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Metabolic assessment of metformin treatment in the colon cancer cell line SW1116

Abstract

Cancers have different metabolic profiles, with varying dependency on glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). The metabolic profile of cancer influences it sensitivity to treatments. Metformin, an anti-diabetic drug, affecting cellular metabolism has been found to have preventive, therapeutic and anti-proliferative effects on cancer. As the suggested target for metformin is mitochondrial complex I, cancer cells relying more on OXPHOS may be more sensitive to metformin treatment. However, the dosage used for metformin treatment is important and studies show that the highest achieved metformin concentration is in the gastrointestinal tract (GI) tract. This suggests that cancers of GI tract could be responsive to metformin treatment and show therapeutic and anti-proliferative effects. This project was designed to investigate how colorectal cancer cell lines of opposing metabolic phenotype responded to metformin treatment within the therapeutic range of metformin and in a physiological glucose background.

SW1116, a mitochondria dependent colorectal cancer cell line was used to study the effects of metformin. Viability protein and gene expression of metabolic proteins was studied. The glucose receptor GLUT1 was used as a marker for metformin induced effects. All experiments were conducted in two different growth media using high (25mM) and low (5 mM) glucose.

Metformin treatment caused a significant reduction in proliferation of SW1116 cells. GLUT1 protein and gene expression showed no significant increase on reduction of glucose but, a significant increase was observed with metformin treatment in high and low glucose. Organic Cation Transporter 1 (OCT1) gene expression was found to decrease with metformin treatment. Uncoupling Protein 2 (UCP2) gene expression was found to decrease in low glucose levels and it decreased further on metformin treatment. UCP2 gene expression was found to increase with metformin treatment in high glucose.

SW1116 cells were found to be sensitive to metformin. GLUT1 and UCP2 could be biomarkers to understand the metabolic alterations in cells after addition of metformin. OCT1, responsible for metformin uptake, could also be a biomarker to understand the sensitivity of cancer to metformin.

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List of Abbreviations:

Abbreviation	Full Name
TCA	Tricarboxylic Acid Cycle
OXPHOS	Oxidative Phosphorylation
ATP	Adenosine Triphosphate
GTP	Guanosine Triphosphate
GLUT	Glucose Transporter
MCT	Monocarboxylate transporter
PDC	Pyruvate Dehydrogenase Complex
PDK	Pyruvate Dehydrogenase Kinase
SUCLA	Succinate-CoA ligase ADP-forming beta subunit
UCP	Uncoupling Protein
ETC	Electron Transport Chain
CRC	Colorectal Cancer
FAP	familial adenomatous polyposis
MAP	MUTYH-associated polyposis
DMSO	Dimethyl sulfoxide
RIPA	Radioimmunoprecipitation Assay
SDS-PAGE	Sodium Dodecyl Sulfate -Polyacrylamide Gel Electrophoresis
PVDF	Polyvinylidene Difluoride
HRP	Horseradish Peroxide
ECL	Enhanced Chemiluminescence
BCA	Bicinchoninic Acid
QPCR	Quantitative PCR
PCR	Polymerase Chain Reaction

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1. Introduction

1.1 Cancer Metabolism

Cancer (*Greek word for crab*), a term coined by Hippocrates, describes a large group of diseases which exhibit some of the most complex biological phenomena [1] [2] [3] [4]. Cancer cells possess a mutated genome that allows them to attain unique characteristics which are not observed in normal somatic cells. These traits are regarded as cancer hallmarks[2]. The established hallmarks of cancer cells are: apoptotic resistance, limitless proliferative potential, sustained proliferative signalling, growth suppressor aversion, angiogenesis, and metastasis[2] [3]. However, these six are not the only unique characteristics a cancer cell may possess[3]. Research shows cancer cells alter their metabolism and this has been proposed as another hallmark[3] [4].

Cancer cells proliferate actively and therefore, require surplus amount of energy and biosynthetic precursors[3] [4]. In order to obtain these, cancer cells need to change their metabolic profile[3] [4]. Nobel laureate Otto Warburg suggested that cancer cells meet their energy demands by increasing glucose uptake and further research supported this hypothesis[5] [3] [4]. In later years, research revealed that cancer cells also depend on amino acids to meet their nitrogen requirements and glutamine plays a crucial role by providing essential tricarboxylic acid (TCA) cycle intermediates [6] (Refer Figure 1.1). Thus, glucose and glutamine are energy sources for cancer cells[3] [4]. Fatty acids are required for formation of membrane and also signalling molecules, therefore, they are not preferred as a primary energy source[7].

1.1.1 Glucose Metabolism and Cancer

Otto Warburg first observed that metabolism of cancer cells is different compared to normal somatic cells[5]. He postulated "Warburg hypothesis" which states that cancer cells consume more glucose than normal cells and rely on glycolysis for energy production, not on oxidative phosphorylation (OXPHOS); even in presence of adequate oxygen levels (aerobic glycolysis)[5]. This led him to conclude that the respiratory organelles, mitochondria, of cancer cells are damaged[5].

Contrary to Warburg's hypothesis, further research confirmed that glucose requirement of cancer cells is high but, mitochondria are not completely damaged and have the potential to carry out OXPHOS[8]. The dependency on OXPHOS-generated energy in cancer cells is low

compared to normal somatic cells[8]. However, some cancer cells have been found to be dependent on mitochondria for their tumorigenic and potential and metastatic potential[9] [10]. Metabolic profile of cancers is different, with variable dependency on glycolysis and OXPHOS[11].

Normal cells carry out OXPHOS (produce 36 ATP/ glucose molecule) and cancer cells mainly depend on glycolysis (produce 2 ATP/ glucose molecule)[12]. Therefore, it seems, to make up for the inefficiency of ATP production in glycolysis, cancer cells take up more glucose than normal cells[3].

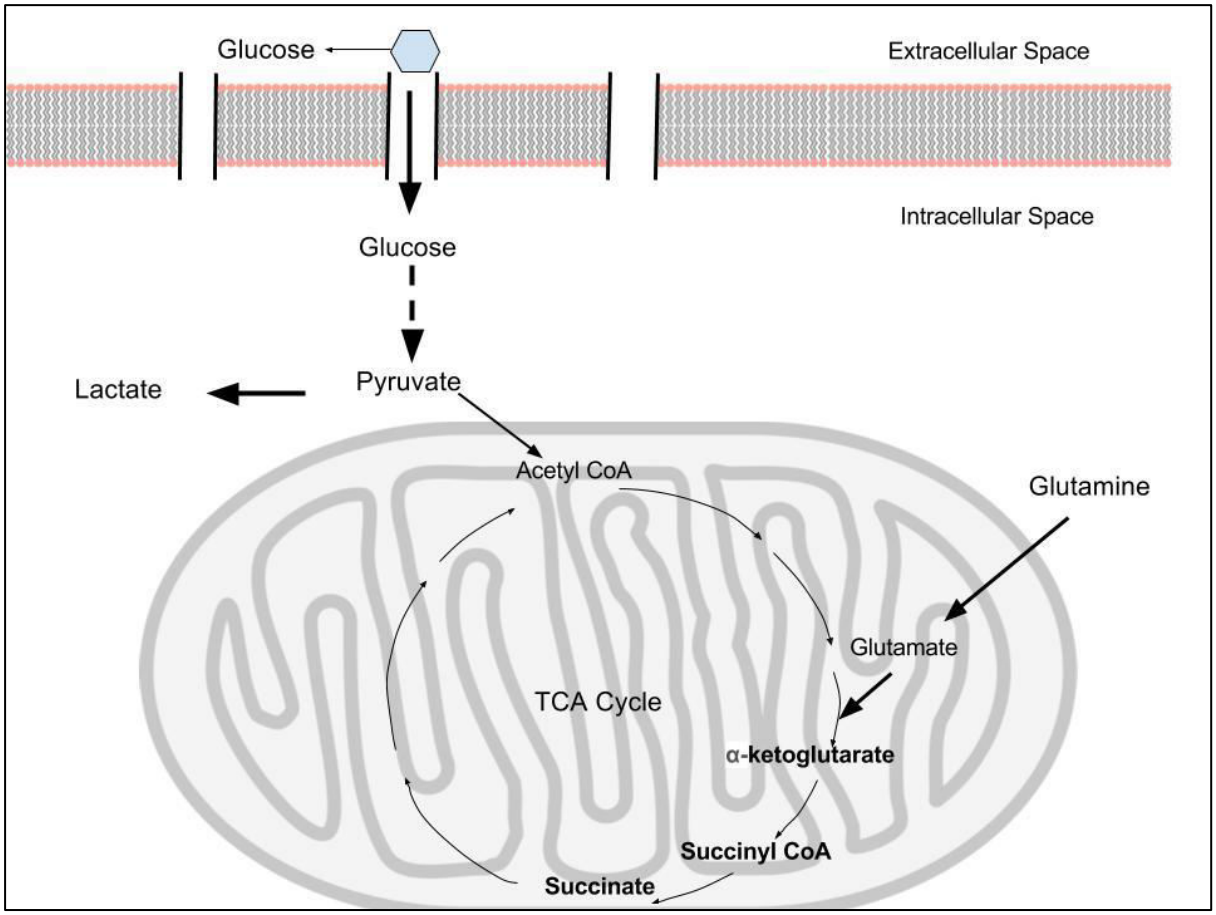

Figure 1.1. 1: Glucose metabolism pathway.
This pathway is altered in cancer cells. Large fraction of glucose taken up by cells forms lactic acid and small part gets directed to tricarboxylic acid (TCA) (as represented by weight of arrows). Glutamine uptake increases and it enters TCA cycle to meet cellular demands.

1.1.3 Components of Metabolism as Potential Biomarkers

The National Cancer Institute (NCI) website defines biomarkers as "*a biological molecule found in blood, other bodily fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or a disease*"[13]. Proteins are one of the classes of biomarkers and in cancer, protein biomarkers have been found to detect disease recurrence and response to treatments[14]. Components of the glycolysis-OXPHOS pathway may function as biomarkers and they are explained below.

Cancer cells have been found to elevate expression of glucose transporters (GLUTs), and GLUT1 studies in relation to cancer have suggested its use as a biomarker[15] [16] [17]. Research has indicated that high GLUT1 expression levels indicate a weak chance of survival[18].According to Warburg Hypothesis, an increase in activity of glycolysis, would lead to lactic acid build up and it would need to be eliminated from the cell. Lactic acid or monocarboxylate transporters (MCTs) especially MCT4 has been studied in association with cancer and its expression level has been affiliated with accelerated cancer growth and increase in glycolysis[19] [20] [21]. Pyruvate dehydrogenase kinase (PDK) inactivates pyruvate dehydrogenase complex (PDC), the enzyme responsible for conversion of pyruvate to Acetyl CoA and high levels of PDK2 are found to be associated with high lactic acid levels and therefore, Warburg effect manifestation[22] [23]. Succinyl-CoA synthetase is an enzyme that converts Succinyl CoA to Succinate and this is where ATP/GTP is formed in TCA cycle, which makes it a key enzyme[12]. Succinate-CoA ligase ADP-forming beta subunit, a part of Succinyl-CoA synthetase and is encoded by *SUCLA2* gene[24] and mutations in this gene are associated with various mitochondria related disorders including cancer[25]. Uncoupling proteins (UCP), also known as mitochondrial leak proteins are present on inner mitochondrial membrane[26]. They allow flow of protons back into the mitochondrial matrix and thereby reduce ATP formation[26]. UCP2 , a member of UCP family is found to be differently expressed in cancer cells and they have been suggested as targets for therapy[27]. OCT1 is a member of Organic Cation Transporters (OCTs) and research has shown that a change in OCT1 expression levels could indicate cancer advancement[28]. The positions of these metabolic components can be seen in Figure 1.1.2.

Therefore, these metabolic elements (GLUT1, MCT4, PDK2, SUCLA2, UCP2 and OCT1) could serve as biomarkers to determine the metabolic profile of cancer cells. Additionally, they could be used to predict response to metabolism-targeting drugs.

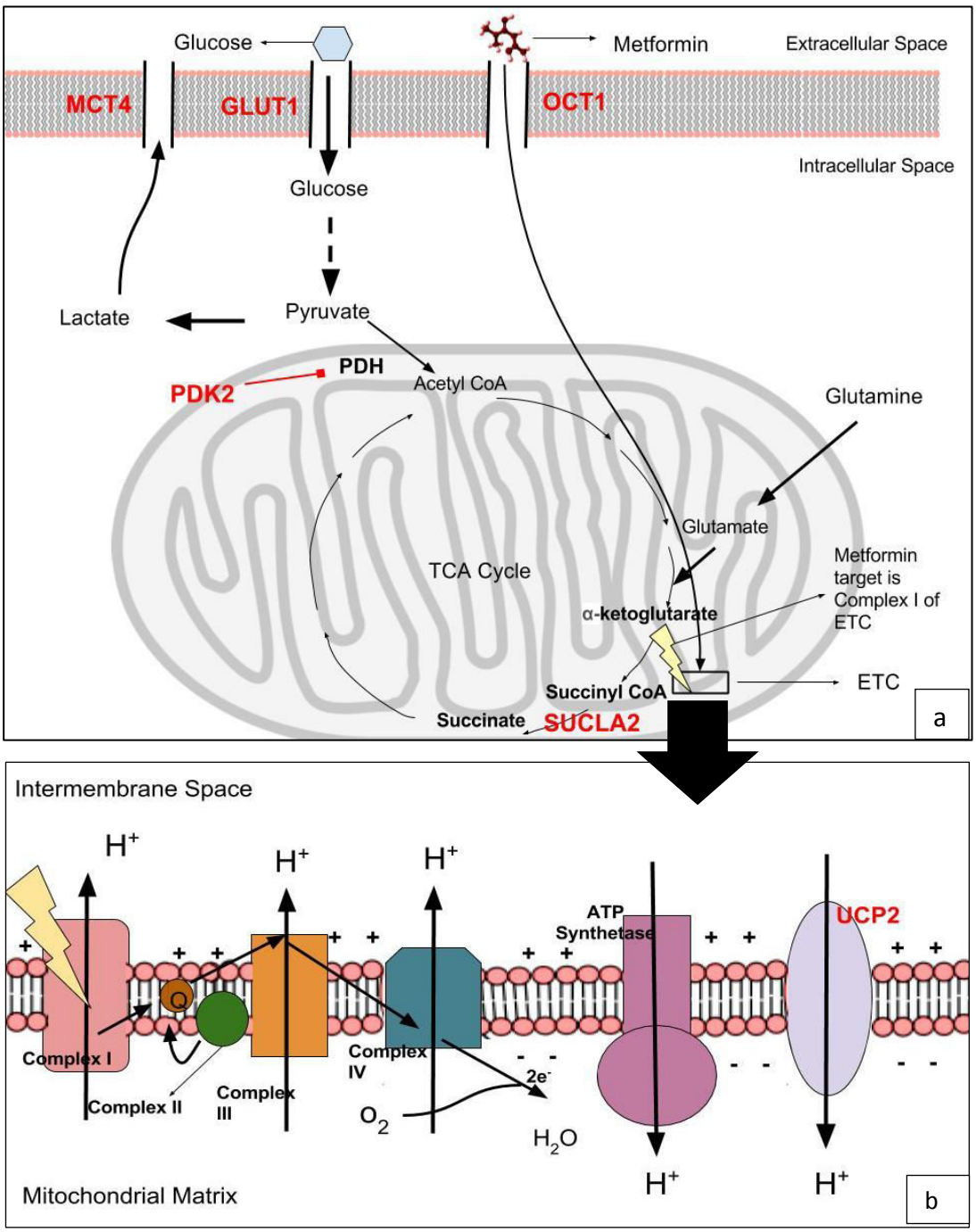

Figure 1.1. 2: Suggested Biomarkers and their position in the metabolic pathway.
Figure 1.1.2 (a) shows the position of GLUT1, MCT4, OCT1, PDK2 and SUCLA2 in the metabolic pathway. Figure 1.1.2 (b) is an enlarged part of electron transport chain (ETC) present on the inner mitochondrial membrane to show location of UCP2 in metabolic pathway.

1.2 Cancer and Metformin

1.2.1 Metformin Overview

Metformin was introduced over 50 years ago and today it is a widely prescribed oral drug against type 2 diabetes[29]. Although metformin has been out in the market for over half a century, its mode of action and target are not clearly understood. Studies have strongly suggested that metformin targets Complex I of Electron Transport Chain (ETC) in mitochondria[30 31]. Metformin has fewer health risks compared to other available anti-diabetic treatments and was found to have a beneficial effect in treatment of various diseases and disorders, including cancer[32]. Effect of various anti-diabetic therapeutic agents was studied and metformin was found to be effective in lowering cancer associated risks [33]. Sulfonylurea and insulin (other treatments for diabetes) did not have any therapeutic or preventive effect on cancer, rather insulin was found to put patients at a higher cancer risk[34].

1.2.2 Metformin and Cancer

Effect of metformin on cancer has been studied extensively and a lot of the work has shown that metformin has a preventive and therapeutic effect on cancer[35]. Research work of Noto H. and colleagues revealed that metformin treatment reduces the risk of developing different cancers[36]. A study on triple negative breast cancer patients reported that metformin put patients at less risk of metastasis[37] and another study suggested that metformin reduced risk of developing ovarian cancer[34]. Research has found contradictory results as well[38].

1.2.3 Metformin effects vary with different cancers

Research (*in vitro* studies) on effects of metformin have been carried out at very high metformin concentrations, some concentrations higher than 10mM [39 40 41]. These concentrations are multiple times higher than metformin concentrations that can be achieved in the body and this may have led to contradictory results. The achievable metformin concentrations in different parts of the body is different and it is found to be highest in gastrointestinal tract[42 43]. Concentration of metformin is achievable in plasma is about 30µM and it can increase upto 300 times in intestine (absorption takes place in intestine)[43 44].

It has been studied that although glycolytic in comparison with normal somatic cells, cancer cells differ in their energy metabolism pathways[11]. Since metformin affects Complex I of

mitochondria, studies suggest that metformin would affect cancer cells which are more dependent on OXPHOS for ATP generation[45].

Organic cationic transporters (OCTs) which are present on the surface of the cells are responsible for metformin uptake, and certain polymorphs of OCT1 disallow cells to take up metformin effectively. Therefore, cells in possession of these OCT1 polymorphs will not be responsive to metformin[46]. Therefore, effect of metformin on cancer is likely to be affected by the location of tumour in body, tumour metabolic profile and OCTs on target tumour cells.

1.3 Colorectal Cancer

1.3.1 Colorectal Cancer (CRC) overview

Colorectal cancer (CRC) incidences and related deaths have increased the last decades making CRC one of the most predominant type of cancer worldwide[47]. It is responsible for around 10% of cancer related deaths, with higher occurrence in developed nations[47]. The majority of CRC is found to be a linked to lifestyle, and only a few percent are directly linked to mutations of genetic origin. [47]. A diet high in red meat consumption, eating less fibre, sedentary lifestyle, obesity and smoking have all been found to increase CRC risk[47]. The hereditary nature of CRC is termed Lynch syndrome, familial adenomatous polyposis (FAP) and MUTYH-associated polyposis (MAP), which all have been found to be more genetically vulnerable to CRC risk[48]. Furthermore, diabetes is often closely connected to the lifestyles described above and also a risk factor for developing colorectal cancer[49].

1.3.2 Metformin and CRC

Cardel M and colleagues studied the effects of metformin on CRC risk. The study was conducted for ten years (2000 through 2009) and the subjects were type II diabetes patients. Their study indicated that metformin treatment was able to reduce CRC occurrence and the study also found out that the effects of metformin on CRC vary with gender [50]. A meta-analysis of five studies indicated a decrease in colorectal neoplasms and CRC cases in type II diabetes patients. It also suggested that metformin had antineoplastic and antiproliferative effects[51]. Study conducted by Garret CR and colleagues showed a significant increase in overall survival rate of CRC in patients who had received metformin treatment (82.5 months) compared with patients who had not received any metformin treatment (60.9 months)[52]. Research by Lee J H and colleagues showed that there was a decrease in CRC deaths in diabetic patients who had received metformin treatment[53]. A study published in 2014 showed that metformin had inhibitory effects on growth of colon cancer cells. The study also observed that metformin had an inhibitory effect on the migration of colon cancer cells, and in combination with 5-fluorouracil and oxaliplatin these effects were more pronounced [54]. These studies indicate that metformin has a protective and preventive effect on CRC.

1.3.3 SW1116 Overview

SW1116 cell line is a slow growing colorectal adenocarcinoma cell line derived from a 73 year old patient, and was established over 50 years ago[55]. SW1116 cells have mutations in

KRAS, TP53 and APC, according to the distributor[56]. Comparatively, with other colon cancer cell lines, SW1116 is known to be a slow growing cancer cell line[55 57]. Previous tests in our lab (unpublished) indicate that SW1116 cells have a more OXPHOS dependent metabolic profile.

1.4 Objectives and Outline

1.4.1 Objectives

The objective of this MSc project was to study the effects of metformin in OXPHOS-dependent SW1116 cells. This was done as a comparative study where another MSc student ran parallel experiments in another colorectal cell line which had previously been determined to be more glycolytic (SW948). Comparing these two cell lines would give us an indication whether the metabolic phenotype of the cancer cell plays a role in metformin susceptibility.

1.4.2 Outline

Proliferation assay was performed to study the behaviour of SW1116 cells in high glucose growth medium [25 mM (4500mg/L] and low glucose growth medium [5mM (1000mg/L)]. Viability assay was performed to study the effect of various metformin concentrations on the cells grown in high and low glucose. Also, to study the time dependent effect of metformin on SW1116 cells the effect of metformin was studied for 24 hr and 48 hr treatment. Depending on the results, concentrations showing significant results were chosen for the experiment.

Since GLUT1 is responsible for glucose uptake, and can be a measure of a nutrient stress response due to metabolic drugs, GLUT1 protein expression was studied using Western-Blot, Confocal Microscopy and Flow Cytometry. Results obtained with different methods were compared. Gene expression for the following proteins was studied: Glucose Transporter (GLUT1), Lactic Acid Transporter (MCT4), Organic Cation Transporter (OCT), Pyruvate Dehydrogenase Kinase (PDK2), Succinyl CoA Synthetase (SUCLA2) and Uncoupling Proteins (UCP2). (Refer Figure 1.2)

Lastly, the mitochondria, which are thought to be the main targets of metformin was studied by assessing mitochondrial mass per volume after treatment.

2. Materials and Methods

2.1 Retrieval and maintenance of SW1116 cells

SW1116 cells were recovered from cryotank, their passage number (number of times the cell population was reseeded) was 8 (P-8). SW1116 cells were cryopreserved in medium containing Dimethyl sulfoxide (DMSO) at -196°C. At cryogenic temperatures, DMSO acts as cryoprotectant by preventing formation of ice crystals; however, it is toxic at room temperature. Exposure to DMSO for a long period of time at room temperature damages cells. Rapid thawing ensures maximum healthy cell population.

Cryovial was recovered from the tank and held in a water bath at 37°C until 75% to 80% of the vial was thawed. T-75 cell culture flask was kept ready with 10 mL cell growth. The cryovial was shaken and the contents were transferred to the flask with a micropipette.

Cells were left overnight to allow attachment to flask surface. Medium was changed the following day to remove any DMSO content in the medium.

Cell growth medium was Glucose content 4500 mg/L Dulbecco's Modified Eagle Medium (DMEM) with 10% Foetal Bovine Serum (FBS) 4 mM L-Glutamine and Penicillin-Streptomycin (100 units/mL, 100µg/mL). In T-75 flasks, at 37°C at 5% CO_2 concentration.

2.1.1 Passage of Cells

Adherent proliferating cells occupy substrate in the cell culture flask, which gets exhausted after a while. This leads to high cell death rate, and change in behaviour pattern of cells due to lack of nutrients and accumulation of metabolic by-products. A fraction of the cell population needs to be reseeded into a new cell culture flask with growth medium which is called passage and the number of times it is done is called a passage number.

Trypsin breaks the protein bonds between cells and substrate and thus, used to detach cells from substrate while reseeding. Serum has trypsin inhibitory property.

1. SW1116 cells were grown in T-75 cell culture flasks, until the cells were 65-70% confluent.
2. Medium was aspirated off, cells were washed with PBS, and 2.5 mL trypsin was added.
3. Cells were incubated in PBS for about 12 minutes in trypsin.
4. 5 mL of medium was added to stop trypsin activity.

5. 2.5 mL of the cells suspension was transferred to a new cell culture flask.

2.1.2 Cell count determination by haemocytometer

Haemocytometer is a glass slide with a chamber to load cell suspension and a grid to facilitate counting of cells. Trypan blue was used to count cells. Live cells manage to exclude trypan blue as they have an intact membrane, and thus are unstained**,** whereas dead cells with a disrupted cell membrane are stained blue. For all experiments needing cell seeding, the number of viable cells were counted. Trypan Blue was 2X, 100µl of this dye was added to 100µl of cell suspension in an eppendorf tube. Standard protocol to count cells was followed.

1. The haemocytometer was prepared by application of water to two sides of the glass coverslip and it was slid over the cytometer. Cell suspension was loaded onto haemocytometer.
2. Cells within the set of sixteen squares in one corner were counted (Refer figure 2.1.2). This was repeated for the remaining set of 16 squares and the average number was calculated (x).

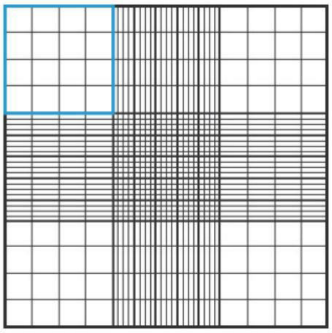

Figure 2.1. 1: Schematic representation of a haemocytometer.
(http://www.abcam.com/protocols/counting-cells-using-haemocytometer).

3. Cells on the right border and bottom border were counted. Cells on the other two borders of the squares were not counted (Refer figure 2.2)

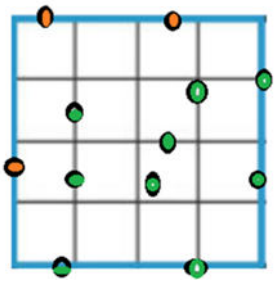

Figure 2.1. 2: Schematic representation of set of 16 squares in each corner.
Cells coloured in green were counted and cells in orange were not.

4. Calculation: $\boldsymbol{n = x \times t \times 10^4}$

   n = Number of cells/mL

   x = average number of cells in a set of 16

   t = dilution factor of trypan blue

   10^4 = dimensions

2.1.3 Storage of SW1116 cells by freezing

Cells may change their behaviour after a few passages or get contaminated while handling. So, while cells were at low passage number (P-11), they were frozen and stored at -196°C (in liquid nitrogen tank) until further use. All the experiments were performed between P-11 to P-23. After crossing P-23, a new vial was taken.

1. Cells were trypsinized and cell suspension was prepared (Refer section 2.1.1)
2. Freezing medium was prepared (15 mL). The components are as follows:
   a) DMEM with 10% FBS = 12 mL
   b) FBS = 1.5 mL
   c) DMSO (10%) = 1.5 mL
3. Cells were counted using a haemocytometer (Refer Section 2.1.2) and transferred to 15 mL centrifuge tubes and centrifuged at 900 rpm and resuspended in freezing medium such that there were 1 x 10^6 cells/mL.
4. Aliquots of 1mL of cell suspension were made cryovials and stored in a cryobox at -20°C for an hour, followed by cryobox with isopropanol for storage at -80°C overnight and then cells were stored at -196°C until further use.

2.2 Cell Proliferation Assay

Principle:

To assess the growth of SW1116 cells in growth medium with different glucose concentrations, they were allowed to proliferate in high (4500 mg glucose/L) and low (1000 mg glucose/L) glucose levels for 72 hours. Cell number was calculated for time intervals at 6, 12, 24, 48 and 72 hours. Cell number was determined after each specified time intervals using a haemocytometer and trypan blue.

FBS, Glutamine and Penicillin-Streptomycin concentrations in both low and high glucose medium were same.

Procedure:

2.2.1 Culture of Cells:

1. Cells were trypsinized (Refer section 2.1.1) and counted using a haemocytometer (Refer section 2.1.2)
2. 6×10^5 cells were resuspended in 6 mL high glucose medium and 6×10^5 cells 6 mL in low glucose medium.
3. In a 6 well plate, 1 ml of the cell suspension and 2 ml of fresh medium was added in each well. Thus, each of the 6 wells had 100,000 cells.
4. Two such plates were prepared one for high glucose and one for low glucose.

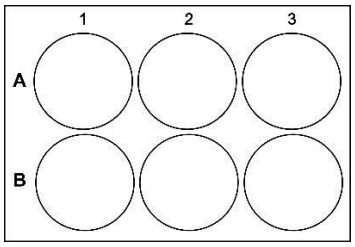

Figure 2.2. 1: Schematic representation of 6 well plate. (http://www.cellsignet.com/ media /plates/6.jpg)

5. The wells were labelled as 6 hours, 12 hours, 24 hours, 48 hours, 72 hours and backup. These cells were then incubated at 37˚C. The cell number was determined after the above mentioned time intervals.

2.2.2 Proliferation Assay:

1. After the time interval of 6 hours, cells from one of the wells were trypsinized (Refer Section 2.1.1) with 0.5 mL trypsin and 1.5 mL trypsin to stop trypsin activity.
2. Cells were counted using haemocytometer (Refer Section 2.1.2).
3. This same protocol was followed for 12, 24, 48 and 72 hours.

2.2.3 Cell Proliferation Calculation:

1×10^5 cells were seeded therefore, this was the number of cells at 0 hours. This was used a reference point (for both high and low glucose) and was set as 100.

Increase or decrease in cell population with time was compared to 0 hours and adjusted according to the following formula:

$$\boldsymbol{x = \frac{n \times 100}{10^5}}$$

n = number of cells at 6hr, 12 hr, 24 hr, 48 hr or 72 hr

10^5 = number of cells at starting point (0 hr)

100 = number of cells at starting point after calibration

x = Number of cells after calibration

2.3 Viability Assay

Principle

MTS a colorimetric viability assay was used to study effect of metformin on viability of SW1116 cells grown in high (4500 mg glucose/L) and low (1000 mg glucose/L). Metformin concentrations used were 0.1 mM, 0.5 mM, 1.0 mM, 3.0 mM and 5.0 mM. MTS Assay was performed to determine the preferred concentrations to use for further experiments. Abcam MTS cell proliferation Assay Kit was used and the protocol provided with the kit was followed.

Procedure:

2.3.1 Culture of cells:

1. Cells were trypsinized (Refer Section 2.1.1), counted (Refer section 2.1.2) and cultured at 4×10^3 cells/200 µL/well.
2. Cells were incubated overnight to allow cells to attach to the surface. Cells were treated the following day.

2.3.2 MTS Assay

1. The following scheme was used:

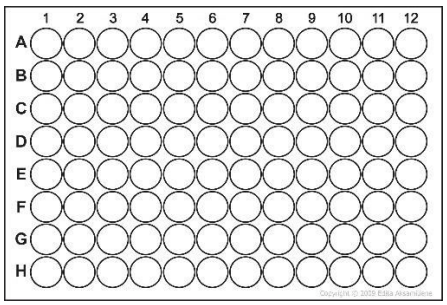

Figure 2.3. 1: Schematic representation of 96 well plate. (http://www. cellsignet. com/ media/plates/96.jpg)

Table 2.3. 1: Loading scheme for MTS Assay experiment

Wells	Treatment	Wells	Treatment
A	High Glucose Control	F1	Low Glucose Control
A2	High Glucose 0.1 mM Metformin	F2	Low Glucose 0.1 mM Metformin
A3	High Glucose 0.5 mM Metformin	F3	Low Glucose 0.5 mM Metformin
A4	High Glucose 1.0 mM	F4	Low Glucose 1.0 mM Metformin

	Metformin					
A5	High Glucose Metformin	3.0	mM	F5	Low Glucose 3.0 mM Metformin	
A6	High Glucose Metformin	5.0	mM	F6	Low Glucose 5.0 mM Metformin	

2. All the treatments were run in triplicates (Rows B, C High Glucose and G, H Low Glucose). Both high and low glucose had a background control (Growth Medium + MST [no cells]).
3. Two such plates were prepared to study effects after 24 hours and 48 hours.
4. After incubation time was completed, 20 µL/well MTS reagent was added to each well and incubated for 4 hours at 37°C in standard culture conditions.
5. The plate was shaken for 30 seconds, and absorbance values were recorded on a plate reader at OD=490 nm to determine proliferation.

2.3.3 Cell Viability Calculation

Blank was adjusted. Cell viability was calculated in percentage.

Control samples, not treated with metformin, were set as 100%. . Control (high and low Glucose) samples were used as references for cell viability assessment in metformin treated samples (high and low glucose respectively).

Percent viability for all cells was calculated using the following formula:

$$\%\ \boldsymbol{cell\ viability\ of\ target\ sample} = \frac{\boldsymbol{absorbance\ value\ for\ target\ sample} \times \mathbf{100}}{\boldsymbol{absorbance\ value\ for\ control\ sample}}$$

2.4 Determination of GLUT1 by SDS-PAGE and Western Blot

Principle:

Cells were lysed for protein extraction by Radioimmunoprecipitation Assay (RIPA) Buffer. RIPA buffer was supplemented with Halt Phosphatase Inhibitor Cocktail (protease inhibitor) to prevent protein degradation. Protein quantitation was carried out by Bicinchoninic Acid (BCA) Assay. Determination of protein concentration in the lysate solution is necessary since protein concentration can affect the Sodium Dodecyl Sulfate -Polyacrylamide Gel Electrophoresis (SDS-PAGE) run and results. Low concentration of proteins will provide a signal too weak to detect and high concentration of proteins will clog the gel and disallow the run of any proteins.

Proteins were analysed using SDS-PAGE. SDS (anionic detergent) imparts negative charge to amino acids and separates them on basis of size. Stain free gels were used, which allows visualization of bands on UV activation and this property of visualization without staining is retained on Blotting.

Proteins were transferred to a Polyvinylidene Difluoride (PVDF) membrane by Western Blotting (electroblotting) and analysed further to study GLUT1 expression. Membrane was incubated with antibodies specific to GLUT1. The amount of antibodies bound to the membrane would be directly proportional to the amount of protein present. To enhance the signal, membrane was incubated with Horseradish Peroxide (HRP) conjugated antibodies (secondary antibodies) against the primary antibody. Thus, amount of HRP present is proportional to amount of target protein, which was detected by Enhanced Chemiluminescence (ECL).

Procedure:

2.4.1 Culture of cells:

SW1116 cells trypsinized (Refer Section 2.1.1), counted (2.1.2) and seeded grown in 6 well plates at a density of 1 x 10^6 cells per well the following loading scheme was used:

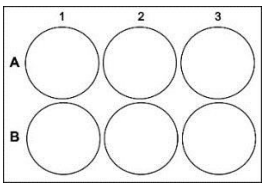

Figure 2.4. 1: Schematic representation of the 6-well plates (http://www.cellsignet.com/media /plates/6.jpg).

Table 2.4. 1: Loading scheme for SDS-PAGE and Western Blot Experiment

Well	Treatment
A1	High Glucose Control
A2	High Glucose Metformin treated (Metformin concentration 0.5 mM)
A3	High Glucose Metformin treated (Metformin concentration 3.0 mM)
B1	Low Glucose Control
B2	Low Glucose Metformin treated (Metformin concentration 0.5 mM)
B3	Low Glucose Metformin treated (Metformin concentration 3.0 mM)

Two plates for the above mentioned scheme were prepared. One plate was incubated for 24 hours and one plate for 48 hours.

2.4.2 Lysate Preparation:

Procedure 1:

1. Cells were trypsinized (Refer Section 2.1.1) and transferred to centrifuge tubes and centrifuged (900 rpm for 5 min).
2. Medium was aspirated off. PBS was added, cells were centrifuged (900 rpm, 5 min) and PBS was aspirated off.
3. RIPA buffer was added to the cells (500 µl for each 1 x 10^7 cells).
4. They were incubated for 2 minutes at room temperature and then centrifuged at 40,000rpm for 5 min at 4°C. The supernatant contained proteins and it was transferred to a new vial and stored at -20°C until further use. This was set-1.

Procedure 2:

1. Medium was aspirated off and plates were kept on ice.
2. Cells were washed with PBS, it was aspirated off and cells were incubates 250 µl RIPA Buffer for 10 minutes.
3. Plastic cell scrapers were used to scrape off the surface and the solution was transferred to vial. The solution was centrifuged for 5 minutes at 40,000 rpm at a temperature of 5°C.

4. The supernatant contained proteins and it was stored at -20°C for further use. This was set-2.

Protein amount in both the sets was estimated By BCA method and Western Blot was performed.

2.4.3 Bicinchoninic Acid (BCA) Assay:

Pierce BCA Protein Assay Kit from ThermoScientific was used. The protocol provided with the kit was followed (Refer Appendix).

2.4.4 Sodium Dodecyl Sulfate -Polyacrylamide Gel Electrophoresis (SDS-PAGE):

1. Stain Free Gels were prepared (Refer Appendix) and standard SDS-PAGE protocol was performed.
2. The gels were assembled, upper buffer chamber and gel tank were filled with 1X Running Buffer.
3. Protein samples were prepared to have 10 µg of proteins with a total volume of 28 µl after inclusion of loading buffer.
4. Entire 28 µl of the prepared protein was loaded onto the gel. 5 µl of protein ladder was loaded in one of the wells.
5. SDS-PAGE was run for 85 -90 minutes at 100 V and the run was stopped as soon as the dye reached the edge of the gel.
6. Gel was exposed to UV to activate the gel and visualize the separation of proteins.
7. After visualization of bands, the stacking gel and dye front was removed. The resolving gel which contained bands of separated proteins was used for Western Blot.

2.4.5 Transfer of Proteins to Membrane:

1. The gel was left in Blotting Buffer for 10 minutes, while PVDF membranes were activated with methanol and rinsed with distilled water followed by transfer buffer.
2. Foam pads and filter papers were soaked in blotting buffer.
3. Western Blot Cassette was assembled as follows: Red plate - foam pad - filter papers - membrane - gel- filter papers - foam pad - black plate.

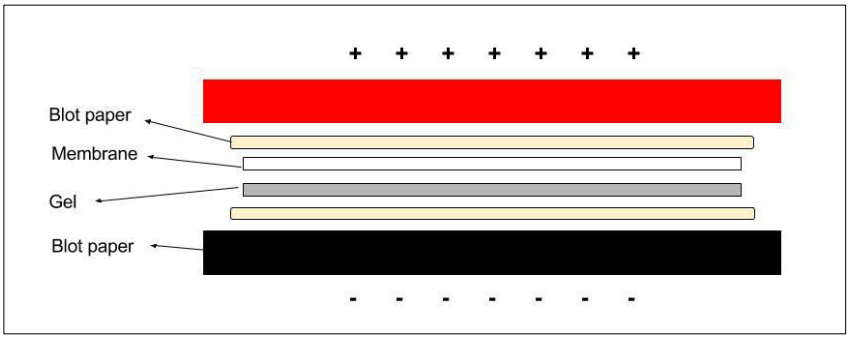

Figure 2.4. 2: A scheme of Western Blot Cassette

4. This cassette was placed in an electrode assembly which was then placed in a western blot tank with the red side towards the red mark and Black side towards the black mark. An ice pack was placed inside the tank and a magnetic bar.
5. Blotting buffer was filled up to the desired level and the entire tank was placed in a box containing ice and box was placed on a magnetic stirrer so as to ensure that the temperature remains uniform throughout the run.
6. Western Blot was run at constant voltage with 100 V and 2 ampere for 1 hour. The membrane was then checked for successful transfer.

2.4.6 Detection of GLUT1 with Antibodies:

1. The membrane was incubated in blocking buffer of 3% skim milk prepared in 1X TBST for 2 hours a room temperature.
2. Primary Antibody solution was prepared in 1.5% skim milk in 1X TBST. The ratio of antibody to milk TBST was 1:100,000. Membranes were incubated in primary antibody solution overnight at 4°C.
3. Membranes were washed with TBST once for 10 minutes and four times for 5 minutes each.
4. Secondary Antibody solution was prepared in 1.5% skim milk in 1X TBST. The ratio of antibody to milk was 1:100,000. Membranes were incubated I the secondary solution for 2 hours at room temperature.
5. Membranes were washed with TBST once for 10 minutes and four times for 5 minutes each.
6. Parafilm was spread out on a tray and ECL detergents 1 and 2 were mixed in a ratio of 1:1 and membranes were placed on the ECL solutions. They were incubated for a minute and then analysed. Two kinds of images were obtained – one before blocking and one after ECL. Image after ECL was normalized against the image obtained before blocking.

7. Membranes were normalized (Refer Appendix) and GLUT1 intensity was measured using ImageLab.

2.4.7 GLUT1 Intensity Calculation

High Glucose Control was used a reference as GLUT 1 levels in this sample are not affected by metformin or low glucose. Therefore, % intensity of GLUT1 in high glucose control was considered to be 100%. Following formula was used to calculate GLUT1 intensity in % for target samples.

$$\boldsymbol{GLUT1\ Intensity\ of\ target\ sample}\ (\%) = \frac{\boldsymbol{GLUT1\ intensity\ target\ sample} \times \mathbf{100}}{\boldsymbol{GLUT1\ Intensity\ of\ High\ Glucose\ control}}$$

2.5 Determination of GLUT1 by Immunostaining

Principle

Immunostaining uses protein binding specificity of antibodies to study presence and localization of protein of interest. SW1116 cells were stained with GLUT1 specific antibodies attached to a fluorophore and imaged using confocal microscopy. The amount of protein present would be directly proportional to fluorescence intensity.

Procedure

2.5.1 Culture of cells:

1. A 24 well plate was used. Sterile circular coverslips were placed in wells.
2. Cells were trypsinized and counted. 10,000 cells per well were cultured. A 150 µL solution containing 10,000 cells was pipetted onto coverslips in the wells and allowed to stand without disturbing it for 30 minutes inside the hood and the plate closed with a lid. This was done to minimise the loss of cells from the coverslip into the wells
3. 350 µL was added to the wells to make a volume of 500 µL.
4. Cells were incubated overnight to allow attachment.
5. Rows A and B were treated the following day, and rows C and D the day after, so that they were treated for 48 and 24 hours. The loading scheme was:

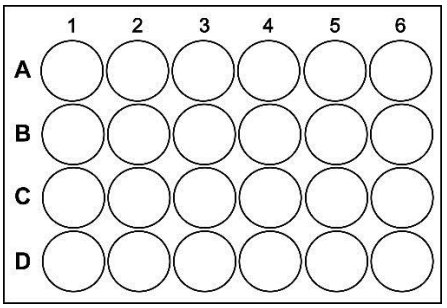

Figure 2.5. 1: A schematic representation of a 24 well cell culture plate (http:// www.cell signet.com/media/plates/24.jpg)

Table 2.5. 1: Loading scheme for Immunostaining experiment

Wells	Treatment	Wells	Treatment
A1	High Glucose Control	B1	Low Glucose Control
A3	High Glucose 0.5 mM Metformin	B3	Low Glucose 0.5 mM Metformin
A6	High Glucose 3.0 mM Metformin	B5	Low Glucose 3.0 mM Metformin
C1	High Glucose Control	D1	Low Glucose Control
C3	High Glucose 0.5 mM Metformin	D3	Low Glucose 0.5 mM Metformin
C6	High Glucose 3.0 mM Metformin	D5	Low Glucose 3.0 mM Metformin

6. After completion of incubation time, Immunostaining was carried out.

2.5.2 Immunostaining:

1. Cells were washed with PBS and fixed by 4%PFA (PFA temperature 37°C) for 30 minutes at room temperature and cells were washed with PBS twice.
2. GLUT1 is a surface receptor so, permeabilization was not required.
3. Coverslips were incubated in blocking solution for an hour.
4. 1X Primary antibody solution was prepared in blocking buffer and added to cells (90 µL/ coverslip) and incubated at 4°C overnight.
5. Antibody was removed. Cells were washed 4x with PBS (4x5minutes).
6. GLUT1 primary antibody was not conjugated with a fluorophore, therefore secondary antibody was required. Fluorophore (Alexa fluor647) conjugated secondary antibody solution, was prepared, added to cells and incubated for 1 hour at room temperature in dark.
7. Antibodies were removed. Cells were washed 4x with PBS (4x5minutes).
8. Nuclear Staining was performed using Hoechst (2µg/mL) for 2-3 minutes before washing.
9. Cells were washed 4x with PBS (4x 5 minutes). All PBS was aspirated off.
10. Mowiol 4-88 (w/DABCO) was used to fix coverslips on microscope slides. 6 µL of mowiol was taken up by reverse pipetting, to avoid air bubble formation.
11. Coverslip was placed on the drop of mowiol such that the cells were sandwiched between slide and coverslip. They were allowed to dry overnight and stored at 4°C in dark until imaging.

2.5.3 Obtaining Images on Confocal Microscope:

1. Cells were observed under 60X oil objective.

2. The excitation spectra for both Hoechst and Alexa Fluor 647 was set as 352nm and 650 nm respectively. Hoechst is blue and Alexa Fluor is Near Infrared. The emission for Hoechst is 461nm and Alexa Fluor is 665nm.
3. To order to obtain comparable images, parameters like Laser power and Pixels was kept constant. Laser power for Hoechst (Channel 1) was 2.43 and for Alexa Fluor 647 (Channel 2) was 2.43. The image pixels were constant at 512 × 512.
4. Z-Stack Images (step length was 0.75 µm) were obtained so that entire cells could be studied while calculating GLUT1 intensity. Just an individual image of cell section taken at random would disregard the remaining cell and provide erroneous and unreliable results.

2.5.4 Calculation of GLUT1 Intensity

1. The software used to take the above pictures was NIS elements software. GLUT1 estimation was done using ImageJ.
2. ImageJ was used to analyse the images and determine GLUT1 intensity.
3. Z stacks were compressed to have one image that contained mean intensity of all the images in the sack.
4. Images were opened in different channels so as to only view Hoechst in one and GLUT1 in the other.
5. Nucleus area in Hoechst image was traced out and the intensity for the nucleus was noted. This was done for 10 nuclei and mean intensity for nucleus was calculated. Then, the total Hoechst intensity in each image was measured and divided by mean nucleus intensity to determine the number of cells in that image.
6. For the same image, total GLUT1 intensity was measured and divided be the number of cells in the image. Thus, GLUT1 intensity per cell was determined and it was compared between samples with High Glucose Control as the reference.
7. GLUT1 values were calculated in percentage with High Glucose Sample as the reference sample as described in Section 2.4.7.
8. The following formula was used:

$$\boldsymbol{GLUT1\ Intensity\ of\ target\ sample}\ (\%) = \frac{\boldsymbol{GLUT1\ intensity\ target\ sample} \times \mathbf{100}}{\boldsymbol{GLUT1\ Intensity\ of\ High\ Glucose\ control}}$$

2.6 Determination of GLUT1 by Flow Cytometry

Principle:

Flow cytometer is a high throughput tool for cell analysis and measurement of multiple parameters including protein expression. Cells in suspension are forced through a narrow beam and each cell is analysed.

Cells were stained with fluorophore conjugated GLUT1 antibody and run on a flow cytometer. The fluorophore worked as a label, resulting fluorescence intensity was measured on the flow cytometer which was proportional to GLUT1 intensity. Flow cytometry protocol provided by lab engineer was followed.

Procedure:

2.6.1 Culture of Cell

Cells were cultured in the same way as described in Section 2.4.1.

2.6.2 Fixation of cells:

1. Cells were trypsinized (Refer Section 2.1.1), counted (Refer section 2.5.2), transferred to a tube (5×10^5 cells/ tube) and centrifuged (900 rpm for 5 minutes) to obtain a pellet.
2. They were treated with 4% formaldehyde at 37°C for 10 minutes, followed by 1 minute incubation on ice.
3. Since GLUT1 is a membrane protein, permeabilization of cells was not required.
4. Cells were stored at 4°C until Immunostaining was carried out.

2.6.3 Immunostaining of cells:

1. Cells were centrifuged (900 rpm, 5 minutes) and supernatant was discarded.
2. 1 mL of Incubation Buffer was added and cells were resuspended, centrifuged (900 rpm, 5 min) and supernatant was discarded. This step was repeated once.
3. 1X primary antibody solution was prepared in incubation buffer, cells were resuspended in 100 µL of antibody solution and incubated for 1 hour at room temperature.
4. After incubation time was completed, cells were washed (Refer Step 2) twice.
5. Primary (GLUT1) antibody was not conjugated with a fluorophore (Alexa fluor647), therefore flurophore conjugated secondary antibody was required.
6. 1X secondary antibody solution was prepared in incubation buffer, cells were resuspended cells in 100 µL solution and incubated for 30 minutes.
7. Cells were washed (Refer Step 2) twice, resuspended in PBS and run on flow cytometer.

2.6.4 Gating Cells and Analysis:

1. Cells were analysed using BD Accuri C6 software.
2. A graph of FSC-A (Forward Scatter Area) against FSC-H (Forward Scatter Histogram) was obtained. FSC-A values were linear and FSC-H values were log.
3. Using gating tools, only events recorded as a plateau were chosen, events that formed a linear line on graph were considered as debris (Refer Figure 2.6.1).

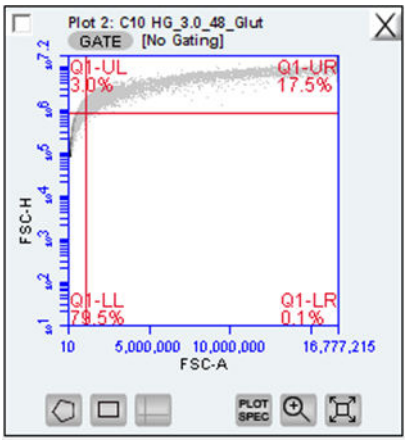

Figure 2.6. 1: Screenshot of flow cytometer events.
Recorded as a graph of FSC-A against FSC-H. Only events recorded on Q1-UR (Quadrant 1-Upper Right) were plot and gated further to isolate single cell population.

4. Area that recorded single cells as events was further gated off and only these events were analysed for GLUT1 intensity (Refer Figure: 2.6.2).

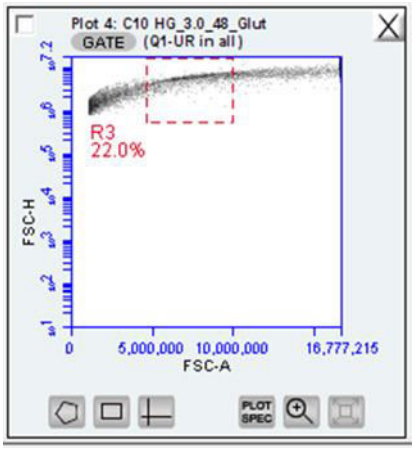

Figure 2.6. 2: Screenshot of data from Q1-UR.
A graph of FSC-A against FSC-H was plot and area of graph that has single cell population was gated as R3.

5. Median Values for GLUT1 intensity were measured for single cell population obtained in R3 gate.

2.6.5 GLUT1 Intensity Calculation

Events recorded as single cell events were analysed. A background negative control sample was run, to detect background autofluoresence. Fluorescence value obtained for background negative sample was subtracted from fluorescence values of all other test samples.

GLUT1 values were calculated in percentage with High Glucose Sample as the reference sample as described in Section 2.4.7.

Since GLUT1 Antibody was conjugated with Alexa Fluor647, intensity values were measured with FL-4 detector.

The following formula was used:

$$\boldsymbol{GLUT1\ Intensity\ of\ target\ sample}\ (\%) = \frac{\boldsymbol{GLUT1\ intensity\ target\ sample} \times \mathbf{100}}{\boldsymbol{GLUT1\ Intensity\ of\ High\ Glucose\ control}}$$

2.7 Gene Expression

Principle:

Effect of low glucose and presence of metformin in medium on gene expression was studied by Quantative PCR (QPCR). RNA was extracted from cells and it was used to synthesize cDNA by reverse transcription, since mRNA is not stable over long period. Also, the QPCR mix contains DNA dependent DNA polymerase and so the reaction is meant for DNA use. cDNA was used as template for QPCR to study expression of genes involved in glucose metabolism and ETC. QPCR was performed using SYBR Green dye, which gives fluorescent signal on binging to dsDNA.

Change in gene expression was measured by relative quantification. Relative quantification involves comparison of sample genes to a reference genes (also known as housekeeping genes). Reference genes are believed to be expressed at same levels under all conditions. Gene expression was calculated by $2^{-\Delta\Delta Ct}$.

Genes studied are included in Table 2.7.1

Table 2.7. 1: The genes studied and their corresponding protein products

Gene	Protein Product
UCP 2	Uncoupling Protein 2 (UCP 2)
SLC2A1	Glucose Transporter 1(GLUT1)
SLC22A1	Organic Cation Transporter 1 (OCT1)
SLC16A3	Lactate Transporters (MCT)
SUCLA2	ADP-forming succinyl-CoA synthetase (SUCLA2)
PDK2	Pyruvate Dehydrogenase Kinase 2 (PDK2)

Procedure:

Experiment kits from Qiagen were used and procedures described in the kit manuals were followed.

2.7.1 Culture of cells:

Cells were cultured in the same way as described in Section 2.4.1.

2.7.2 RNA Extraction:

1. RLT Buffer was added to lyse cells and they were scraped off. The resultant cell lysate was pipetted onto QIA shredder in a 2 mL tube and centrifuged (2 min, full speed).
2. 96% ethanol was added to the flow through.
3. The resultant sample was added to RNeasy spin column and centrifuged (15 sec and 10,000 rpm). Flow through was removed and RW1 buffer was added to the column, and centrifuged for (15 sec and 10,000 rpm) and the flow through was removed.
4. RPE buffer was added to the column and centrifuged for (15 seconds, 10,000 rpm) and flow through was discarded, RPE buffer was added again to RNeasy spin column and centrifuged (2 minutes and 10,000 rpm) to wash the spin column membrane.
5. Spin column was placed in a new collection tube and 40 µL of RNase-free water was added to the column and centrifuged (1 min and 10,000 rpm) to elute RNA which was stored at -80°C and it was quantified with a NanoDrop. cDNA was prepared after quantification.

2.7.3 RNA Quantitation:

1. RNA samples were kept on ice and allowed to thaw.
2. Two pedestals of the NanoDrop were cleaned with RNase free water and a special tissue paper and it was to set NanoDrop to Blank (since RNase free water was used to elute out RNA).
3. 2 µL of sample was used for analysis. Amount of RNA present in the sample was recorded by NanoDrop. RNA concentration in the sample was determined by NanoDrop.
4. 1 µg of RNA was required to prepare cDNA.

2.7.4 cDNA Preparation:

1. 10 tubes were prepared each for 10 different samples.
2. gDNA Wipeout Buffer was used to remove genomic DNA traces present in the sample.
3. Amount of template RNA which contained 1 µg of RNA was added. RNase free water was added to make the volume upto 14 µL. This solution was incubated at 42°C for 2 minutes.

4. Reverse Transcription Master mix was prepared (10% extra was prepared since some is lost on the walls of tube and on pipette tips) in the following way:

Table 2.7. 2 Components in reaction mixture for cDNA preparation

Component	Amount
Quantiscript Reverse Transcriptase (contains RNase inhibitors)	11 µL
Quantiscript RT Buffer, 5X (Contains Mg^{2+} and dNTPs)	44µL
RT Primer Mix	11 µL
Total	66 µL

5. 6 µL of the above Reverse Transcription Master mix was added to each tube, and incubated at 42°C for 15 minutes.
6. After incubation, the tubes were incubated for 95°C for 3 minutes to inactivate Reverse Transcriptase.
7. Volume is made upto 100 µL with RNase and DNase free water. This leads to a concentration of 10 ng/µL.
8. It was stored at -20°C until QPCR was performed.

2.7.5 Quantitative PCR:

1. Primers for the above mentioned genes were validated before use (Refer Appendix).
2. Volume of QPCR reaction mixture was 25 µL.

Table 2.7. 3: List of reagents and their required volumes for QPCR

Serial Number	Component	Volume
1.	2X PCR Master Mix (SYBR Green)	12.5 µL
2.	10X Primers	2.5 µL
3.	cDNA 10 ng	1 µL
4.	H_2O	9 µL
	Total	25 µL

3. PCR plates were loaded in the manner mentioned in the templates below. Two plates were prepared so results could be obtained in duplicates.
4. QPCR reaction was carried out in 96 well plate. Experiment was run in duplicates. (Refer Appendix for loading scheme)
5. Roche Thermal cycler QPCR programme was set to the following settings:

Table 2.7. 4: QPCR cycling conditions

Step	**Time**	**Temperature**	
PCR Initial Heat Activation	15 min	95°C	
3 Step Cycling:			
Denaturation	15 s	94°C	40 cycles
Annealing	30 s	55°C	
Extension	30 s	72°C	
Data Acquisition from software			

2.7.6 Calculation Relative Quantification of genes:

Target genes *were SLC2A1, SLC22A1, SLC16A3, SUCLA2, PDK2* and *UCP* while *HSP90* and *RRN* were reference genes. Certain unavoidable human errors especially sample lost while pipetting could cause less sample to be introduced to wells and this deviates results. The reference genes are expressed same under all conditions and are used to normalize the sample and minimise errors.

QPCR was performed on Roche Lightcycler96 and Ct values were obtained for reference and housekeeping genes.

Ct values were used to quantify gene expression by relative quantification. Ct value of both reference genes was subtracted from Ct value of target genes. ΔΔCt values were calculated using high glucose control as a reference sample. It is unaffected by metformin and reduction of glucose level. To calculate fold increase negative values of ΔΔCt were expressed as power of 2 ($2^{-\Delta\Delta Ct}$) [58].

For High Glucose Control, $2^{-\Delta\Delta Ct} = 2^0 = 1$. This indicates no increase. For the other target samples, the increase or decrease was calculated according to the above description.

2.8 Flow Cytometry for mitochondria

Principle:

Flow Cytometry was also performed to assess mitochondria mass/volume of cells. Antibodies against a receptor protein which was translocase of outer mitochondrial membrane 20 (TOMM20) were used to stain mitochondria and analyse them.

Procedure:

2.8.1 Culture of Cell

Cells were cultured in the same way as described in Section 2.4.1.

2.8.2 Fixation of cells:

Cells were fixed (Refer section 2.6.2).

2.8.3 Permeabilization of cells:

1. After fixation, cells were centrifuged (5 min, 900 rpm) and PBS + Formaldehyde solution was removed.
2. 90% Methanol was added and cells were incubated on ice for 30 minutes.
3. Cells were stored at-20°C until Immunostaining was carried

2.8.4 Immunostaining of cells:

1. Cells were immunostained (Refer Steps 1-4; Section 2.6.3).
2. TOMM20 antibody was conjugated with a fluorophore (Alexa fluor488) and hence, secondary antibody was not required.
3. After incubation time was done, cells were washed 3 times, resuspended in PBS and run on flow cytometer.

2.8.5 Gating Cells and Analysis:

Cells were gated and analysed in the same was as described in Section 2.6.4.

2.8.6 GLUT1 Intensity Calculation:

As described in Section 2.6.5, only single cell events were analysed. A background negative was used to subtract background autofluoresence.

TOMM20 values were calculated in percentage with High Glucose Sample as the reference sample as described in Section 3.4.7.

TOMM20 antibody was conjugated with Alexa fluor488 and therefore, FL1 detector was used as excitation peak is at 493 nm and emission peak is at 519 nm.

Formula used for calculation of mitochondrial mass was:

$$\boldsymbol{TOMM20\ intensity\ of\ target\ sample}\ (\%) = \frac{\boldsymbol{TOMM20\ intensity\ target\ sample} \times \mathbf{100}}{\boldsymbol{TOMM20\ Intensity\ of\ High\ Glucose\ control}}$$

3. Results

3.1 Cell Proliferation in High and Low Glucose

3.1.1 General Observations

Cells grown in low glucose were visibly less dense than cells grown in high glucose. The colonies formed by cells grown in low glucose were smaller than cells in high glucose (Both probably due to slower growth).

The number of cells found floating in low glucose medium was 2 to 3 times higher than cells grown in high glucose.

A comparatively high number of necrotic cells were found at 6 hours in low glucose medium.

The cells grown in low glucose medium had a more rounded appearance when compared to cells in high glucose which had a very pavement like appearance.

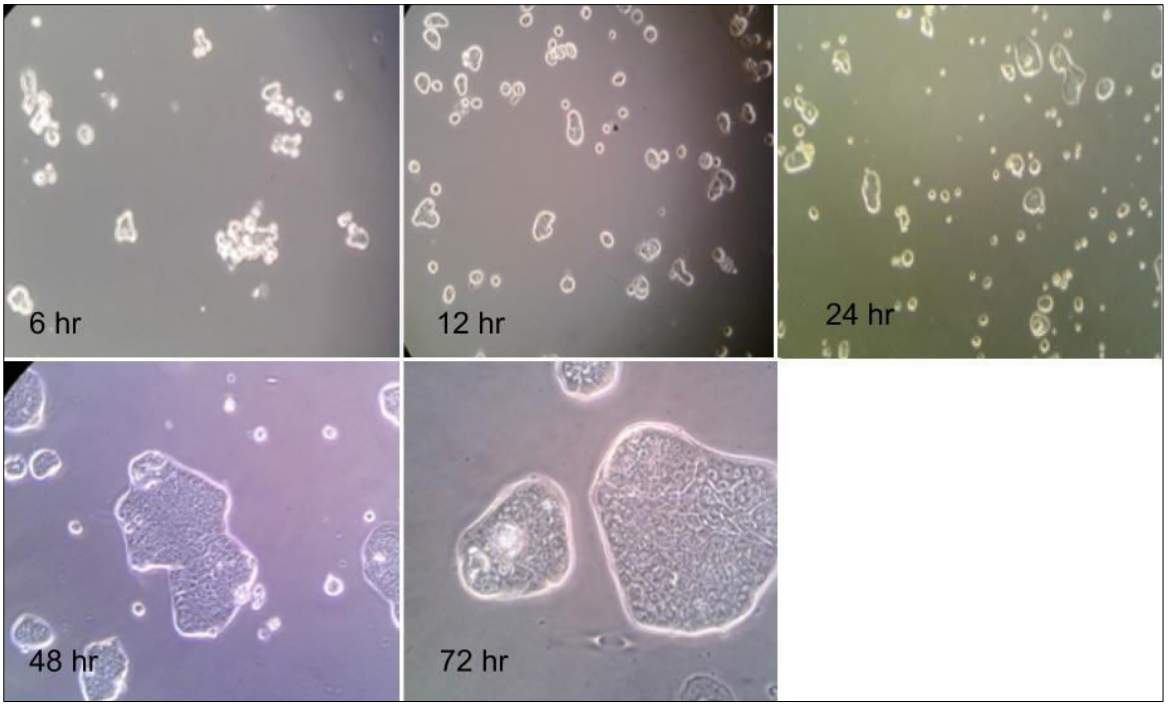

Figure 3.1. 1: Images of cells grown in low glucose medium. Number of hours indicate the time at which they were taken. Observed under 20X.

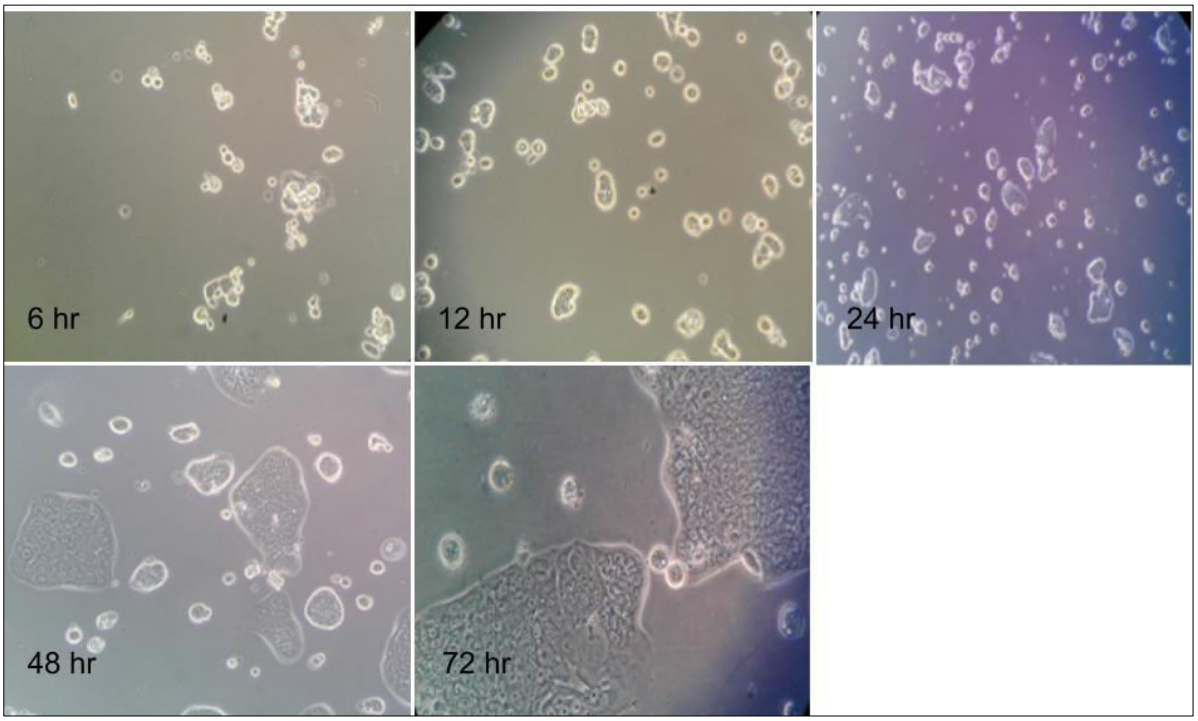

Figure 3.1. 2: Images of cells grown in high glucose medium. Number of hours indicate the time at which they were taken. Observed under 20X.

3.1.2 Proliferation Assay

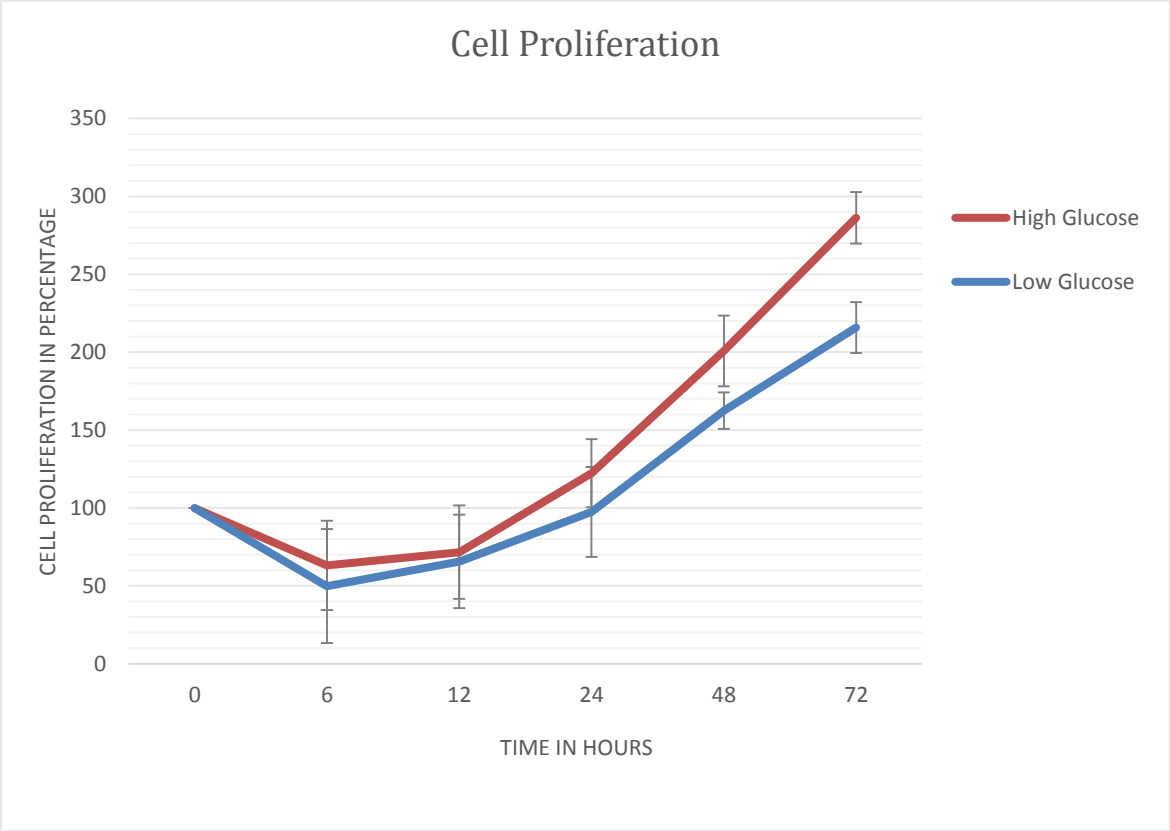

Figure 3.1. 3: Cell proliferation in high (4.5g/L) and low (1g/L) glucose.

Number of cells was found to decrease initially (Fig 3.1.3) (Refer Appendix for result table). This was observed in both high and low glucose medium. Proliferation rate in high glucose was greater than proliferation rate in low glucose. After 72 hours, growth rate in high glucose was 286.25% and in low glucose it was 215%.

In high glucose, the number of cells doubled around 48hr and in low glucose around 68 hr. The number of SW1116 in low glucose was 75% of the number of cells present in high glucose after 72 hours.

3.2 Effect of Metformin on Cell Viability

MTS assay was performed to test the effect of metformin on cell viability in high and low glucose.

3.2.1 Effect of Metformin in High Glucose Growth medium:

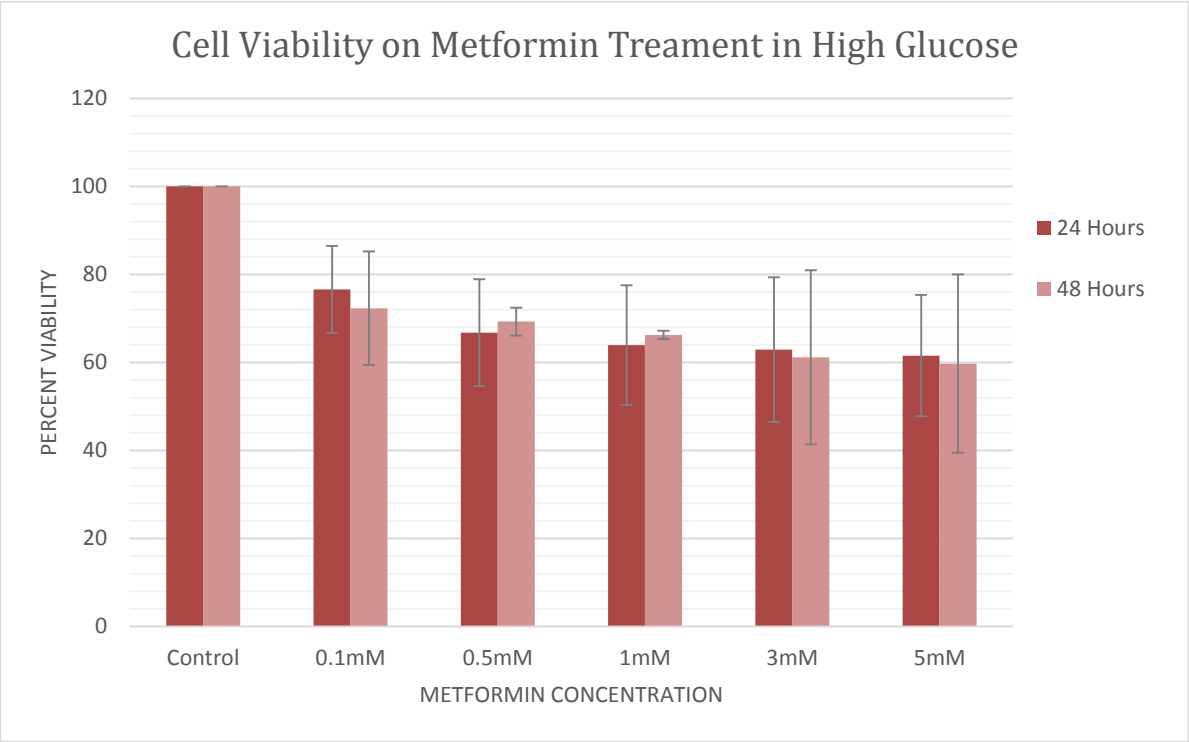

Figure 3.2 1: Percent cell viability of SW1116 cells in high glucose growth medium after metformin treatment of 24hr and 48 hr.

Metformin treatment was found to decrease the viability of SW1116 cells in high glucose (Fig 3.2.1) (Refer Appendix for result table). There was a decline in cell viability with increase in metformin concentration. Viability of cells in all metformin treatments was approximately 2% to 4% higher in 24 hour treatment than 48 hour treatments. However, for 0.5 mM treatment sample, viability was 3% higher in 48 hr treatment than 24 hr treatment.

3.2.2 Effect of Metformin in Low Glucose Growth medium:

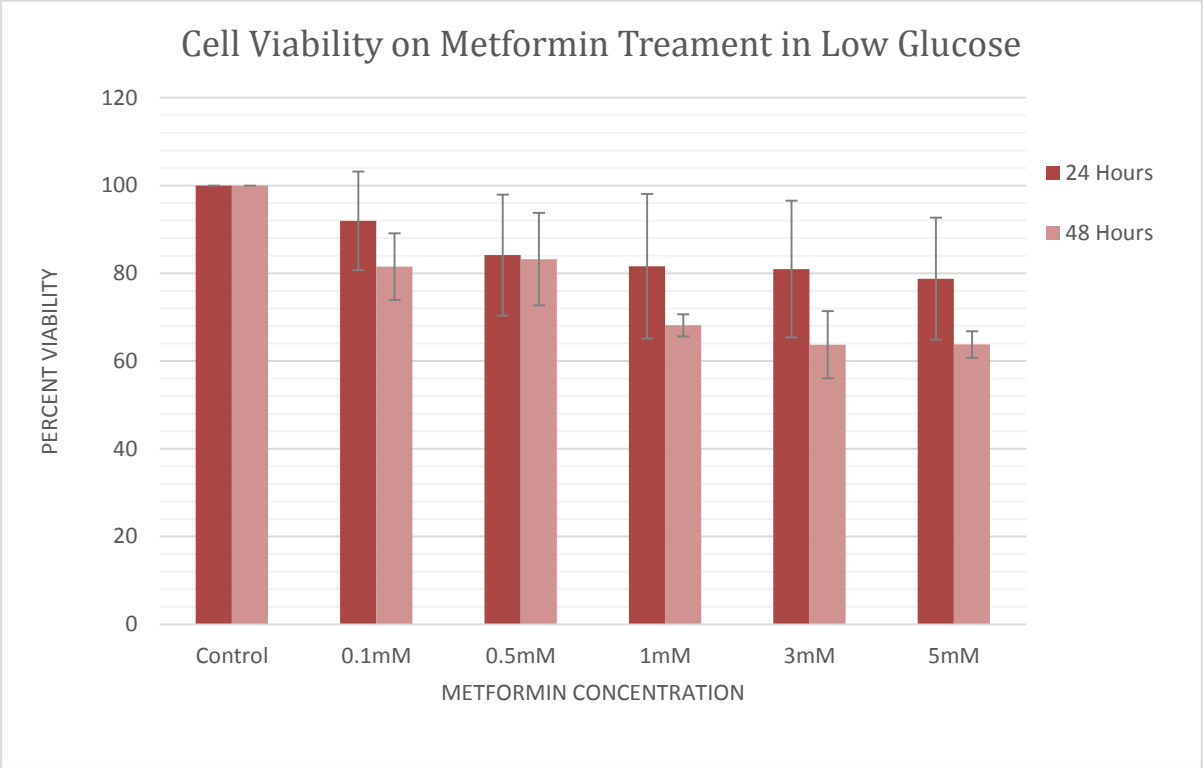

Figure 3.2 2: Percent cell viability of SW1116 cells in low glucose growth medium after metformin treatment of 24hr and 48 hr.

Metformin treatment was found to decrease the viability of SW1116 cells in low glucose (Fig 3.2.2) (Refer Appendix for result table) growth medium and the trend was similar to high glucose. There was a decline in cell viability with increase in metformin concentration. Viability of cells in all metformin treatments was approximately 2% to 13% higher in 24 hour treatment than 48 hour treatments. The difference in viability of 24 and 48 hours was big in low glucose treatment.

It was found that after metformin treatment, cell viability in low glucose was higher than cell viability in low glucose.

0.5mM and 3.0 mM concentrations were used for further experiments. These concentrations were used in both high and low glucose growth medium, and after 24 and 48 hr of treatment.

3.3 Effect of Metformin on GLUT1 Protein Expression (Western Blot)

Effect on GLUT1 (Biomarker) protein expression was studied after metformin treatment of different concentrations (preferred concentrations from MTS Assay). The study was performed under the same conditions (24 and 48 hr treatment, and low and high glucose medium). The studies that follow were all performed under these conditions.

Table 3.3. 1: List of samples studied and corresponding abbreviations used for the samples.

Sample	**Sample Abbreviation**
High Glucose 24 hr 0.5 mM metformin Treatment	HG24_0.5mM
High Glucose 24 hr 3 mM metformin Treatment	HG24_3.0mM
High Glucose Control	HG_C
High Glucose 48 hr 0.5 mM metformin Treatment	HG48_0.5mM
High Glucose 48 hr 3 mM metformin Treatment	HG48_3.0mM
Low Glucose 24 hr 0.5 mM metformin treatment	LG24_0.5mM
Low Glucose 24 hr 3 mM metformin treatment	LG24_3.0mM
Low Glucose Control	LG_C
Low Glucose 48 hr 0.5 mM metformin treatment	LG48_0.5mM
Low Glucose 48 hr 3 mM metformin treatment	LG48_3.0mM

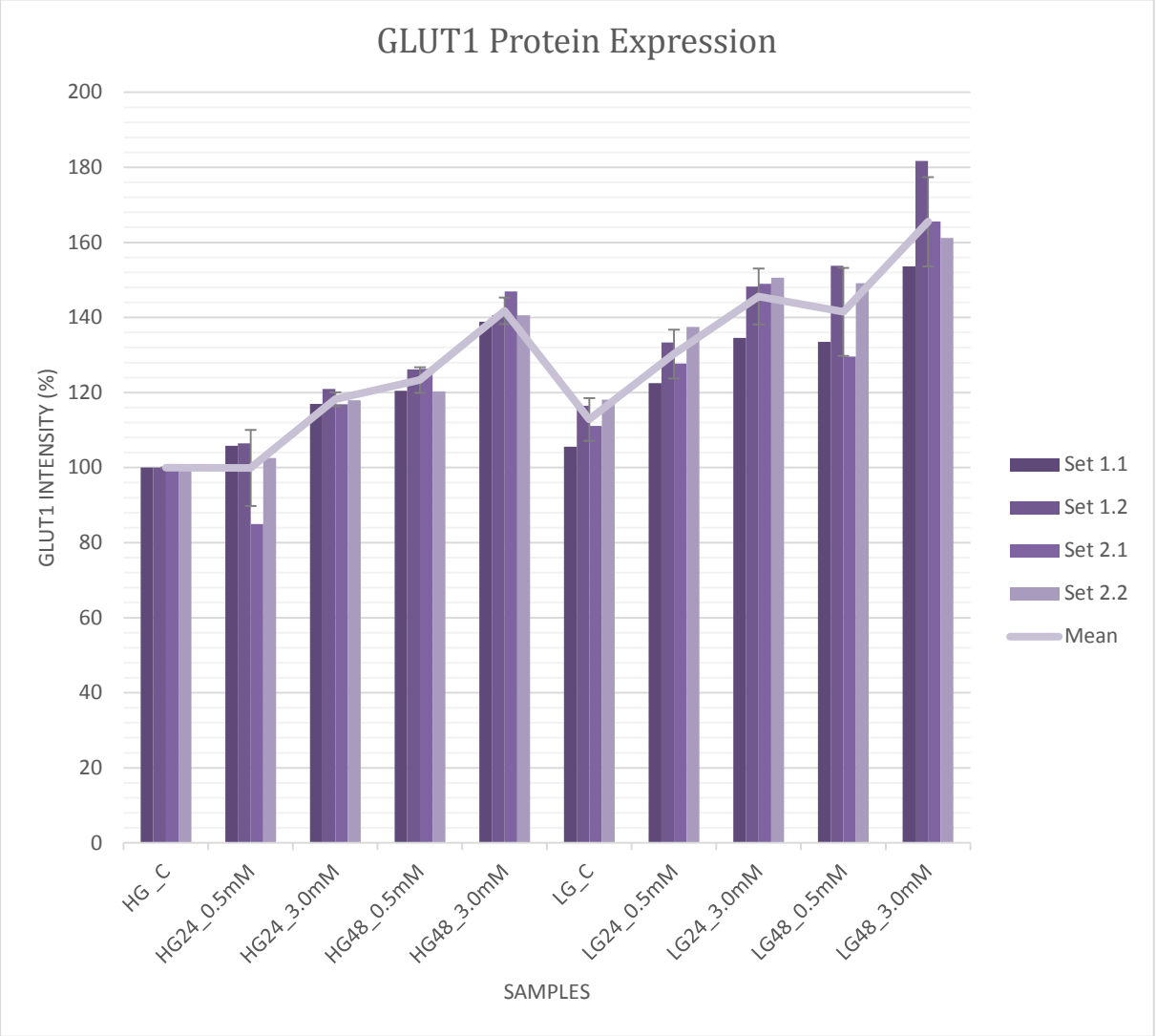

Figure 3.3. 1: % relative GLUT1 values for all samples. It also shows the mean and standard deviation for all the samples.

GLUT1 protein expression was found to increase in low glucose conditions and on metformin treatment (Fig 3.3.1) (Refer Appendix for result table). Increase in GLUT1 protein expression in low glucose medium was found to be small. Decrease in glucose concentration alone did not have a significant effect on the GLUT1 expression. However, a significant increase in GLUT1 protein expression was found after metformin treatment in both high and low glucose medium.

GLUT1 expression increased with increase in metformin concentration. Metformin treatment of 3.0 mM metformin caused a greater increase in GLUT1 protein expression than 0.5 mM metformin treatment. GLUT1 protein expression was found to be higher after 48 hr treatment

than 24 hr treatment. GLUT1 protein expression was highest at 3.0 mM metformin treatment for 48 hr in low glucose growth medium.

3.4 Determination of GLUT1 Protein Expression and Location (Immunostaining)

Immunostaining using GLUT1 antibody was used to determine the protein expression of GLUT1 on cell membrane and whether the detected increase found in GLUT1 levels by Western Blot was also observed here.

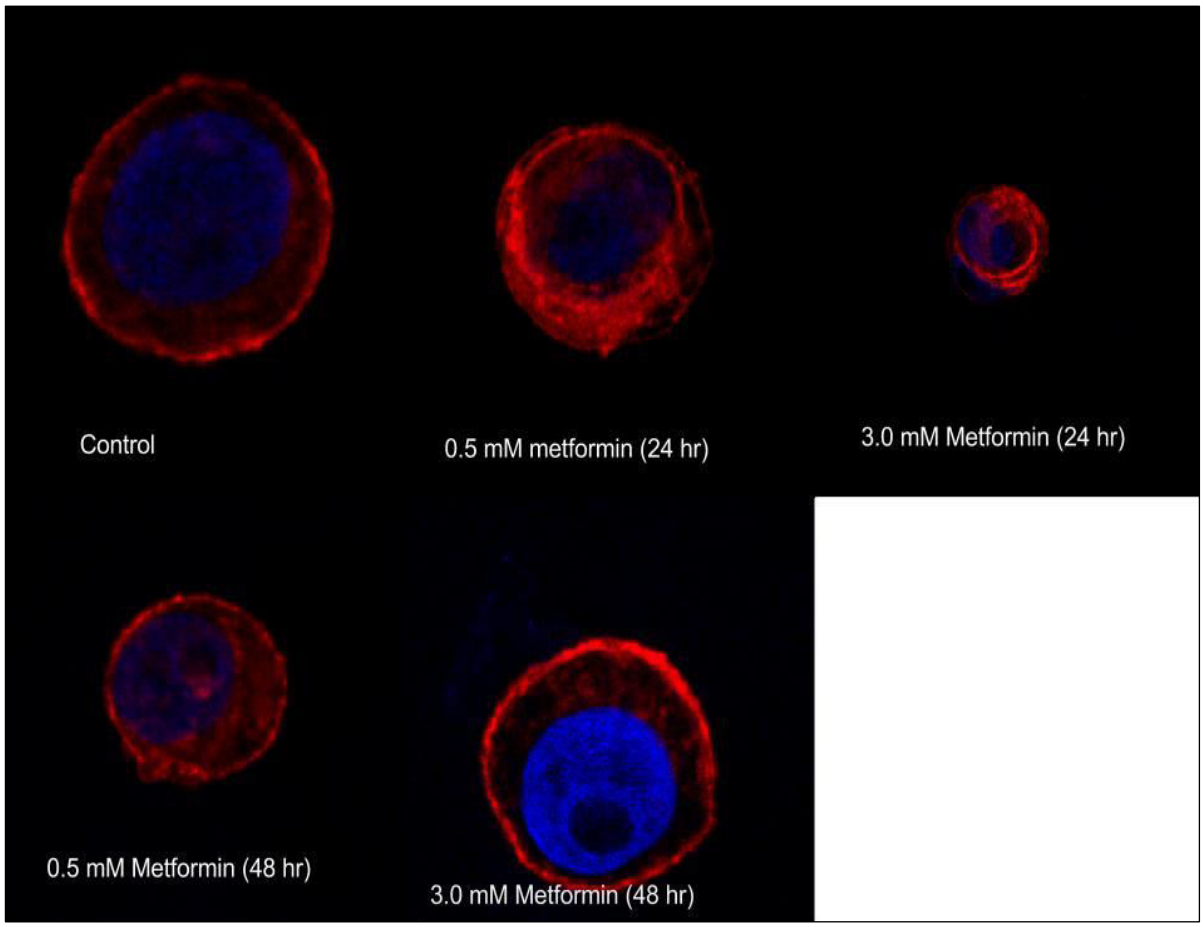

Figure 3.4. 1: Confocal images of SW1116 cells grown in low glucose.
Cells have been stained with Nuclear Stain Hoechst (Blue) and GLUT1 antibody (Red). Conditions of treatment are mentioned in boxes for respective image. Images taken under 60X oil objective .

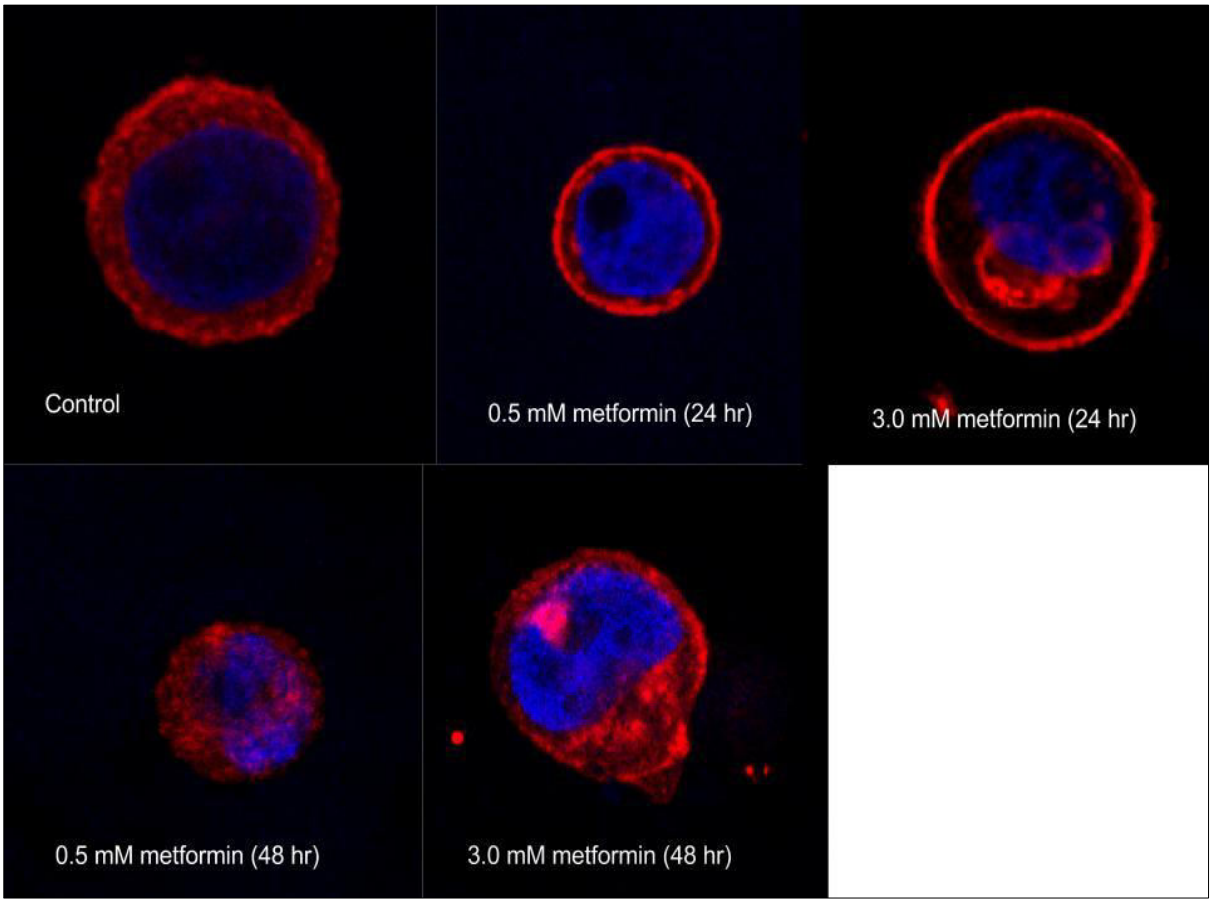

Figure 3.4. 2: Confocal images of SW1116 cells grown in high glucose.
Cells have been stained with Nuclear Stain Hoechst (Blue) and GLUT1 antibody (Red). Conditions of treatment are mentioned in boxes for respective image. Images taken under 60X oil objective.

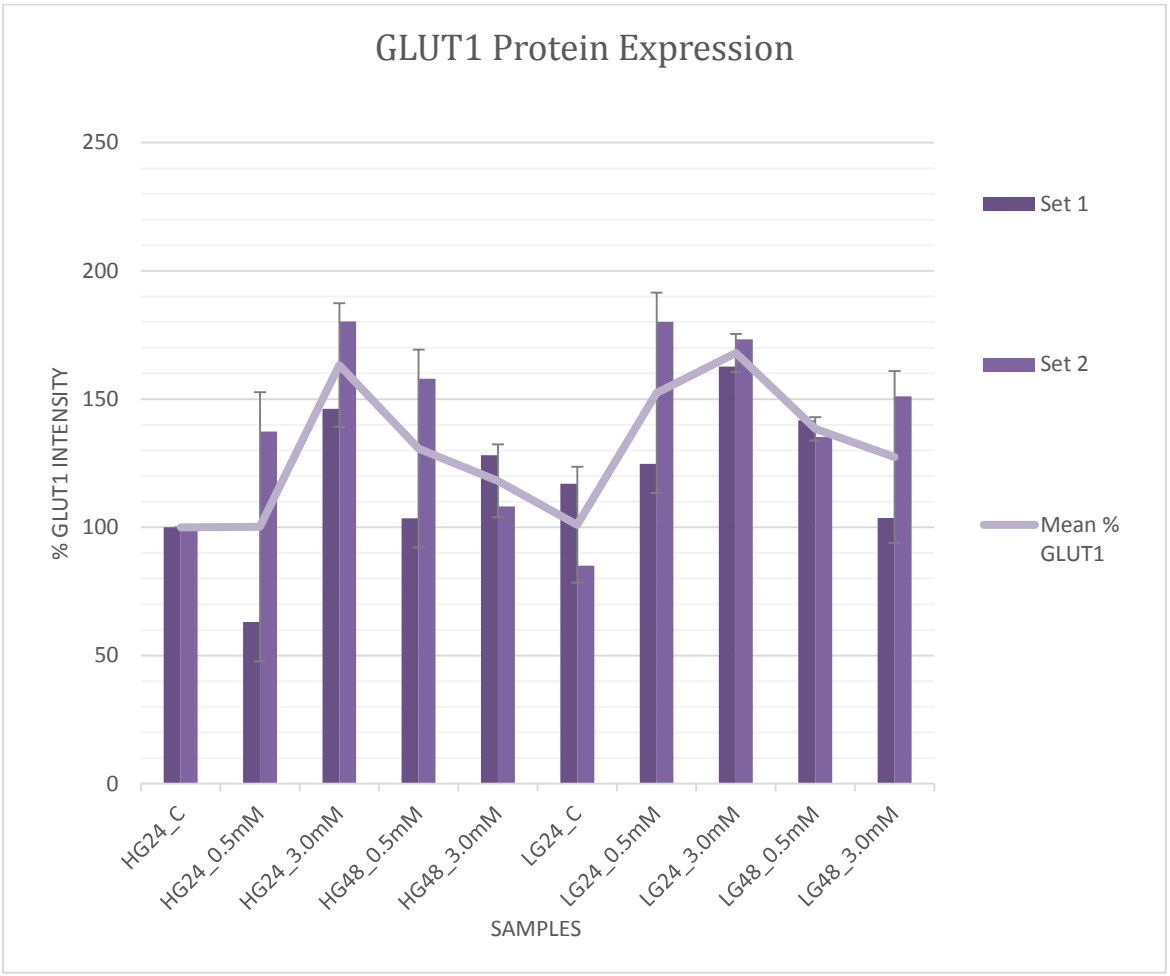

Figure 3.4. 3: % GLUT1 intensity per cell of Set1 and Set2, Mean % GLUT1 intensity and standard deviations for all samples.

Here, we observed a similar effect. GLUT1 protein expression was found to increase in low glucose medium and after metformin treatment in high and low glucose (Fig: 3.4.3). The degree of GLUT1 increase was similar in almost all test samples.

Samples treated for 48 hr with 3.0 mM metformin concentration in high and low glucose showed an increase in GLUT1 expression when compared with normal. However, the degree of increase was different, it was found that the GLUT1 protein expression was lower when measured by Immunostaining (Confocal Microscopy). For 48 hr, 3.0 mM treatment in high glucose the GLUT1 expression was 23% higher by Western Blot and for low glucose it was 38% higher by Western Blot.

3.5 Determination of GLUT1 Protein Expression (Flow Cytometery)

A high throughput protein expression analysis (flow cytometry) was performed to evaluate GLUT1 expression in 0.5×10^6 number of cells for each treatment.

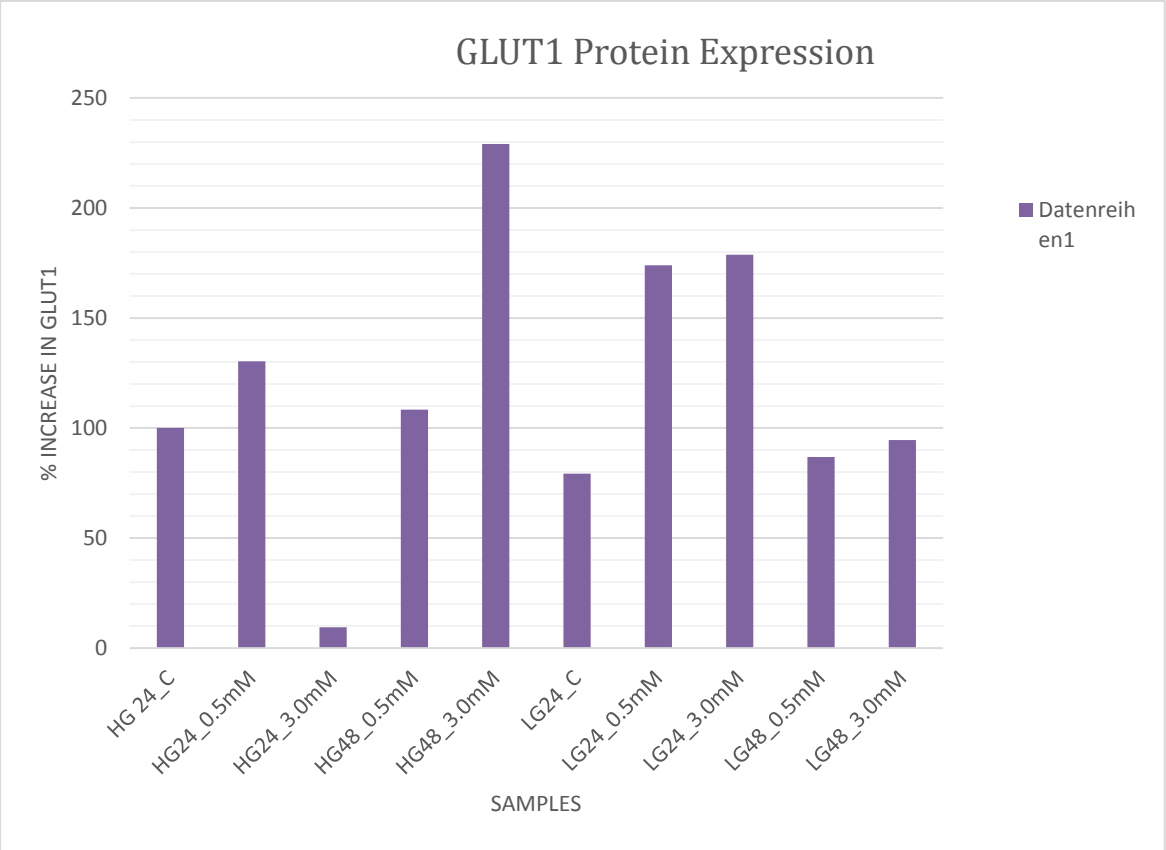

Figure 3.5. 1: % relative GLUT1 values determined by Flow Cytometery.
Similar trends in GLUT1 protein expression are marked with red boxes.

Here we observed that, protein expression levels of GLUT1 were found to increase with metformin treatment in most samples. However for 3.0 mM metformin treatment for 24 hr showed a marked decrease in GLUT1 protein level. GLUT1 levels were also found to be considerably low for cells grown in low glucose medium with no metformin, and cells treated with 0.5 mM and 3.0 mM metformin for 48 hr. These results are not in agreement with results obtained for Western Blot.

Comparison of GLUT1 protein expression results indicated by Western Blot, Confocal Microscopy and Flow Cytometry:

Table 3.5. 1: GLUT1 intensities (in percentage) as indicated by Western Blot, Confocal Microscopy and Flow Cytometry.
Comparable results by the three methods are marked by a red box.

Sample	GLUT1 intensity in percentage		
	Western Blot	Confocal Microscopy	Flow Cytometry
HG_C	100.0	100.0	100.0
HG24_0.5mM	99.9	100.2	130.4
HG24_3.0mM	118.2	163.3	9.4
HG48_0.5mM	123.3	130.7	108.4
HG48_3.0mM	141.7	118.1	229.1
LG_C	112.8	101.0	79.2
LG24_0.5mM	130.3	152.4	174.0
LG24_3.0mM	145.6	168.0	178.7
LG48_0.5mM	141.5	138.4	86.9
LG48_3.0mM	165.5	127.4	94.5

Results obtained GLUT1 protein expression by Western Blot and Confocal Microscopy are consistent for control and 0.5 mM 24 and 48 hr metformin treatments in both high and low glucose. The results obtained in high and low glucose growth medium for 3.0 mM 24 and 48 hr metformin treatment are not consistent with a difference of over 25% in GLUT1 protein expression in these samples. Although, the results obtained for Flow Cytometry indicate an increase in GLUT1 expression with metformin addition, the level of increase does not correlate with the GLUT1 protein increase indicated by Western Blot and Confocal Microscopy.

3.6 Gene Expression

Study of gene expression of other metabolic biomarkers under the same treatment conditions used for GLUT1 protein expression was done by qPCR.

SLC2A1

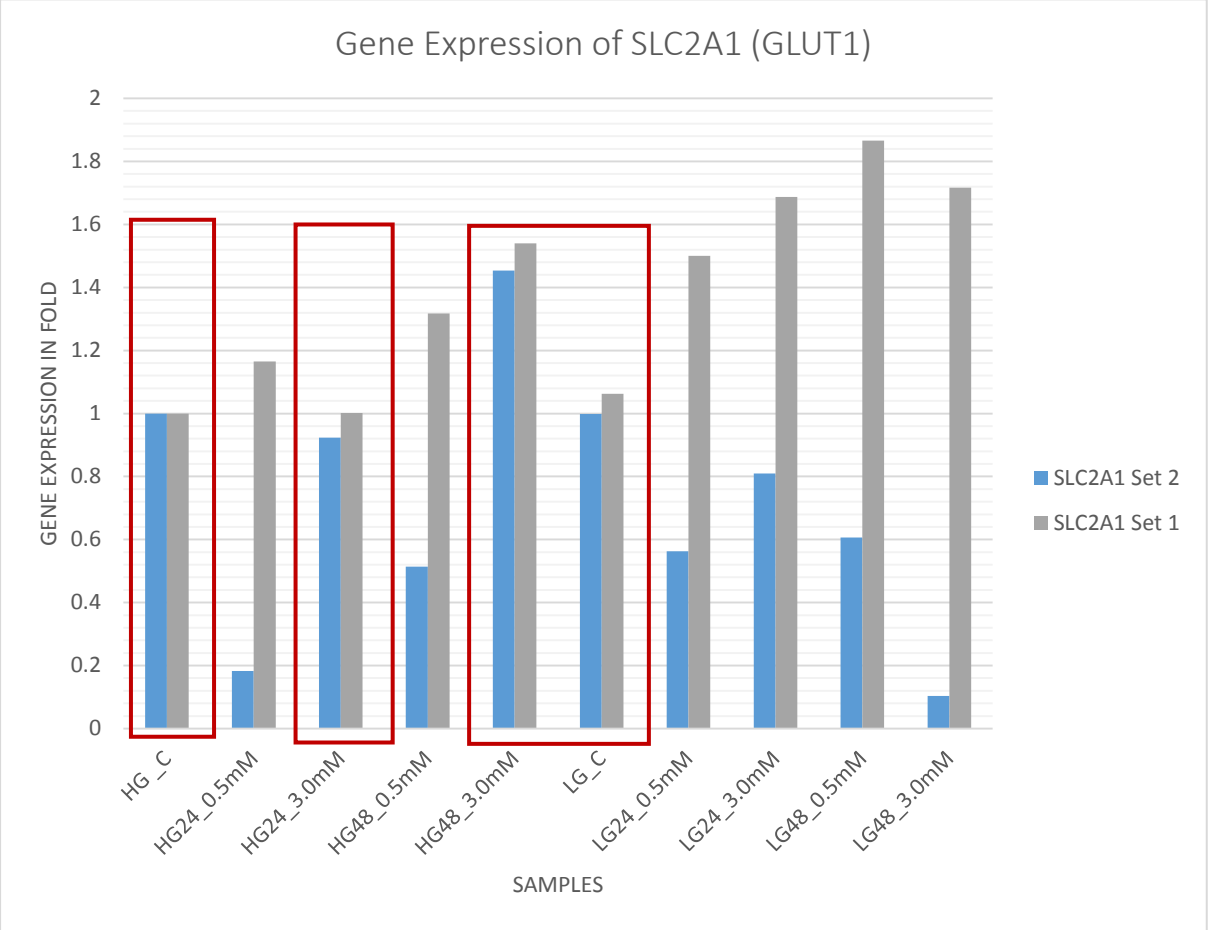

Figure 3.6. 1: SLC2A1 gene expression in 2^{-ddCt}.
Results showing similar trends are marked with a red box.

We observed that, SW1116 cells showed an increase in SLC2A1 (GLUT1) gene expression with metformin treatment in all test samples for Set 1, this was comparable to GLUT1 protein expression studied by Western Blot and Confocal Microscopy.

However, for Set 2 some treatment samples showed increase and some showed decrease in SLC2A1 gene expression, therefore, not all results obtained were comparable.

SLC22A1

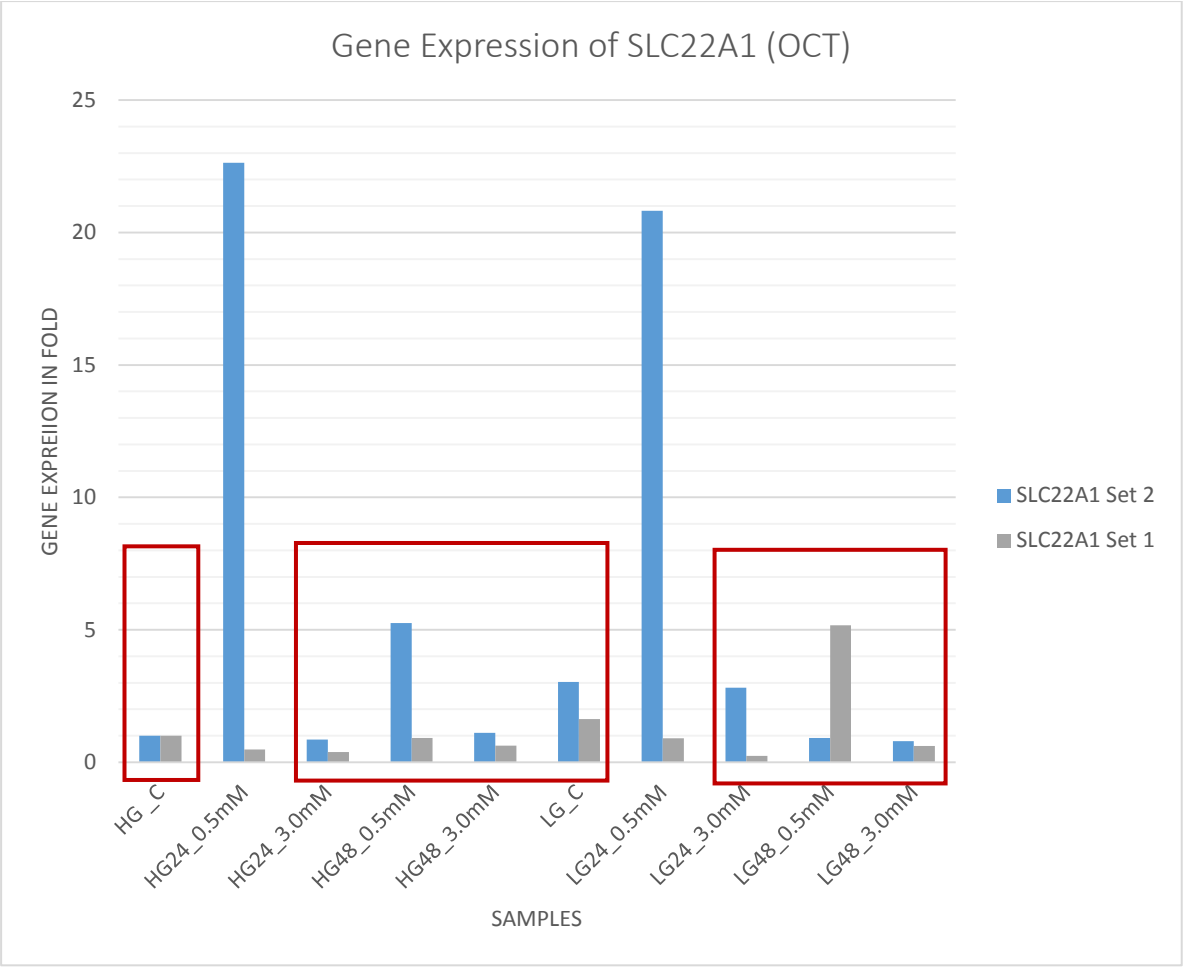

Figure 3.6. 2: SLC22A1 gene expression in 2^{-ddCt}.
Results showing similar trends are marked with a red box.

Metformin affects SLC22A1 (OCT) gene expression in SW1116 cells. In most cases, a decrease in SLC22A1 gene expression was observed with metformin treatment. The decrease in OCT1 gene expression was proportional to increase in increase in metformin concentration.

Results obtained for metformin treatments for 0.5 mM and 3.0 mM metformin treatments at 24 and 48 hours in high glucose growth medium were comparable. Results obtained for low glucose control and 3.0mM treatment for 48 hr were comparable. All of them showed a decrease in SLC22A1 gene expression after metformin treatment.

A major increase in SLC22A1 gene expression was observed at 0.5 mM metformin 24 hr treatment in high and low glucose for Set 2. The increase in gene expression was over 20 fold. These readings could not be compared with results of Set1.

SLC16A3

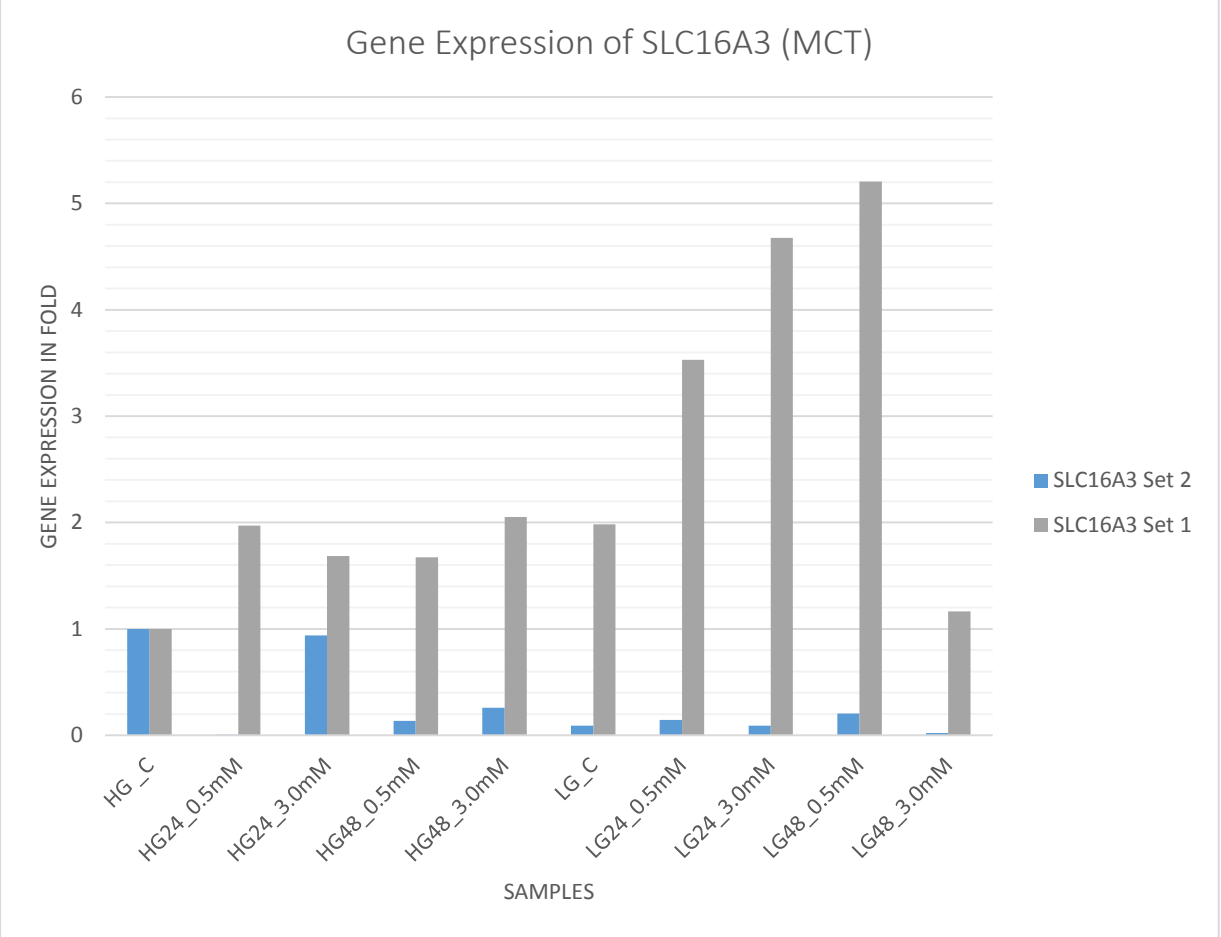

Figure 3.6. 3: SLC16A3 gene expression in 2^{-ddCt}.

We observed that the gene expression decreased of *SLC16A3* in one set of readings and the other set showed an increase in SLC16A3 gene expression.

In the set that showed decrease in gene expression on addition of metformin in high glucose, over tenfold reduction in *SLC16A3* gene expression on reduction of glucose. Addition of metformin decreased the gene expression further in low glucose medium. The decrease observed was proportional to the increase in metformin treatment.

In the set that showed increase in gene expression on addition of metformin in high glucose, twofold increase in *SLC16A3* gene expression on reduction of glucose. Addition of metformin increased the gene expression further in low glucose medium. The increase observed was proportional to the increase in metformin treatment. However, the 48 hr 3.0 mM metformin treatment sample in low glucose did not show any increase in *SLC16A3* gene expression.

UCP2

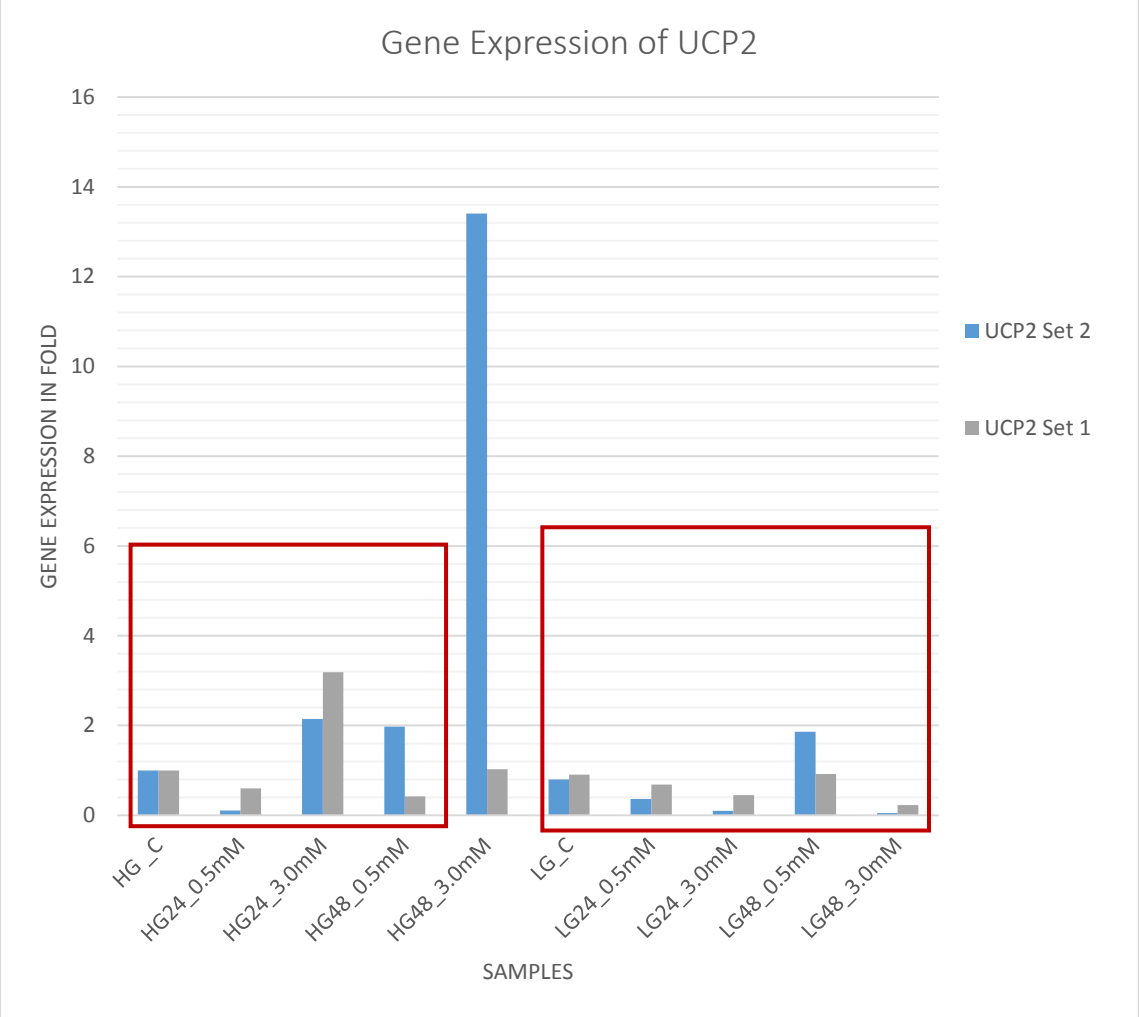

Figure 3.6. 4: UCP2 gene expression in 2^{-ddCt}.
Results showing similar trends are marked with a red box.

We observed that the *UCP2* gene expression was affected by metformin treatment. An increase in expression was observed with metformin treatment in high glucose growth medium. The increase in gene expression increased with increase in concentration of metformin treatment. A decrease in UCP2 gene expression was observed with reduction of glucose levels and a further decrease in gene expression was observed with metformin treatment in low glucose growth medium. An increase in metformin concentration led to a further decrease in expression of the gene.

UCP2 gene expression observed for 48 hr 3.0 mM metformin treatment shows no ncrease in gene expression for one set and shows and increase of over thirteen fold in another set. These results were very different.

SUCLA2

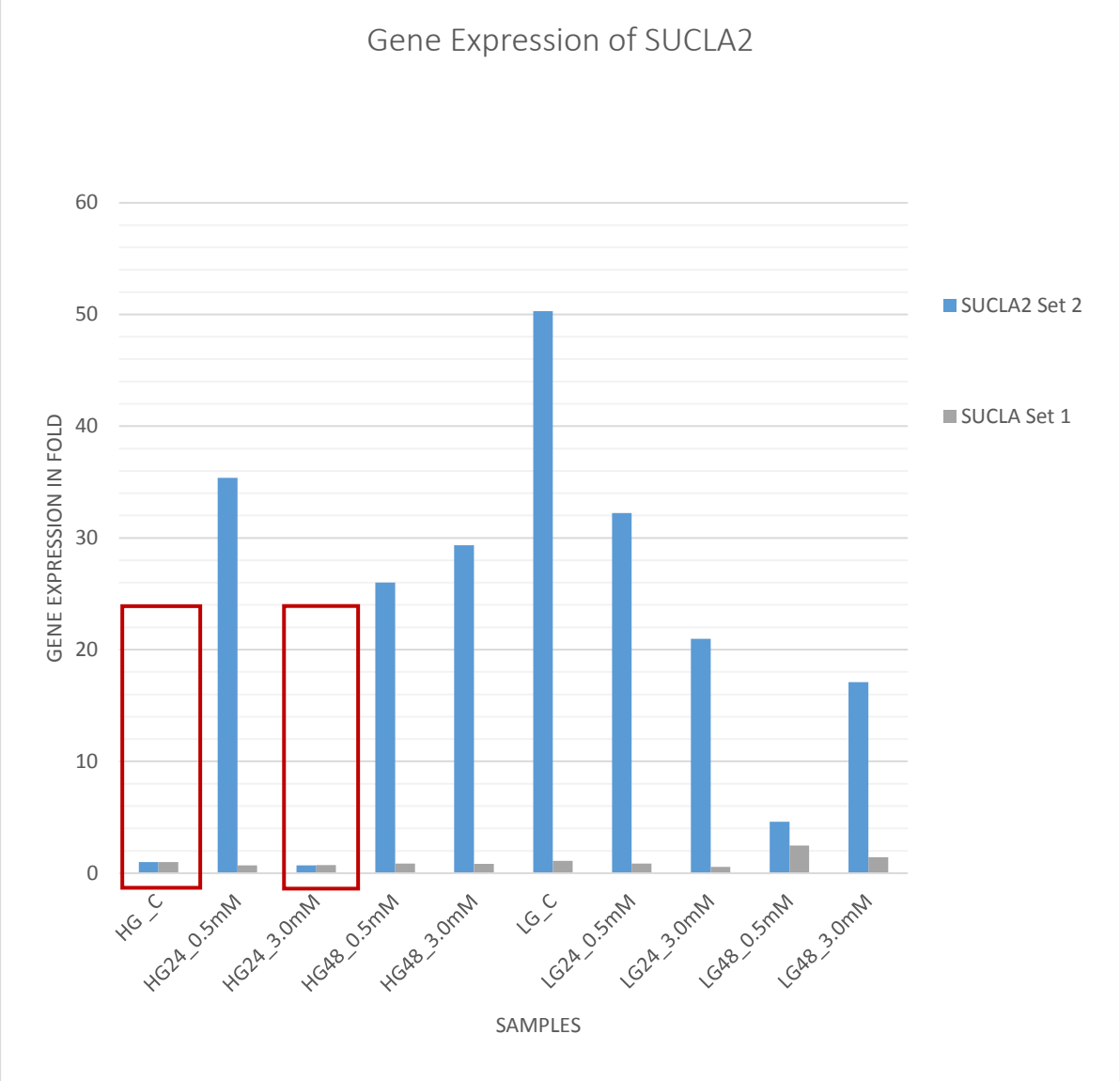

Figure 3.6. 5: SUCLA2 gene expression in 2^{-ddCt}.

We observed that the gene expression decreased of *SUCLA2* in one set of readings and the other set showed an increase in SLC16A3 gene expression.

In the set that showed decrease in gene expression on addition of metformin in high glucose, no significant change in *SUCLA2* gene expression on reduction of glucose. Addition of metformin decreased the gene expression low glucose medium. The decrease observed was proportional to the increase in metformin treatment. It was found to increase slightly for 48 hr metformin treated low glucose samples.

In the set that showed increase in gene expression on addition of metformin in high glucose, over fifty fold increase in *SUCLA2* gene expression on reduction of glucose. Addition of metformin increased the gene expression in low glucose medium, but it was lower than gene expression for low glucose control. The increase observed was not proportional to the increase in metformin treatment. However, the 48 hr 3.0 mM metformin treatment sample in low glucose did not show a very high increase in *SUCLA2* gene expression. The highest increase in *SUCLA2* gene expression was observed on reduction of glucose alone.

PDK2

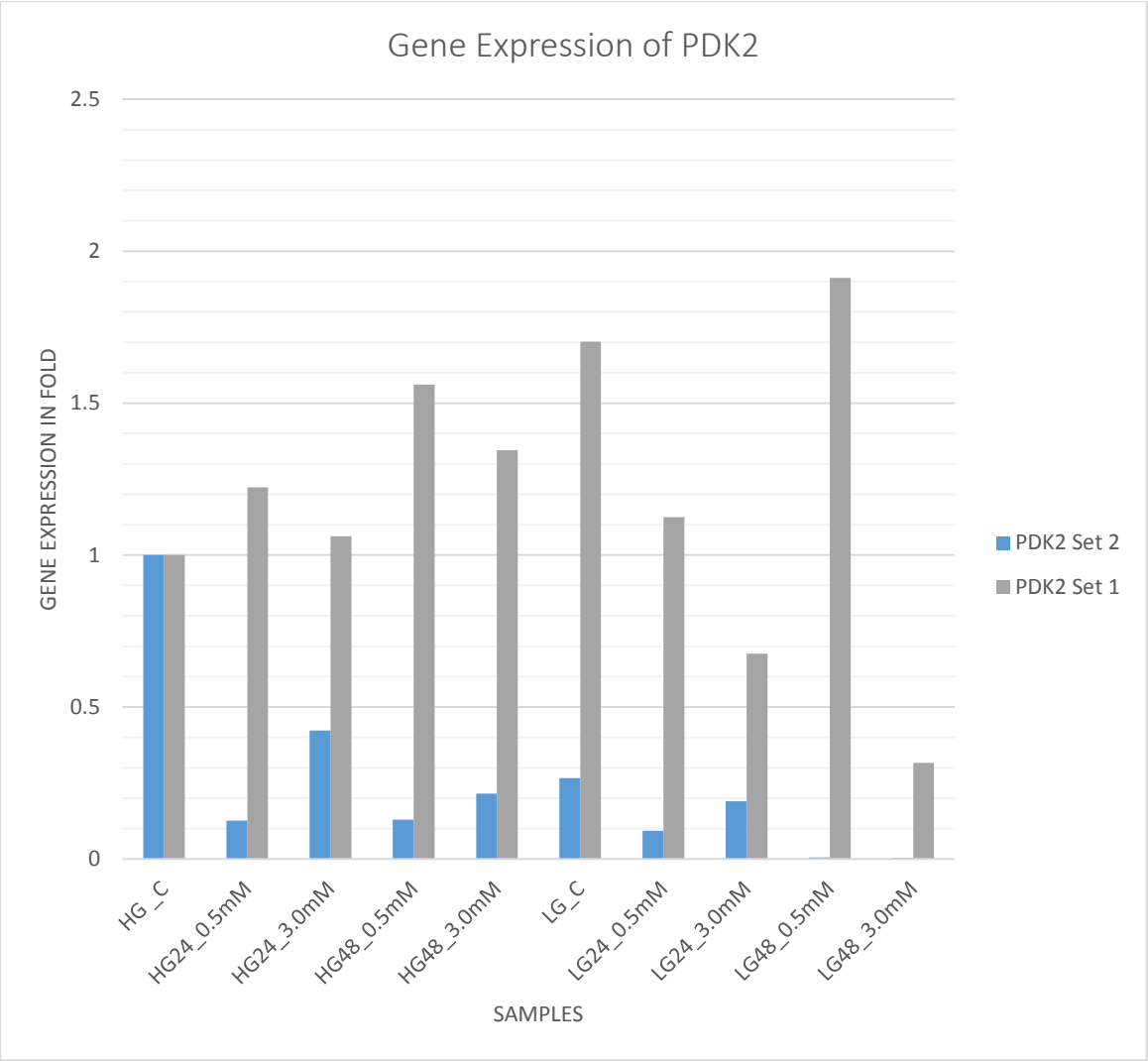

Figure 3.6. 6: PDK2 gene expression in 2^{-ddCt}.

We observed that the gene expression decreased of *PDK2* in one set of readings and the other set showed an increase in *PDK2* gene expression.

In the set that showed decrease in gene expression on addition of metformin in high glucose, approximately five fold reduction in *PDK2* gene expression on reduction of glucose. Addition of metformin decreased the gene expression further in low glucose medium. The decrease in *PDK2* gene expression was greater for metformin concentrations for 0.5 than it was for 3.0 mM treatment.

In the set that showed increase in gene expression on addition of metformin in high glucose, twofold increase in *SLC16A3* gene expression on reduction of glucose. Addition of

metformin increased the gene expression further in low glucose medium. The increase observed was proportional to the increase in metformin treatment. However, the 48 hr 3.0 mM metformin treatment sample in low glucose did not show any increase in *SLC16A3* gene expression.

3.7 Flow Cytometery Mitochondria

To assess the volume of mitochondria in cells after treatment a mitochondrial specific antibody (TOMM20) was used in flow cytometry.

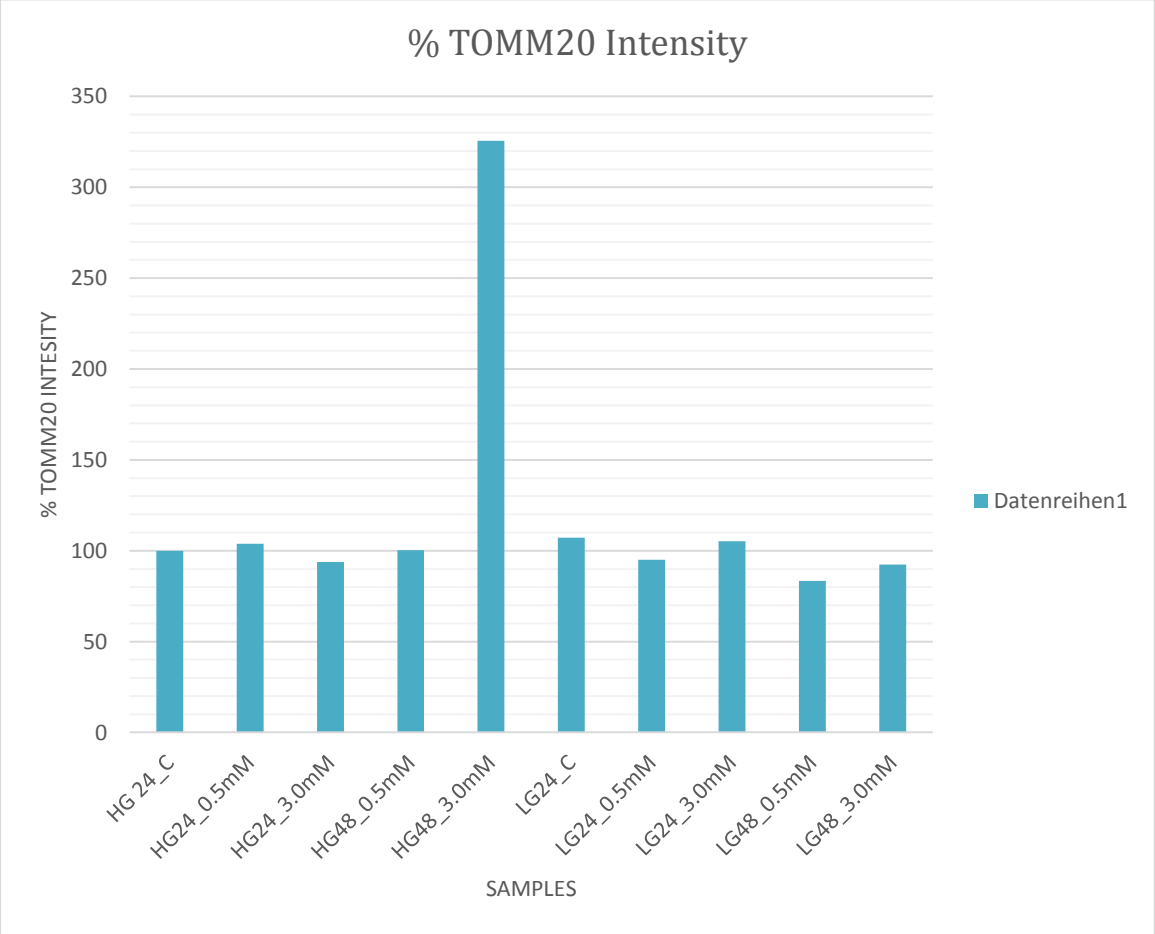

Figure 3.7. 1: TOMM20 Intensities for all samples measured by Flow Cytometry.

This method is not indicative of the mitochondrial activity but merely of mitochondrial mass. No significant change in mass of mitochondria was found after metformin treatment in high and low glucose growth medium. Cells treated with 3.0 mM metformin for 48 hr show over 300% increase in the mitochondria mass.

4. Discussion

4.1 Cell Proliferation on reduction of glucose

Although almost all cancer cells increase their glucose uptake, the degree of uptake could vary between cancers[11].Therefore, it is important to know the dependency of cancer cell line on glucose. In comparison with other colorectal cancer cell lines SW1116 is one of the most slow growing cancer cell lines[55 57]. SW1116 population was found to double at 48 hr in high glucose medium and in low glucose the number of cells doubled at around 70 hr. Number of cells at 72 hr in high glucose growth medium was approximately 25% higher than number of cells at 72 hr in low glucose growth medium. Although the cell line is slow growing, the results suggest a considerable dependency on glucose by SW1116 cells. However, reduction of glucose had a slightly altered behaviour and appearance of cells as described in section (3.1.1) which indicated that the cells were stressed.

The population doubling time is found to be around 68 hours on the EMBL-EBI website[59], however these cells are usually grown in Leibovitz's L-15 Medium (according to SW1116 datasheet)[60] which contains D-Galactose as major carbon source. Medium used here was DMEM which contains glucose as a carbon source. Research by Rossignol *et. al.* indicated a decrease in cell proliferation of HeLa cell lines grown in galactose medium, proliferation was faster in glucose medium, and cells grown in galactose medium seemed to rely more on OXPHOS[61]. Therefore, the slower rate of proliferation in galactose medium could be due to lower net ATP production via galactose – glucose-1-phosphate – pyruvate pathway[12]. The proliferation of SW1116 cells in low glucose was similar to proliferation rate mentioned on EMBL-EBI website. This suggests that the cells grown in low glucose depend more on OXPHOS for energy requirement.

Also, the passage number at which this was obtained was not mentioned. Cells transform and change their behaviour over time. This may be the reason why the proliferation rate does not match with proliferation rate mentioned on EMBL-EBI page.

4.2 Cell viability on metformin treatment

Reduction in cell viability was observed with metformin treatment; the cell viability reduced with increase in metformin concentration. Viability did not seem time dependent in high glucose as the difference between viability values obtained at 24 and 48 hours had a

difference of up to 4%. However, for low glucose it seemed time dependent as the difference between viability values for 24 and 48 hours was up to 17%.

The concentrations chosen for further experiments were 0.5 mM and 3.0 mM of metformin.

Metformin concentration of 5.0 mM would be very difficult to achieve in a human body, and would be present only in heavy metformin doses. Research has shown that the average metformin concentration in the body would be 30μM and in the gut it would be 30 to 300 times higher. Metformin treatment of 0.1 mM was not chosen since it did not show a significant effect on cells grown in low glucose. Metformin treatment of 1.0 mM was not chosen since cell viability was not significantly different than cell viability observed in 0.5 mM metformin treatment.

The standard deviation for values is ±16 and ±19 for some readings. This could be explained because cells undergoing apoptosis can also reduce MTS salt, therefore in some cases apoptotic cells also contributed to coloured product formation caused deviations in the results.

4.3 Metformin treatment caused increase in GLUT1 Protein Expression

Enhanced glucose uptake is possible by increase in glucose transporters on cell surface. GLUT1 expression has been linked to various cancers, though not all. Some types of cancers do not show increase in GLUT1 but, adenocarcinomas have shown increase in GLUT1 expression[16].

GLUT1 protein expression was studied by Western Blot, Confocal Microscopy and Flow Cytometry. GLUT1 protein expression was found to increase with reduction in glucose concentration, however, the increase was not significant. Metformin treatment in high and low glucose concentration showed an increase in GLUT1 expression. This suggests that cells depend on mitochondria for energy production. Targeting mitochondria caused an increase in expression of proteins responsible for glucose uptake. A lung cancer study has suggested that GLUT1 increase causes an increase in aerobic glycolysis[62]. This indicates that the cells might have altered their metabolism. However, there is some discrepancy in the results obtained by the three techniques.

Although an increase in GLUT1 intensity was obtained in all samples, some samples show a lower GLUT1 intensity when measured by confocal microscopy than western blot. Studies have indicated that GLUT1 transporter is not a membrane protein alone, but also exists in cytoplasmic form. Many cancers (adenocarcinoma included) have shown an elevated cytoplasmic GLUT1 expression[16]. Cells were not permeabilized for GLUT1 study in confocal microscopy and flow cytometry and therefore the GLUT1 specific antibody did not have access to cytoplasmic protein. This seems to be the reason for lower intensity obtained in some samples for confocal microscopy. Confocal microscopy allowed the visualization and location of GLUT1 protein, but it was not a high throughput method for quantitative determination of GLUT1 protein expression.

The GLUT1 intensity measured by flow cytometry show an increase in GLUT1 with metformin treatment, but the level of increase does not correlate with the increase measured by western blot and confocal microscopy. This could be explained, as cells were not permeabilized and therefore, cytoplasmic GLUT1 levels could not be detected. In addition to this, the experiment was performed with 5×10^5 cells/ 500 µL and the loss of cells was very high during sample preparation for flow cytometry as it involved multiple washing steps. Therefore, only a small fraction of the cells was analysed and these could be the reasons why the level of GLUT1 increase does not correlate in all the samples.

4.4 Gene Expression

GLUT1:

We found no significant increase in *GLUT1* expression with low glucose levels, however there was a significant increase in *GLUT1* expression after metformin treatment. The results obtained for *GLUT1* gene expression correlate to the results obtained for *GLUT1* protein expression.

OCT1:

The study showed that metformin treatment led to a decrease in *OCT1* gene expression. Metformin gets actively taken up by *OCT1* present on cell surface and very little can diffuse passively[29]. It was found that *OCT1* expression decreased with an increase in concentration. One explanation could be that at high concentrations of metformin, passive diffusion of metformin probably increases and leads to a decrease in *OCT1* expression. This could be a stress response of cells due to cation accumulation. Another explanation could be that

reduction on *OCT1* levels is response of SW1116 cells to stress induced by metformin. Study on ovarian cancer cells showed that low levels of *OCT1* limit the metformin activity [63]. Reduction in OCT1 levels could also be protective response of cells in response to metformin.

MCT4:

We found that metformin affects the *MCT4* gene expression and results for both biological sets was found to be different. One set of readings showed an increase in *MCT4* gene expression and another set showed a decrease in *MCT4* gene expression. Increase in MCT4 could be explained as increased dependency on glycolysis leads to lactic acid build up (Warburg Hypothesis). As a consequence, *MCT4* expression is increased to transport excess lactic acid out of the cell. A study on pancreatic cancer showed that high levels of *MCT4* expression is associated with higher glycolytic metabolism[21]. Therefore, an increase in *MCT4* expression could indicate a shift in cancer metabolism from OXPHOS to glycolysis.

Another set of readings, show a decrease in *MCT4* expression with metformin treatment. One possibility is that there was something wrong with control sample. Since gene expression in all the target samples is determined on comparison with control, a decrease is observed. This is just one possibility and repetition of the experiment would confirm the theory.

UCP2:

Our study showed that *UCP2* gene expression was affected by metformin treatment. We found that metformin treatment led to an increase in *UCP2* gene expression in high glucose and a decrease in low glucose.

An increase in UCP2 gene expression could be explained as increase in *UCP2* leads to reduction of membrane potential, and a reduction in ATP production from by mitochondria[26]. A study by Esteves P, *et al* suggests that an increase in UCP2 could be linked to transition from glycolytic to OXPHOS[64]. Another study by Donadelli M and colleagues has also suggested that a UCP2 over expression indicates that cells favour glycolysis[27]. Therefore, *UCP2* increase suggests a transition from OXPHOS to glycolysis. This seems to comply with the above results for other genes, and goes on to indicate that the increase in *UCP2* on metformin addition could be pushing the cells to a more glycolytic profile.

UCP2 gene expression decreased in low glucose and there was a further decrease in *UCP2* expression on metformin addition. This could be explained as low glucose control does not have metformin, the decrease in *UCP2* gene expression could be response of cells to maintain energy requirements by relying more on mitochondria. It is possible that the cells may also be using amino acids as alternative substrates to keep the ATP production going. Increase in GLUT1 along with decrease in UCP2 seems to suggest that the cells in low glucose are trying to maintain their energy demands by change in these expressions. A further decrease in UCP2 on metformin treatment coupled with GLUT1 increase could be measures taken by the cell to maintain energy production, by relying on mitochondria.

SUCLA2

We found that one set of SUCLA2 gene expression results showed an increase on metformin treatment and another showed decrease in gene expression. The decrease in *SUCLA2* expression could indicate a metabolic alteration in cellular metabolism and a decrease in dependency on TCA cycle. A study published in 2013 and another in 2016 by different research groups suggested that *SUCLA2* inhibition reduced dependency of cells on TCA[65 66]. Thus, it seems that the cells shift their metabolism from OXPHOS to glycolysis. One research study showed increased *SUCLA2* gene expression in metastatic muscle lesion[67]. Therefore the observed increase in *SUCLA2* could be due to stress where the cells may require energy and metabolic precursors from TCA; as higher amount of *SUCLA2* would expedite the TCA cycle.

PDK2

We found that metformin treatment caused an increase in *PDK2* gene expression in one set of results and a decrease in another set. *PDK2* inhibits PDH and therefore prevents entry of pyruvate into TCA cycle[22]. Therefore, an increase in *PDK2* could be due to altered cellular metabolism and a shift from OXPHOS to glycolysis. Research has shown that p53 related tumours increase *PDK2* which leads to increase in glycolysis[23]. This indicated that the cells were turning glycolytic after metformin treatment. There was another set of readings, that showed a decrease in *PDK2* expression with metformin treatment. It is possible that there was something wrong with control sample. Gene expression in all the target samples is determined on comparison with control therefore, a decrease is observed. This is just one possibility and repetition of the experiment would confirm the theory.

Gene expression results obtained for metformin treatment suggested that it alters the gene expression of proteins involved in metabolism and therefore, metabolism in cells. However, there was a significant difference in level of gene expression obtained in both sets and for many genes the results showed opposite trend. Repetition of experiments in future will be able to give more robust results and confirm theories.

For high glucose, the cells were proliferating much faster than they were in low glucose (as indicated by proliferation assay), as the proliferation rate was higher, they were more affected when mitochondria was targeted. For low glucose, decrease in UCP2 on metformin addition along with an increase in GLUT1 suggested cells were a bit stressed and they tried meet energy requirements. This could be the reason for low proliferation as suggested by proliferation assay and MTS Assay on metformin addition. The results also suggest that cells need to rely on other energy substrates.

4.5 Mitochondrial Biogenesis on metformin treatment

Research has shown that mitochondrial biogenesis is altered in cancer cells[68]. However, our study found no significant change in mitochondrial mass per volume on metformin treatment. The experiment was performed only once, and many cells were lost during flow cytometry. Therefore, these readings and not very robust and repetition of the experiment is to obtain more robust readings.

5. Conclusions and Future Perspectives

5.1 Conclusions

The mitochondria-dependent SW1116 cells were found to be sensitive to metformin. From the proliferation assay and interpretation of gene expression results it can be said that metformin affected cells grown in high glucose more than it affected cells grown in low glucose.

The increase in GLUT1 protein and gene expression after metformin treatment and no significant increase in GLUT1 in low glucose level alone, suggested that GLUT1 would be a useful biomarker to determine the response of cells to metformin treatment

UCP2 gene expression was found to be affected by metabolic stress where both metformin and glucose levels were able to affect its expression. Therefore, based on the study it would be difficult to state if UCP2 alone would be good biomarker to evaluate effect of metformin. However, UCP2 seems to be a good biomarker to determine the energy dependency on mitochondria and in combination with GLUT1, UCP2 expression was able to indicate the response of cells to metformin.

The decrease in OCT1 gene expression upon metformin treatment suggested that SW1116 cells reacted to the metformin induced stress by reducing the expression of metformin transporters (OCT1). This indicated that OCT1 could be a good biomarker to determine susceptibility and response of cells to metformin treatment.

Gene expression studies on MCT4, PDK2 and SUCLA2 did not yield robust results. Therefore, whether or not they could be used as biomarkers to determine response to metformin treatment could be determined after repetition of experiments.

Similarly, no significant change was observed on mitochondrial biogenesis in response to metformin treatment and since the experiment was performed only once, no conclusions could be drawn from it.

5.2 Future Perspectives

The suggestion, previously mentioned in 'Discussion', that cancer cells have altered their metabolism is only based on gene expression results and we had not performed any experiments to study the metabolic switch in cancer cells. Further research could be conducted to study the metabolic alteration in cancer cells after metformin treatment with the Seahorse Mito Stress Test.

In low glucose, UCP2 decrease together with GLUT1 increase indicated that cells were starved and they were probably dependent on amino acids and perhaps fatty acids as well for energy production. Further research on amino acid and fatty acid involvement in energy metabolism might confirm the theory.

Metformin may also affect fatty acid synthesis as it is related to TCA cycle. Citrate formed in TCA cycle can get exported out of mitochondria into cytosol and can be used for fatty acid synthesis[7]. Therefore, the glucose taken up by cells may get channelled into fatty acid synthesis. As metformin targets mitochondria and causes shift in metabolic profile of cells, it may also have an effect on fatty acid synthesis which is crucial for cancer cells for formation of lipid bilayer and signalling molecules[7]. Future research could be done to study the effect of metformin on fatty acid synthesis.

6. References

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Appendix

1. Cell Proliferation Assay

Cell proliferation in High Glucose Growth Medium:

Table A1.1: Number of cells per well, the mean and proliferation rate in percentage.

Time in hours	Number of cells per well in High Glucose			Mean	Proliferation in Percentage
	Set 1	Set 2	Set 3		
0	100000	100000	100000	100000	100
6	60000	36250	93300	63183	63
12	70000	42500	102500	71667	72
24	115000	105000	147000	122333	122
48	175000	217500	210000	200833	201
72	280000	273750	305000	286250	286

Cell proliferation in Low Glucose Growth Medium:

Table A1.2: Number of cells per well, the mean and proliferation rate in percentage.

Time in hours	Number of cells per well in Low Glucose			Mean	Proliferation in Percentage
	Set 1	Set2	Set3		
0	100000	100000	100000	100000	100
6	30000	27500	92000	49833	50
12	70000	33750	93300	65683	66
24	100000	67500	125000	97500	98
48	173300	150000	164000	162433	162
72	200000	232500	215000	215833	216

2. Cell Viability by MTS Assay

High Glucose: 24 hour Treatment

Table A2.1: Average absorbance values for 24 hr treatment samples in high glucose medium of each experiment set.

Average absorbance	**Control**	**0.1 mM**	**0.5 mM**	**1.0 mM**	**3.0 mM**	**5.0 mM**
Set 1	0.4267	0.296	0.266	0.227	0.229	0.219
Set 2	0.455	0.330	0.262	0.27	0.256	0.255
Set 3	0.497	0.437	0.4	0.394	0.406	0.384

Table A2.2: Table includes % viability of cells for 24 hr treatment samples in high glucose growth medium of each experiment set. It shows the mean values for % proliferation of cells and the standard deviation.

Percent Viability	**Control**	**0.1mM**	**0.5mM**	**1mM**	**3mM**	**5mM**
Set 1	100	88	80	79	82	77
Set 2	100	72	57	59	56	56
Set 3	100	69	62	53	51	51
Mean	100	77	67	64	63	62
Standard deviation	0	10	12	14	16	14

High Glucose: 48 hour treatment

Table A2.3: Average absorbance values for 48 hr treatment samples in high glucose of each experiment set.

Average absorbance	Control	0.1 mM	0.5 mM	1.0 mM	3.0 mM	5.0 mM
Set 1	0.604	0.419	0.405	0.405	0.352	0.366
Set 2	0.532	0.324	0.361	0.346	0.228	0.208
Set 3	0.4235	0.366	0.309	0.291	0.361	0.354

Table A2.4: Table includes % viability of cells for 48 hr treatment samples in high glucose growth medium of each experiment set.

Percent Viability	Control	0.1mM	0.5mM	1mM	3mM	5mM
Set 1	100	69	67	67	58	61
Set 2	100	61	68	65	43	39
Set 3	100	86	73	67	82	80
Mean	100	72	69	66	61	60
Standard deviation	0	13	3	1	20	20

Low Glucose: 24 hour Treatment

Table A2.5: Average absorbance values for 24 hr treatment samples in low glucose growth medium of each experiment set.

Average absorbance	Control	0.1 mM	0.5 mM	1.0 mM	3.0 mM	5.0 mM
Set 1	0.769	0.784	0.701	0.669	0.667	0.653
Set 2	0.394	0.370	0.366	0.372	0.366	0.349
Set 3	0.514	0.411	0.351	0.324	0.326	0.323

Table A2.6: Table includes % viability of cells for 24 hr treatment samples in low glucose growth medium of each experiment set. It shows the mean values for % proliferation of cells and the standard deviation.

Percent Viability	Control	0.1mM	0.5mM	1mM	3mM	5mM
Set 1	100	102	91	87	87	85
Set 2	100	94	93	95	93	89
Set 3	100	80	68	63	63	63
Mean	100	92	84	82	81	79
Standard deviation	0	11	14	16	16	14

Low Glucose: 48 hour Treatment:

Table A2.7: Average absorbance values for 48 hr treatment sample in low glucose growth of each experiment set.

Average absorbance	Control	0.1 mM	0.5 mM	1.0 mM	3.0 mM	5.0 mM
Set 1	0.66	0.544	0.475	0.4687	0.462	0.422
Set 2	0.802	0.711	0.744	0.538	0.530	0.535
Set 3	0.896	0.659	0.761	0.595	0.494	0.544

Table A2.8: Table includes % viability of cells for 48 hr treatment samples in low glucose growth medium of each experiment set. It shows the mean values for % proliferation of cells and the standard deviation.

Percent Viability	Control	0.1mM	0.5mM	1mM	3mM	5mM
Set 1	100	82.47475	71.9697	71.0101	69.92424	63.93939
Set 2	100	88.62786	92.80665	67.04782	66.11227	66.73597
Set 3	100	73.52203	84.90177	66.3818	55.16941	60.69193
Mean	100	81.54154	83.22604	68.14657	63.73531	63.7891
Standard deviation	0	7.59603	10.51906	2.502147	7.659223	3.024819

3. GLUT1 Protein Expression (Western Blot):

BCA:

Preparation of Working Reagent: Working Reagent A was mixed with Working Reagent B in A ratio of 1:50 ratio, to prepare the Working Reagent (WR).

Preparation of Diluted Albumin (BSA) Standards: One of the provided ten ampules containing BSA was used to prepare the following dilutions.

Table A3.1: Dilutions for protein standard samples.

Vial	Volume of Diluent	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

Procedure Summary (Microplate Procedure, Standard Protocol)

1. 20µL of sample and 160 µL of WR was pipetted into a microplate.
2. The plate was incubated in dark at 37°C for 30 minutes.
3. Plate was allowed to cool down and put on a shaker for 30 seconds.
4. Absorbance was read at 562nm on a plate reader. Blank value was assigned and the protein concentration was calculated by the software.

Protein Lysates Set 1:

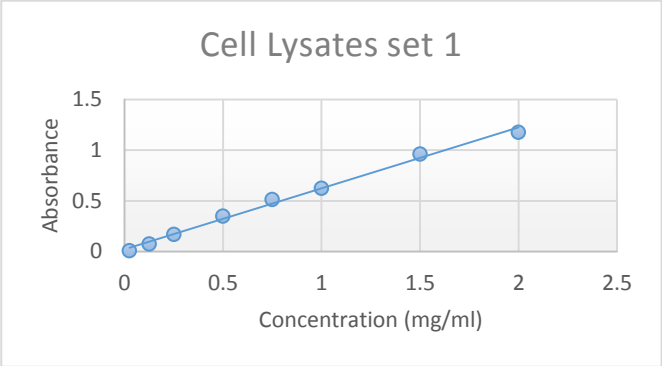

Figure A3.1: Standard Graph of BCA for protein lysates set1.

Table A3.2: Readings of Standard BSA Assay Protein Lysates Set 1:

Sample	Concentration (mg/ml)	Wells	Value	Mean Value	SD
1	2	A1	1.058	1.178	0.107
		B1	1.263		
		C1	1.214		
2	1.5	A2	1.014	0.962	0.045
		B2	0.936		
		C2	0.937		
3	1	A3	0.626	0.625	0.039
		B3	0.585		
		C3	0.663		
4	0.75	A4	0.491	0.514	0.032
		B4	0.55		
		C4	0.499		
5	0.5	A5	0.306	0.348	0.07
		B5	0.429		
		C5	0.31		
6	0.25	A6	0.159	0.169	0.009
		B6	0.177		
		C6	0.169		
7	0.125	A7	0.077	0.074	0.003
		B7	0.07		
		C7	0.075		
8	0.025	A8	0.005	0.009	0.006
		B8	0.016		
		C8	0.006		

Protein Lysates Set 2:

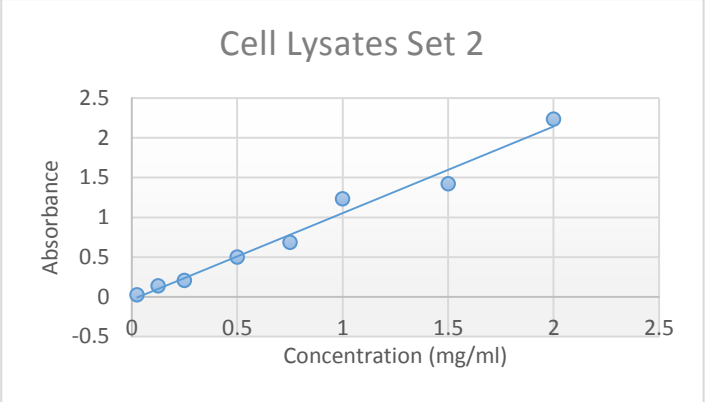

Figure A3.2: Standard Graph of BCA for protein lysates set2.

Table 5.3.3: Readings of Standard BSA Assay Protein Lysates Set 1:

Sample	Concentration (mg/ml)	Wells	Value	Mean Value	SD
1	2	A1	1.582	2.232	0.612
		B1	2.318		
		C1	2.796		
2	1.5	A2	0.776	1.419	0.937
		B2	0.988		
		C2	2.494		
3	1	A3	1.989	1.229	0.66
		B3	0.798		
		C3	0.901		
4	0.75	A4	0.28	0.684	0.383
		B4	0.731		
		C4	1.041		
5	0.5	A5	0.436	0.497	0.143
		B5	0.66		
		C5	0.396		
6	0.25	A6	0.335	0.202	0.117
		B6	0.157		
		C6	0.115		
7	0.125	A7	0.124	0.137	0.019
		B7	0.127		
		C7	0.159		
8	0.025	A8	0.072	0.023	0.045
		B8	-0.016		
		C8	0.013		

Table A3.4: Protein Concentrations for respective samples were calculated from standard graph

Sample Abbreviation	Set 1		Set 2	
	Absorbance	**Protein Concentration (mg/ml)**	**Absorbance**	**Protein Concentration (mg/ml)**
HG24_0.5mM	0.508	0.808	0.508	0.808
HG24_3.0mM	0.488	0.774	0.488	0.774
HG_C	0.474	0.751	0.474	0.751
HG48_0.5mM	0.532	0.847	0.532	0.847
HG48_3.0mM	0.321	0.496	0.321	0.496
HG_C	0.301	0.463	0.301	0.463
LG24_0.5mM	0.432	0.681	0.432	0.681
LG24_3.0mM	0.356	0.555	0.356	0.555
LG_C	0.425	0.67	0.425	0.67
LG48_0.5mM	0.306	0.471	0.306	0.471
LG48_3.0mM	0.373	0.582	0.373	0.582
LG_C	0.368	0.574	0.368	0.574

Gel Preparation:

Stain free gels were cast using solutions from the kit. Gels prepared were of 1 mm thickness and components were prepared and added according to the manual provided along with the kit. Table A3.5: Table of contents and composition for one gel.

Component	Amount Added	
	Resolving	Stacking
Acrylamide solution A	3 ml (Resolver Solution)	1 ml (Stacker Solution)
Acrylamide solution B	3 ml (Resolver Solution)	1 ml (Stacker Solution)
10% APS	30 µl	10 µl
TEMED	3 µl	2 µl

Normalization and analysis of membrane:

1. Normalization of proteins and intensity calculation of GLUT1 was done using ImageLab Software.
2. Stainfree image and chemiluminescent images were linked and lanes were defined manually in stainfree image.
3. To get similar background profile in all lanes, rolling disc size was adjusted. The lanes were copied from stainfree image and pasted on chemiluminescent image.
4. For normalization, from Analysis tool box Normalization option was chosen. Under Normalization Channel, Stainfree Blot was chosen and the method to normalize was chosen as total protein normalization.
5. The marker need to be excluded from the results. From Analysis tool box, Molecular Weight Analysis Tools was chosen and marker lane was selected to exclude that data.
6. By clicking analysis table, information table was obtained which gave Normalization factor and Intensity.

This was exported to excel and data was analysed using High Glucose Control as a reference.

Table: A3.6 : The following table includes intensity readings for GLUT1 after normalization of the blot.

Sample	Intensity			
	Set 1.1	Set 1.2	Set 2.1	Set 2.2
HG _C	2231594	13865153	86503319	144363256
HG24_0.5mM	2466583	15754723	119092257	166101274
HG24_3.0mM	2109429	13025828	101854002	140805566
HG48_0.5mM	2929455	18308414	149639551	198008108
HG48_3.0mM	2177076	13748617	97626524	140396679
LG_C	2584850	17363847	130075638	193553178
LG24_0.5mM	2838256	19310845	151742756	212052125
LG24_3.0mM	2226479	15173389	113164792	166353484
LG48_0.5mM	3239646	23664503	168685246	226955570
LG48_3.0mM	2222795	15425543	115125567	165869313

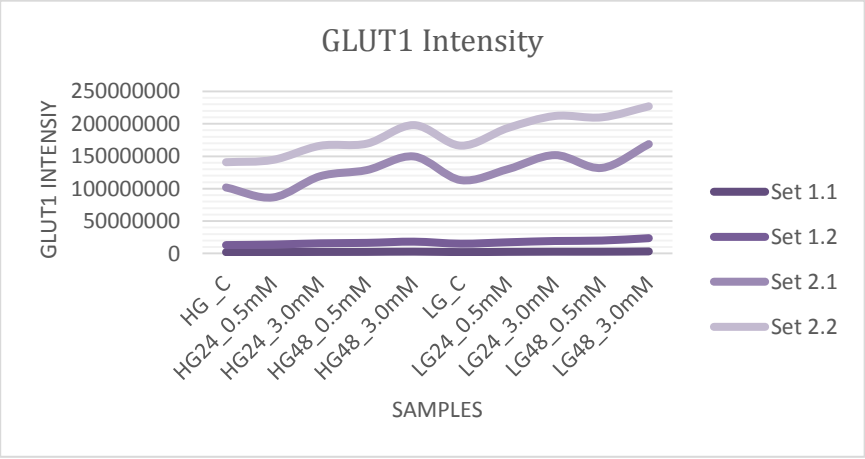

Figure 5.3.4: Figure 3.3.2 is a graph of all measured relative intensities for GLUT1 for Western Blot after normalization.

GLUT1 intensity readings were then used to calculate percent relative intensity where, High Glucose Control was used as reference.

Table A3.7: % relative GLUT1 intensities for all samples.

Sample	Percent Increase in GLUT1 Intensity					Standard Deviation
	Set 1	Set 2	Set 3	Set 4	**Mean**	
HG_C	100	100	100	100	**100**	0
HG24_0.5mM	105.7914	106.4435	84.92874	102.5267	**99.92258**	10.1417
HG24_3.0mM	116.9313	120.9499	116.9245	117.965	**118.1927**	1.902049
HG48_0.5mM	120.4397	126.1699	126.3739	120.3436	**123.3318**	3.39625
HG48_3.0mM	138.8743	140.5547	146.9157	140.6252	**141.7425**	3.542506
LG_C	105.5489	116.4869	111.1049	118.1441	**112.8212**	5.703892
LG24_0.5mM	122.5379	133.3032	127.7079	137.4613	**130.2526**	6.513158
LG24_3.0mM	134.5509	148.2504	148.9807	150.5992	**145.5953**	7.42806
LG48_0.5mM	133.4956	153.7477	129.6155	149.1373	**141.499**	11.74231
LG48_3.0mM	153.5793	181.6737	165.6147	161.1837	**165.5128**	11.86501

4. GLUT1 Protein Expression (Immunostaining)

Table A4.1 Values of GLUT1 Intensities per cell. The mean value of GLUT1 Intensities per cells and Percent of GLUT1 Intensity for Set 1

Samples	S 1	S 2	S 3	Mean Set 1	Percent of GLUT1 Intensity Set 1
HG24_C	51499.99	44060.55	57177.05	50912.53	100
HG24_0.5mM	30277.84	46922.19	19173.79	32124.6	63.09764
HG24_3.0mM	47105.91	124514.5	51706.4	74442.29	146.216
HG48_0.5mM	21460.93	62681.48	73889.15	52677.19	103.4661
HG48_3.0mM	107053.3	51298.32	37370.22	65240.6	128.1425
LG24_C	85577.57	57011.81	36052.42	59547.26	116.9599
LG24_0.5mM	160288.7	13022.57	17280.93	63530.72	124.7841
LG24_3.0mM	66185.24	63390.49	118942.4	82839.37	162.7092
LG48_0.5mM	121730.9	50414.63	44245.58	72130.39	141.6751
LG48_3.0mM	48474.74	41794.01	68129.1	52799.28	103.7059

Table A4.2 Values of GLUT1 Intensities per cell. The mean value of GLUT1 Intensities per cells and Percent of GLUT1 Intensity for Set 2

Samples	S 1	S 2	S 3	Mean Set 2	Percent of GLUT1 Intensity Set 2
HG24_C	50507.01	45721.12	47402.03	47876.72	100.0011
HG24_0.5mM	145269.7	29084.22	22939.35	65764.41	137.3635
HG24_3.0mM	52508.63	45116.3	161328.7	86317.86	180.2939
HG48_0.5mM	16019.16	202937.4	7944.402	75633.65	157.9776
HG48_3.0mM	46844.34	69582.18	38816.29	51747.6	108.0863

LG24_C	67821.18	35451.17	18909.86	40727.4	85.06816
LG24_0.5mM	196947.7	40306.63	21421.06	86225.12	180.1002
LG24_3.0mM	76460.43	36305.76	136042.8	82936.32	173.2308
LG48_0.5mM	113617.3	58001.72	22516.38	64711.8	135.1649
LG48_3.0mM	35623.74	51510.82	129878.9	72337.82	151.0935

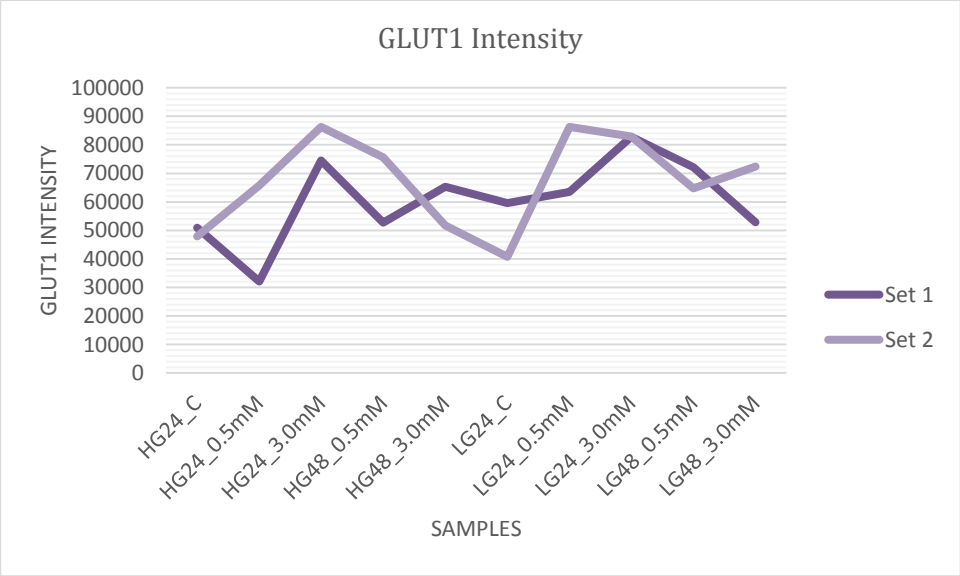

Figure A4.1: GLUT1 Intensitie/cell of Set1 and Set2

Table A4.3: % GLUT1 Intensities per cell of Set1 and Set2, Mean of % GLUT 1 intensities obtained for both the sets and Standard Deviation

Samples	**Set 1**	**Set 2**	Mean % GLUT1	**STDV**
HG24_C	100	100.0011	100.0005	0.00077
HG24_0.5mM	63.09764	137.3635	100.2306	52.51387
HG24_3.0mM	146.216	180.2939	163.255	24.09668
HG48_0.5mM	103.4661	157.9776	130.7218	38.54545
HG48_3.0mM	128.1425	108.0863	118.1144	14.18191
LG24_C	116.9599	85.06816	101.014	22.55089
LG24_0.5mM	124.7841	180.1002	152.4421	39.1144
LG24_3.0mM	162.7092	173.2308	167.97	7.439891
LG48_0.5mM	141.6751	135.1649	138.42	4.603442
LG48_3.0mM	103.7059	151.0935	127.3997	33.5081

5. GLUT1 Protein Expression (Flow Cytometer)

Table A5.1: GLUT1 Intensity Median FL4-A Values, GLUT1 Intensity Median FL-4 values after subtraction of negative control sample, and GLUT1 Intensity values in percentage.

Samples	**Median FL4-A**	**Median FL4-A (minus negative abs)**	**GLUT1 intensity in percentage**
HG_C	33,039.00	5,560.00	100
HG24_0.5mM	34,729.00	7,250.00	130.395683
HG24_3.0mM	27,999.00	520.00	9.35251799
HG48_0.5mM	33,503.50	6,024.50	108.354317
HG48_3.0mM	40,218.00	12,739.00	229.118705
LG_C	31,884.50	4,405.50	79.2356115
LG24_0.5mM	37,155.50	9,676.50	174.03777
LG24_3.0mM	37,417.00	9,938.00	178.741007
LG48_0.5mM	32,310.00	4,831.00	86.8884892
LG48_3.0mM	32,733.00	5,254.00	94.4964029
HGC_neg_control	27,479.00		

6. Gene Expression

The quantity of RNA in each sample was determined.

Table A6.1: Quantity of RNA in each sample and A260/A280 ratio for each sample.

Sample	Set 1		Set 2	
	ng/µl	A260/A280	ng/µl	A260/A280
HG_C	197.5	2.02	225.2	2.02
HG24_0.5mM	352.8	2.04	166.7	2.01
HG24_3.0mM	324	2.05	185.8	2.02
HG48_0.5mM	136.6	1.99	125.8	2.02
HG48_3.0mM	154.4	2.01	202.6	2.04
LG_C	377.7	2.04	226.6	2.02
LG24_0.5mM	371.8	2.04	152.3	2.03
LG24_3.0mM	267.9	2.03	171.4	2.01
LG48_0.5mM	270.9	2.03	154.4	2.01
LG48_3.0mM	188.7	2.03	101.9	2.00

Primer Validation Results:

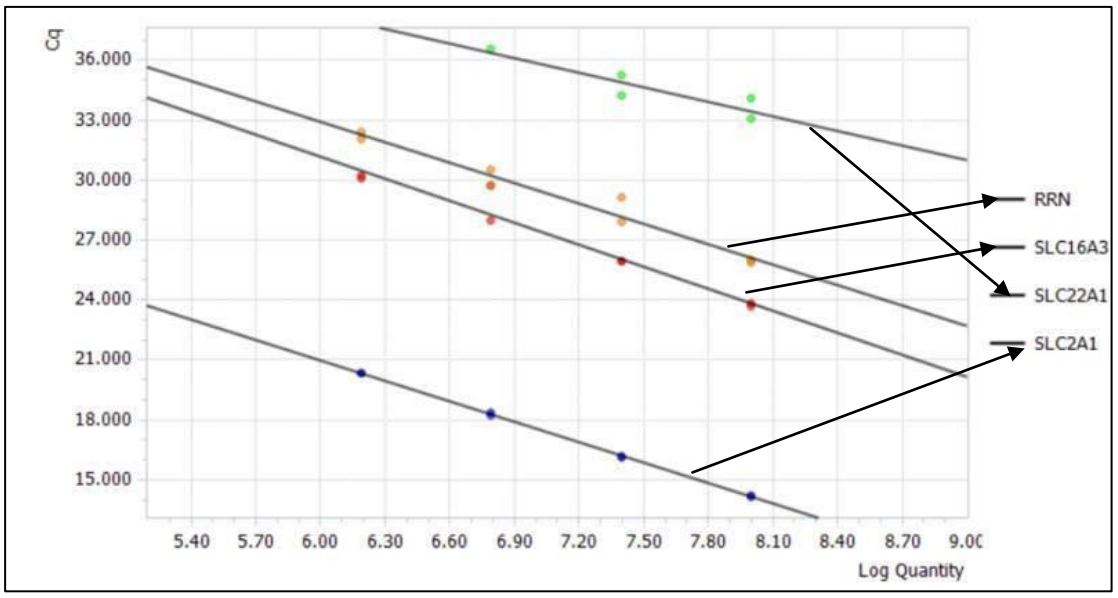

Figure 6.1: Validation results for *RRN, SLC16A3, SLC22A1 and SLC2A1*

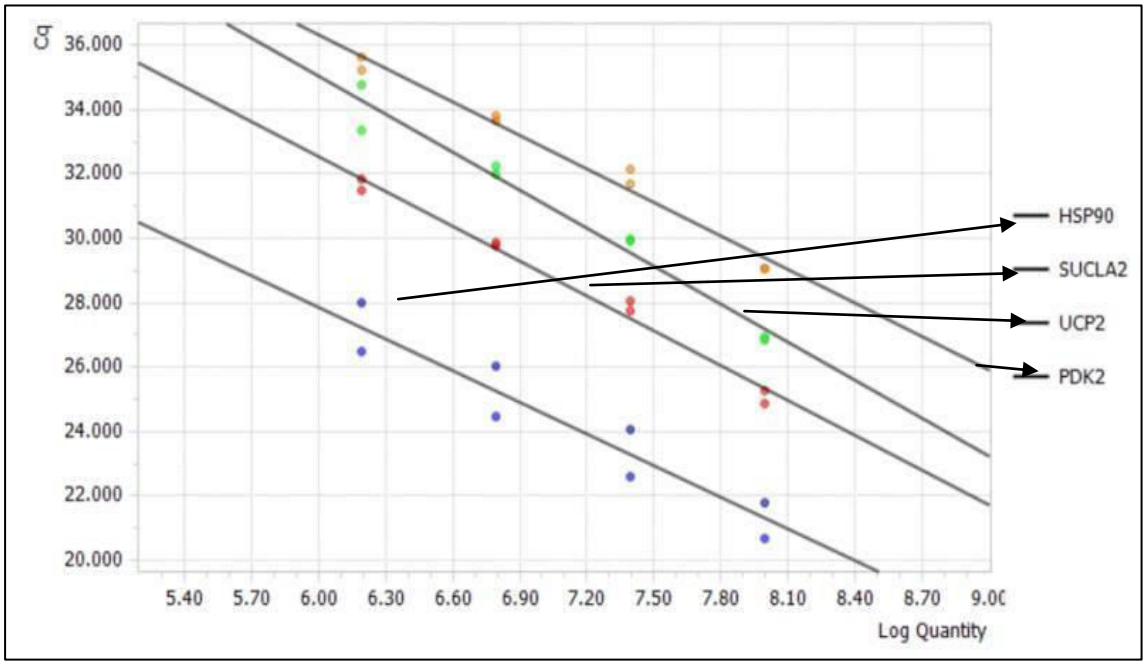

Figure 6.2: Validation results for *HSP60, SUCLA2, UCP2 and PDK2*

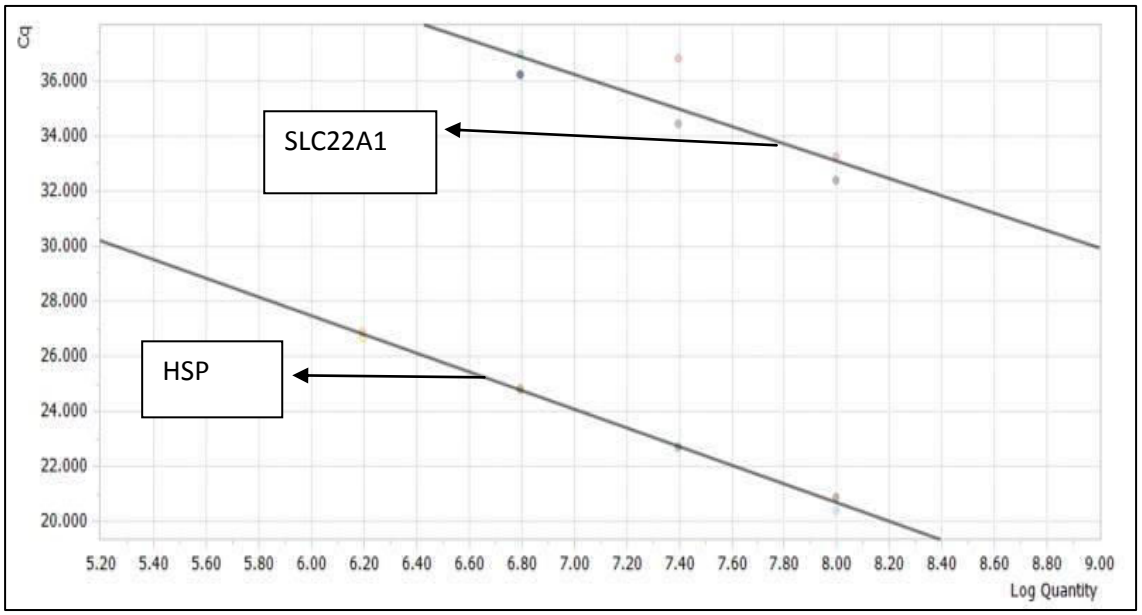

Figure 6.3: Validation results for *HSP and SLC22A1* performed again.

Table A6.2: Abbreviations for samples:

Sample	Code	Sample	Code
High Glucose Control	Sample 1 (S1)	Low Glucose Control	Sample 6 (S6)
High Glucose 0.5 mM metformin 24 hr	Sample 2 (S2)	Low Glucose 0.5 mM metformin 24 hr	Sample 7 (S7)
High Glucose 3.0 mM metformin 24 hr	Sample 3 (S3)	Low Glucose 3.0 mM metformin 24 hr	Sample 8 (S8)
High Glucose 0.5 mM metformin 48 hr	Sample 4 (S4)	Low Glucose 0.5 mM metformin 48 hr	Sample 9 (S9)
High Glucose 3.0 mM metformin 48 hr	Sample 5 (S5)	Low Glucose 3.0 mM metformin 48 hr	Sample 10 (S10)

Loading Scheme for Gene expression qPCR Reactions:

Plate 1:

HSP90 S1	HSP90 S2	HSP90 S3	HSP90 S4	HSP90 S5	HSP90 S6	HSP90 S7	HSP90 S8	HSP90 S9	HSP90 S10	HSP90 -ve control	BLANK
HSP90 S1	HSP90 S2	HSP90 S3	HSP90 S4	HSP90 S5	HSP90 S6	HSP90 S7	HSP90 S8	HSP90 S9	HSP90 S10	HSP90 -ve control	BLANK
SUCLA2 S1	SUCLA2 S2	SUCLA2 S3	SUCLA2 S4	SUCLA2 S5	SUCLA2 S6	SUCLA2 S7	SUCLA2 S8	SUCLA2 S9	SUCLA2 S10	SUCLA20 -ve control	BLANK
SUCLA2 S1	SUCLA2 S2	SUCLA2 S3	SUCLA2 S4	SUCLA2 S5	SUCLA2 S6	SUCLA2 S7	SUCLA2 S8	SUCLA2 S9	SUCLA2 S10	SUCLA20 -ve control	BLANK
UCP2 S1	UCP2 S2	UCP2 S3	UCP2 S4	UCP2 S5	UCP2 S6	UCP2 S7	UCP2 S8	UCP2 S9	UCP2 S10	UCP2 -ve control	BLANK
UCP2 S1	UCP2 S2	UCP2 S3	UCP2 S4	UCP2 S5	UCP2 S6	UCP2 S7	UCP2 S8	UCP2 S9	UCP2 S10	UCP2 -ve control	BLANK
PDK2 S1	PDK2 S2	PDK2 S3	PDK2 S4	PDK2 S5	PDK2 S6	PDK2 S7	PDK2 S8	PDK2 S9	PDK2 S10	PDK2 -ve control	BLANK
PDK2 S1	PDK2 S2	PDK2 S3	PDK2 S4	PDK2 S5	PDK2 S6	PDK2 S7	PDK2 S8	PDK2 S9	PDK2 S10	PDK2 -ve control	BLANK

Plate2:

RRN18S S1	RRN18S S2	RRN18S S3	RRN18S S4	RRN18S S5	RRN18S S6	RRN18S S7	RRN18S S8	RRN18S S9	RRN18S S10	RRN18S -ve control	BLANK
RRN18S S1	RRN18S S2	RRN18S S3	RRN18S S4	RRN18S S5	RRN18S S6	RRN18S S7	RRN18S S8	RRN18S S9	RRN18S S10	RRN18S -ve control	BLANK
SLC2A1 S1	SLC2A1 S2	SLC2A1 S3	SLC2A1 S4	SLC2A1 S5	SLC2A1 S6	SLC2A1 S7	SLC2A1 S8	SLC2A1 S9	SLC2A1 S10	SLC2A1 -ve control	BLANK
SLC2A1 S1	SLC2A1 S2	SLC2A1 S3	SLC2A1 S4	SLC2A1 S5	SLC2A1 S6	SLC2A1 S7	SLC2A1 S8	SLC2A1 S9	SLC2A1 S10	SLC2A1 -ve control	BLANK
SLC16A3 S1	SLC16A3 S2	SLC16A3 S3	SLC16A3 S4	SLC16A3 S5	SLC16A3 S6	SLC16A3 S7	SLC16A3 S8	SLC16A3 S9	SLC16A3 S10	SLC16A3 -ve control	BLANK
SLC16A3 S1	SLC16A3 S2	SLC16A3 S3	SLC16A3 S4	SLC16A3 S5	SLC16A3 S6	SLC16A3 S7	SLC16A3 S8	SLC16A3 S9	SLC16A3 S10	SLC16A3 -ve control	BLANK
SLC22A1 S1	SLC22A1 S2	SLC22A1 S3	SLC22A1 S4	SLC22A1 S5	SLC22A1 S6	SLC22A1 S7	SLC22A1 S8	SLC22A1 S9	SLC22A1 S10	SLC22A1 -ve control	BLANK
SLC22A1 S1	SLC22A1 S2	SLC22A1 S3	SLC22A1 S4	SLC22A1 S5	SLC22A1 S6	SLC22A1 S7	SLC22A1 S8	SLC22A1 S9	SLC22A1 S10	SLC22A1 -ve control	BLANK

Table A6.3: Effect of Metformin on UCP2 gene expression in terms of fold increase and decrease is included in the table.

Sample	**2^{-ddCt}**	
HG_C	1	1
HG24_0.5mM	0.108819	0.600818
HG24_3.0mM	2.143547	3.187665
HG48_0.5mM	1.972465	0.421177
HG48_3.0mM	13.40779	1.026334
LG_C	0.799683	0.905948
LG24_0.5mM	0.363493	0.68302
LG24_3.0mM	0.096388	0.449066
LG48_0.5mM	1.862835	0.920188
LG48_3.0mM	0.050241	0.224533

Table A6.4: Effect of Metformin on SLC2A1 gene expression in terms of fold increase and decrease is included in the table.

Sample	**2^{-ddCT}**	
HG_C	1	1
HG24_0.5mM	0.183011	1.164734
HG24_3.0mM	0.923382	1.001734
HG48_0.5mM	0.514057	1.317223
HG48_3.0mM	1.453973	1.539541
LG_C	0.998269	1.062527
LG24_0.5mM	0.562529	1.500039
LG24_3.0mM	0.809442	1.687632
LG48_0.5mM	0.606046	1.866066
LG24_3.0mM	0.103306	1.717131

Table A6.5: Effect of Metformin on SLC22A1 gene expression in terms of fold increase and decrease is included in the table.

Sample	**2^{-ddCT}**	
HG_C	1	1
HG24_0.5mM	22.62742	0.484645
HG24_3.0mM	0.861546	0.386221
HG48_0.5mM	5.259771	0.912249
HG48_3.0mM	1.113422	0.633976
LG_C	3.036691	1.632972
LG24_0.5mM	20.82147	0.90125
LG24_3.0mM	2.818642	0.237336
LG48_0.5mM	0.918594	5.169411
LG24_3.0mM	0.798298	0.61132

Table A6.6: Effect of Metformin on SLC16A3 gene expression in terms of fold increase and decrease is included in the table.

Sample	**2^{-ddCT}**	
HG_C	1	1
HG24_0.5mM	0.006151	1.972465
HG24_3.0mM	0.939523	1.68471
HG48_0.5mM	0.136787	1.673073
HG48_3.0mM	0.259715	2.052668
LG_C	0.090402	1.982746
LG24_0.5mM	0.144586	3.530812
LG24_3.0mM	0.091189	4.675109
LG48_0.5mM	0.203415	5.205367
LG24_3.0mM	0.020978	1.164734

Table A6.7: Effect of Metformin on SUCLA2 gene expression in terms of fold increase and decrease is included in the table.

Samples	**2^{-ddCt}**	
HG_C	1	1
HG24_0.5mM	35.38338	0.702222
HG24_3.0mM	0.699793	0.715736
HG48_0.5mM	25.99208	0.857079
HG48_3.0mM	29.34413	0.825019
LG_C	50.30047	1.08862
LG24_0.5mM	32.22258	0.854114
LG24_3.0mM	20.96629	0.557676
LG48_0.5mM	4.586838	2.483716
LG24_3.0mM	17.08905	1.416666

Table A6.8: Effect of Metformin on PDK2 gene expression in terms of fold increase and decrease is included in the table.

Samples	**2^{-ddCt}**	
HG_C	1	1
HG24_0.5mM	0.126306	1.22264
HG24_3.0mM	0.423373	1.062527
HG48_0.5mM	0.129408	1.561032
HG48_3.0mM	0.214641	1.344901
LG_C	0.266554	1.702317
LG24_0.5mM	0.093105	1.125058
LG24_3.0mM	0.190122	0.675955
LG48_0.5mM	0.00565	1.911891
LG24_3.0mM	0.003065	0.316439

7. Mitochondrial Biogenesis (by Mitochondrial staining)

Table 5.7.1: Median Values, Median values after subtraction of negative absorbance and Percent intensity of TOMM20.

Samples	**Median FL1-A**	**Median FL1-A (minus negative abs)**	**Relative TOMM20 intensity in percentage**
HG 24_C	317,566.00	311,412.00	100
HG24_0.5mM	329,374.50	323,220.50	103.791922
HG24_3.0mM	298,259.00	292,105.00	93.8001747
HG48_0.5mM	318,479.00	312,325.00	100.293181
HG48_3.0mM	1,020,067.50	1,013,913.50	325.58588
LG24_C	339,762.50	333,608.50	107.127696
LG24_0.5mM	302,034.00	295,880.00	95.0123952
LG24_3.0mM	333,985.00	327,831.00	105.272437
LG48_0.5mM	266,088.00	259,934.00	83.4694874
LG48_3.0mM	293,647.00	287,493.00	92.3191785
HGC_neg_control	6,154.00	0.00	

List of Reagents and Material

Product name	Product number	Company	Notes
Proliferation Assay			
Dulbecco's modified eagle medium high glucose	D5671-500ml	Sigma	Added pen/strep and glutamine
Dulbecco's modified eagle medium low glucose 1g/L D-Glucose	21885-025	Gibco	Added pen/strep only
Trypsin-EDTA T4049	SLBR8652V	-	
Phosphate Buffered Saline Tablet	P4417-50TAB	Sigma	One tablet added to 200 mL distilled water
Trypan Blue	K940—100mL	AMRESCO	
Foetal Bovine Serum	50115/0045B	-	
T-75 flasks		Falcon	
Cell Viability			
MST Reagent	ab197010	abcam	
Dulbecco's phosphate buffered saline	L0615-500	Sigma	
Metformin hydrochloride	PHR1084-500mg	Sigma-Aldrich	
Ascent software	v 2.6	Thermo labsystems	
Muktiskan ascent	-	Thermo scientific	
SDS PAGE-Western Blot			
Ammonium per sulfate	A4675.0100	Sigma	
Tris ultrapure	T1000-1	Saveen werner ab	
Hcl			
Methanol	1.06009.2500	Emsure	
NaCl	31434n-1kg-r	Sigma-aldrich	
Tween 20	P1362	Melford	

Microbiology Skim Milk powder	1.15363.0500	Merk	
TritonX	A4975.0100	Applichem	
Glycine	G0809.1000	Duchefa biochemie	
SDS		Sigma	
TEMED	CASNr 110-18-9	BIO-RAD	
Antibody Solution Rb mAb to GLUT1	ab 115730	Abcam	
Antibody Solution Goat pAb to Rb IgG (HRP)	ab 97051	Abcam	
ECL Western Blotting Substrate	32106	ThermoScientific Pierce	
Prestained Protein Ladder	26619	ThermoScientific	
BCA Protein Assay Kit	23227	ThermoScientific Pierce	
TGX Stain-Free Fastcast Acrylamide Kit	161-0185	ThermoScientific	
Immunostaining			
Tween			
PFA	P6148-500G	Sigma-Aldrich	
Antibody Solution Rb mAb to GLUT1	Ab 195020	Abcam	
Antibody Solution Donkey pAb to Rb IgG (Alexafluor647)	Ab150075	Abcam	
q PCR			
RNeasy Mini Kit ()50	Cat. No. 74104	Qiagen	
QIAshredder (50)	Ref 79654	Qiagen	
Nanodrop	-	Thermofisher	
Reverse Transcription Kit	Cat. No. 205311	Qiagen	
QuantiTect Primer Assay	Ref: 205311	Qiagen	

Lightcycler 96	5815916001	Roche	
Quantitect Primer Assay Hs_HSP?AB1_2_SG	Qt0167?790	Qiagen	
Quantitect Primer Assay Hs_RRN18S_1_SG	QT00199367	Qiagen	
Quantitect Primer Assay Hs_UCP_1_SG	QT00014140	Qiagen	
Quantitect Primer Assay Hs_SUCLA2_1_SG	QT00102788	Qiagen	
Quantitect Primer Assay Hs_SLC16A3_1_SG	QT00085855	Qiagen	
Quantitect Primer Assay Hs_SLC22A1_1_SG	QT00019572	Qiagen	
Pcr-pl 96w low pr weiss Roche	781365	Life science	Free of DNase and RNase
TE Buffer	Gift from Lutz Lab		
Quantitect Primer Assay Hs_SLC2A1_1_SG	QT00068957	Qiagen	
Quantitect Primer Assay pdk2	QT00038262	Qiagen	
Lightcycler software	V 1.1.0.1320	Roche diagnostics international ltd	
Flow cytometry			
C6 flow cytometer	-	Accuri c6	
Antibodies for TOMM20	Abcam		
Antibodies for GLUT1	(same as immunostaining)		