The Interplay Between Beta-Hydroxybutyrate, Insulin and Glucose on Regulating MCF7 Cell Cycle Status

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Abstract

The most common and aggressive cancer in women is breast cancer, and some forms may even pose a high risk for fatality. Many advanced cancers, including breast cancer are characterized by glucose dependency, leading to oxidative stress and cellular proliferation. The Ketogenic diet (KD), a prospective nutritional adjuvant therapy for cancer, encompasses a repertoire in aiding to treat epileptic seizures, improve insulin sensitivity and reduce adiposity. This study demonstrates an *in-vitro* approach to stimulate the KD in order to evaluate the effects on MCF7 breast cancer cell-cycle progression. Under the external conditions of decreased blood insulin, decreased circulating glucose and increased beta-hydroxybutyrate (βHB), all factors which accompany consumption of the KD, revealed preliminary evidence displaying the expression of cell cycle regulating proteins responsible for reducing cellular proliferation. Results illustrate that in depleted glucose conditions, expression of Akt signalling pathway is downregulated, which is responsible for cell survival. AMPK, an energy sensing pathway is upregulated in response to βHB supplementation in low glucose states, which stabilizes downstream p27 protein that assists in halting cell cycle progression. Together, these findings provide evidence that creating a negative growth environment for MCF7 cells has the potential to implicate cellular energy and attenuate cell proliferation.

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List of Abbreviations

AKT	Protein kinase B
AMPK	Adenosine monophosphate-activated protein kinase
AMEM	Alpha's modification of eagle's medium
ATP	Adenosine triphosphate
APC/C	Anaphase promoting complex/cyclosome
βHB	Beta-hydroxybutyrate
CAK	CDK activating kinase
CDK	Cyclin dependent kinase
CKI	Cyclin dependent kinase inhibitor
COX IV	Cytochrome c oxidase subunit 4
DNA	Deoxyribonucleic acid
ER+	Estrogen receptor positive
ER-	Estrogen receptor negative
ETC	Electron transport chain
FFA	Free fatty acid
FBW7	F-box and WD repeat domain-containing 7
G0	Gap 0 phase
G1	Gap 1 phase
G2	Gap 2 phase
GSH	Glutathione
IGF1	Insulin-like growth factor 1
INK4	Inhibitor of kinase 4

KD	Ketogenic diet
KIP	Kinase inhibiting protein
M	Mitosis phase
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NOX	NADPH oxidases
PI3K	Phosphatidylinositol 3-kinase
PR+	Progesterone receptor positive
PR-	Progesterone receptor negative
PVDF	polyvinylidene difluoride
Rb	Retinoblastoma
ROS	Reactive oxygen species
S	Synthesis phase
TAG	Triacylglycerol
TCA	Tricarboxylic acid cycle
TNF-α	Tumor necrosis factor-alpha

1.0 Review of Literature

1.1 Cell Cycle Overview

Cell growth is tightly controlled by the cell cycle, a specific and tightly regulated sequence of events. Early in the cell cycle, cells are challenged with a decision, based on an assessment of growth conditions that will ultimately determine their fate. In an ideal situation cells will enter the cell cycle and complete a round of replication or, if conditions are unfavourable, the cell will withdraw from the cell cycle and terminate the division cycle[1]. If the environment is stable and favours cellular division, the cell will progress through highly regulated checkpoints, marking its progression through a new cycle of division. Stages follow the order of G0 (rest/quiescence), G1 (checking of environment), S (synthesis and replication of genetic strands), G2 (environmental growth) and M (mitosis and cellular division). A mitogenic signal from the external environment triggers the cell to enter G1, which causes activation of cyclins and cyclin-dependent kinases (CDKs) present in the cell[2]. CDKs are considered among the most important proteins involved in cell cycle control. They are a family of multi-functional enzymes that modify protein substrates involved in the progression of cell cycle[2]. CDKs elicit their functions by phosphorylating their respective target substrate proteins by transferring phosphate groups from Adenosine triphosphate (ATP) to specific amino acid sequences on the substrate[2]. Cyclins are also a set of family proteins, which act primarily as facilitator/modifier proteins when CDKs bind to them forming a functioning active cyclin/CDK complex. CDKs are regulated by phosphorylation, with the specific site of phosphorylation (or dephosphorylation) determining whether the protein be “turned on” or “turned off”, thereby ultimately determining its function[3]. During cell cycling, activated CDKs phosphorylate their respective target proteins which advances the cell division process. Cyclin D plays a critical role in the initiation of cellular

division in response to a mitogenic growth signal through activation of CDK4/6[3]. A series of steps need to occur before having an active cyclin D paired to CDK4/6 complex. Cdc25A, a protein phosphatase, removes the inhibitory phosphorylation on tyrosine 15 imparted by the Wee1 kinase during quiescence. CDK4/6 also undergoes phosphorylation by cyclin activating kinase (CAK) on threonine 172, as part of its complex and tightly regulated activation as part of progression through G1. During quiescence CDK4/6 is inactivated by binding with the inhibitor of kinase 4 (INK4) protein, part of the cyclin dependent kinase inhibitor (CKI) family [2]. This inhibitory binding is disrupted by the $p27^{KIP1}$ protein binding to the cyclin D-CDK4/6 complex. This binding of p27 to the cyclin D-CDK4/6 complex serves a secondary function, that being the sequestration of p27 from its primary target, cyclin E-CDK2 complex (figure 1). When p27 is sequestered by cyclin D CDK4/6, it reduces the availability of free p27, the primary inhibitor of cyclin E/CDK2 complex[4]. This helps the cell transition towards the G1/S transition by making it easier for Cyclin E protein expression to increase above threshold necessary to overcome Kip inhibitors like p27 and p21. Meanwhile CDK2 is activated, in part, by the removal of Wee1 phosphorylation of CDK2 on tyrosine 15 by cdc25A[4]. Cyclin E and CDK2 bind together to form an active complex that phosphorylates numerous target proteins whose cumulative functions promote deoxyribonucleic acid (DNA) replication and S-phase entry. In order to become active, the level of expression of cyclin E/CDK2 needs to increase beyond the level p27. Once activated, the cyclin E/CDK2 complex phosphorylates p27 on Threonine-187 which facilitates the ubiquitin-dependent degradation of p27 protein, thereby removing the cell cycle inhibition imparted by p27 and allows the cell to properly progress from G1 into S-phase[5] (figure 2).

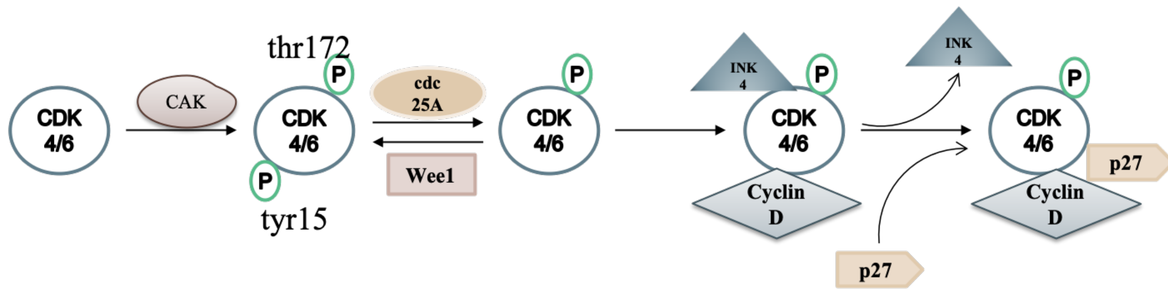

Figure 1: Illustrating the activation of cyclin D CDK4/6

The activation of CDK4/6 by the removal of Wee1 phosphorylation by cdc25A allowing for the phosphorylation of CDK4/6 by CAK on thr172. Cyclin D then binds to CDK4/6 and permits the immediate binding of p27 which causes INK4 to dissociate. The end result is activated cyclin D CDK4/6 and sequestered p27, in order for p27s availability pool to be reduced.

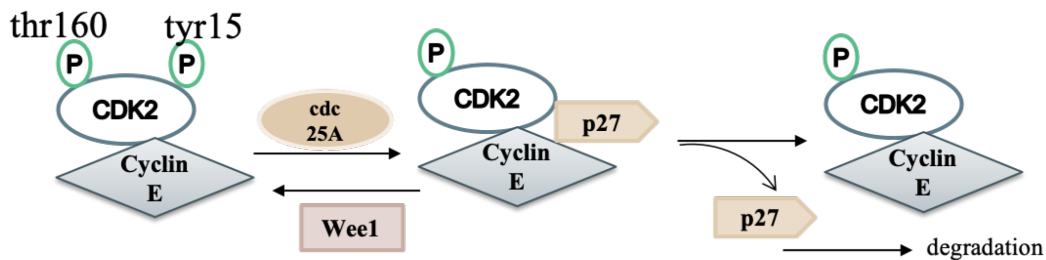

Figure 2: Stages of the activation process of cyclin E CDK2

Similarly, to cyclin D-CDK4/6, the removal of Wee1 phosphorylation on tyr 15 is caused by the phosphorylation/activation of the phosphatase cdc25A. The complete activation of cyclin E CDK2 depends on the stoichiometric balance with its primary inhibitory protein p27. When the level of cyclin E/cdk2 increases above p27, p27 will then be tagged for degradation allowing for S-phase entry of cellular division.

1.1.2 Cell Cycle Regulation

Understanding how proteins activate and facilitate checkpoints of cell proliferation are fundamental to understanding the regulation of cell growth and how certain factors can affect the cycle of cells. Before cell growth is initiated, the cell remains in a biochemical state of quiescence (G0), where it will remain inactive in growth until a commitment is made, normally exerted from an external signal to trigger cell cycle progression[6]. Once activated by a mitogenic growth signal, it takes the cell out of G0 and commits to the cell cycle by entering G1 where cyclin D/CDK 4/6 levels increase and can remain elevated until the end of M phase (cellular division/mitosis)[7]. The role of cyclin D and CDK4/6 complex is to solely phosphorylate retinoblastoma protein allowing for the release of inhibition of E2F transcription factors[8]. The E2F transcription factors transcribe genes whose protein products are involved in multiple functions including DNA binding, replication and transcription. E2F transcription factors are responsible for creating a positive feedback system through its transcribing of cyclin E[9]. Cyclin E and its kinase associate CDK 2, further promote the phosphorylation of retinoblastoma and release more E2F transcription factors[6]. Once the level of cyclin E/CDK 2 protein complexes are increased beyond levels higher than p27 protein, the cyclin E/CDK2 complex becomes active at which point it phosphorylates p27 on threonine-187 which tags p27 for degradation. The cell then propagates rapidly into S-phase and begins to undergo DNA duplication[6]. Once DNA has been replicated in S-phase, the cell prepares for cellular division by not only growing in size but also activating cyclin A/cdk2 complexes. By the end of DNA duplication in S-phas,e cyclin E is ubiquitinated by Fbw7 protein (F-box and WD repeat domain-containing 7), targeting cyclin E for protein degradation. Cyclin A begins to accumulate and bind to CDK1/2, marking the end of S-phase and leading the cell into G2, where DNA integrity is checked before cellular division[5]. Cyclin A-CDK1/2 activation is a rate limiting component that is required for initiation of

prophase and cell cycle progression, expressed at the G2/M boundary[10]. This checkpoint is crucial as it serves as the crucial checking stage to ensure that DNA mutations do not remain through mitosis allowing the accurate replication of DNA. Detected mutations would result in the cell cycle arrest within G2 in order to repair DNA or, if the DNA damage is not repairable, initiate cell death/apoptosis. If the integrity of the DNA is checked and no mutations are present, cyclin B-CDK1 will begin to activate leading the cell cycle into mitosis[10]. Mitosis consists of 5 stages that produce two identical copies of the genome and two identical daughter cells. Cyclin B-CDK1 is responsible for the immediate hyper-phosphorylation of lamins which work to dissolve the nuclear membrane in preparation for complete prophase[11]. The next four stages immediately follow prophase, which are prometaphase, metaphase, anaphase and telophase, where the newly duplicated chromosomes arrive at opposite poles and begin to decondense while forming their nuclear envelope, as the poles push away forming two identical cells. Exit from mitosis requires CDK inactivation by ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C)[12]. APC/C is activated during prometaphase by mitotic phosphorylation and binding of its activator, Cdc20. This initiates cyclin A degradation in G2 while cyclin B is stabilized at the spindle checkpoint. Upon completion of mitosis, the destruction box on cyclin B is exposed to APC/C, resulting in the degradation of cyclin B-CDK1 in addition to securing, which liberates the enzyme seperase that is involved in binding the sister chromatids and drives inactivation, mitotic exit and cytokinesis[12].

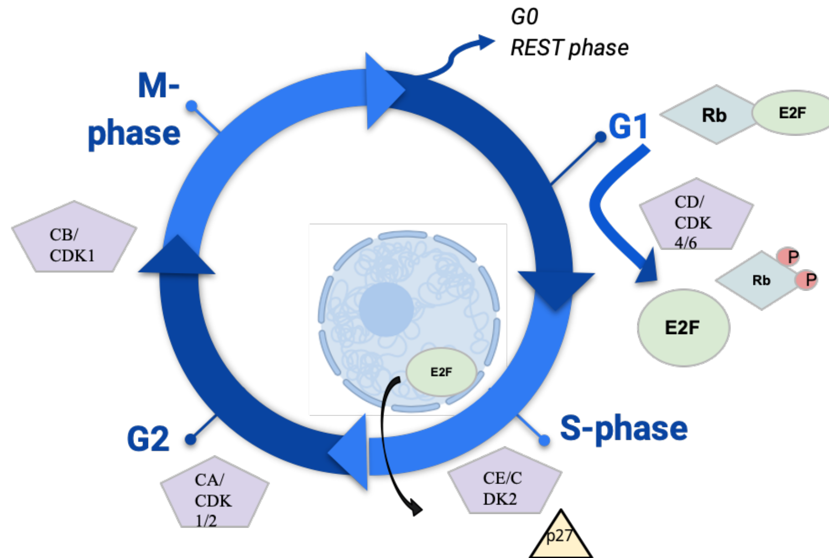

Figure 3: Stages of cell cycle transition in a mammalian cell

Cell remains in a quiescence phase until acted upon by a mitogenic signal. A growth signal induces the G_1/S phase transition by causing the activation of cyclin D CDK4/6 complex and thus reducing the availability p27 has on cyclin E CDK2. Cyclin D CDK4/6 and Cyclin E CDK2 are responsible for phosphorylating Rb which in turn is dissociated from E2F transcription factors. E2F factors proceed to initiate DNA replication and propagate S-phase entry by inducing the transcription of more cyclin E. G_2/M phase transition is dependent mainly on cyclin B CDK1 which is responsible for the hyper-phosphorylation of complexes required for nuclear replication. The degradation of these cyclin and CDK complexes will only occur once cellular division has occurred and no other mitogenic signals are sensed.

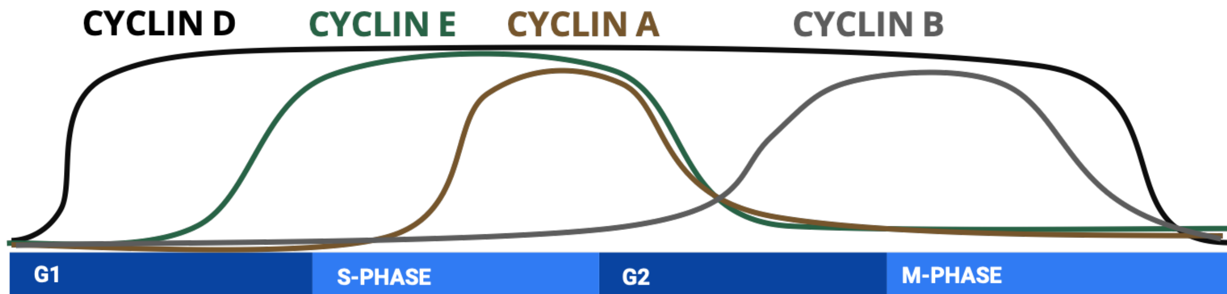

Figure 4: Progression of cyclin complexes throughout cell cycle.

Once the cell senses a mitogenic signal in the environment, the cell exists its quiescence phase and begins to grow in size as cyclin D rises in G_1 and remains high throughout the entire process of cellular division. In S-phase, constant growth is imperative as the chromosomes duplicate, and cyclin E takes on this responsibility to provide the positive feedback loop that causes the propagation to complete S-phase. Cyclin A works to maintain the integrity of the replicated DNA in G_2 before entering mitosis. In M-phase, cyclin B triggers a cascade of steps that ultimately result in the separation of chromatids, which two new daughter cells emerge from.

1.2 Cancer

Behind the idiopathic state and complexity of all cancers, there lies a limited number of 'critical' events that propel normal cells to become tumour cells which enter into a state of uncontrollable cell division and invasion. Cancer cells are often no longer responsive to the normal signals that control for cellular growth and are known to evade programmed cellular death. The human body displays a great degree of highly regulated order and stability at every level. Any instability within the genome or alterations to the cellular environment that affects cell cycle regulation will inevitably cause disorder and disease. Unlike point-mutation genetic diseases such as downs syndrome, cancer is a sporadic disease dependent on alterations in numerous environmental factors, oncogenes, tumour-suppressor genes and stability genes leading to tumorigenesis[13]. Mammalian cells have multiple safeguards to protect them against potential lethal effects of genetic mutations. In cancer, genetic mutations lead to the loss of homeostasis and failure to control cellular division drives the accumulation of further genetic mutations, thereby exacerbating the situation. Some of the 'hallmarks' that underlie the characteristics of cancer are; angiogenesis induction, unresponsiveness to growth suppressors, activating invasion and metastasis, enabling unregulated replication, evading cell death, sustained proliferative signaling[14] and metabolic adaptations including the Warburg effect[15]. Recognition of the specific mutations that are underlying any individual cancer will expand the opportunities for the development of new cancer treatments.

The Canadian Cancer Society reports that the lifetime probability of developing cancer in both adult males and females is exactly 1 in 2.3[16]. This is important to note as the prevalence of cancer has increased since past years and the incidence has decreased considerably from 1 in 3 adults being diagnosed less than a decade ago. Besides the undeniable improvements in

diagnostics, treatment, care management and prevention that are allowing survival rates to increase, the problem still exists that increasing individuals, whether healthy, unhealthy, old or young are diagnosed with and die from cancer. It is well hypothesized that since humans are living longer, the more DNA errors build up as time passes, increasing the risk of developing cancer[17], but this fact alone does not account for these trends.

Although many cancer advances have been made in all realms, from tests and screening to diagnostics and treatment, the fact exists that continued research is a necessity for the further improvement to both quantity and quality of life. This sporadic disease can differ from person to person based on personal history and the multitude of combinations of physical and environmental alterations coupled with the almost infinite combination of mutations. To add to the explanation of why many continue to die, the physiological response to cancer and cancer treatments will vary largely from person to person, mirroring the diversity of genetic instability and profile of cancers that can impact degree of proliferation and therapeutic resistance. Therefore, it seems highly unlikely that one treatment or therapy will be sole solution for the cure for cancer.

As a reflection of the diversity of cancer profiles, there are a number of emulating cancer treatment routes. Most therapies implemented by medical practitioners have a similar objective, which is to administer a treatment that controls the growth, spread and recurrence of the disease. Currently there are limited routes of treatment for patients, and these treatments can be divided broadly into three groups, local therapy, systemic therapy, or targeted therapy[18]. Local therapy involves a specific area of the body that can be operated on during surgery for removal of cancerous cells or administer radiation directly to cancer cells, and the combination of the two are also possible[18]. Radiation therapy is classified into electromagnetic and particulate radiation[19]. Through the mechanism of oscillating high energy particles, such as x-rays, gamma

rays, electrons, or protons, in order to target and destroy or damage cancer cells[19]. When targeting cancer cells, radiation works by making small breaks in the DNA of the cell with the hope of activating the internal cell death mechanisms elicited by extensive DNA damage and inability to repair the damage. Although this mechanism can keep cancer cells from growing, nearby normal cells can be affected by radiation and affect non-tumour cells. In addition, numerous cancers have impairments in the DNA damage repair pathway (apoptosis), thereby rendering unable to respond to this type of therapeutic intervention. Thus, there are limits to the broad application of radiation therapy as a modality for universal cancer care.

Systemic therapy involves full body exposure of drugs, termed chemotherapy, which is administered through the bloodstream to reach cancer cells[18]. Chemotherapy, a cocktail of drugs absorbed by all body cells whether they exhibit cancerous or normal phenotypes[18]. There are many types of breast cancers, as such there are different chemotherapy drug combinations that work depending on the specific alterations. Estrogen and progesterone receptor positive cancers are viewed generally as the most responsive to chemotherapy as they provide a hormone receptor on the membrane surface which drugs may target once in the bloodstream[20]. Breast cancers without these receptors tend to be more aggressive and highly metastatic as anti-estrogen therapies have no surface receptors to respond to anti-estrogen drugs[20]. There are other chemotherapy drugs that do not utilize the estrogen and progesterone receptors and are designed to kill dividing cells. The problems with these types of drugs is that the associated side effects are endless, very much due to the fact that they do not preferentially target cancer cells and equally compromise healthy dividing cells which results in the loss of hair, nails, weight and appetite, increased pain and fatigue, increased risk for blood clots and cognitive problems[21].

Targeted therapy includes the use of drugs to target specific molecules on the surface of or inside cancer cells, such as receptors or proteins on the surface of the membrane[18]. The

advantage of molecule targeted drugs is that they allow for the functional inhibition designed to prevent further growth and spread of cancer cells. For breast cancer, targeted therapy using medication will be prescribed if the cancerous cells display estrogen and progesterone hormone positive receptors (ER+/PR+) which allow the drug to recognize the receptor proteins on the surface of the cell. Targeted drugs specific to ER+/PR+ cancers include a variety of aromatase inhibitors and anti-hormone blocking drugs that treat obese post-menopausal women with metastatic cancer[22]. Aromatase inhibitors such as letrozole work by blocking the enzyme aromatase, which is responsible in converting the hormone androgen into estrogen. By doing so, the availability and production of estrogen in the body will be reduced and so will the ability to stimulate the growth of the hormone receptor positive breast cancer cells[23]. Results from the Breast International Group in 2019 suggested that these hormone therapy drugs validated the prognostic significance of lowered risk of reoccurrence in postmenopausal women with ER+/PR+ and early breast cancer[24]. With any drug, comes side effects and targeted therapy drugs also come with a long list of adverse side effects such as, increased risk of severe osteoporosis, weight gain, hormonal imbalance[25].

1.2.1 Reactive Oxygen Species and Cancer

Cancer cells struggle with exposure to persistent metabolic oxidative stress in comparison to normal cells, which is largely due to their inherent hallmark of mitochondrial dysfunction and NADPH oxidases (NOX) activation[26]. Reactive oxygen species (ROS) are radicals that have an unpaired electron, making them highly reactive. High levels of ROS in cancer cells can result from increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased activity of oxidases, cyclooxygenases, lipoxygenases and thymidine phosphorylase[27,28,29]. To understand the impact ROS molecules has on cancer cells, it is important to know that ROS is a

crucial component of the cellular redox signalling network in normal cells. Low levels of cellular oxidation by superoxide or hydrogen peroxide activates correct mitogenic signalling and proliferation[30]. ROS, particularly hydrogen peroxide, can act as a second messenger in cellular signaling by regulating protein activity through reversible oxidation of receptor tyrosine kinases[31]. It then participates in a crucial step in early G1-phase as the oxidation event progresses the cell cycle to S-phase[32]. The second part to the progression to S-phase is the cellular level of glutathione (GSH), a cellular antioxidant that remains low and increases as oxidation events occur and this response is necessary for cells to progress from G1 to S-phase[32]. The intrinsic redox cycle proves to be a cell cycle regulator at the level of the electron transport chain (ETC) in the mitochondria when processing bursts of ROS. When conditions extend periods of prolonged cellular oxidation, such that seen in continuously proliferating cancerous cells where they feature increased metabolic activity/energy demands where the mitochondrial dysfunction free radicals and potentially trigger DNA damage and cell death [30].

1.3 Obesity

The prevalence of overweight and obesity has drastically risen over the past years in North American society. A recent study led by Imperial College London and published in The Lancet highlights a ten-fold increase in obesity among children and adolescents worldwide. The study projected that by 2022, there will be more obese children than underweight children aged 5 to 19[33]. According to Health Canada, in 2007-2009 one in four adults were obese and in recent years the ratio has grown, with one in three Canadian adults being classified as obese[34]. Global data has shown that mean body mass index (BMI), an indicator of body fat, has increased considerably from 1980 to 2008,and the trend continues to grow and is varied between nations[35]. It is known that being overweight or obese can have serious and detrimental impacts on many aspects of human health. Cardiovascular diseases, musculoskeletal disorders, type 2 diabetes, cancers (endometrial, breast and colon), and even sleep apnea and depression can all be traced back to obesity as the main risk factor and contributor[36].

In its most simplistic lens, obesity is the result of an imbalance between high intake of calories and/or a lowered energy expenditure, with the resulting positive energy balance leading to an accumulation of adipose tissue[37]. Originally adipose tissue was considered to simply be a storage organ for excess energy in the form of triglycerides, but decades of research growth has established that adipose tissue is a complex and an essential metabolic endocrine organ with the ability to control body function. In response to excessive energy intake adipose tissue expands existing cells by increasing the storage of lipids (hypertrophy) and when that is in adequate new adipocytes are formed through the differentiation of pre-adipocytes[37].

1.3.1 Adipose Tissue as an Endocrine Organ

Over the past decade, biochemical studies have shown the that a major role of adipose tissue is controlling systemic body functions by secreting hormones responsible for a wide variety of physiological actions including appetite, glucose balance, insulin sensitivity, aging, fertility and body temperature[38].

Fat accumulation and fat storage is determined by the balance between fat synthesis (lipogenesis) and fat breakdown (lipolysis or fatty acid oxidation). Lipogenesis is a process, which mainly occurs in adipose tissue and as well in the liver, whereby excess energy is stored by the creation of fatty acids which are then available as energy reserves[37]. Lipolysis involves the breakdown of triglycerides and thereby liberating their stored energy for use in metabolism[37]. Adipocyte triacylglycerols (TAGs) comprise more than 90% of the cellular content of white adipocytes and serves an important role in insulation and protection of organs[39]. Lipogenesis is responsive to changes in diet where excess energy from high caloric intake occurs and paired with a high carbohydrate regimen, this will result in high glucose levels in the blood which will stimulate the release of insulin and inhibit release of glucagon, making glucose a substrate for lipogenesis[40]. In the response to a changing environment or in times of metabolic stress, fasting, prolonged exercise or diet for example, the body's demand for energy increases above circulating glucose level, triggering fat metabolism. During lipolysis, hormone-sensitive lipase is responsible for the hydrolysis of the TAG droplets to form three FFAs and one glycerol molecule to be used as an energy source by other cells[37]. Glycerol is shuttled back to the liver for the use of oxidation or gluconeogenesis, the FFA are immediately bound to albumin and carried in the blood stream to be delivered to the liver, muscle and any non-hepatic tissues for oxidation[37]. β-

Oxidation is the process in which the FFA are converted into acetyl coenzyme A molecules to be used in the ETC of the mitochondrial matrix, as another modality of energy for cells[41].

In addition to energy storage, adipose cells secrete many hormones, growth factors, enzymes, cytokines, complement factors and matrix proteins[42]. Adipose tissue also express numerous surface receptors and are thus susceptible to endocrine regulation by many processes including food intake, fat metabolism, energy expenditure, immunity and blood pressure homeostasis[43]. Cell function regulation involves a complex network of endocrine, paracrine, and autocrine signals that influence the response of many tissues, a few being the hypothalamus, pancreas, liver, skeletal muscle, kidneys, endothelium and the immune system[43]. Data show that there are some inherent differences between the two subtypes of adipose tissue, those being visceral and subcutaneous. Visceral adipose tissue has a unique profile of adipocytokine production compared to subcutaneous adipose, for example higher concentrations of interleukin-6 and plasminogen activator inhibitor 1[37]. Over secretion of these adipocytokines and reduced secretion of beneficial adipocytokines like adiponectin, may be one of the reasons that accumulation of visceral adipose tissue is thought to be a more detrimental contributor in some lifestyle related obesity-dependent diseases. Given clinical findings, the reduction of visceral fat may be an essential preventive measure and efficient therapeutic procedure to help regulate the onset of metabolic syndrome and cardiovascular disease[44]. Subcutaneous adipose tissue secretes a different profile of endocrine, paracrine and autocrine factors compared to visceral adipose tissue including TAG, glycerol, leptin, adiponectin, resistin, proinflammatory cytokines like tumor necrosis factor alpha, insulin-like growth factor 1 and angiotensinogen[42,43]. The endocrine role of adipose tissue is best characterized by leptin, insensitivity to leptin, or leptin deficiency causes type 2 diabetes, hyperphagia, morbid obesity, neuroendocrine abnormalities, cardiovascular disorders and immune dysfunction[45]. Importantly, during the development of

obesity adipocyte hypertrophy, immune cell function is altered, and most immune cells and macrophages begin to express inflammatory phenotypes[46]. Marking obese related immune dysfunction.

Independent of adipose tissue, the prevalence of obesity as a disease has reached epidemic proportions. This preventable health morbidity is detrimentally associated with numerous other ailments including metabolic syndrome, cardiovascular disease, dyslipidemia, insulin resistance, diabetes, fatty liver, sleep apnea and cancer[47,48]. Importantly, with excess adipose tissue, adipocytes have shown to cause an inflammatory and prothrombotic response, which can further increase the risk of strokes[49]. Data suggests that obesity for women near menopause has increased risk of breast cancer whereas weight loss approaching menopause is associated with decreased risk of breast cancer[50]. This is particularly important for health care professionals when creating a treatment plan and implementing interventions that would help the patients who also suffer from obesity as a co-morbity. It is well established for many diseases that weight loss can result in the significant reduction in the risk level of these comorbid conditions.

1.4 Obesity and Breast Cancer

The link between obesity and risk of cancer is evident and the influence of the obesity epidemic may play a role in the acceleration and development of malignancy. Historically, cancer research was focused on specific gene mutations and amplifications within each cancer keeping therapy concentrated on the internal alterations relevant to each unique cancer. Obesity focused research led to theories that there is a window of susceptibility which can mediate and exacerbate the onset of tumor progression. Excess body fat is shown to increase the risk of colorectal, post-menopausal breast, pancreatic, kidney and many more cancers[51]. A study in 2019 exposed breast cancer (MCF7) cells to normoglycemia, hyperglycemia and obese conditions in order to evaluate a role of glucose on the metabolic malfunctions in these cancer cells[52]. Results from the study revealed an increase in the aggressiveness of the cells microenvironment in the diabetogenic obese condition (high-glucose) and increased tumor proliferation[52]. High amounts of refined and natural sugars found in the diet combined with low caloric expenditure can result in an exacerbation of mutagenic effects and hormonal imbalance[53]. The common feature of cancer cells is altered metabolism which favours increased glucose uptake and fermentation of glucose to lactate, even in the presence of mitochondria[15]. This is called aerobic glycolysis, a mechanism referred to as the Warburg effect[54]. The Warburg effect has been documented and studied for over 90 years. Tumours are shown to rely heavily aerobic glycolysis, which is associated with exponential growth, decreased rates of mitochondrial respiration and decreased levels of catalytic enzymes used in the tricarboxylic-acid cycle (TCA)[55]. A study in 2014 examined the increased aerobic glycolysis response in yeast cells in the presence of oxygen, they found that high glucose maximized growth rate and energy production[55]. When measuring rates of oxygen and carbon dioxide, it revealed that as cells

multiplied and cell count increased, oxygen consumption level per cell, decreased[55]. This highlights the Warburg shift to aerobic glycolysis as an adaptive metabolic approach to support the increased energy demands that accompany elevations in cellular proliferation, regardless of oxygen availability.

Breast cancer is the most common malignancy and is the leading cause of cancer-related death among women worldwide[56]. Although advancements have been made in screening for breast cancer earlier in life, the incidence of new cases is currently estimated to be 1 in 8 Canadian women that will develop and 1 in 33 will die from breast cancer[57]. The risk factors for breast cancer in women are numerous but more studies and recent research has shown that diet, physical inactivity, obesity and weight gain are among the strongest risk factors for cancer development and progression, regardless of menopausal status[58]. Visceral adipose in obese women is correlated with the development of insulin resistance and metabolic syndrome, both of which increase the risk of cancer and recurrence in cancer patients[59]. Work done by Baker and Fabian showed the association between visceral adipose tissue and circulating adipose stromal cells in the facilitation of angiogenesis and tumour metastasis[59]. They hypothesize that adipose stromal cells can be a useful biomarker in clinical trials of obese breast cancer patients undergoing a weight loss intervention plan[59].

Obesity-related risk factors such as insulin resistance, IGF1, leptin, TNF-alpha have all been shown to increase activation of the PI3K (phosphatidylinositol 3-kinase)/Akt (protein kinase B) signaling pathway[60]. When taken together, cancer cells and several obesity-related systemic alterations may contribute to favour glucose metabolism, which promotes proliferation by activating and driving excessive stimulation of the PI3K/Akt/mTOR (Mammalian target of rapamycin) signaling pathway[61]. Activation of oncogenes such as Mdm2 and/or PI3K/Akt blocks the function of the pro-apoptotic tumour suppressor protein p53[60]. A study investigated whether

metabolic alterations induced by adipose cells on breast cancer cells, and revealed that co-culture with either mouse or human mammary adipocytes increased viability of MCF7 cells[62]. When the adipocytes were cultured in high glucose media, a two-fold increase in cell growth, a doubling of IGF1 release and a stimulation of mitogenic signaling pathways in MCF7 cells were all observed, compared to the reverse effects when cultured in low glucose media[62].

1. 5 Ketogenic diet and obesity

Genetic predisposition in combination with inactive lifestyles and high caloric intake leads to weight gain. The ketogenic diet is a nutritional intervention that reduces caloric intake by restricting the intake of carbohydrates, increasing fat and maintaining protein consumption[63]. Recent studies have shown high fat/low carbohydrate diets are effective in reducing body fat and improve risk factors that are associated with obesity[64]. The ketogenic diet imparts better patient retention, greater weight loss, decreased triglyceride levels and increased high-density lipoprotein which are all desirable in the treatment of obesity and hyperlipidemia[65]. The goal of this diet is to shift metabolism from carbohydrate to fat as the primary source for energy/ATP production. When individuals are "starved" of carbohydrates, ketone bodies become the major source of energy that supply all non-hepatic cells, becoming primary source for ATP production[66,67]. This switch in metabolic fuel is termed *ketosis*[64].

A state of ketosis is reached after days of fasting or when the body has been in a state of carbohydrate starvation, at which point the glucose reservoir has been depleted and the body resorts to lipolysis, the liberation of energy through the metabolism of triglycerides from lipids stored in adipose tissue, as primary body energy source[67]. The breakdown of triglycerides produces 3 fatty acids and 1 glycerol molecule. The 3-carbon glycerol molecule will be used to undergo gluconeogenesis to make glucose for specialized cells such as the red blood cells that require glucose to function. The fatty acids will be carried bound to albumin to the liver where they are catabolised in enzymatic reactions to produce ketone bodies[41]. Ketone bodies are transported throughout the body and enter non-hepatic cells as a source of fuel[68]. There are three major ketone bodies, beta-hydroxybutyrate, acetoacetate and acetone (figure 5). These are central to providing non-glucose energy as a substrate used in all extra-hepatic tissue (including brain

cells) during carbohydrate restriction[69]. In the mitochondria, ketone bodies undergo beta-oxidation to generate ATP production and force aerobic respiration[67].

A 2007 study demonstrated that obese diabetic subjects on the ketogenic diet experienced significant decreases in blood glucose, total cholesterol, low density lipoprotein cholesterol, triglycerides and urea levels with an associated increase in HDL levels over 56 weeks. The ketogenic diet has shown to have beneficial effects for type 2 diabetics. Clinical research of overweight and obese participants with type 2 diabetes were put on either the ketogenic diet or a low-caloric diet for 24 weeks[70]. Effective results were seen predominately in the group on the ketogenic diet, all health parameters including total cholesterol, low-density lipoprotein cholesterol, body weight, BMI and blood glucose levels were significantly decreased following the 24-week study[70]. This study shows the beneficial effects of the ketogenic diet in obese patients with type 2 diabetes, in order to improve glycemic control. Meta-analysis of randomised controlled trials shows strong supportive evidence that the ketogenic diet in weight loss therapy is effective when adhered to[71]. The ketogenic diet will benefit obese patients and improve metabolic conditions by the process of utilizing the adipose reservoir.

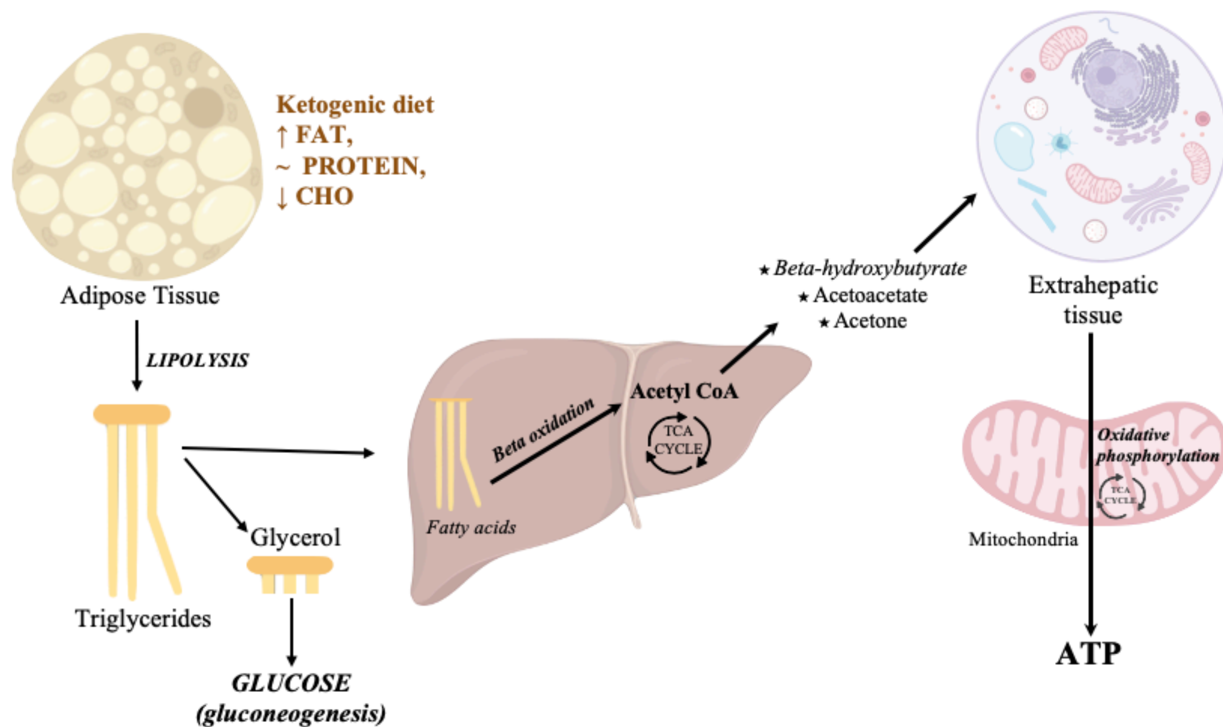

Figure 5: ATP production by the utilization of triglycerides from fat stores in adiposity

Once the body is deprived of carbohydrates, it will shift metabolisms in order to keep up with energy expenditure. This metabolism relies on the conversion of fat into ketone bodies which will be utilized in the TCA cycle, at the level of the mitochondria, for vast ATP production. The ketone body found in abundance is beta-hydroxybutyrate, it will proceed to be the main energy substrate for as long as the breakdown of fat from adipose tissue is occurring in the absence of carbohydrate.

1.6 Ketogenic diet and Cancer

Almost a century ago German physiologist Otto Warburg observed that cancer cells had increased rates of glycolysis despite the adequate availability of oxygen, a characteristic termed aerobic glycolysis[54,15]. In epithelial cells, glucose enters the cell primarily via glucose transporter 1 (GLUT1) and undergoes glycolysis where it produces 2 ATP molecules and pyruvate. Two primary fates of pyruvate are being either converted to lactate anaerobically, via lactate dehydrogenase, or entering the mitochondria where it generates 36 ATP molecules via oxidative phosphorylation. Many cancer cells exhibit an increased expression of GLUT1 transporters which allows for enhanced glucose uptake resulting in greater intracellular glucose availability and a reliance on glucose for energy generation. Mitochondrial integrity has been shown in cancer cells to be dysfunctional, leading to reductions in the oxidative capacity and ultimately reducing the amount of ATP molecules generated from the mitochondria and corresponding increases of pyruvate entering the lactic acid fermentation pathway[72]. In cancer, this shift in metabolism is known as the *Warburg Effect*[54,15] and is considered one of the hallmarks of malignant transformation. Independent of oxygen availability, aerobic glycolysis is the main form of ATP production to provide energy in cancer cells (figure 6). This reliance on glycolytic flux occurs very early in tumorigenesis and less dependence on oxidative phosphorylation in the mitochondria is a beneficial phenotypic alteration, allowing cells to thrive as the tumour grows and will ultimately exist in a hypoxic state[73,74]. This transformation allows cancer cells to compensate for oxidative phosphorylation deficiencies in order to maintain sufficient energy for tumour invasion, viability and growth[75]. As a consequence of impaired energy metabolism, diminished oxidative phosphorylation together with fermentation leads to the accumulation of ROS. ROS are carcinogenic and mutagenic agents that are largely responsible for the genomic

instability seen in tumor cells[76]. Physiologically relevant concentrations of estrogen have been shown to generate ROS in ER-positive breast cancer cells, which induces DNA synthesis and phosphorylation of kinases and transcription factors[77].

The ketogenic diet is emerging as a potentially effective adjuvant therapeutic avenue for managing malignant cancers, including breast cancer. The excess in ketone bodies produced as a source of energy following ketogenic diet consumption reduces the reliance on glucose for energy production, which can potentially make cancer cell survival tenuous in a number of ways. Firstly, cancer cells cannot effectively use fatty acids or ketone bodies for ATP synthesis through oxidative phosphorylation due to structural impairments and dysfunctional machinery of their mitochondria[78]. Secondly, once taken up by cells ketone bodies cannot be fermented, thus driving cells to utilize aerobic means for energy production which is problematic since tumours tend to be hypoxic and are known to possess dysfunctional mitochondria[79]. Bartmann et al. showed β-hydroxybutyrate, the major ketone body produced in humans during ketosis, could not stimulate breast cancer growth in cell culture[80]. The study showed that β-hydroxybutyrate could indeed increase oxygen consumption rates, proof in principle that ketone bodies can force aerobic metabolism in cancer cells. A study by Freedland and Mavropoulos observed three groups of mice consuming either a ketogenic diet, low-fat diet or "Western" diet, showed that mice on the ketogenic diet not only lose weight but had decreased levels of IGF1 and decreased hepatic fatty infiltration relative to the other dietary groups[81]. Decreasing IGF1is thought to be beneficial to an obese patient with insulin resistance by decreasing cellular proliferation signals, such as PI3K/Akt signalling[82]. Another key component in cancer cell cycle is the role of mTOR signalling pathway. A hallmark of mTOR is the duel-specificity mechanism to phosphorylate Akt on either serine or threonine amino acid sites, triggering downstream regulators such as,

fatty acid synthesis, protein synthesis, cell survival and proliferation, and autophagy[83]. It is frequently shown that mTOR is upregulated in most carcinomas such as breast, prostate, liver, renal and lung cancers[83]. The continuous upregulation of mTOR leads to the phosphorylation of Akt and promotes cancer cell migration and proliferation and suppression of autophagy[83]. Activity of AMP-activated protein kinase (AMPK) increases when intracellular energy levels are required to be maintained, thus inhibiting energy consuming regulators, such as fatty acid synthesis and protein synthesis, and promote activation of mitochondrial content, fatty acid oxidation and autophagy through mTOR suppression[84]. It has been shown that apoptotic cells potently upregulate AMPK and target inhibition of Akt activation causing inhibition of cell growth and targets induction of apoptosis and necrosis in the cell[85]. It becomes evident that the crosstalk between AMPK and Akt phosphorylation levels drives the fate of cellular division and proliferation. A previous study examined expression levels of pS6 and pAkt, markers of mTOR pathway activation in hippocampus and liver tissue of rats fed the ketogenic diet whom were induced with status epilepticus[86]. In the study, quantitative findings via western blot revealed that the ketogenic diet increased AMPK and reduced both pS6 and pAkt protein expression in the hippocampus and liver tissue samples when rats were fed the ketogenic diet[86]. It is worth noting that this study through the ketogenic diet prevented hyperactivation of mTOR signalling pathway and reduced affects prolonged seizures in rats[86].

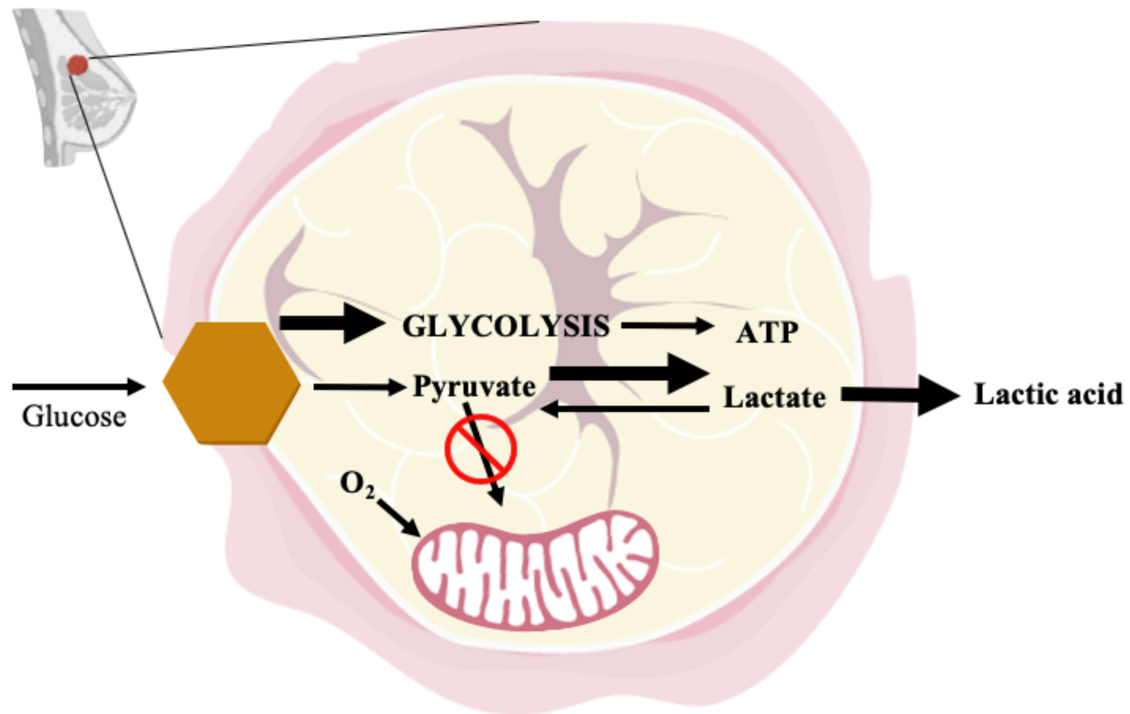

Figure 6: The amplification of a breast cancer cell exhibiting the Warburg effect

In cancer cells, the reliance on glucose for energy production is heavily due to glycolysis. The pyruvate production is converted into lactate, is favoured over the utilization for it in the mitochondria, since the integrity of mitochondria in cancer cells remains dysfunctional. The expected reductions in oxidative capacity can be assumed as the amount of ATP molecules generated from mitochondrial oxidative phosphorylation is reduced. Breast cancer cells promote the fermentation of glucose into lactate, which then accumulates in the blood as lactic acid causing a decrease in pH.

2.0 Research Objectives

Based on the review of the literature, the ketogenic diet (KD) has been shown to be effective in numerous cancer related studies. This *in vitro* study will focus on evaluating whether the primary changes to circulatory factors induced by the KD consumption, such as decreased insulin and glucose concentrations and elevated beta-hydroxybutyrate (βHB), can induce direct effects on cellular growth signalling and shift towards oxidative phosphorylation as the main energy producer of ATP[87]. The importance of this *in vitro* experimental design is to isolate the direct effects of the ketogenic diet and separate them from the effects that are due to reduced adiposity, a task very difficult to accomplish *in vivo*.

2.1 Hypothesis

1. Increasing βHB supplementation levels solely will not be sufficient to convey significant effects on cellular proliferative signalling. Cell cycle regulatory proteins in breast cancer cells will not be affected when βHB is the only alteration to the environment.
2. In order to elicit an inhibitory effect on breast cancer cells when supplemented with βHB, there must also be alterations to reduce insulin and glucose levels. Coupled together, these primary effects seen with consumption of the KD will trigger a change in substrate preference towards the utilization of βHB as energy. A downregulation of phospho-Akt protein expression will be established along with a cross-activation and upregulation in phospho-AMPK and p27 levels which will trigger breast cancer cells to reduce cellular proliferation and trigger apoptotic responses.

3.0 Manuscript

The Interplay Between Beta-hydroxybutyrate, Insulin and Glucose on Regulating MCF7 Cell Cycle Status

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Manuscript Author Contributions

Monica Tawadrous: performed all experiments and data analysis; wrote manuscript.

Dr. Michael K. Connor: supervised project as principal investigator; contributed to the writing and editing of the manuscript.

3.1 Abstract

The Ketogenic diet (KD) has recently attained popularity as a dietary intervention for reducing body adiposity. The diet comprises approximately 90% of dietary calories from fat, 8% from protein, and 2% from carbohydrates. The KD was first developed in the 1920s as a nutritional intervention for patients with epilepsy to manage associated seizures. The diet has now become a prospective nutritional adjuvant therapy for cancer, encompasses a repertoire in aiding to treat epileptic seizures, improve insulin sensitivity, reducing adiposity, and it is now being considered as a nutritional approach for an adjuvant cancer therapy. Cancer cells possess a metabolism that relies predominantly on glucose and glycolysis for energy production, which was first described by Otto Warburg and represents one of the hallmarks of cancer. This study evaluates an in-vitro approach to create the primary changes observed in body circulation that accompany KD consumption on MCF7 breast cancer cell-cycle progression, which are decreased blood insulin, circulating glucose and increased production of ketone body (beta-hydroxybutyrate). Results illustrate that in depleted glucose conditions, expression of Akt signalling pathway is downregulated, which is responsible for cell survival. AMPK, an energy sensing pathway is upregulated in response to βHB supplementation in low glucose states, which stabilizes downstream p27 protein that assists in halting cell cycle progression. Together, these findings provide evidence that creating a negative growth environment for MCF7 cells has the potential to implicate cellular energy and attenuate cell proliferation, through the depletion in glucose availability stores and increased utilization of ketone bodies.

3.2 Introduction

The principle of the ketogenic diet (KD) is a reduction in carbohydrate composition within the diet (2%) with the balance of energy being comprised of fat (≈ 90%) and protein (8%)[66]. The KD has recently gained traction, but it is by no means a new dietary intervention. It was first developed in the 1920s as a nutritional treatment for patients with epilepsy[88]. The reason behind this as treatment can be explained by the depletion in glucose availability in circulation, which forces the preference of ketone body utilization that is shown to improve brain function and reduce seizures. The KD is being studied as a reliable and permanent nutritional intervention to battle obesity and associated metabolic disorders. Previously in the lab, we have shown that adiposity can increase breast cancer proliferation by producing an adipose-dependent microenvironment that favors cell growth by altering the adipokine profile of the 'obese' adipose tissue[89]. With the KD being implemented to decrease adiposity, another beneficial effect would be an alteration in the adipokine secretion profile that accompanies weight loss to one that impairs breast cancer proliferation. However, the make-up of the diet may also have direct effects on cancer cell growth that are independent of those associated with the adipokine secretion profile.

Cancer cells exhibit an enhanced reliance on glucose metabolism for the production of energy compared to non-cancer cells. Accompanying this metabolic shift, cancer cells also prefer to convert pyruvate to lactic acid for the production of ATP, regardless in the presence of oxygen. This mechanism is one of the "hallmarks of cancer" [90] and was first described by Otto Warburg almost a century ago[66,91]. This adaptation is beneficial for cancer cells because despite the fact that they are able to expand their blood supply (capillarization) as the tumor grows, tumors tend to be under vascularized and exist in a hypoxic environment. Thus, the preference of

converting pyruvate to lactate rather than shuttling it into the mitochondrion and through the electron transport chain for ATP production allows tumors to thrive in lower oxygen environments. It becomes difficult for cancer cells to utilize the mitochondria as they harbour mitochondrial mutations leading to impaired or dysfunctional mitochondrial respiration. This, coupled with lower oxygen partial pressures, means that cancers require large amounts of glucose to satisfy the energy demands of proliferation. Due to the conversion of glucose to lactic acid, this produces far less ATP than if the pyruvate molecules generated by glycolysis are converted to acetyl CoA and shuttled through the electron transport chain[91]. The KD drastically reduces carbohydrates consumed, eliciting distinct reductions in blood glucose and insulin secretion[66]. Thus, the KD can potentially starve cancers of their primary energy source. This drastic reduction in glucose availability forces a switch in body metabolism with fat being the primary energy precursor. The increased fats/free fatty acids are taken up by the liver, whereby they are converted into ketone bodies which subsequently enter the blood stream for uptake by non-hepatic cells. This presents a conundrum for a cancer cell as its metabolism is geared to glucose as the primary energy source. The increased ketone bodies in the systemic circulation are taken up into cancer cells and converted into acetyl CoA, which bombards the cell with an accessible energy source that can enter the mitochondrion for aerobic respiration. Given that cancer cells live in a hypoxic environment and suffer from mitochondrial dysfunction, the cell would be forced to utilize the mitochondria for energy production or cease proliferation. This forced shift in metabolism may reverse the "Warburg effect" and result in a situation of increased oxidative stress and lead to an increased production of ROS as a result of improper handling of electron exchange in the electron transport chain. The intended consequence is a reduction in ATP availability, which will activate AMPK and stall proliferation, while potentially inducing programmed cell death pathways (autophagy and/or apoptosis)[92].

Understanding the fundamental impact the ketogenic diet has on cancer cells, highlights the possible adjuvant therapy in order to control cell growth by altering the environment breast cancer cells are situated in. Throughout literature, evidence shows that the KD reduces the rate of which cancers grow both *in-vivo* and *in-vitro*. The research regarding MCF7 cells and their cell cycle protein analysis in response to alterations made to surrounding glucose, insulin and ketone body levels has not been examined. The study will focus specifically on identifying key changes in the cell cycle of MCF7 breast cancer cells when environmental alterations to the medium occur. Changes that address glucose, insulin and βHB, which in turn, will have a positive outcome in order to reduce cellular proliferation of MCF7 cells. Key components in this hypothesis such as cell survival and cellular energy will be examined via Akt and AMPK, respectively.

3.3 Methods and Materials

Cell Culture

MCF7 (Michigan Cancer Foundation-7) cells were cultured in T75 flasks (Fisher Scientific, Whitby, ON) in standard Minimum Essential Medium Eagle, Alpha modification (AMEM; Sigma, Mississauga, ON), supplemented with 5% fetal bovine serum (FBS; Wisent, Montreal, QC), 3% antibiotic and antimycotic (Wisent), 1% sodium pyruvate, 1% non-essential amino acids, and 10μg/ml of human insulin (Sigma) at 37°C and 5% CO_2. Subsequently, cells were seeded in 6-well plates (Sarstedt, Montreal, QC) in standard AMEM.

Insulin, Glucose and ketone body supplementation treatments

Insulin experiments were conducted using 0, 5, and 10 mM of insulin in standard AMEM. Experiments investigating the effects of βHB and glucose were conducted in standard AMEM supplemented with 5mM of insulin.

To assess the direct effects of βHB on MCF7 growth, cells were grown in standard AMEM (5 mM insulin) and treated with physiological levels of β-Hydroxybutyrate (3-14 mM; βHB; Sigma). Cells were treated over 24, 48, and 72 hours prior to harvesting. To assess the role of altering glucose levels on the effects of βHB, MCF7 cells were cultured in glucose and pyruvate-free Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Burlington, ON) and glucose will be supplemented at concentrations of 1.25, 2.5, 5 mM along various concentrations of βHB (3-14 mM). Cells were collected after 48 hours of incubation.

Cell Harvesting

For collection, cells were washed twice with cold phosphate buffered saline (PBS; Wisent), scraped and collected into 1.7mL microcentrifuge tubes and were placed in the centrifuge (2320 x g at 4°C). The pellet was then resuspended in 0.2% TENT buffer (TRIS,

EDTA, NaCl, 0.2% Triton x-100), 1% Phosphatase inhibitor (PHOSstop, Roche, Montreal, QC), and protease Inhibitor (Sigma). Suspended cells were then sonicated and placed in the centrifuge (16168 x g at 4°C). Supernatant lysates was then collected and stored at -80°C.

Immunoblotting

A Bradford Assay was used to determine protein concentrations of cell lysates. 25 μg of protein was subjected to standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated using 12% or 15% gels at 125V for 1-1.5 hours. Following separation proteins were transferred overnight at 40V (4°C) to polyvinylidene difluoride (PVDF) membrane (Bio-rad, Missisauga, ON). Membranes were then blocked for 2 hours in 10% milk, washed in 0.5% TBST (tris-buffered saline with 0.2% Tween-20; Bioshop, Burlington, ON), then incubated overnight with primary antibodies including AMPK, $pAMPK^{T172}$, AKT, $pAKT^{T308}$, $pAKT^{S473}$ (New England Biolabs, Pickering, ON); p27 (BD Biosciences, Mississauga, ON); $p27^{T198}$ (Cedarlane Labs, Cambridge, ON); Cyclin E and COXIV (Abcam, ,MA) in 5% BSA at 4°C with shaking. Membranes were rinsed three times using TBST and incubated at room temperature with corresponding HRP-linked secondary antibodies in 5% skim milk powder in TBST with agitation for 1 hour at room temperature. Membranes were subsequently washed three times in TBST at room temperature and proteins were visualized following addition of an enhanced chemiluminescence substrate and imaged using a Kodak In Vivo FX Pro Imager (Carestream, Rochester, NY). Proteins were quantified using Carestream software. All proteins were corrected for uneven loading using β-Actin (Abcam).

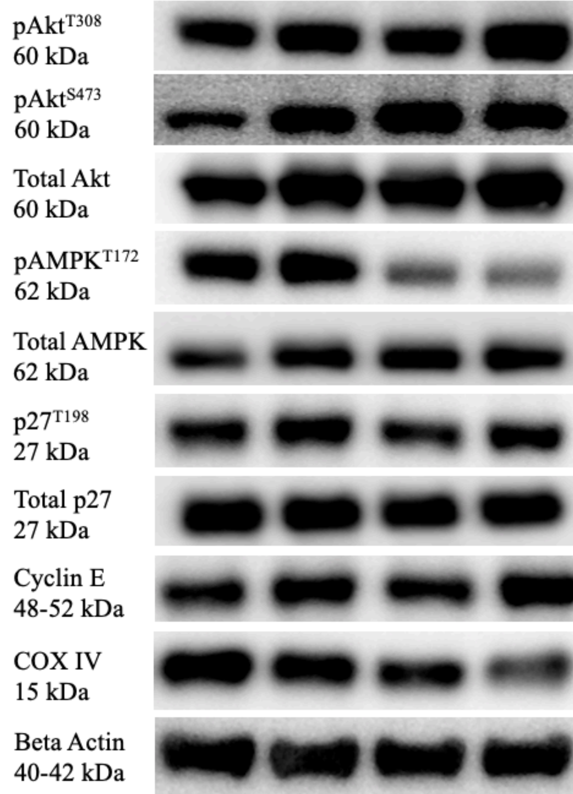
Statistical Analyses

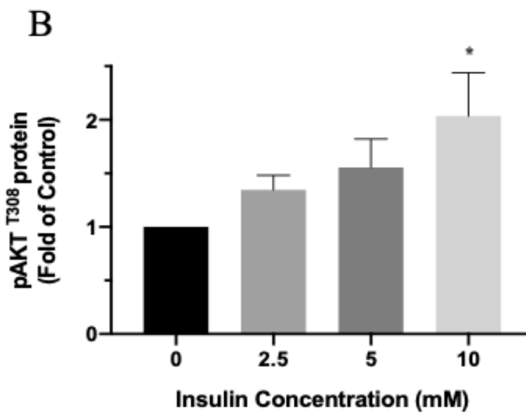
Statistical analyses were conducted using GraphPad Prism 5 software. One-way ANOVA was used to determine significant differences treatment main effects and Tukey's post-hoc test was used to detect differences between individual data points. Means will be considered statistically significant when $p \leq 0.05$.

3.4 Results

3.4.1 Insulin dosage on MCF7 cells after 48 hours upregulates cell cycle proteins

One of the major body chemistry adaptations to KD consumption is a reduction in blood insulin levels. Furthermore, insulin has been characterized as an eliciting proliferative effect. As such, initial experiments examined the effects of reducing insulin exposure on various proteins that control proliferation (Fig. 7A-J) and a measure of cellular mitochondrial content (COXIV, Fig. 7J). Reducing insulin concentrations resulted in dose-dependent reductions in $pAkt^{T308}$ and $pAkt^{S473}$ protein levels when examining concentrations from 10mM down to 2.5mM and 0mM (Fig. 7B-C). Similarly, cyclin E protein expression was also lower as insulin levels were decreased from 10mM (Fig. 7E). Decreasing insulin did not elicit any significant changes in $AMPK^{T172}$, p27 and $p27^{T198}$ protein levels (Fig. 7F, H-I). Decreasing insulin exposure appeared to increase mitochondrial content as evidenced by the increase in the protein levels of mitochondrial marker COX IV (Fig. 7J). There was no observed change in total AMPK and total AKT protein levels across all insulin doses (Fig. 7G).


pAkt^{T308}
60 kDa
pAkt^{S473}
60 kDa
Total Akt
60 kDa
pAMPK^{T172}
62 kDa
Total AMPK
62 kDa
p27^{T198}
27 kDa
Total p27
27 kDa
Cyclin E
48-52 kDa
COX IV
15 kDa
Beta Actin
40-42 kDa
A. Insulin: 0mM 2.5mM 5mM 10mM


B
pAKT ^{T308} protein (Fold of Control)
Insulin Concentration (mM)
0
2.5
5
10
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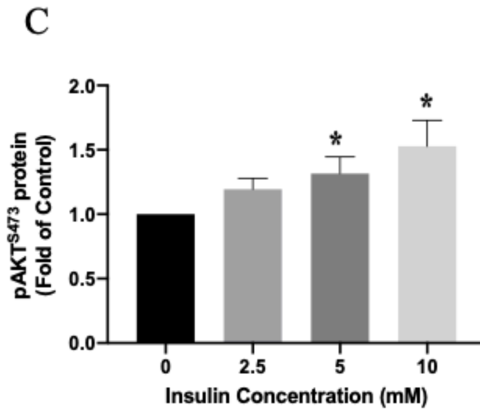
C
pAKT^{S473} protein (Fold of Control)
Insulin Concentration (mM)
0
2.5
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10
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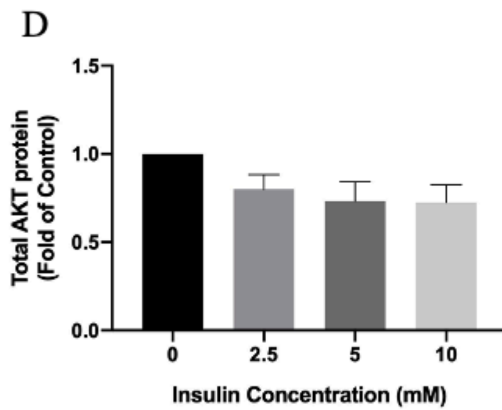
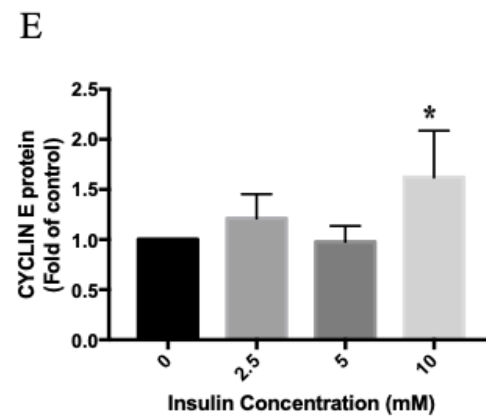

D
Total AKT protein (Fold of Control)
Insulin Concentration (mM)
0
2.5
5
10


E
CYCLIN E protein (Fold of control)
Insulin Concentration (mM)
0
2.5
5
10
*

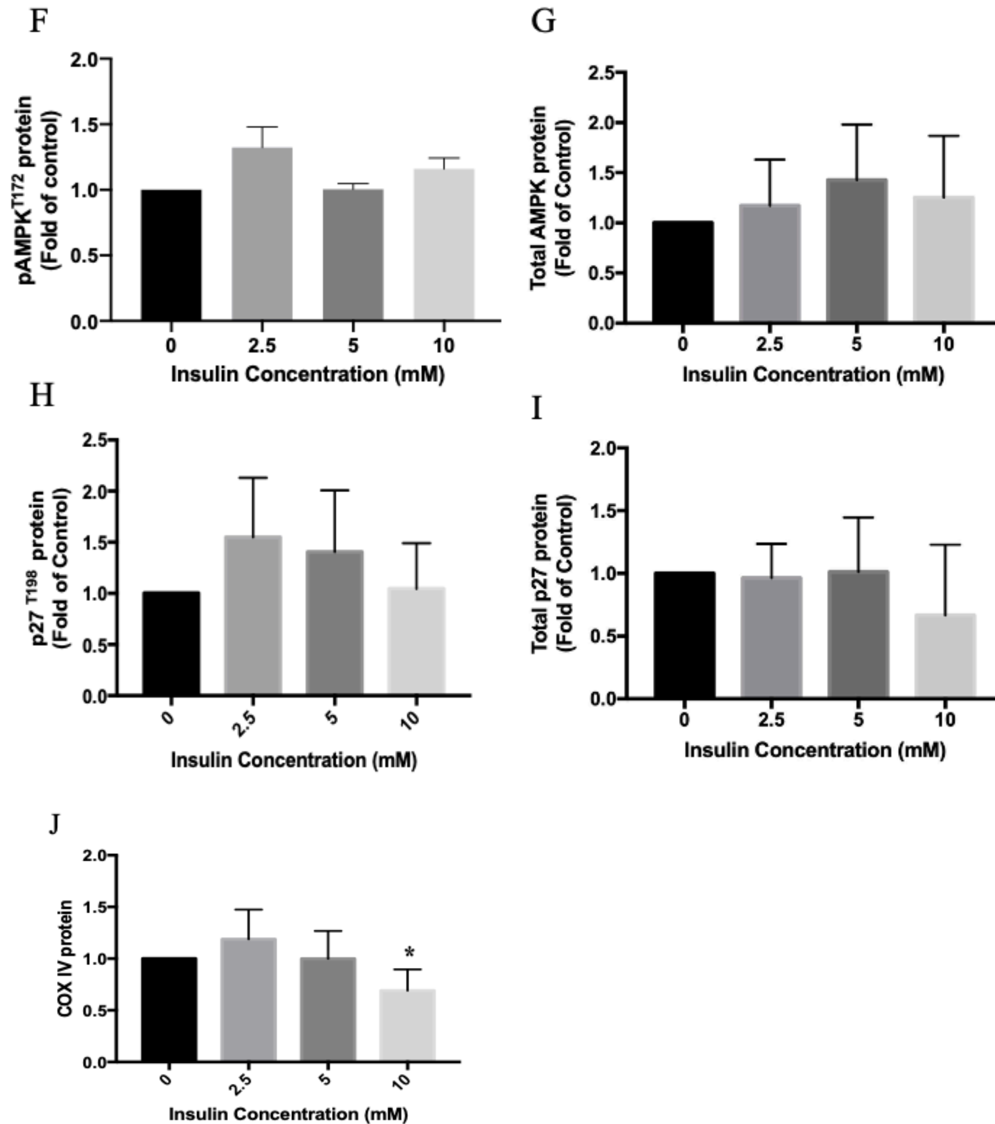
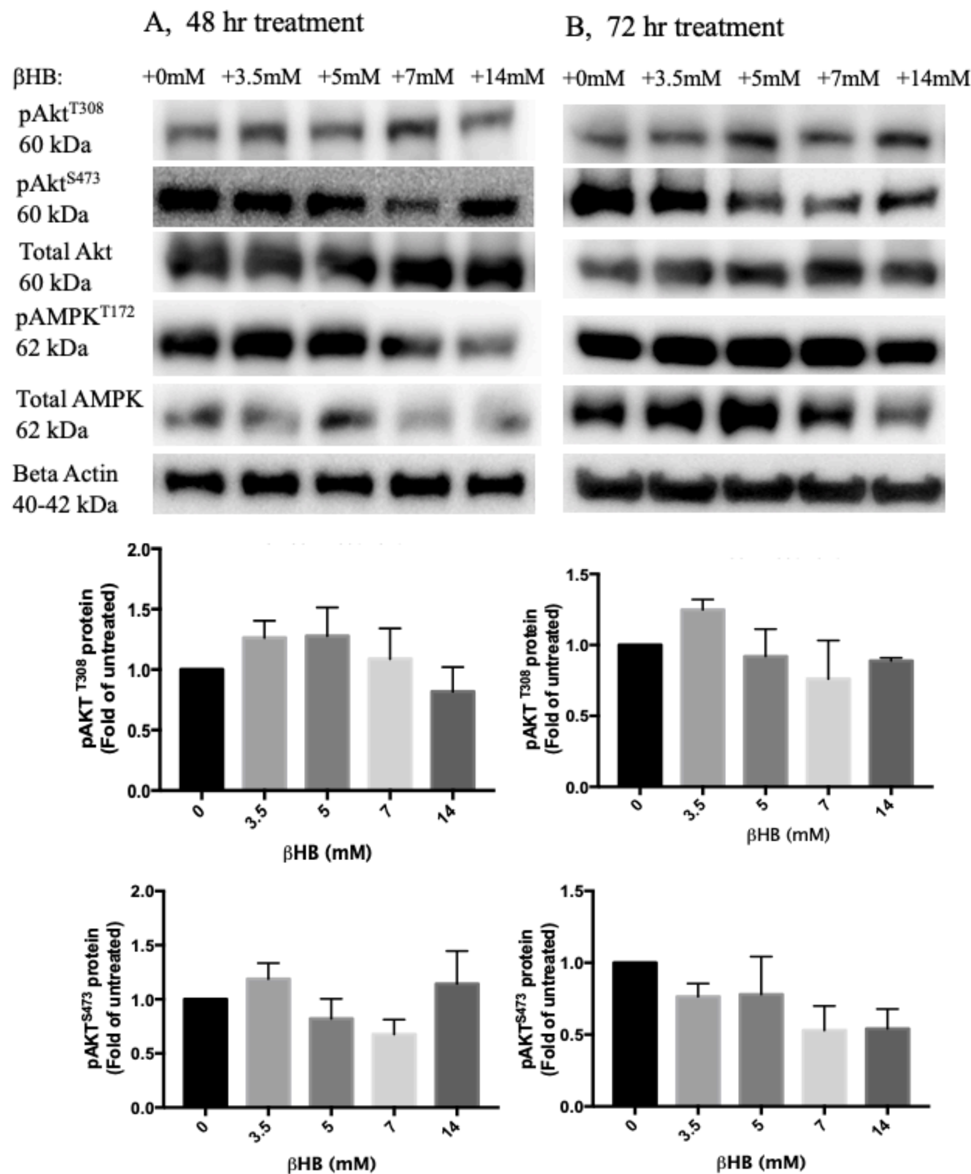


Figure 7, Insulin dosage on MCF7 cells after 48 hours upregulates activation of phosphorylated Akt. Expression in $pAkt^{T308}$ (p = 0.0448) and $pAkt^{S473}$ (p = 0.0390) (**B, C**) compared to zero insulin conditions. Cyclin E was significantly upregulated at 10mM of insulin (p = 0.0113) (**E**). Cox IV decreases expression in high insulin levels compared to the lower doses (p = 0.0349) (**J**). pAMPK ^{T172}, total AMPK, $p27^{T198}$ and total p27 show no significant changes under these dosages (**F-I**). All values reported are mean with SEM bars; $p<0.05$ compared to control (n=6,8).

3.4.2 βHB does not elicit adaptations in AKT or AMPK signaling pathways in the presence of high glucose concentrations

KD consumption increases the concentration of the ketone βHB in the peripheral circulation. To evaluate the direct effects of βHB on the expression of cell signaling and cell cycle regulatory proteins, MCF7 were treated with βHB in 5mM insulin high glucose (10 mM) medium for 48 hrs (Fig. 8A). Surprisingly, even at high levels of βHB (14 mM) the effects on protein expression were not evident. Similarly, at 72hrs βHB had no affect (Fig 8B).



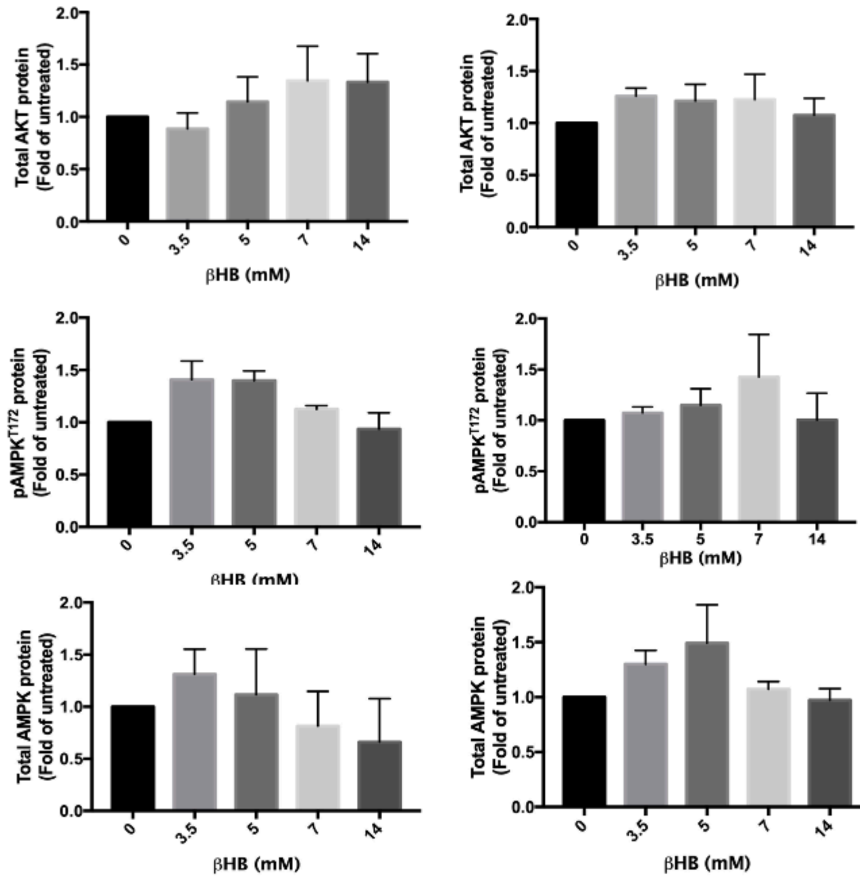


Figure 8, βHB dosage in 5mM insulin and high glucose media does not elicit adaptations in AKT or AMPK signaling pathways

βHB treatments in 5mM of insulin over 48 and 72 hours. Statistical significance was not attained during these time course examinations. Although trends can be seen, the addition of βHB is not seen to impact regulation in the presence of high (10mM) medium glucose (n=4).

3.4.3 βHB and cell cycle regulatory proteins after 72-hour treatment in high glucose media

Western blots show the expression of cyclin E, $p27^{T198}$, total p27 and Cox IV in 10mM glucose with βHB supplementation. Expression levels of cyclin E, Cox IV and total p27 do not exhibit any cell cycle changes in response to βHB (figs. 9B, D-E). While $p27^{T198}$ in figure 9C ($p=0.0454$), a downstream protein residue phosphorylated by AMPK, is upregulated at 7mM of βHB, this was not exhibited in its upstream regulator AMPK in figure 8. Overall, these cell cycle regulatory proteins are unchanging throughout βHB treatment and have no impact on the growth even in the 72-hour time course.

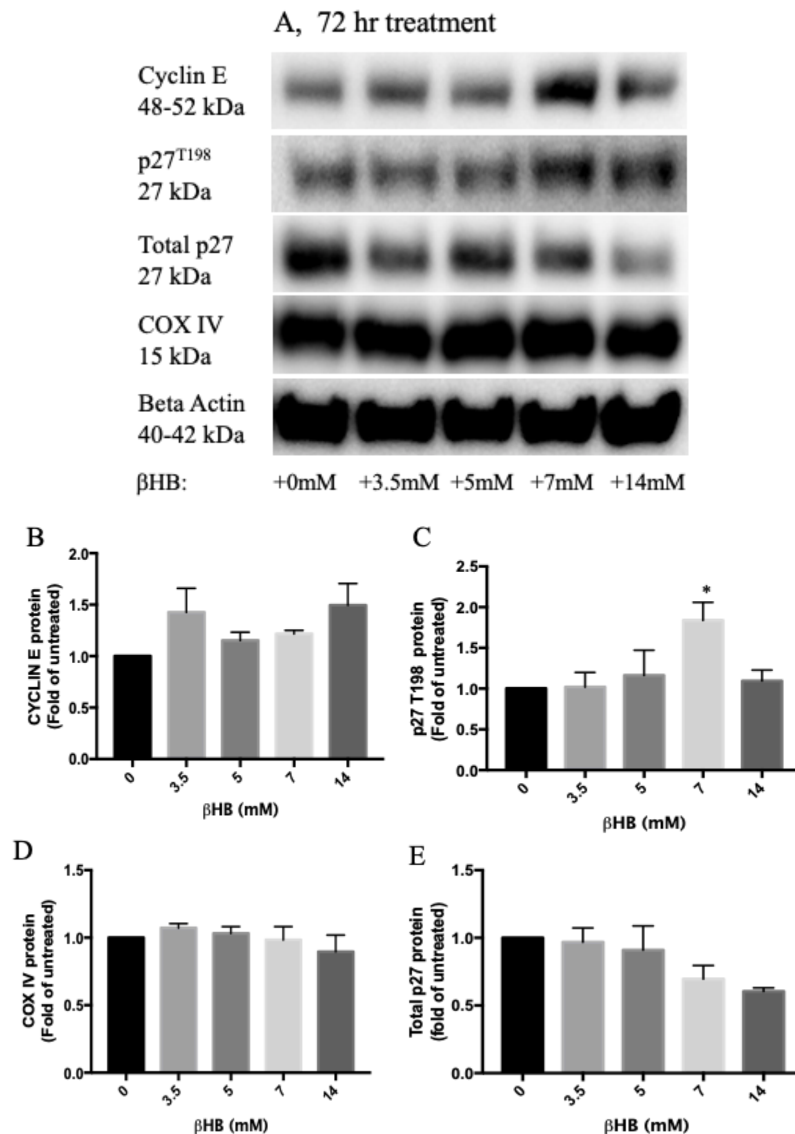


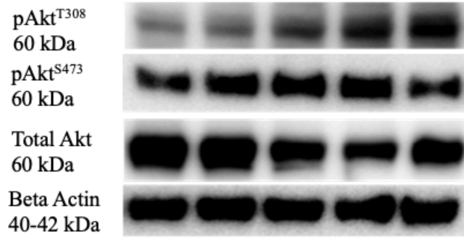
Figure 9, βHB dosage in 5mM insulin and high glucose media is shown to impact phosphorylated p27 but have no adaptations on cyclin E or cox IV

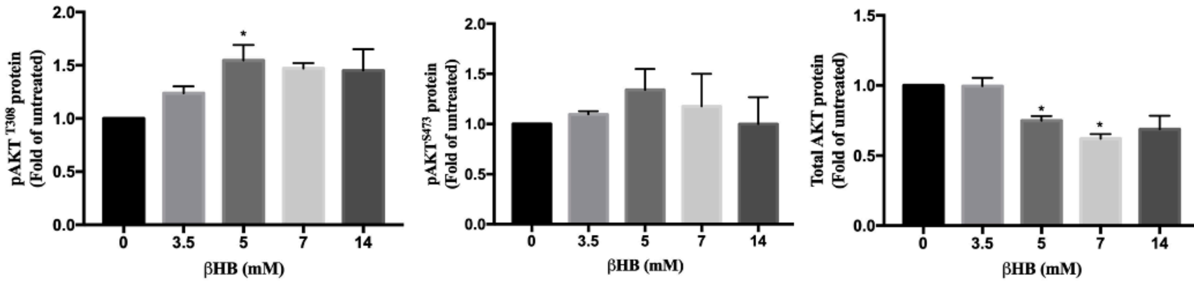
βHB treatment in 5mM of insulin over 72-hours. **A,** western blots showing the expression of cyclin E, Cox IV, $p27^{T198}$ and total p27 protein. No evident significant changes were seen in pooled data of cyclin E **B**, Cox IV **D** and total p27 **E**. $p27^{T198}$ shows significance for 7mM βHB relative to 0mM βHB ($p=0.0454$) ($n=4$).

3.4.4 βHB decreases the activation of Akt as glucose levels decline

Given that during nutritional ketosis both insulin and glucose levels are decreased, we set out to evaluate whether βHB elicits more evident effects as glucose levels decline. When exposed to βHB in high glucose (10 mM) conditions, MCF 7 cells appeared to be unresponsive to ketone bodies (Fig. 9). Reducing glucose to below 5 mM there was an apparent deactivation of the Akt pathway, as evidenced by reductions in $pAkt^{T308}$ and Akt^{S473} (Fig. 10A-D). The effects of βHB appeared to be more extreme and predictable at the lowest glucose concentrations. Total Akt levels were not affected by βHB.

A





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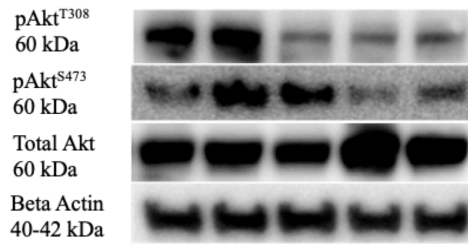
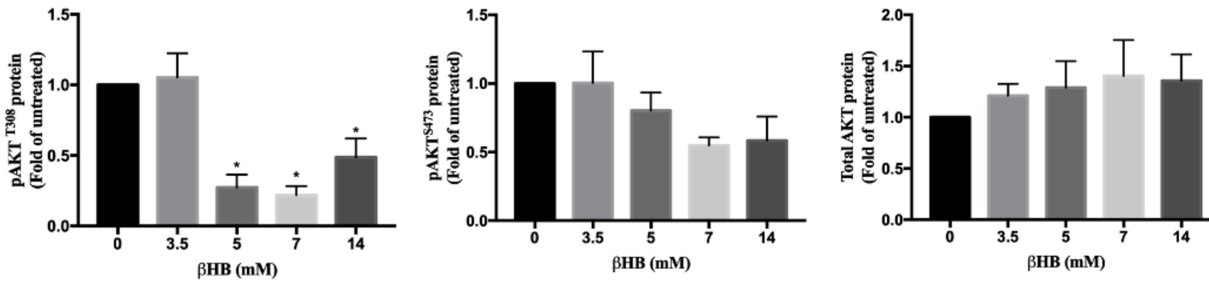
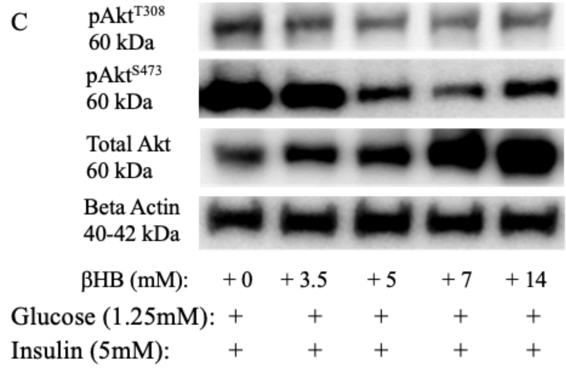
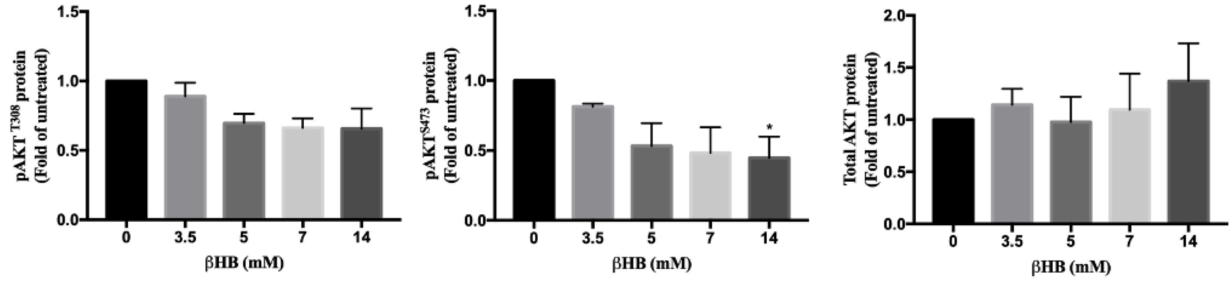


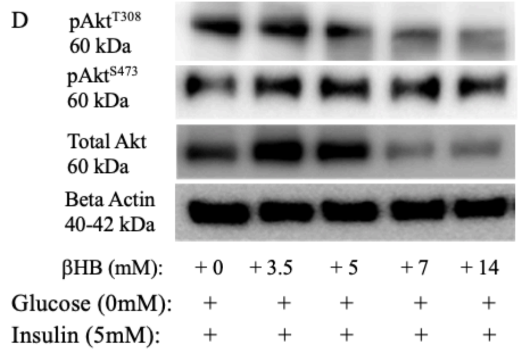


C





D



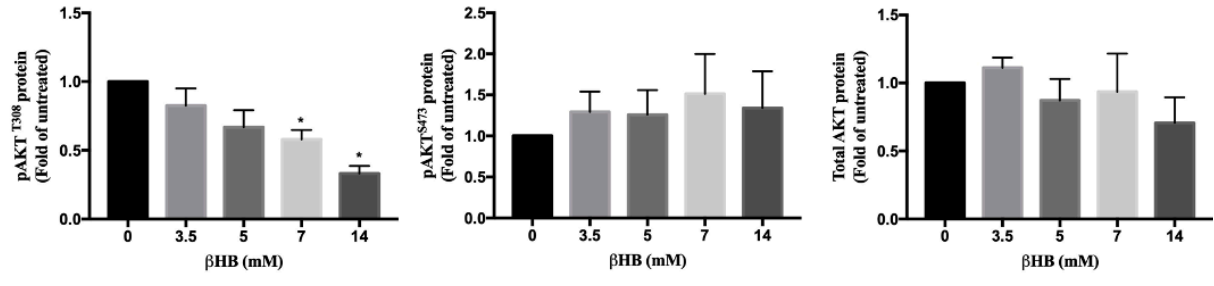
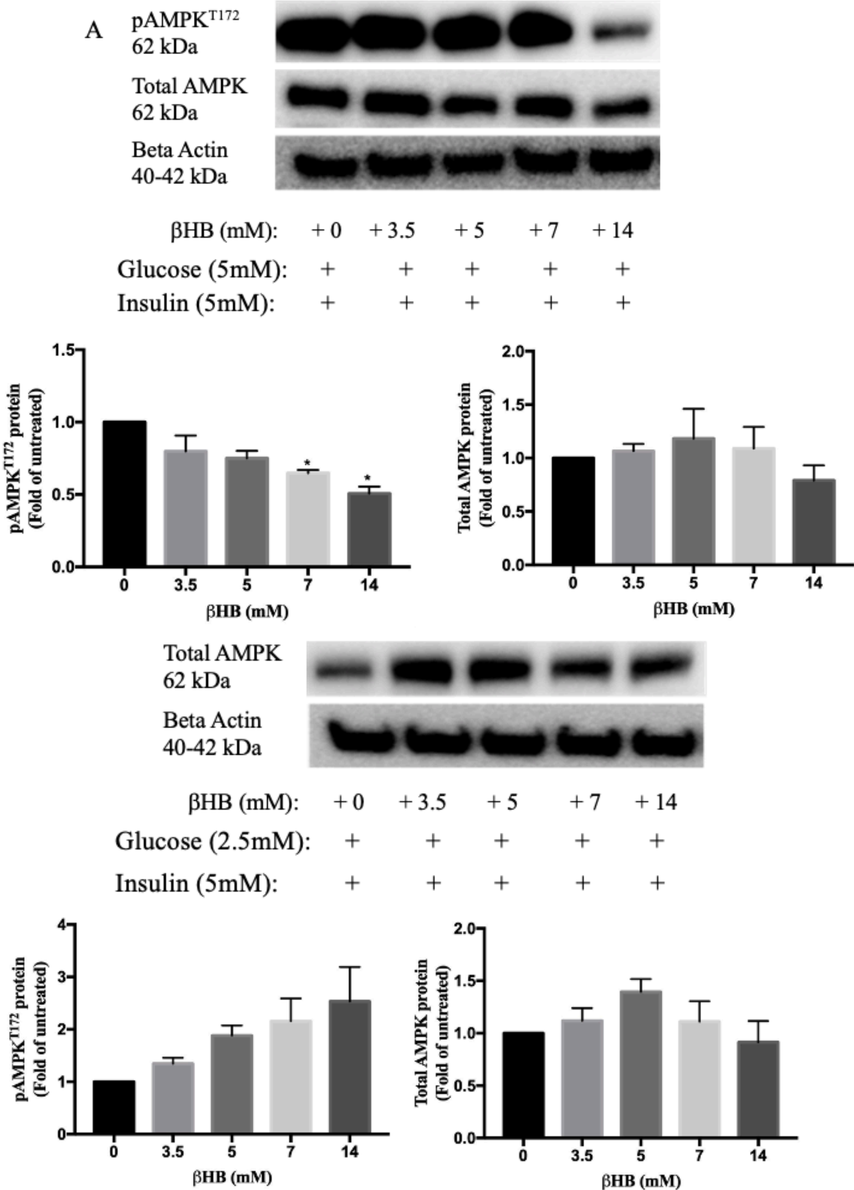
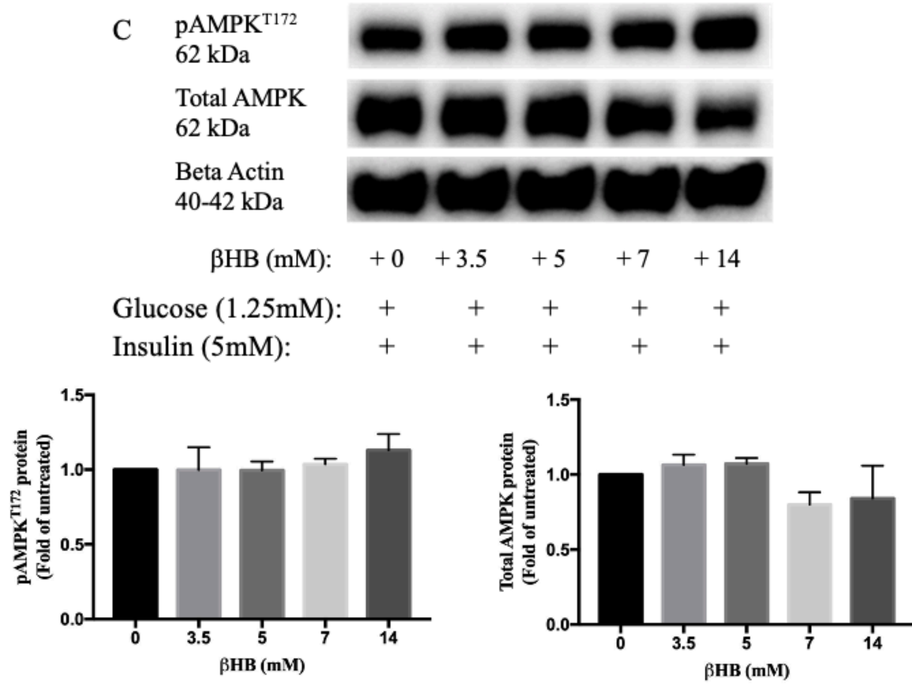


Figure 10, A – D, βHB decreases the activation of phosphorylated Akt in low glucose levels Akt^{T308} expression in glucose concentrations of 0, 1.25, 2.5 and 5mM. MCF7 cells responded with a significant decrease in Akt^{T308} expression at 2.5mM, 1.25mM and 2.5mM (**B**, p=0.0004, **C**, p=0.0293, **D**, p=0.0010) when the level of βHB increased. Whereas, in high glucose media at 5mM, as expected, a significant increase in Akt^{T308} protein expression (**A**, p=0.0290). Akt^{S473} protein expression in media containing 1.25mM of glucose showed significant decreases as ketone bodies increased in media, with p=0.0224 (**C**). In trials with 5mM, 2.5mM and 0mM glucose, saw no significant reductions in Akt^{S473} protein level. In groups 0, 1.25 and 2.5mM of glucose, total Akt expression did not lead to any significant alterations to expression levels. Total Akt expression in 5mM of glucose **(A)** expressed significant reductions in protein phosphorylation (p=0.0003). All values are mean ± S.E.M; *p<0.05 (n=4).

3.4.5 AMPK demonstrates a biphasic response to βHB as glucose concentrations decrease

When glucose was decreased to 5mM there was a reduction in $pAMPK^{T172}$ in response to βHB (Fig. 11A). As MCF7 cells were progressively starved of glucose, their preferred energy source, βHB increased $pAMPK^{172}$ protein levels (Fig. 11B-D). As expected, no change is seen in total AMPK levels in response to βHB (Figs. 11C-D).




C
pAMPK T172
62 kDa
Total AMPK
62 kDa
Beta Actin
40-42 kDa
βHB (mM): + 0 + 3.5 + 5 + 7 + 14
Glucose (1.25mM): + + + + +
Insulin (5mM): + + + + +
pAMPK T172 protein (Fold of untreated)
1.5
1.0
0.5
0.0
0 3.5 5 7 14
βHB (mM)
Total AMPK protein (Fold of untreated)
1.5
1.0
0.5
0.0
0 3.5 5 7 14
βHB (mM)

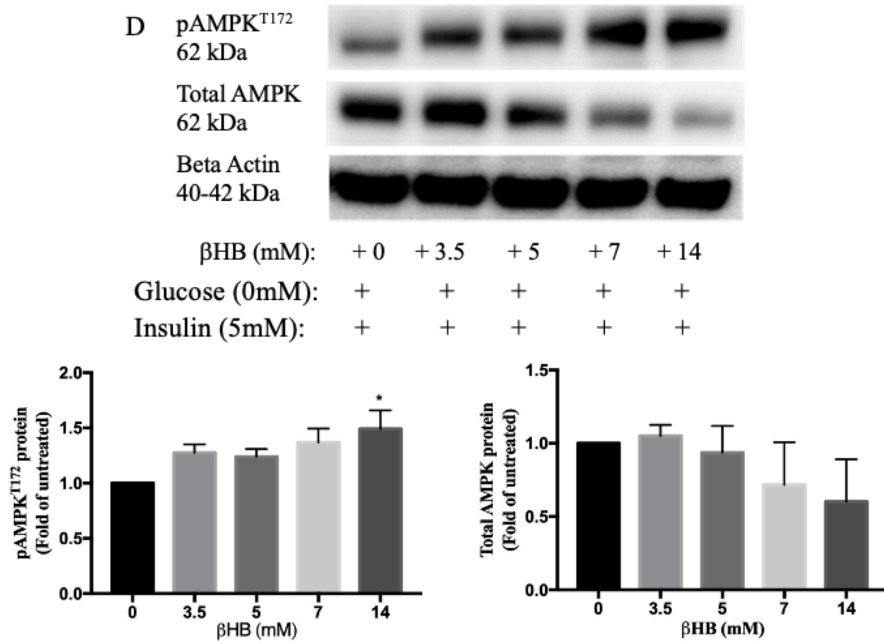
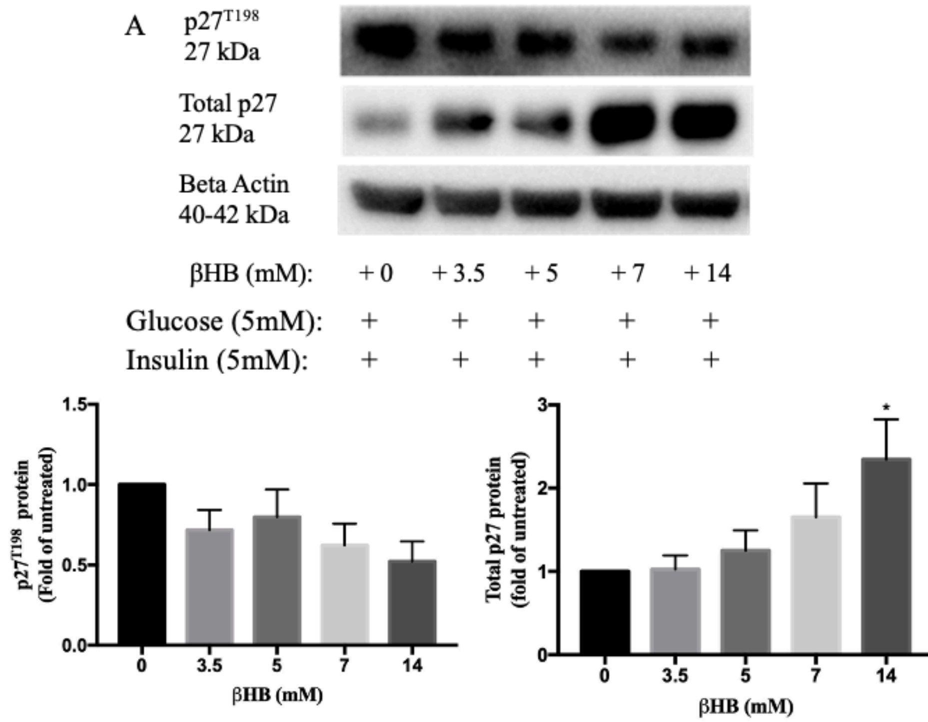

D
pAMPK T172
62 kDa
Total AMPK
62 kDa
Beta Actin
40-42 kDa
βHB (mM): + 0 + 3.5 + 5 + 7 + 14
Glucose (0mM): + + + + +
Insulin (5mM): + + + + +
pAMPK T172 protein (Fold of untreated)
2.0
1.5
1.0
0.5
0.0
*
0 3.5 5 7 14
βHB (mM)
Total AMPK protein (Fold of untreated)
1.5
1.0
0.5
0.0
0 3.5 5 7 14
βHB (mM)

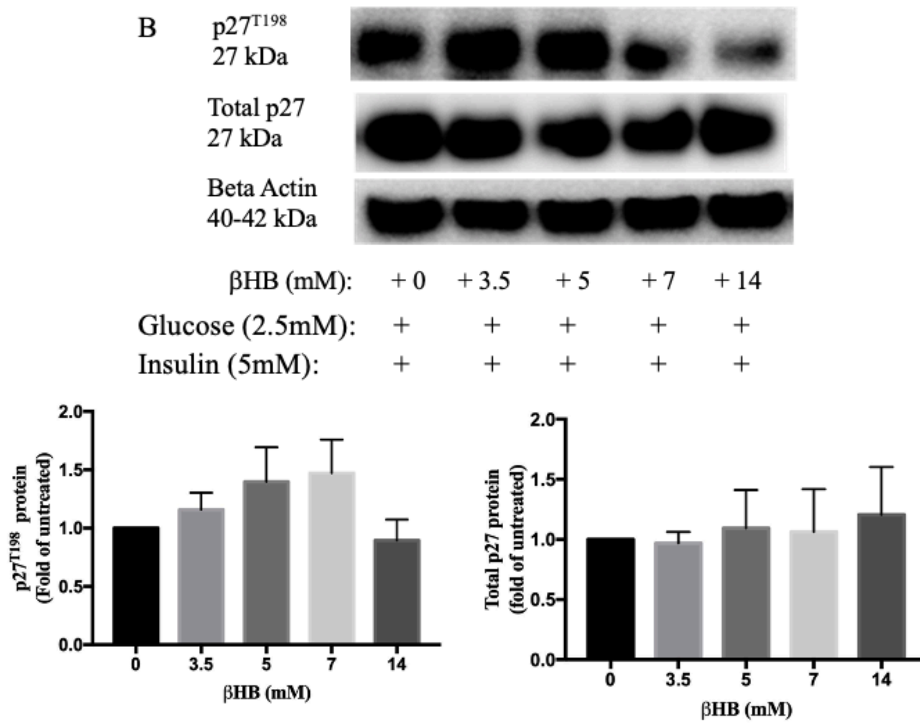
Figure 11: AMPK responds distinctively to βHB as glucose concentrations decrease

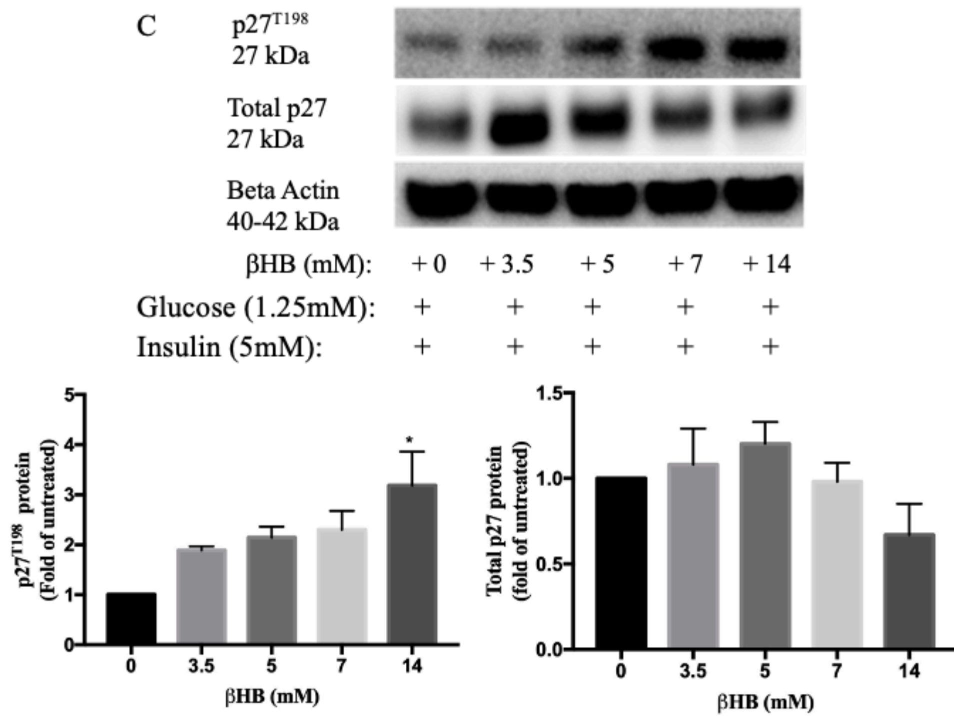
As glucose dose changed to 5mM, there is a significant downregulation in pAMPK^{T172} expression in a dose-dependent pattern as βHB concentration increases (**A**, p=0.004). No significant changes were seen in glucose groups 2.5mM and 1.25mM (**B and C**, respectively). What can be gathered from glucose dose 2.5mM **(B)** is the upregulation trend that occurs in a dose-dependent manner in response to elevated ketone bodies. When glucose was depleted further to 0mM, pAMPK^{T172} is upregulated as the concentration of βHB increases (**D**, p=0.0441). No significant changes were observed in total AMPK levels in any glucose conditions. All values are mean ± S.E.M; *p<0.05 (n=4).

3.4.6 βHB induced activation of AMPK leads to p27 phosphorylation

Phosphorylation of p27 at T198 by AMPK has been previously shown to stabilize the protein and promote cell cycle arrest[93]. Given that βHB activates AMPK we measured the response of p27 and its phosphorylation at T198 in response to increased ketone exposure. At 5mM glucose (fig. 12A) there was a reduction in p27^{T98} and an increase in total p27. As glucose concentrations decreased this pattern changed with p27^{T198} protein levels increased, specifically at 1.25mM glucose condition (fig. 12C) and total p27 levels decreased with βHB treatment (fig. 12B-D). Taken together these results suggest that βHB induce alterations in the p27 pool.


A
p27T198
27 kDa
Total p27
27 kDa
Beta Actin
40-42 kDa
βHB (mM): + 0 + 3.5 + 5 + 7 + 14
Glucose (5mM): + + + + +
Insulin (5mM): + + + + +
p27T198 protein (Fold of untreated)
Total p27 protein (fold of untreated)
0 3.5 5 7 14
βHB (mM)
*


B
p27T198
27 kDa
Total p27
27 kDa
Beta Actin
40-42 kDa
βHB (mM): + 0 + 3.5 + 5 + 7 + 14
Glucose (2.5mM): + + + + +
Insulin (5mM): + + + + +
p27T198 protein (Fold of untreated)
Total p27 protein (fold of untreated)
0 3.5 5 7 14
βHB (mM)


C
p27T198
27 kDa
Total p27
27 kDa
Beta Actin
40-42 kDa
βHB (mM): + 0 + 3.5 + 5 + 7 + 14
Glucose (1.25mM): + + + + +
Insulin (5mM): + + + + +
p27T198 protein (Fold of untreated)
Total p27 protein (fold of untreated)
βHB (mM)
0 3.5 5 7 14

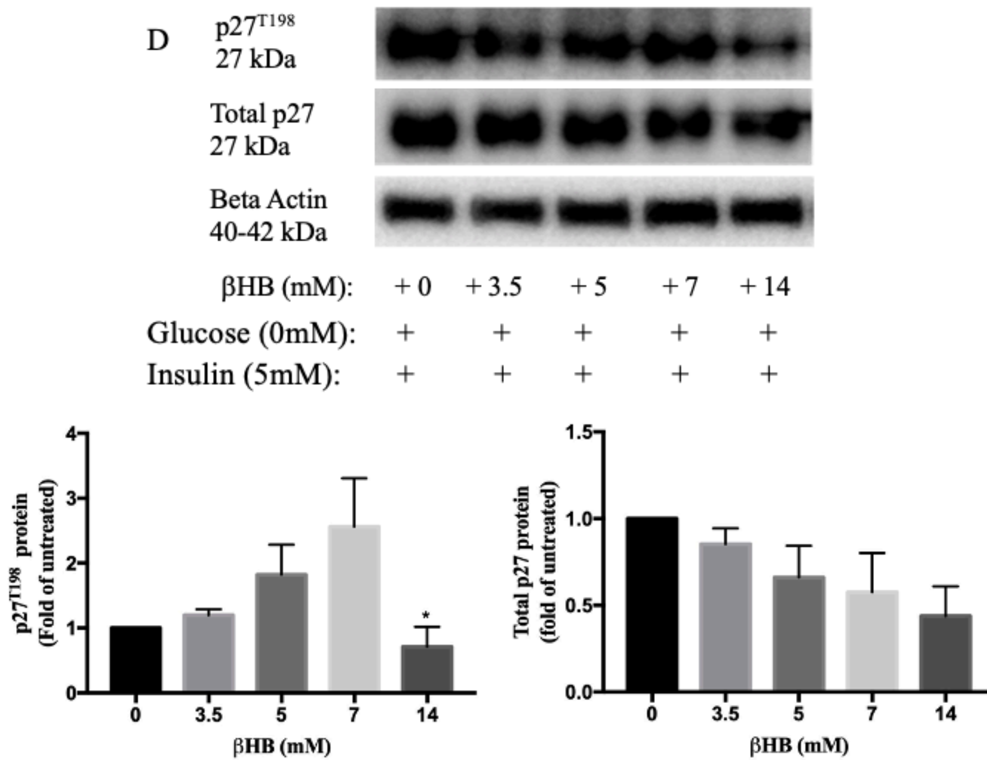

D
p27T198
27 kDa
Total p27
27 kDa
Beta Actin
40-42 kDa
βHB (mM): + 0 + 3.5 + 5 + 7 + 14
Glucose (0mM): + + + + +
Insulin (5mM): + + + + +
p27T198 protein (Fold of untreated)
Total p27 protein (fold of untreated)
βHB (mM)
0 3.5 5 7 14

Figure 12: In low glucose levels, βHB induced activation of AMPK leads to p27 phosphorylation

Downregulation of $p27^{T198}$ is seen in 5mM glucose conditions but were not statistically significant **(A)**. As the shift of energy substrate is occurring in a more glucose depleted environment, insignificant changes can be seen in 2.5mM conditions **(B)**. At 1.25mM glucose, **C** illustrates the dose-dependent increase in $p27^{T198}$ protein levels in response to increased βHB levels (p=0.0050). $p27^{T198}$ expression increased steadily across 0, 3.5 and 7mM βHB concentrations until a significant decrease at 14mM of βHB in no glucose condition (**D**, p=0.0402). Total p27 protein expression did not significantly decrease in low glucose states **(C-D)** but trended downward in response to βHB treatment. As expected, total p27 expression was upregulated in glucose 5mM as βHB treatments were added (**A**, p=0.0405). All values are mean ± S.E.M; $^{*}p<0.05$ (n=4).

3.4.7 βHB supplementation decreases cyclin E protein expression

Cell cycle status is dependent on numerous regulator proteins. We evaluated the effects of βHB on the G1 promoting protein cyclin E. At 5 mM glucose cyclin E is unaffected by βHB (Fig. 13A). However, as glucose level drop below 5mM, cyclin E protein levels decrease as βHB concentration increases (Fig. 13B-D).

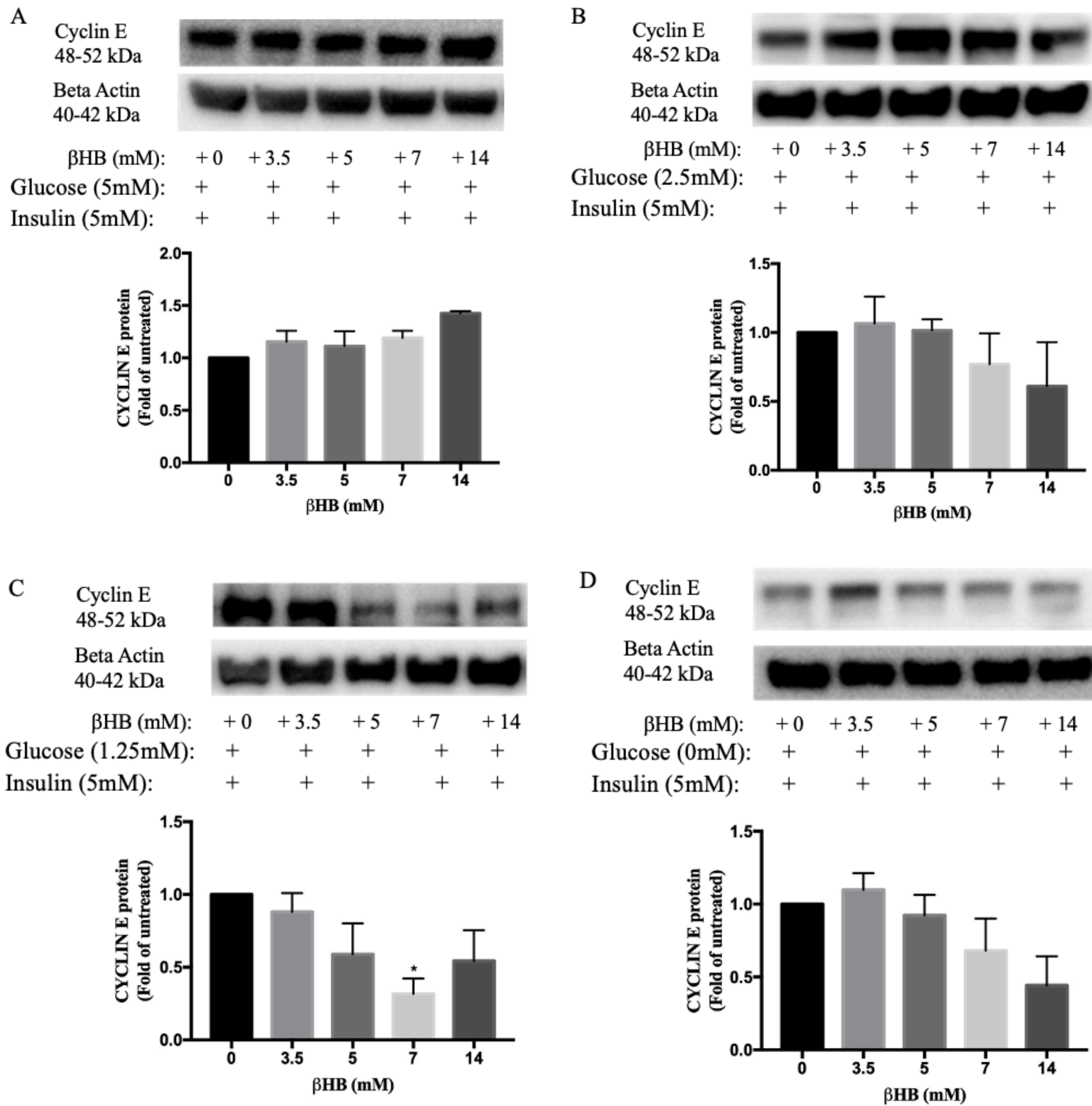


Figure 13: As glucose conditions decrease and βHB supplementation increases, cyclin E protein expression downregulates

In 5mM glucose, βHB treatments had no impact on cyclin E expression **(A).** As glucose is depleted in conditions of 2.4mM, 1.25mM and 0mM, cyclin E expression is downregulated with ketone supplementation **(B-D)** with significance seen in 1.25mM glucose group **(C**, p=0.0429). All values are mean ± S.E.M; *p<0.05 (n=4).

3.4.8 βHB treatments do not affect mitochondrial content

We also measured the levels of COXIV protein, a standard marker of mitochondrial content. At lower glucose concentrations βHB activated AMPK (Figure 11), an important step in the initiation of cellular mitochondrial biogenesis. Furthermore, starving the cells of glucose while flooding it with βHB may shift the cellular metabolism to rely more on aerobic means to generate ATP. As MCF7 cells were starved of glucose and treated with βHB there were no effects on COXIV protein expression after 72 hours of treatment (Figures 14A-D).

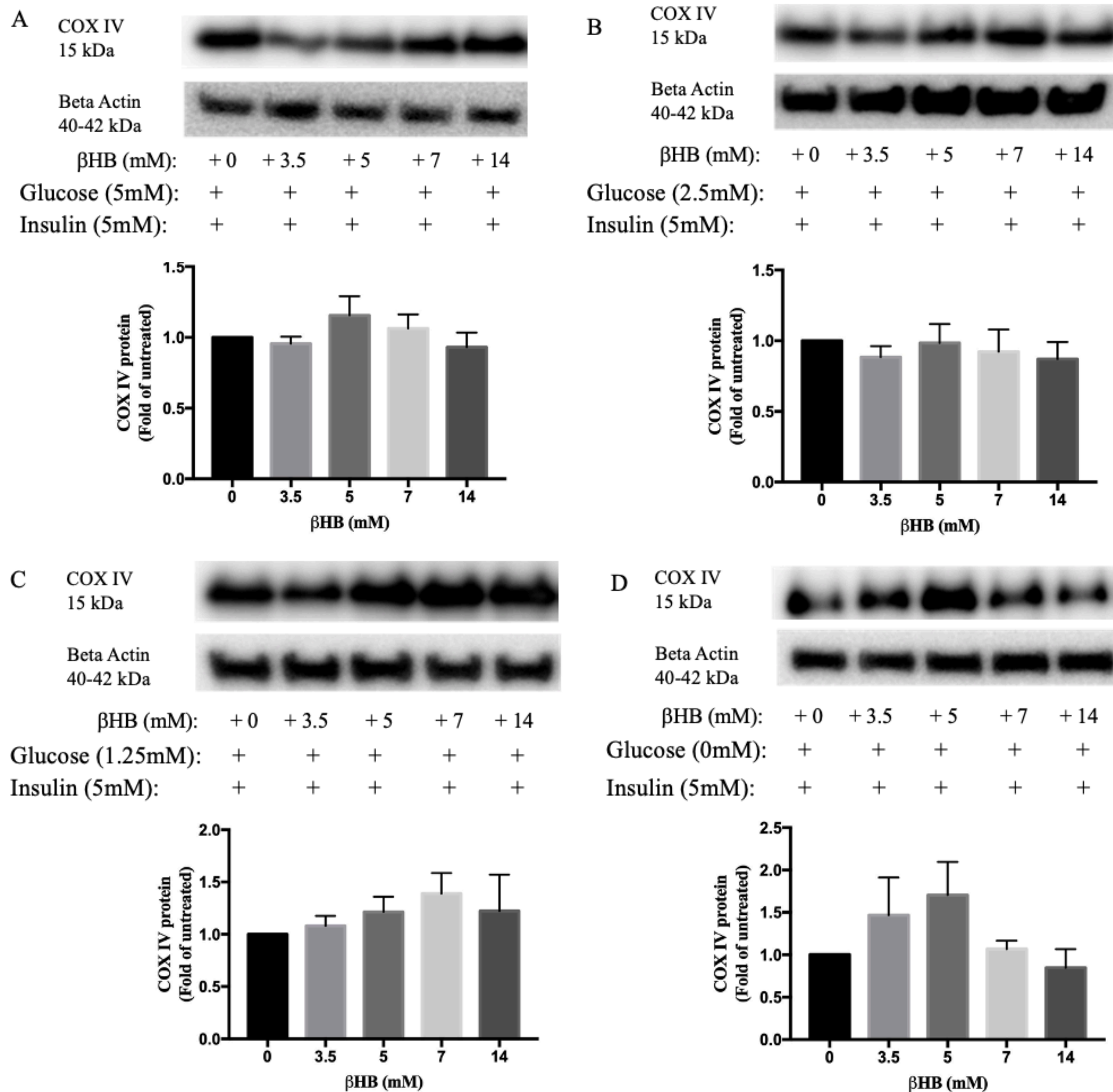


Figure 14: βHB exposure does not elicit mitochondrial content changes

βHB treatments in media that contained 5mM and 2.5mM of glucose had no significant difference in COX IV expression **(A and B)**. The expression of COX IV presented trends in glucose conditions of 1.25mM and 0mM but it was evident that no significant differences had been reached **(C and D)**. All values are mean ± S.E.M; *$p<0.05$ (n=4).

3.5 Discussion

With the incidence of breast cancer and fatality rates continually on the rise, there is an increasing need to determine novel interventions to improve patient prognosis. It is blatantly evident in literature that a reduction in adipose tissue decreases the growth of breast cancers. One dietary intervention that has risen to prominence in obesity management is the ketogenic diet, comprised mainly of high fat and low carbohydrate. The body responds to the KD by increasing circulating ketone bodies which can be used as a primary source of fuel in metabolically active cells. We used a cell culture model to isolate the effects of the three main physiological changes followed by the ketogenic diet. Decreased insulin, decreased circulating glucose and increased production of ketone bodies (mainly βHB) are the conditions that predominately change following adherence to the ketogenic diet. By isolating these factors to MCF7 cells, we study the specific link of cell cycle regulation within breast cancer cells. An early 2000 study examined the effects of the ketogenic diet over 24 weeks and observed significant reductions in body weight, body mass index, triglycerides, low density lipoprotein cholesterol and blood glucose[94]. In a more recent study, effects of glucose levels on the proliferation of breast cancer cells were examined[95]. They found that breast cancer cells grown in high glucose medium promoted the proliferation of cells versus cells grown in low glucose media[95]. These two reports are few of many that support the rationale that changes made to the microenvironment leads to alterations made to tumour growth. Manipulating specific variables that aid in cancer cell cycle regulation is beneficial to creating an adjuvant cancer therapy. Thus, effectiveness of physiological dosages of βHB are only evident in a low glucose and low insulin environment. This means that administration of βHB alone will not serve as a potential therapy, all physiological changes induced by the KD consumption need to occur. This is in line with previous research in rats

where βHB administration to obese animals actually enhanced breast cancer growth[94,95]. Our results suggest that adding βHB alone only adds an exogenous energy source and the reduction in blood glucose availability is necessary to affect proliferation, likely due to an internal metabolic shift of the Wartburg effect.

Morphological observations of MCF7 cells were photographed pre and post 48-hour βHB treatment (supplemental figure 16). Interpretations upon images revealed in low insulin and low glucose environments, MCF7 breast cancer cells respond to βHB more distinct. This is described by the swelling and increased light exposure on the parameter of the cell. This feature has been seen in previous studies that portray MCF7 cells upon swelling, in which cellular membrane does not change in size[96]. In this study, it was shown that 7mM of βHB in the absence of glucose and 1.25mM of glucose could cause this cellular membrane swelling feature, signifying no change in size to the MCF7 cells. In comparison to βHB treatment in high glucose media, MCF7 cells are seen to proliferate and grow in size regardless the presence of ketone bodies. High glucose media can show MCF7 cells proliferate rapidly over a short period of time. Versus low glucose media, where the migration of MCF7 cells are slower at reaching maximum confluency[97].

Insulin elicits direct mitogenic effects through its action on cancer cell growth, invasion and cancer angiogenesis, as well as its role in promoting estrogen and IGF response in healthy and malignant breast cancer cells[98,99]. As figure 7A-B highlights MCF7 cell cycle effects when insulin levels increase, Akt pathway and cyclin E protein increase activation and promote cell invasion. When tested effects of βHB on MCF7 cells in conditions of high glucose, figure 8, we no change in the phosphorylation of cell cycle promoting proteins. This can be highlighted through past studies which saw high glucose conditions was responsible for the increased

viability, proliferation and invasion in MCF7 cells[100]. As insulin and glucose are both mediators in the mitogenic activation of the Akt pathway, cancer cells respond with increase glycolytic and lactate rates as a way to produce ATP for energy[101]. Importantly, when the level of glucose was restricted to physiological fasting levels, 1.1 - 2.5mM[102] and insulin held at 5mM, results demonstrate that βHB had an effect on $pAkt^{T308/S473}$ and thus, cell cycle proliferation (figure 10A-C). Results show the phosphorylation and activation of Akt pathway, which is a marker of mTOR activation, decreases as the range of βHB increases. The idea of restricting insulin and glucose and providing MCF7 cells with an alternative energy precursor, allows us to make an association between the ketogenic diet and reducing cancer cell invasion. A study involving a rat model fed the ketogenic diet presented a downregulation of mTOR activation, Akt being the downstream target protein[86]. Following the ketogenic diet, consumption for βHB increases and renders cancer cells less reliant on glucose and the need for aerobic glycolysis, as the shift to mitochondrial respiration and oxidative phosphorylation acts as a natural barrier in reducing cancer cell growth subsequently because of the dysfunction to the mitochondria.

AMPK is a crucial energy sensor governing cell metabolism. As the AMPK pathway and PI3K/Akt cascade control and coordinate bioenergetics and cell viability, they do so with opposing regulatory effects as they respond to extracellular conditions. Insulin promotes the uptake of glucose and inhibits AMPK by inducing the phosphorylation of Akt[103]. This finding supports our result in figure 7F, as no changes was seen in the activation of AMPK when MCF7 cells were cultured in high insulin conditions. In turn, when insulin levels were dropped to 2.5mM, phosphorylation of $AMPK^{T172}$ is upregulated. Likewise, when insulin and glucose were both depleted, phosphorylated AMPK expression was upregulated. This result is similar to a study which applied this treatment of low insulin and low glucose to endothelial cell growth, in

which endothelial cells exhibited low AMPK levels[104]. AMPK acts as a cellular trigger to inhibit cell proliferation by activating ATP producing catabolic pathways and decreasing ATP-consuming anabolic mechanisms in an effort to boost cellular ATP stores[105]. Sources from previous research have shown low glucose conditions induces S-phase cell cycle arrest in endometrial cancer cells with increased phosphorylation of AMPK, a result consistent with what we see in MCF7 cells[84]. Research involving a mouse model carrying astrocytoma cells, a form of cancer found in the brain or spinal cord, found that mice on a KD increased mean survival time via the upregulation of AMPK compared to mice on a standard diet[106]. Ketosis activates a metabolic checkpoint and arrests cellular growth in conditions of low ATP levels, increases factors that activate AMPK and inhibits factors that decrease AMPK[107]. The increase in AMPK levels at low glucose with supplementation of βHB supports the rational that shifting the energy source of MCF7 breast cancer cells may reduce the high energy consuming processes and therefore reduce the cancer cell growth rate.

Previous studies have indicated that p27 phosphorylated on terminal threonine 198 by AMPK triggers its stabilization[108]. It has been seen through studies that modulation of AMPK signaling alters p27 expression levels[93]. In accordance with this, an early 2000 study saw that both AMPK signalling and p27 levels in pre-neoplastic cells increased upon exposure to various nutritional and chemopreventive anti-cancer agents[109]. A more recent study from 2016 examined the effects of a potent anti-cancer compound and its derivative agent, spliceostatin, on HeLa S3 cells and solidified prior foundations that upregulated p27 expression levels supressed G1 to S-phase transition, arresting cells in G1 stage[110]. As the results demonstrated previously in figure 11A-C where AMPK levels are upregulated as a result of βHB exposure. Figure 12A-C illustrated the upregulation of $p27^{T198}$ in a similar trend as its upstream protein regulator,

AMPK^{T172}. Confirming the notion that activation of AMPK stabilizes p27 in the cell and prevents its degradation, contributing to the overall p27 levels. MCF7 breast cancer cells cultured in 5mM insulin and 1.25 – 2.5mM of glucose represented a dose-dependent increase in p27^{T198} as the level βHB supplementation increased. The nutritional approach to suppress cancer cells via activation of AMPK pathway can also be justified using this model, depleting the cellular environment of insulin and glucose while shifting the metabolism to ketosis. An early study investigated the effects of p27 deletion in diet-induced atherogenesis and they established a link between decreased p27 protein expression and excessive arterial cell proliferation[111]. Simply, the formation of fatty plaques in the arteries express the growth suppressor p27 in very low levels allowing cellular proliferation[111]. Using this notion of diet induced impact on p27 expressions can be applied to cancer cells in a ketogenic diet. While these findings in p27 have limits in drawing conclusions, it still remains unknown if βHB is responsible for the specific increase in activation of p27. If so, βHB holds a valuable key in reversing the Warburg effect in cancer cells and allow a cellular environment that supresses cellular growth.

Overall, we showed that the primary alterations that occur in circulation as a result of consumption of the ketogenic diet have merit to slow down the proliferation of MCF7 breast cancer cells *in-vitro*. Evidence found here, illustrates that proliferative and survival pathways, pAkt$^{T308/s473}$ decrease in response to a change in metabolism caused by the extracellular environment. Our findings showed that the notion of depleting glucose and insulin in the environment of cancer cells and providing ketone bodies as the primary source of cellular energy may promote mitochondrial respiration and potentially reverse the Warburg shift allowing for less reliance on anaerobic glycolysis. It now becomes hypothesized that the possible reversal in the Warburg shift may cause cancer cells to ineffectively utilize the ketone bodies as the

alternative energy substrate, triggering cell cycle exit or even inducing cell death by accumulation of ROS. Thus, the ketogenic diet may lead to a suitable nutritional adjuvant cancer therapy by manipulating the external growth environment in order to supress MCF7 cell cycle progression.

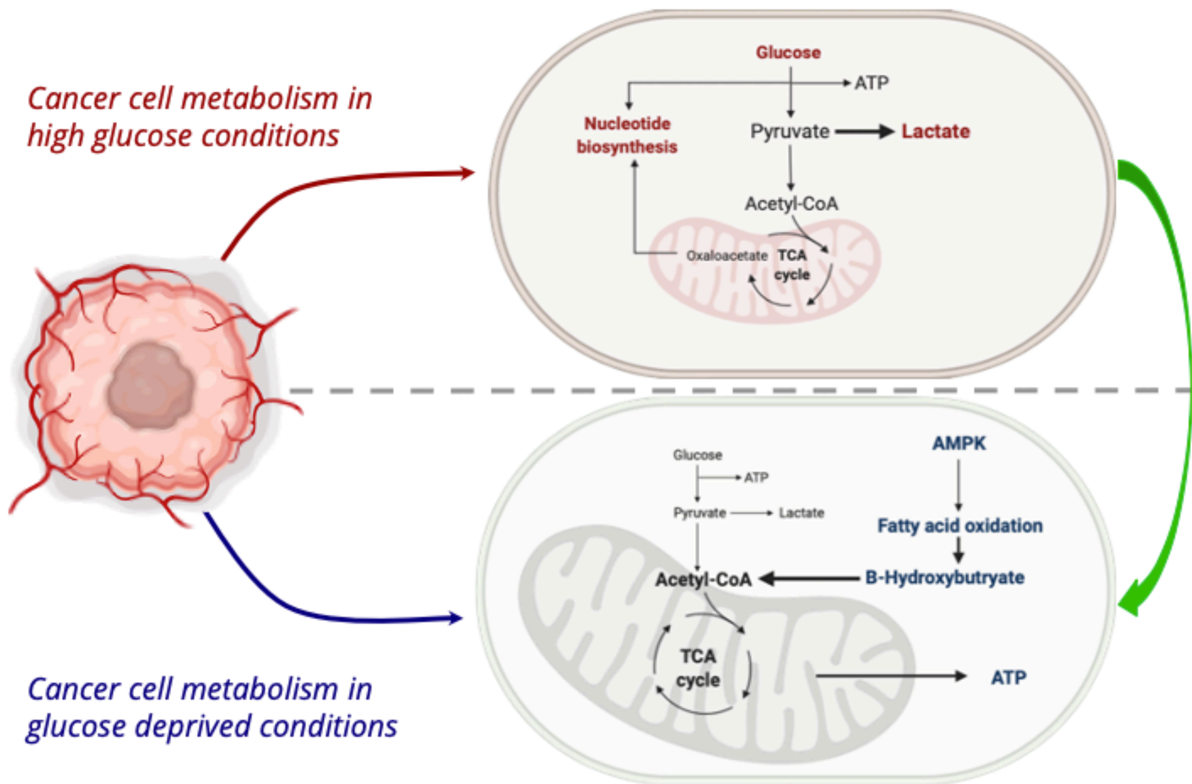


Figure 15: Bypass of the Warburg shift via ketone bodies and decreased glucose in order to promote aerobic metabolism and initiate cell cycle exit

When a cancer cell is in high glucose conditions, regardless of the presence of oxygen, aerobic glycolysis will be favoured as the metabolism pathway to produce energy. This mechanism is referred to as the Warburg shift and in doing so, cancer cells see increased cellular division and increased lactic acid production. The model this study presents, attempts to reverse the Warburg effect via ketone bodies and a decreased glucose state in order to promote aerobic metabolism by utilizing mitochondrial oxidative phosphorylation.

4.0 Limitations and Future Directions

4. 2 Limitations

Beta-hydroxybutyrate and cellular response – An important piece of this research model is the biological relevance, that all variables manipulated are produced and controlled by the human body. When manipulating βHB levels, normal ketosis levels are seen anywhere between 0.5mM – 8mM[112]. We measured effects of 14mM βHB in order to highlight maximum dose effect on MCF7 cells. This proved to be beneficial as it revealed that 14mM βHB creates a cytotoxic environment for cultured cancer cells. The impact it had on the study was demonstrated through statistical analysis, where the sample size included 14mM βHB, making the data too variable, causing possible significance to be silenced in certain experiments. Future adjustments here would be to increase experiment repetitions for a larger sample size.

Implementing a ketosis state in cell culture model – when investigating the ketogenic diet as a nutritional intervention on cell culture model, there is an oversimplification of the interaction between cancer cells and the supplemented growth media. In a simplistic outlook, the tumour microenvironment in humans is much more complex than the microenvironment for cultured cells, as the human body has many factors that can influence the behaviour of cancer cells. Although other researchers are looking at the link between cancer and the ketogenic diet in animal models, this current area involving MCF7 cells, glucose, ketone bodies, Akt and AMPK pathways is rare to find in literature. Therefore, this first needs to be understood in a controlled cell culture model that excludes the impact and complexity of the body and adipokine profiling.

Time impact on low glucose conditions – in high glucose and βHB dosage experiments, 48- and 72-hours post-treatment effects were analyzed. This is important as previous research showed that increasing exposure time will develop stronger effects, similarly as the ketogenic

diet will show a ketosis state in humans 3 days after acclimatization to the diet. However, in low glucose conditions only 48 hours post-treatment was experimented for and not 72 hours. I believe that if the same experiments were repeated over 72 hours in glucose conditions between 0 – 2.5mM then that would give enough time for increased effects in cell cycle regulating pathways.

4. 2 Future Directions

The foundation for the study has been developed over time and with the groundwork thus far, applying further designs can benefit its field. Firstly, a cell cycle profile can be measured via propidium iodide staining assay in order to quantify cell cycle arrest at desired time points post-treatment. An analysis would examine cell cycle phases at low glucose conditions across increasing βHB levels.

Mitochondrial ROS is hypothesized to play a role in this study. To show whether the accumulation of ROS will trigger cell exit. Mitochondrial ROS can be detected using 5-chloromethyl fluorescein diacetate (CMFDA) staining assay in order to measure ROS activity. Either microplate reader or flow cytometer machine can be used to measure and quantify ROS emissions.

Beta-hydroxybutyrate, one of three ketone bodies hold a lower pH. To evaluate the condition of the growth media before and after supplementation of βHB, the acidity should be tested before treatment of βHB and before cell harvesting. This should be measured by means of utilizing ketone pH strips, in order to test the effects of βHB on lowering the pH of cultured cell media.

A final design experiment that can be done is measuring the oxygen consumption rate of MCF7 cells as the exposure to βHB increases and the reliance on glucose decreases. This can be done using the Seahorse XF assay to quantify the cellular oxygen consumption rate of both mitochondrial respiration and glycolysis in real time to assess whether oxygen increases in the presence of βHB treatments and low glucose conditions.

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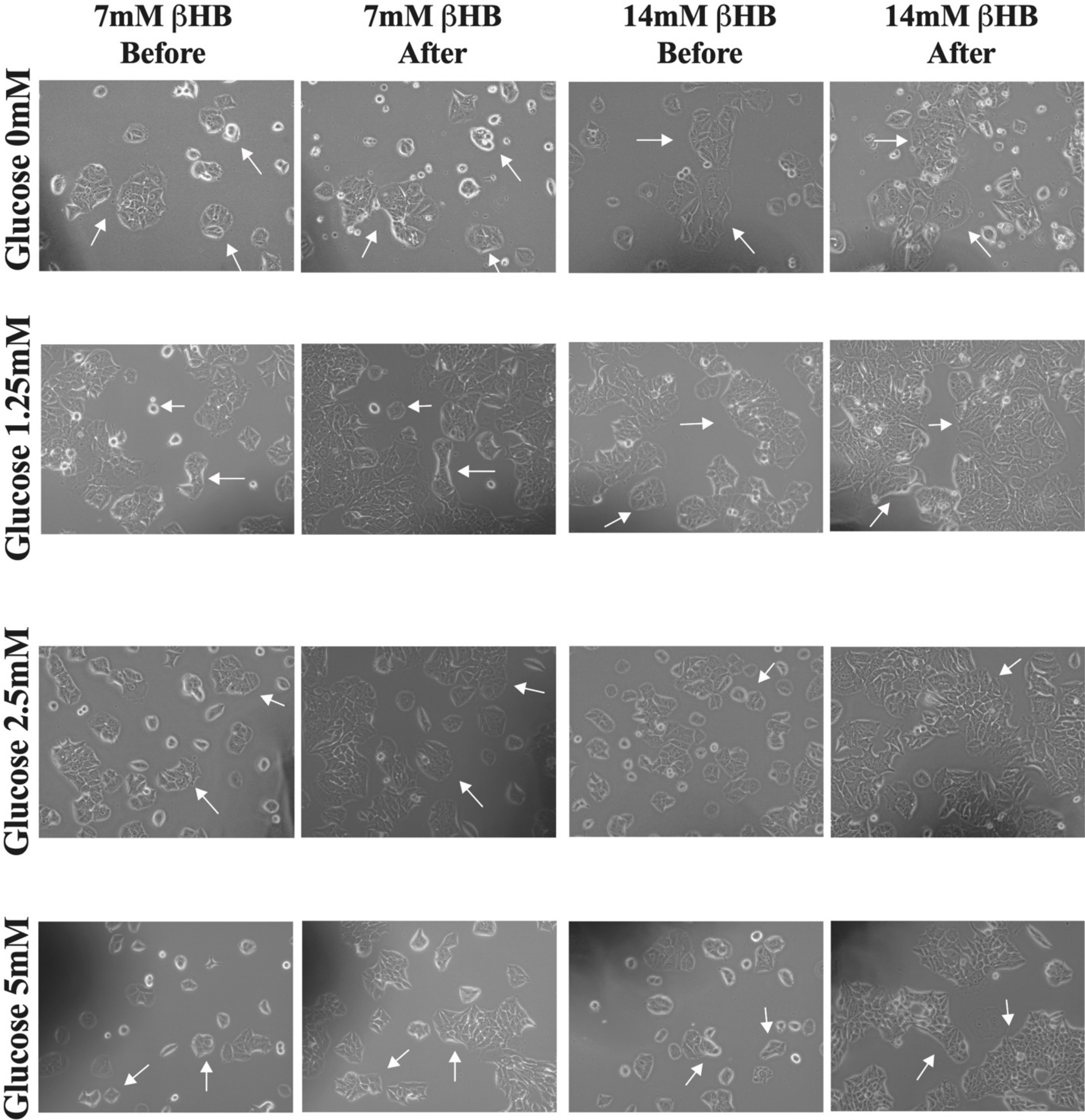
6.0 Appendix

Supplemental figure 16: βHB induced modification in cellular growth response



Supplemental figure 16: Microscopic images of MCF7 cancer cells treated with 7mM and 14mM βHB across four glucose conditions

βHB treatments of 7mM and 14mM induces cellular growth change. It is shown at the range that mimics most similarly to physiological ketosis is 7mM of βHB treatment. The results demonstrate that after 48 hours of culture in 5mM insulin and across varying glucose groups, there are visible effects on MCF7 cells are proliferating at a much slower rate. Observing 0, 1.25 and 2.5mM of glucose before and after 7mM βHB treatment, we can see slight cellular growth but not as rapid as in 14mM βHB. Compared to extreme βHB levels of 14mM, shown to trigger a cellular increase in growth. The extreme dose of βHB demonstrates a possible adverse response that can be seen when MCF7 cells are grown in this high βHB environment. Nonetheless, specifically in lower glucose conditions with βHB present, gross cellular abnormalities begin to appear more prominently than in higher glucose conditions. Arrows indicate examples of growth size before and after 48 hours of βHB treatments.