis rate within those cells. The DMSO groups appear to have similar ECAR values which sit lower than the DEHP groups. Following Oligomycin injection, all samples increase their ECAR values, suggesting an increase in glycolysis; NG + DEHP remains the sample with the highest ECAR while the DMSO groups still appear to be very similar and lower. Following the second injection, there is a decrease in all samples of ECAR while again, NG DMSO remains highest and the DMSO groups appear similar. The final injection had varying results in each sample group, within the NG + DEHP and NG + DMSO there was initially an increase following the Rotenone/Antimycin A, however over the timepoints, the ECAR fell back to around the sample values as before the injection. Similarly, HG + DEHP increased, but remained increased across each timepoint after the final injection. Finally, within the HG + DMSO group, the ECAR continued to decrease following the final injection.

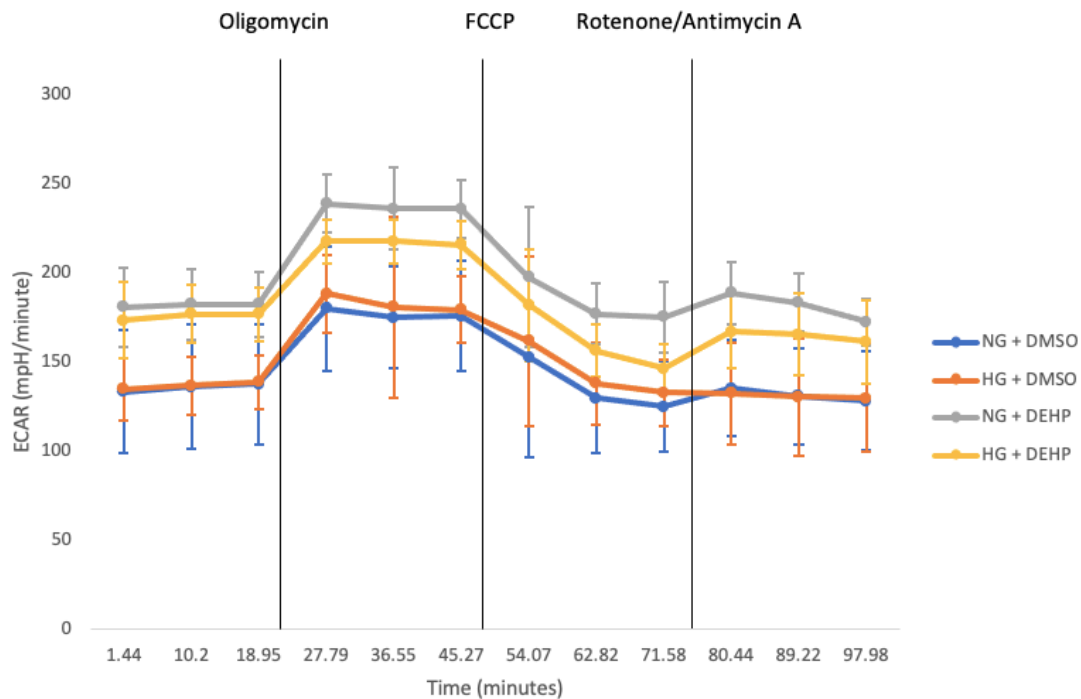


Figure 62: Extracellular acidification rate (ECAR) profile for cells for cells grown under normal glucose treated with diethyl hexyl phthalate (NG + DEHP) (n= 4) and high glucose and diethyl hexyl phthalate (HG + DEHP) (n= 4) in comparison to the normal glucose and DMSO (NG + DMSO) (n= 4) and high glucose and DMSO control (HG + DMSO) (n= 4). N numbers represent technical replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or 2000µM of di(ethylhexyl) phthalate (DEHP). Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF[®]24 Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Within the H₂O₂ samples and their control groups, as shown in Fig.63, before nay injections, NG + H₂O₂ had the highest ECAR and therefore glycolysis rate within the cells; the remaining 3 appear similar, with NG being slight lower than the rest. Following the first injection, Oligomycin, there is an increase in the ECAR values within all the samples, with NG + H₂O₂ remaining the highest while the others remain similar with NG still falling slightly lower. Following the injection of FCCP, the water controls show a decrease in ECAR, suggesting the acceleration of the last stages of the electron transport chain stimulated by this injection resulted in a decrease in glycolysis. However, within the H₂O₂ groups, there was initially a decrease, but then the ECAR values within the samples increased again over the remaining timepoints, suggesting the glycolysis had increased within the cells. Following the final injection, within the water control groups, similar observations are seen as to those described after the FCCP injection. Within the DMSO, while the initial timepoint after the final injection there was an overall increase compared to just before.

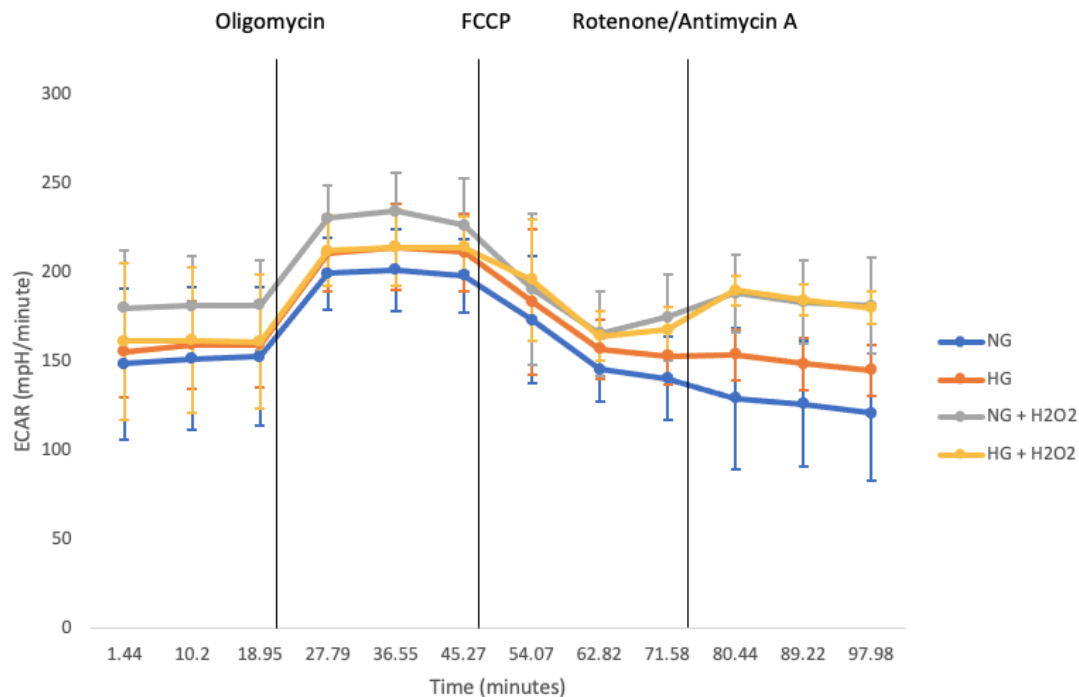


Figure 63: Extracellular acidification rate (ECAR) profile for cells grown under normal glucose treated with hydrogen peroxide (NG + H₂O₂) (n= 4) and high glucose and hydrogen peroxide (HG + H₂O₂) (n= 4) in comparison to the normal glucose (NG) (n= 4) and high glucose controls (HG) (n= 4). N numbers represent technical replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% water treatments or 20μM of Hydrogen Peroxide (H₂O₂). Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF^e24 Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Within the MCL-5 cell line (Fig. 64), before injections, NG + DMSO had the highest ECAR and therefore glycolysis rate while HG + 1-NP had the lowest; the remaining two had very similar rates. Following the first injection, there was an increase within all samples of glycolysis rate due to the decrease in mitochondrial respiration rate caused by the oligomycin. While the samples with the highest and lowest ECAR rates remained after the first injection, there is a larger distinction between the middle two groups, with HG + DMSO now slightly higher in ECAR rates than NG + 1-NP. This order is also seen following the second, FCCP, injection, where all samples saw a decrease within the ECAR rates; within the HG + DMSO group, this decrease appears slight compared to the other samples. Following the final injection, all but HG + DMSO saw an increase in ECAR values; this would suggest that as the mitochondria has shut down, the cell is once again increasing glycolysis to compensate energy production.

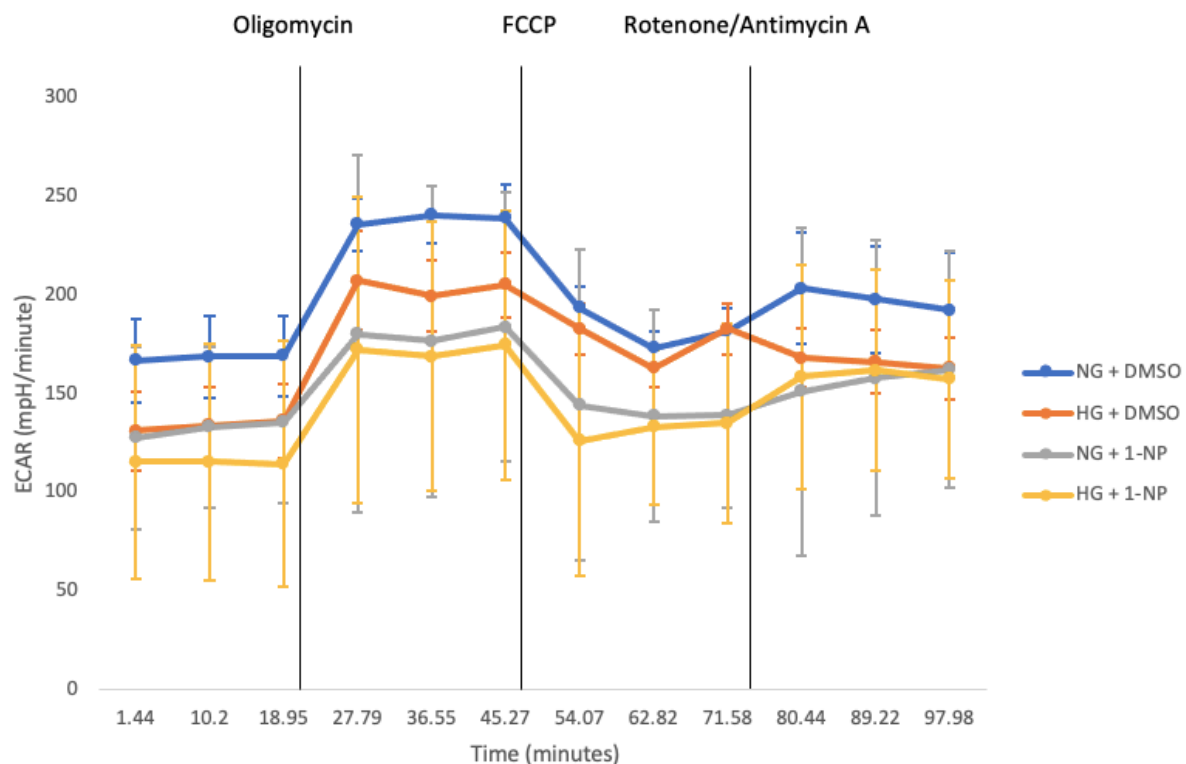


Figure 64: Extracellular acidification rate (ECAR) profile for cells grown under normal glucose treated with 1-nitropyrene (NG + 1-NP) (n=6) and high glucose and 1-nitropyrene (HG + 1-NP) (n=6) in comparison to the normal and DMSO (NG + DMSO) (n=6) and high glucose and DMSO controls (HG + DMSO) (n=5). N numbers represent technical replicates. Normal glucose samples were cultured in 10mM of glucose, high glucose samples were cultured in 29mM, both glucose groups either received 1% (v/v) DMSO treatment or 80.9µM of 1-nitropyrene (1-NP). Samples were diluted to 400,000 cells/ml using Seahorse Mito Stress Test medium, 40,000 cells were placed in the 24 well Cell Culture Microplate plate pre-treated with Cell Tak and spun to adhere to the bottom. Following incubation, samples were run XF^e24 Seahorse Bioanalyzer where they received an oligomycin, FCCP and Rotenone/Antimycin injections respectively.

Chapter 7. Glossary

Fluorescence Microscopy- A imaging technique which uses fluorescent dye to allow imaging of certain aspects of the cell dependent on dye through the excitation of fluorophores and detection of a fluorescence signal.

Next Generation Sequencing- A fast sequencing technique that allows parallel sequencing of fragments of DNA samples to determine the sequence.

Seahorse Bioanalyzer- A technique which allows real time analysis of cellular metabolism through the detection of oxygen Consumption Rate (OCR) and Extra Cellular Acidification Rate (ECAR)

Basal Respiration- Oxygen consumption used to meet cellular ATP demand. Shows energetic demand of the cell under baseline conditions.

ATP Production- Shows ATP produced by the mitochondria that contributes to meeting energetic demand.

Proton Leak- Shows ATP produced by the mitochondria that contributes to meeting energetic demand or can be used to regulate mitochondrial ATP production.

Maximal Respiration- Shows the maximum rate of respiration that the cell can achieve.

Spare Capacity- Shows the cells ability to respond to demand and can indicate cell fitness or flexibility.

Non-mitochondrial respiration- Oxygen consumption remaining following shut down of mitochondrial respiration.

qt-PCR- A PCR technique where the amplification and detection of data is combined into one step.

Low frequency variation- Variation that occurs within the genome at a lower frequency.

Hyperglycaemia- Blood glucose levels are too high. An individual is said to be hyperglycaemic when they have a blood glucose level higher than 7.8mmol/l before a meal and 8.5mmol/l two hours post meal.

Homoplasmic- All copies of mtDNA present are identical.

Heteroplasmic- The presence of more than one type of mtDNA within an individual, cell or mitochondrion

Copy number- The number of copies of a particular gene or genetic region in the genome

Chapter 8-Bibliography

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