TITLE

In vitro quantification: long-term effect of glucose deprivation on various cancer cell lines

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KEYWORDS

Cancer, long-term glucose deprivation, *in vitro*, metabolic treatment.

ABSTRACT

Objective

Although metabolic treatment of highly glycolytic cancers and metastases is becoming an important research field, the effects of such treatments are not fully quantified yet. In this article we attempt to quantify the effect of long-term glucose deprivation (similar to Ketogenic Diets etc.) on cancer cells using *in vitro* tests.

Research methods and procedures

Two tumorigenic cell lines were used, namely a metastatic breast and a cervical cancer cell line. The non-tumorigenic control cell line was an immortalized breast cell line. All the cell lines were stabilized at a typical average human blood glucose (BG) level of 6mmol/L. The cell lines were then exposed to the therapeutic BG level of 3mmol/L for 90 days.

Results

The tests showed that glucose deprivation restricted the different cancer cell lines' growth more than that of non-tumorigenic cells. The different cell lines were also differentially affected, which suggests that long-term glucose deprivation will not be equally effective for different types of cancer.

The highly glycolytic breast cancer cell line was most adversely affected, with cell growth decreasing to 30% after 26 days. Cell growth was stable at this level for up to 22 days. Furthermore, all of the other cancer cell lines were similarly affected. This *in vitro* data could help to direct future human *in vivo* tests to find the most therapeutic time (cancer cells at their most vulnerable) for additional short-term adjuvant therapies.

Partial recovery of proliferation occurred after 90 days. Therefore, as expected, the results also show that without an adjuvant treatment, full extinction cannot be reached with the proposed long-term metabolic treatment.

Conclusion

The need for more clinical data on long-term glucose deprivation treatments for cancer is well described in literature. This paper attempts to add to the available pool of knowledge.

BACKGROUND

There is an increase in the understanding of the benefits of strict glucose control during the treatment of highly glycolytic cancers and metastases (HGCM) [1,2].

Various nutritional strategies are used to decrease circulating blood glucose (BG) levels and elevate ketone bodies for therapeutic purposes [3]. These nutritional strategies include fasting [4,5], restricted ketogenic diet (KD-R) [6], calorie restriction [6] and ketone supplementation [7] in combination with the use of diabetic medication (e.g. metformin).

The most established of these nutritional strategies, the KD-R, has shown therapeutic effects on various types of cancer when used in combination with adjuvant therapies [3,8–13]. Fasting, as used by anorexic patients, has shown a vastly reduced co-occurrence of HGCM [14].

These studies point to a therapeutic window created by the high glucose uptake of certain cancer cell types compared to that of non-cancer cells. Despite the large therapeutic evidence of the benefits of nutritional strategies, a very recent review (2017) by Winter et al. state that: *"…nutritional strategies targeting glycemic modulation to exploit the observed tumor glucose-dependency have not yet been thoroughly investigated in clinical trials and existing clinical data is limited* [3]*."*

We agree with Winter et al. [3]. Typical *in vitro* studies of glucose deprivation on cancer cells investigated the short-term effects, where glucose deprivation ranged from 5 hours [15], 2 days [16–18] to 7 days [19]. As the effect of KD-R is over months, we need data for at least 90 days to see initial and longer-term effects.

More importantly, in previous studies e.g. [15–19] the following procedure was usually followed: after initial cell growth in Dulbecco's modified Eagle's medium (DMEM) at a glucose level of 25.52mmol/L, the cells were exposed to severely low glucose levels (0-2.5mmol/L) [15–19].

The glucose levels in DMEM are thus usually four times higher than the average human physiological BG concentration of approximately 6mmol/L (HbA1C of 5.3% [20]). Therefore, the cancer cells are not given adequate time to stabilize at typical human BG levels before being given the final "push" to the therapeutic BG level. A therapeutic level of 0mmol/L is also not practical. Thus, the above-mentioned models are not good models for investigating practical long-term glucose deprivation effects.

In this paper we will endeavour to add to the existing pool of *in vitro* clinical data for long-term glucose deprivation strategies. Some of the questions we want to answer are the following:

1. Can we conduct *in vitro* tests with cancer cells at typical *in vivo* physiological BG concentrations? *In vitro* tests are usually done at four times higher BG levels [21]. As the aim of the investigation is BG control, the BG baseline should be correct to have confidence in the results.
2. We know that the ketogenic diet (KD) does not work for all cancer patients [10]. Therefore, we want to investigate what the effect is of glucose deprivation on different cancer cell lines that possess varying glucose demands.

3. At what cell growth rate (for metabolic treatments) will cancer cell lines recover and after what treatment period?
4. When will the *in vitro* cancer line be at its most vulnerable? Therefore, when does a potential therapeutic window for short-term adjuvant therapies open? This *in vitro* data could direct future human *in vivo* tests as to when cancer growths are at their most vulnerable.

We made a significant assumption in the present investigation that will need verification through additional research. We had to assume a minimum achievable BG level for a combination of KD-R, fasting and the use of BG lowering medication e.g. metformin.

An average BG level as low as 1mmol/L has been reported for fasting [22]. BG levels as low as 4mmol/L were reported for cancer patients on a KD [3]. Values for a combination of fasting, KD-R and metformin use could not be found. For the present research a value of 3mmol/L was assumed as a starting point.

The question may be asked why mouse models are not used for the present glycolytic study [23]. One important reason is the difference between the BG microenvironments between mice and humans [21,23,24].

Studies with cancer-bearing mice and humans show that in humans the BG environment increases by 1.15-fold [25] while in mice, very shortly after invasion by highly glycolytic cancers and metastases (HGCM), BG decreases by 2.1-fold [26]. This can *inter alia* be attributed to the large difference in metabolic parameters between humans and mice [24].

For instance, mice have a 7.5-fold greater basal metabolic rate than humans [24], with a 10-15 fold higher relative basal glucose turnover rate [24]. This means that rodents are under much greater metabolic constraints than humans.

With such a high basal glucose demand in mice, together with the added high glucose demand of HGCM, it is important to either sustain adequate glycogen storages or to use very high levels of energy expensive gluconeogenesis in order to provide for the upregulated glucose demand.

However, mice have 2-fold less liver and 4.5-fold less muscle glycogen stores than humans [24]. Furthermore, the mouse will have to eat excessively in order to supply the added BG demand of the cancer via gluconeogenesis [24]. Therefore, the dramatic reported decrease in cancer-bearing mice's BG levels after invasion by highly glycolytic cancers.

Glucose availability to cancer cells (which is the effect we want to study) is thus much lower in mice compared to in humans. Metabolic (glycolytic) effects on highly glycolytic cancers can therefore not always be accurately extrapolated to human responses from mice studies.

Furthermore, we would not have full control over BG levels in mice. On the other hand, we will have full control over the *in vitro* microenvironment. BG control in a cancer model is important as human BG control is much tighter than that of mice [21,23]. We thus hypothesise that properly formulated *in vitro* experiments could potentially be more relevant for obtaining long-term metabolic data for humans than using *in vivo* mice models.

METHODS AND MATERIALS

Preamble

The average glucose microenvironment of healthy humans is approximately 5.8mmol/L (HbA1C of 5.3% [20]). As BG levels for cancer patients are usually higher [25] a value of 6mmol/L is used as a starting point.

Standard cultivation of cancer cells is done in high glucose Dulbecco's modified Eagle's medium (DMEM) at a glucose level of 25.52mmol/L [15–17,19]. This represents a 4-fold

higher concentration above physiological conditions, which could cast doubt over the validity of the proposed glucose treatment results. Furthermore, glutamine levels in standard cultivation of cancer cells is 4mmol/L, which are also 4-8 times higher than human physiological plasma glutamine levels of 0.5-0.9mmol/L [27–29].

Tumorigenic and non-tumorigenic cell lines were grown in DMEM before being exposed to physiological average glucose and glutamine levels of 6mmol/L and 0.6mmol/L respectively, until cell growth stability was reached (experimental condition 1). Growth stability was regarded as a period of very little variation in relative cell growth. Thereafter, cells were exposed to low glucose levels of 3mmol/L until cell growth stability was again reached (experimental condition 2).

Tests were conducted on four cell lines i.e. three tumorigenic and one non-tumorigenic cell line. Of the tumorigenic cell lines, two were metastatic breast cancer cell lines namely, the highly glycolytic M.D. Anderson metastasis breast cancer (MDA-MB)-231 and the less glycolytic Michigan Cancer Foundation (MCF-7) cells. The third cancer cell line was the Henrietta Lacks (HeLa) cervical cancer cell line. The non-tumorigenic cell line used was a spontaneously immortalised non-tumorigenic breast cell line (MCF-10A).

In this study we used the same methods as in previous studies including morphology- and proliferation experiments [30,31]. These methods were described in detail in previous papers [30,31]. Crystal violet staining and spectrophotometry [30,31] were again used here to interpret cell density, proliferation and cell viability.

Cell culture procedure

Cells were grown and maintained in 25 cm^2 tissue culture flasks in a humidified atmosphere at 37°C, 5% CO_2 in a Forma Scientific water-jacketed incubator (Ohio, United States of America). Cells were cultured in DMEM with 25.52mmol/L glucose, 4mmol/L L-glutamine,

and 1mmol/L sodium pyruvate, supplemented with 10% dialysed heat-inactivated foetal calf serum (FCS) (56°C, 30min), 100U/ml penicillin G, 100µg/ml streptomycin and fungizone (250µg/l).

Negative control for tumorigenic cell lines:

Negative control was cultured in DMEM with 25.52mmol/L glucose and 4mmol/L L-glutamine supplemented with 10% dialysed heat-inactivated FCS (56°C, 30 min), 100U/ml penicillin G, 100µg/ml streptomycin and fungizone (250µg/l).

Negative control for the non-tumorigenic cell line:

The non-tumorigenic breast cancer cell line was cultured in a ratio of 50% DMEM to 50% Ham's F-12 medium (HF12M) containing with 17.56mmol/L glucose and 2mmol/L L-glutamine with the addition of 10% dialysed FCS (56°C, 30 min), 500ng/ml hydrocortisone, 20ng/ml epidermal growth factor (EGF), 100ng/ml cholera toxin, 10µg/ml insulin, 100u/ml penicillin G and 100µg/ml streptomycin and fungizone (250µg/l).

Experimental conditions

Experimental condition 1 for tumorigenic cell lines:

Experimental condition 1 for tumorigenic cell lines consisted of DMEM with 6mmol/L glucose, 0.6mmol/L L-glutamine, and 0mmol/L sodium pyruvate supplemented with 10% dialysed heat-inactivated FCS (56°C, 30 min), 100U/ml penicillin G, 100µg/ml streptomycin and fungizone (250µg/l).

Experimental condition 1 for non-tumorigenic cell line:

Experimental condition 1 for the non-tumorigenic cell line consisted of a ratio of 50% DMEM to 50% HF12M containing with 6mmol/L glucose and 0.6mmol/L L-glutamine with the

addition of 10% dialysed FCS (56°C, 30 min), 500ng/ml hydrocortisone, 20ng/ml EGF, 100ng/ml cholera toxin, 10µg/ml insulin, 100u/ml penicillin G and 100µg/ml streptomycin and fungizone (250µg/l).

Experimental condition 2 for tumorigenic cell lines:

Experimental condition 2 for tumorigenic cell lines consisted of DMEM with 3mmol/L glucose, 0.6mmol/L L-glutamine, and 0mmol/L sodium pyruvate supplemented with 10% dialysed heat-inactivated FCS (56°C, 30 min), 100U/ml penicillin G, 100µg/ml streptomycin and fungizone (250µg/l).

Experimental condition 2 for non-tumorigenic cell lines:

Experimental condition 2 for the non-tumorigenic cell line consisted of a ratio of 50% DMEM to 50% HF12M containing with 3mmol/L glucose and 0.6mmol/L L-glutamine with the addition of 10% dialysed FCS (56°C, 30 min), 500ng/ml hydrocortisone, 20ng/ml EGF, 100ng/ml cholera toxin, 10µg/ml insulin, 100u/ml penicillin G and 100µg/ml streptomycin and fungizone (250µg/l).

Cell proliferation (Crystal violet staining)

Cells were seeded in 96-well plates and incubated at 37°C and 5% CO_2 for 24 hours in a complete growth medium containing DMEM with 25.52mmol/L glucose, 4mmol/L L-glutamine, and 1mmol/L sodium pyruvate supplemented with 10% dialysed heat-inactivated FCS (56°C, 30 min), 100U/ml penicillin G, 100µg/ml streptomycin and fungizone (250µg/l). Long-term proliferation studies were conducted in a 6-well plate.

Cells were then exposed to experimental condition 1. Cells were exposed to these conditions until cell growth stabilised (reached a plateau). After proliferation stabilised in these

conditions, cells were exposed to experimental condition 2 until cell growth stabilised again (reached a plateau).

Upon termination of the experiment, cells were fixed with 1% glutaraldehyde (100 µl) at room temperature for 15 min. The glutaraldehyde was then replaced with 0.1% crystal violet (100 µl) at room temperature for 30 min. Plates were left to dry, thereafter, 0.2% triton X-100 (200 µl) was added to the plates and incubated overnight to solubilize the crystal violet. The plates were read on an EPOCH Microplate Reader (Biotek Instruments, Inc. (Winooski, Vermont, United States of America)) at a wavelength of 570 nm thereafter.

RESULTS AND DISCUSSION

Quantitative data were collected via spectrophotometry from three independent repeats for all experiments. **Figure 1 and 2** represent the calculated averages of percentage cell growth. The error bars illustrate the standard deviations compared to negative controls, which represent cells propagated in complete growth medium for the same time period. (In some cases the standard deviations were so small that it is difficult to see them on the figures.)

Figure 1 shows the response in percentage cell proliferation of all the tested cell lines when glucose concentration was reduced from 25.52mmol/L to 6mmol/L on day zero (experimental condition 1). All cell lines were stable after approximately 30 days. (The non-tumorigenic cell line (MCF-10A) remained stable from day zero).

Data demonstrates that tumorigenic cell lines can proliferate in physiological glucose cell culture media without long-term adverse effects on cell viability. This *in vitro* media condition is a more appropriate model for human *in vivo* conditions than the traditional DMEM conditions. It should thus yield improved confidence in the results of the next (glucose treatment) phase of the clinical trial.

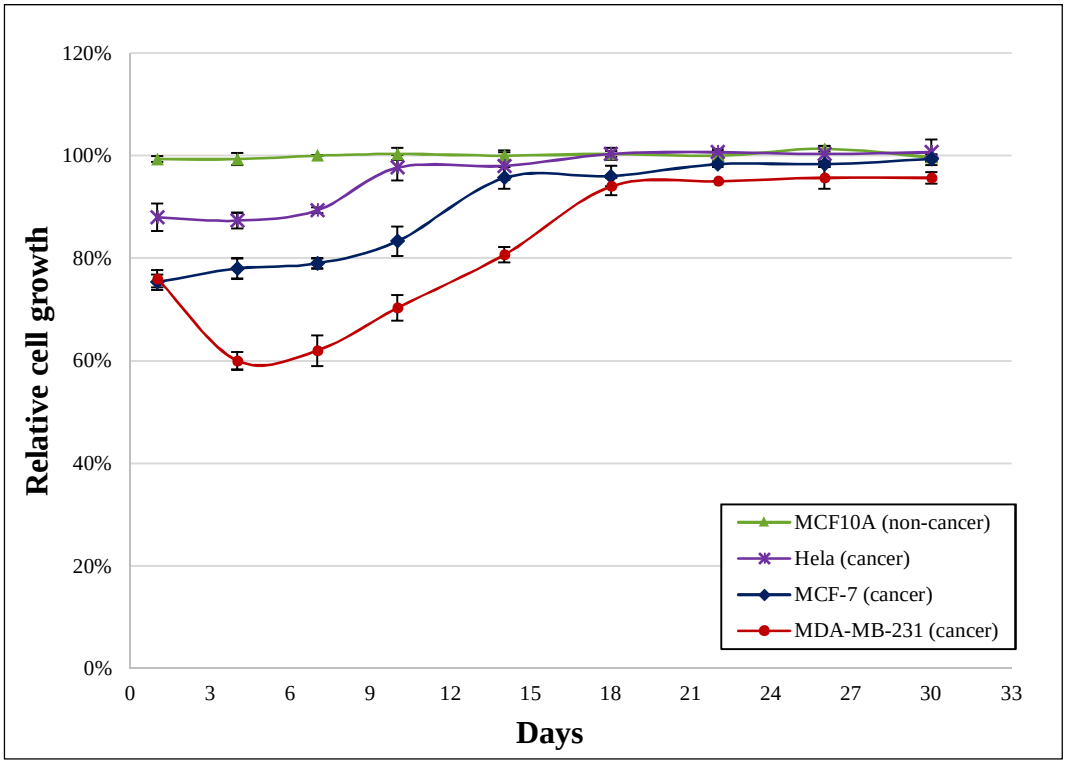

Figure 1. Relative cell growth when cells were exposed to glucose concentration of 6mmol/L and glutamine 0.6mmol/L. (Glucose concentration was 25.52mmol/L and glutamine 4mmol/L before time zero.) Each data point is an average of three or more independent repeats. Cell growth was evaluated using crystal violet staining and spectrophotometry. HeLa, Henrietta Lacks; MCF, Michigan Cancer Foundation; MDA, MD Anderson.

After the cell growth of all cell lines stabilised, cells were exposed to the BG treatment phase (experimental condition 2). This was done at 3mmol/L glucose to mimic a combination of fasting, KD-R and metformin use. **Figure 2** illustrates a decrease in cell growth of all cell lines when compared to cells propagated in complete growth medium. All cell lines were stable at approximately 65 days of exposure.

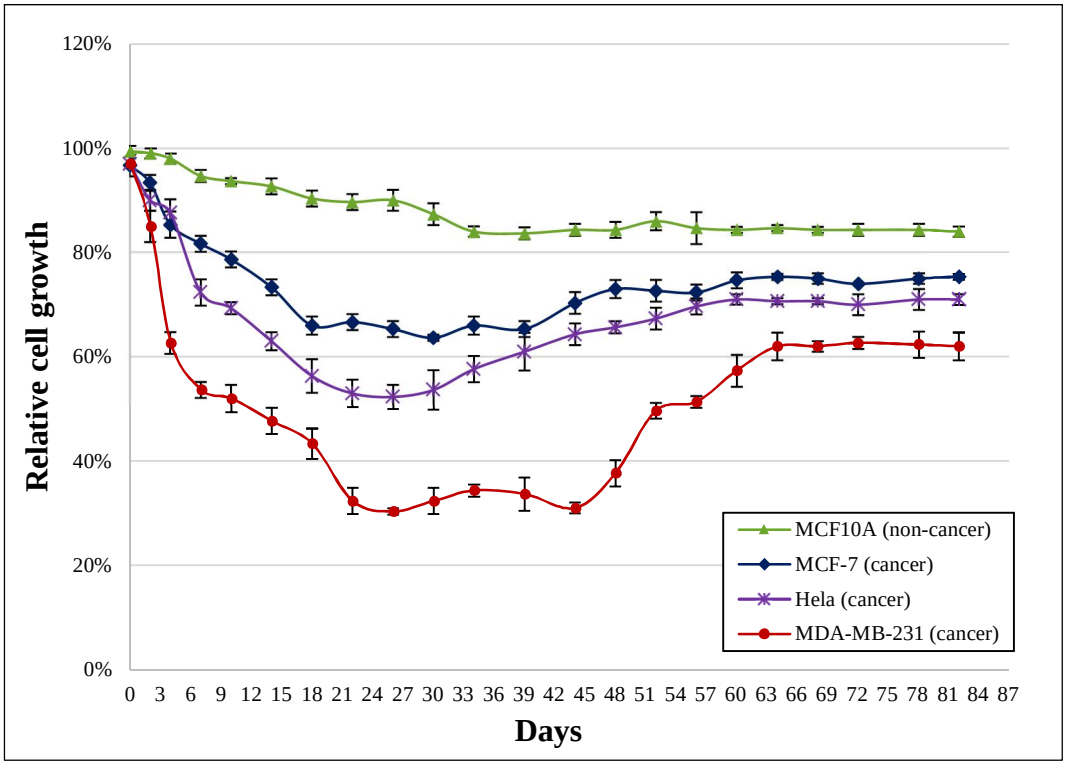

Figure 2. Effects on cell lines with glucose concentration of 3mmol/L after initial exposure to medium containing 6mmol/L glucose and maintaining glutamine at 0.6mmol/L. Each data point is an average of three or more independent repeats. Cell growth was evaluated using crystal violet staining and spectrophotometry. HeLa, Henrietta Lacks; MCF, Michigan Cancer Foundation; MDA, MD Anderson.

There are four important observations that were made in this study. These will be investigated in the rest of this section. The observations are the following:

1. Long-term glucose deprivation affects the investigated cancer cells more than non-cancer cells.
2. Different cancer cell lines are differentially affected by long-term glucose deprivation.
3. There is a period when the *in vitro* cancer cells are at their most vulnerable (lowest cell growth). Such information may be helpful to develop future cancer treatment protocols.
4. All cancer lines recover, but not fully. For full extinction of cancer cells, adjuvant therapies are needed.

A summary of percentage cell growth recovery point, days until cell growth recovery point, percentage minimum cell growth, and days until minimum cell growth is given in **Table 1.** As

expected, the highly glycolytic metastatic cell line (MDA-MB-231) was most affected, with a minimum cell growth of 30% (±0.58%) on day 26 and stabilisation point at 62% (±2.65%).

Table 1. *In vitro* results after cells were exposure to 3mmol/L glucose for 90 days.

Cell line description	Cell growth recovery point (mean ± standard deviation)	Days until cell growth recovery point	Minimum cell growth (mean ± standard deviation)	Days until minimum cell growth
MCF-10A (non-tumorigenic, breast cell line)	84 ± 1.00%	34	84 ± 1.00%	34
MCF-7 (metastatic luminal breast cell line)	75 ± 2.00%	60	65 ± 2.00%	26
HeLa (glycolytic cervical cancer cell line)	71 ± 1.00%	60	52.33 ± 2.31%	26
MDA-MB-231 (highly glycolytic metastatic breast cell line)	62 ± 2.65%	64	30 ± 0.58%	26
Average days until minimum cell growth (excluding non-tumorigenic cell line)				**26**

Furthermore, the non-tumorigenic cell line (MCF-10A) was affected the least, with cell growth remaining stable at 84% (±1.00%) after 34 days. This reduction in cell growth could be similar to the regular "weight loss" experienced during low glucose levels induced *via* restriction of dietary intake and metformin use. It may be concluded that metabolic glucose deprivation could be a safe therapy.

Figure 2 and Table 1 show that cancer cell growth increases again after the minimum levels were reached. This illustrates the need for adjuvant therapies, e.g. chemotherapy, radiation, hyperbaric or other extra metabolic treatments [32].

The most therapeutic time to administer such an adjuvant therapy would be when the cancer is at its most vulnerable. This could be where the least cancer cells survive. **Table 1** shows that for the three cell lines investigated, a potential time for an adjuvant therapy would be at around 26 days after reaching a BG level of 3mmol/L. The most aggressive cancer stayed at its most

vulnerable stage between days 22 and 44, which provides a wide adjuvant therapeutic window. However, the *in vitro* data have to be verified by future human *in vivo* tests.

Figure 2 also shows that the therapeutic effect is higher for the highly glycolytic cell line than for the less glycolytic ones. Future decisions on metabolic treatment should thus be done based on metabolic activity of the cancer (SUVs) rather than on the type of cancer. Glucose metabolic activity can be measured by [^{18}F]fluorodeoxyglucose ([^{18}F]FDG) based positron emission tomography scans (PET) and the cancer cells' respective standard uptake values (SUVs) [33].

CONCLUSION

The need for more clinical data on long-term glucose deprivation treatments for cancers (e.g. fasting, KD-R etc.) is well described in literature. In this paper we attempted to add to the available pool of knowledge. The research showed the following:

1. *In vitro* cancer tests can successfully be done at a human *in vivo* BG microenvironment of 6mmol/L. This provides more confidence in results than the typically used *in vitro* BG microenvironment of 25.5mmol/L.
2. Long-term metabolic treatment affects different cancer cell lines differently. It is suspected that the SUVs of cancers will be used in the future as standard practice to establish the usefulness of glucose deprivation treatment (e.g. nutritional etc.). Future research should establish these SUVs.
3. As full cancer extinction is not possible with long-term BG deprivation alone, adjuvant therapies are needed. Here we made a first *in vitro* attempt to establish the potentially best therapeutic time for any adjuvant therapy. For the cancer cell lines investigated, the 26 day period after a BG level of 3mmol/L is reached, seems to be a sensible

adjuvant time target. This *in vitro* data could help direct future human *in vivo* studies to find the best therapeutic time for adjuvant therapies.

LIST OF ABBREVIATIONS

BG	Blood Glucose
DMEM	Dulbecco's Modified Eagle's Medium
EGF	Epidermal Growth Factor
FCS	Foetal Calf Serum
[^{18}F]FDG	[^{18}F]fluorodeoxyglucose
HeLa	Henrietta Lacks
HF12M	Ham's F-12 Medium
HGCM	Highly Glycolytic Cancers and Metastases
KD	Ketogenic Diet
KD-R	Restricted Ketogenic Diet
MCF-7	Michigan Cancer Foundation (metastatic breast cancer cell)
MCF-10A	Michigan Cancer Foundation (non-tumorigenic immortalised cell line)
MDA-MB-231	M.D. Anderson Metastasis Breast cancer cell line

PET	Positron Emission Tomography
SUV	Standard Uptake Value

ETHICAL APPROVAL AND INFORMED CONSENT

Not applicable since this study was done using commercially available cell lines.

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AUTHORS' CONTRIBUTIONS

All authors participated with the conceptualization of the project and study design. MHV did the laboratory work and data analysis. MHV and AMJ supplied the funding from research grants. Some elements of the study were funded by the first author. EHM, AAM and GEM compiled the initial draft of the manuscript. All authors have assisted in revisions and have approved the final manuscript.

COMPETING INTERESTS

The authors declare there are no competing interests.

AVAILABILITY OF DATA AND MATERIAL

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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