Changes in BHB, Glucose, and Insulin that Mimic Ketogenic Diet-Induced Changes May Affect MCF7 Growth and Metabolism

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Abstract

Cancer is a complex class of diseases that are characterized by out-of-control cell growth in the human body. They cause harm to the body when they form into tumours and are responsible on a global scale for millions of deaths annually. Breast cancer is one of the leading causes of death in women specifically. It has been linked to factors that are associated with obesity such as adipose tissue, high insulin, and many other factors. The ketogenic diet is a low carb, moderate protein, and high fat diet that helps to reduce adipose tissue markers associated with obesity and cancer. Fat metabolism and ketone production may also help to fight cancer cell growth by starving tumours of glucose. More specifically, physiological levels of ketones and glucose seen on a ketogenic diet may also shift cancer cells to a dysfunctional oxidative phosphorylation within the mitochondria while raising levels of cell cycle inhibitors.

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List of Abbreviations:

AdipoR1	Adiponectin receptor 1
ADP	Adenosine diphosphate
Akt	Protein kinase B
AMEM	Alpha's modification of eagle's medium
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
ATP	Adenosine triphosphate
βHB	Beta-hydroxy butyrate
β-OHBD	Beta-hydroxy butyrate dehydrogenase
C	Control
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
COX IV	Cytochrome *c* oxidase subunit 4
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ERK	Extracellular signal regulated kinase
ER+	Estrogen receptor positive
Fbw7	F-box and WD repeat domain-containing 7
FFA	Free Fatty Acid
FOXO	Forkhead box O
G1	Gap 1 phase
G2	Gap 2 phase

GSK	Glycogen synthase kinase
HDL	High-density lipoprotein
HIF-1α	Hypoxia-inducible factor 1-alpha
IGF	Insulin-like growth factor
IL-6	Interleukin-6
INK	Inhibitor of CDK4
IR	Insulin receptor
IRS-1	Insulin receptor substrate 1
KB	Ketone bodies
KIP	Kinase inhibitory protein
KPC	Kip1 ubiquitylation-promoting complex
hKIS	Human kinase interacting stathmin
KD	Ketogenic diet
LDL	Low-density lipoprotein
LKB1	Liver kinase B1 or Serine/Threonine Kinase 1
M	Mitosis
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
Myf	Myogenic factor
NAD+	Nicotinamide adenine dinucleotide
OXPHOS	Oxidative Phosphorylation

PGC-1α	Peroxisome proliferator-activated receptor gamma
PI3k	Phosphatidylinositol-4,5-biphosphate 3-kinase
Pten	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
Raf	Rapidly accelerated fibrosarcoma
Rb	Retinoblastoma
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
S	Synthesis phase
SCOT	Succinyl-CoA-3-oxaloacid CoA transferase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SiRNA	Small interfering ribonucleic acid
Skp1	S-phase kinase-associated protein 1
Skp2	S-phase kinase-associated protein 2
Src	Proto-oncogene tyrosine-protein kinase
TCA	Tricarboxylic acid cycle
TGFβ	Transforming growth factor beta
TSC2	Tuberous sclerosis complex 2
Ub	Ubiquitin

1.0 - Review of Literature:

The cell cycle is a tightly regulated process that can be disrupted by external factors. Factors that disrupt the cell cycle and lead to cancer are higher in the obese population. Work done in our lab (Theriau and Connor, 2017) has shown that fat tissue from lean rats is able to slow down cancer cell growth, compared to obese adipose tissue. Knowing that we can slow down or prevent cancer cell growth by reducing excess adipose tissue and thus overall body fat is extremely helpful in the future direction of cancer research. A prevention model should be carefully examined and given much thought. In this regard, the ketogenic diet has consistently shown to be the most effective method of losing body fat (relative to other diets) and has been shown to reduce markers of cancer cell growth (Jansen and Walach, 2016). The benefits are two-fold: reducing adipose tissue and markers associated with cancer cell growth, as well as reducing glucose and insulin, known growth promoters of cancer cells. Studies done on mice and human tumours show a decrease in tumour size and glucose uptake (Veech, 2004). Thus, research into whether KD can elicit beneficial effects for obese cancer patients represents a novel direction for therapy/prevention.

1.1 - Mammalian Cell Cycle:

The mammalian cell cycle is a process by which cells divide, replicates their DNA and are divided into the two identical daughter cells. This is a tightly regulated process containing specific checkpoints to ensure accurate replication. The ability of cells to duplicate is dependent on Cyclin-Dependent Kinases (CDK) and Cyclins. The interaction between the CDKs (Serine/Threonine kinases) and their binding partners is one of the important factors that determines cell cycle progression and regulation of the transition steps (Figure 1). The cell cycle is split into four phases termed the gap 1 (G1), DNA replication (S), and gap 2 (G2) phases.

Progression through the mammalian cell cycle is dependent on factors that tightly regulate these phases. Prior to transition into S-phase the cell checks whether energy status is sufficient to support division while in G2 (Norbury et al. 1992). The cell determines if DNA damage or replication errors exist, and if they cannot be fixed, the cell will proceed to apoptosis (Figure 1). A biochemically distinct quiescent state (G0) can occur where the cell remains inactive until cell division replication is triggered by an external signal (Caldon et al. 2005).

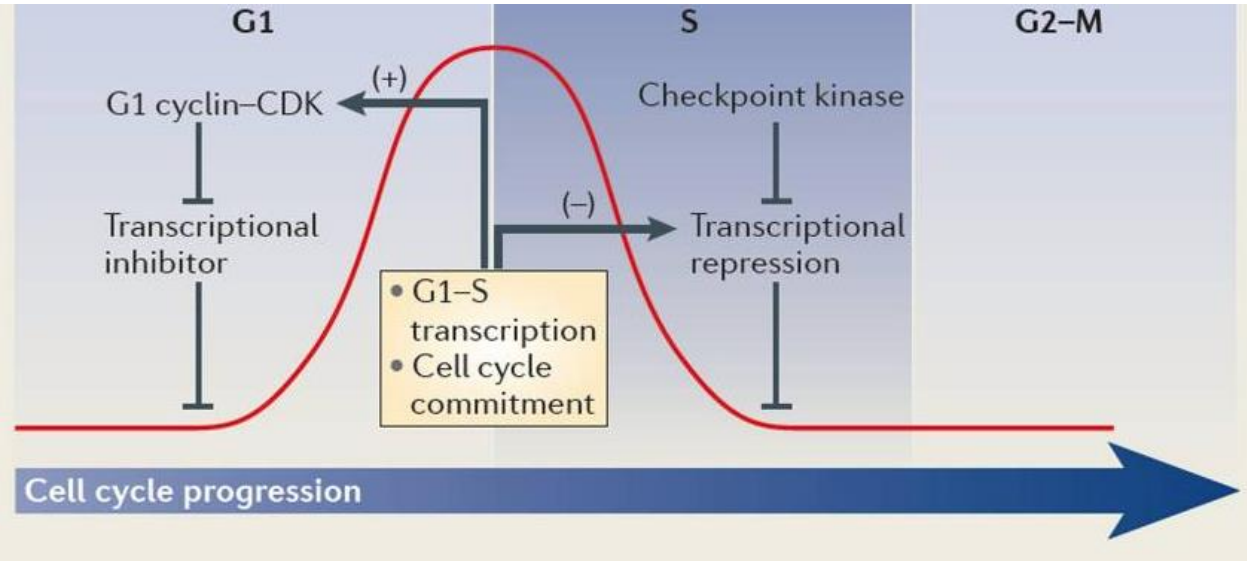

Figure 1: Control of cell-cycle progression from G_1 into S phase. The cell decides whether it will commit during late G_1/early S phase.

Adapted from Bertoli et al. 2013. *Nature Reviews: Molecular Cell Biology.*

1.1.1– Regulation of the Cell Cycle:

Transition between phases of the cell cycle is highly dependent on CDKs and Cyclins. The transitions from G1 to S phase and G2 to M phase are regulated by changes in levels of Cyclins and other post-translational modifiers (kinases and phosphatases). CDKs and Cyclins can be thought of as the engine of the cell cycle that progress the cell through each phase. While CDK proteins tend to stay at fairly constant levels throughout the cell cycle, as the name suggests Cyclins undergo temporal fluctuations in protein levels to regulate progression through the cell cycle. Cyclins are upregulated by transcription factors, which are increased by signaling pathways that are normally activated externally. Regulation of phase transitions depends on specific Cyclin-CDK complexes that target intermediary targets. During G1, the predominant Cyclin–CDK complexes are Cyclin D–CDK4/6 and Cyclin E–CDK2. During S phase Cyclin A–CDK2 is activated while Cyclin A–CDK1 and Cyclin B–CDK1 are turned on during G2 and mitosis (Caldon et al. 2005). Cyclins are generally up-regulated at the transcriptional level, which ultimately increases protein expression. Conversely, the reductions in protein levels are regulated by changing their degradation through ubiquitin mediated proteolysis (Sherr et al. 1999). My thesis will primarily focus on the transition from G1 to S-phase in order to slow cancer progression via regulation of these Cyclin-CDK complexes as deregulation of G1 is evident in the majority of cancers (Tenga and Lazar, 2013).

The cell responds to growth-factor dependent signals during the early G1 phase and mis-activation of CDK-Cyclin complexes can lead to growth in cancer cells. Specifically, the Cyclin E-CDK2 complex represents the checkpoint in the G1/S transition, and entry into the S phase (Koff et al. 1992). As an indication of its importance, Cyclin E is overexpressed in many breast

cancers and correlates with poor prognosis. Loss of Cyclin E-CDK2 regulation plays a role in cancer progression (Gladden et al. 2003).

There are two families of Cyclin-dependent kinase inhibitors known as the INK4 (inhibitors of CDK4) and KIP (kinase inhibitory protein) families. As their name suggests, they have the ability to inhibit the cell cycle during the G1/S transition by constraining the activity of specific CDKs. The inhibitor of CDK4 (INK4) proteins p16INK4a, p15INK4b, p18INK4c, and p19INK4d only bind to CDK4 and CDK6, and no other Cyclin/CDKs. During the G1 phase the kinase inhibitor proteins (KIP) p21 (WAF1/Cip1), p27 (Kip1), and p57 (Kip2) inactivate CDK2 complexes by binding to both Cyclin and CDK subunits, thereby preventing kinase activity. One of the key inhibitors of the G1/S transition is $p27^{Kip1}$, which antagonizes the activity of Cyclin E-CDK2 and Cyclin A-CDK2, preventing premature passage through the G1/S transition (Sherr and Roberts, 1999). $p27^{Kip1}$ impairs cell proliferation in both proliferative and quiescent cells (Xu et al. 1999), plays a crucial role in the G1/S transition through its regulation and localization (Satyanarayana & Kaldis, 2009) and therefore is an important protein with regards to cell cycle regulation.

1.1.2 - $p27^{kip1}$:

$p27^{kip1}$ is a protein that is critical in the control of cell differentiation and cell cycle arrest. The expression of p27 is low in late G1 and stays low through M-phase. Also, p27 is low in the majority of tumours, so much so that it is used as a predictor of patient prognosis (Massagué, 2004, Yang et al. 1998). The protein expression is primarily regulated at a post-translational level during late G1, with targeted degradation of p27 occurring through the ubiquitin-proteasome pathway. The importance of p27 in cell cycle regulation has been demonstrated by

p27 -/- mice which developed specific tumours spontaneously and were more susceptible to tumours in the presence of chemical carcinogens (Nakayama et al. 1996). Low levels of p27 protein expression correlate to poor prognosis and cancer patient survival (Sgambato et al. 2000). p27 forms a tight inhibition complex with CDK2/Cyclin E, independent of p27 phosphorylation state (Xu et al. 1999). During late G1 most of the p27 in proliferating cells is bound to Cyclin D/CDK4-6 and this sequesters p27 from CDK2/Cyclin E, partially removing p27 constraint and allowing the build-up to G1/S transition. Essentially, there is competition between Cyclin D-dependent kinases and CDK2/Cyclin E for Cip/Kip proteins like p27 (Vlach et al. 1997). CDK2/Cyclin E directly phosphorylates p27 on its threonine 187 (T187) residue. The phosphorylation of p27 by CDK2/Cyclin E results in Ub-mediated removal of p27 from the cell which allows cells to transition from G1 to S phase. This process occurs in two steps: p27 is an inhibitor of CDK2/Cyclin E, but that phosphorylation of p27 by CDK2/Cyclin E can inactivate p27 as an inhibitor to promote cell cycle progression. CDK inhibitors are regulated at a post translational level and therefore rely on external signals. The manner in which they are regulated by their environment is of tremendous importance when analyzing the cell cycle in the role of cancer.

p27 is an important independent prognostic factor in breast cancer, characterized by its strong inhibitory activity toward Cyclin E/CDK2, which allows it to directly inhibit similar CDK-Cyclin complexes and arrest cells in G1 (Lloyd et al. 1999). Most studies done on prognostic significance of p27 expression in tumours have reported a decrease in p27 expression in more aggressive tumours (Steeg and Abrams 1997). One study that analyzed p27 expression in breast cancer (202 patients, <1cm in size) found nodal status and low p27 expression to be independent prognostic parameters in various methods of analyses (Tan et al. 1997). Women

whose breast cancer did not spread to any lymph nodes (node negative) and lower levels of nuclear p27 had an increase in cancer relapse risk. There was a staggering 10-fold increase in the risk of relapse when compared to women with increased levels of nuclear p27 expression (Chappuis et al. 2000). The most significant prognostic factor in p27 is that its protein levels are high in most cases of breast carcinomas, and correlated with levels of Cyclin D1 and estrogen receptor, which presents a predictor of survival as they are low in aggressive carcinomas (Chiarle et al. 2000).

1.1.3 - Regulation and degradation of $p27^{kip1}$:

p27 is regulated at the translational level in late G1, early S phase. (Medema et al. 2000). This regulated degradation of $p27^{kip1}$ during G1 and S phase is critical to allowing cell division. While the degradation is moderate during the G1 phase, it is enhanced during the S phase. The ubiquitin-proteasome system responsible for degradation of proteins is part of that regulatory process (Caldon et al. 2006). During G1 and S phase, the degradation of p27 is regulated by two RING-finger E3 ubiquitin ligase-containing complexes (Kamura et al. 2004). These have differing subcellular locations and timing to target p27 for proteasomal degradation via ubiquitylation. The first is a complex with 2 subunits, known as the Kip ubiquitin-promoting complex (KPC). It functions in the cytoplasm during G1 and is made up of KPC1 and KPC2, along with two E2 enzymes Ubc4 and UbcH5A. These are responsible for the initial loss of p27 that activates CDK2/Cyclin E in the nucleus to promote S phase entry. The KPC1 and KPC2 complexes are largely responsible for the initial loss of p27, with the CDK2/Cyclin E being responsible for the remaining nuclear export and degradation of p27. This mechanism is dependent on phosphorylation of p27 on its Serine-10 (S10) residue, known as human kinase

interacting stathmin (hKIS), which is located within the nucleus. It binds to the C-terminal domain of $p27^{kip1}$, phosphorylating S10 to promote its nuclear export to the cytoplasm (Ishida et al. 2000) (Figure 2B). p27 nuclear export is dependent on MAPK activation and is activated in mid-late G. hKIS is largely responsible for regulating cell cycle progression by phosphorylating p27 in response to mitogens. This has been demonstrated by expression of hKIS overcoming cell cycle arrest by $p27^{kip1}$. It is further demonstrated by siRNA depletion of KIS, inhibiting S10 phosphorylation, and promoting growth arrest. Implications of $p27^{kip1}$ as the critical target for KIS p27–/– cells with treatment of KIS siRNA that leads to growth and progression to S phase similar to control treated cells. (Boehm et al. 2002). The second mechanism of p27 degradation is triggered upon phosphorylation of p27 on a threonine residue (T187) by the CDK2/Cyclin E complex. Contrary to KPC1, this occurs in the nucleus during late G1, allowing for the binding of the SFC E3 ubiquitin ligase through the F-box protein Skp2 (Hengst, 2004) (Figure 2A). Typically, phosphorylation of CDK-bound p27 is done by free and active CDK2/Cyclin E, as the p27-bound CDK2 is catalytically inactive due to p27-mediated remodeling of its catalytic cleft and thus removal of ATP (Pavletich, 1999). Besides degradation, phosphorylation of p27 also plays a role in subcellular localization of the protein. Three tyrosine residues (Y74, Y88, Y89) have been associated with proteolysis of p27. A group of Src proteins can phosphorylate these amino acid residues to reduce nuclear p27 by impairing p27 inhibition of CDK2, and thus facilitating CDK2/Cyclin E-dependent p27 degradation (Chu et al. 2007) (Figure 2C).

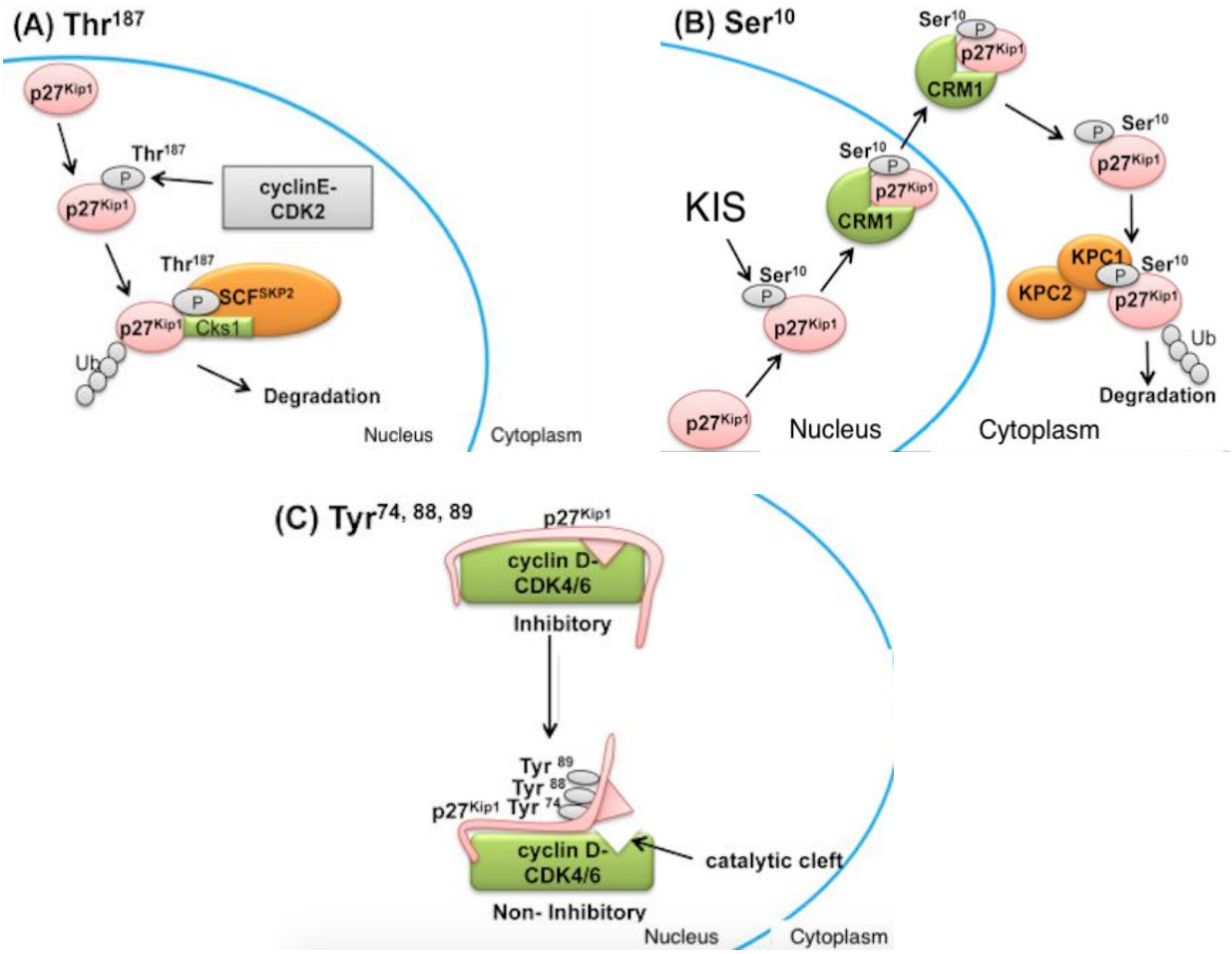

Figure 2: Post-transcriptional regulation of p27. Some of the pathways regulating the phosphorylation, localization, and degradation of p27 have been identified and are illustrated here. **(A)** Degradation by F-box protein Skp2. **(B)** Phosphorylation of S10 by KIS to promote nuclear export to the cytoplasm. **(C)** Src proteins phosphorylate Y88, Y89, and Y74 to facilitate degradation.

Adapted from Hnit et al. 2012. *Int. J. Biochem. Cell Biol.*

1.1.4 – $p27^{kip1}$ and AMPK:

p27 can also be regulated by the energy level of the cell. The ATP levels determine if the inhibition of CDK2/Cyclin E occurs. In lower ATP levels (<50 μm) p27 acts as a CDK inhibitor,

but at higher levels of ATP (>1mM) it is more likely to act as a substrate (Sheaff et al. 1997). While p27 phosphorylation sites are typically reserved for degradation or delocalization, there is a site for stability within the cell. AMPK, which is activated by AMP and LKB1 during conditions where there is a decline in ATP:ADP ratios in the cell, functions as an intracellular energy sensor that regulates cell metabolism and proliferation. It can phosphorylate p27 on the T198 residue to increase stability of the Cyclin-dependent kinase (Figure 3). The phosphorylation of p27 is important in quiescent cells and early G1 as it inhibits CDK2/Cyclin E leading to cell cycle arrest. LKB1-AMPK pathway-dependent phosphorylation of p27 at T198 stabilizes p27, allowing cells to survive metabolic stress from autophagy and growth factor withdrawal. The LKB1-AMPK pathway regulates p27 protein abundance and T198 phosphorylation, with data suggesting that T198 phosphorylation promotes p27 stability under metabolic stress. Additional research has shown a statistical correlation between LKB1 and p27 in human breast cancer cell lines, specifically MFC7 cells (Liang et al. 2007).

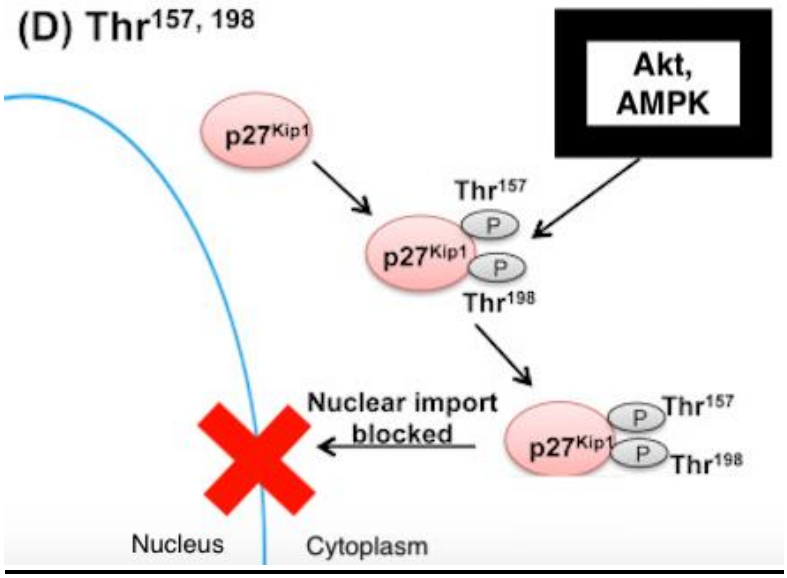

Figure 3: AMPK phosphorylates p27 at T198 to promote stability and prevent nuclear import. Adapted from Hnit et al. 2012. *Int. J. Biochem. Cell Biol.*

1.1.5 - Deregulation of $p27^{kip1}$ in Cancer:

In quiescent cells, p27 protein levels decrease after stimulation with mitogens, with expression of p27 in cultured cells causing cell cycle arrest in G1 (Toyoshima and Hunter, 1994). Mitogenic signals can inactivate $p27^{kip1}$ through many mechanisms including suppression of transcription, inhibition of nuclear accumulation and sequestration by active Cyclin/CDK complexes. When Cyclin E/CDK2 is unhindered it targets p27 for degradation, which removes the inhibitor protein (Giancotti, 2004). This process also requires the F-box protein Skp2, and overexpression of this E3 ligase leads to diminishment of p27 in carcinomas, brain tumours, and lymphomas (Blain et al. 2003). Most of the p27 expression is regulated by mitogenic and anti-mitogenic signaling that works to affect its translation, stability, and localization. Two commonly mutated pathways in human cancer are Ras/Raf/Mek1 and PI3K/Akt (Figure 4). These pathways directly impact the activity and abundance of p27 (Blain et al. 2003). Studies have even shown that the abundance of p27 in tumours is correlated with ubiquitin-dependent proteolysis. Overexpression of PI3K or loss of *Pten* also directly increase Skp2 levels (Mamillapalli et al., 2001). Many studies have been done on the overexpression of Ras, Cyclin E, and activation of MAPK. The results show that it reduces p27 stability while increasing p27 phosphorylation and degradation (Pruitt and Der, 2001). The mislocalization of p27 to the cytoplasm in breast cancer directly affects its activity (Baldassarre et al. 1999). Poor long-term survival in breast cancer has been directly correlated with cytoplasmic p27 (Liang et al. 2002). The inability of cytoplasmic p27 to inhibit downstream nuclear Cyclin-CDK targets prevents cell cycle arrest. Ras overexpression contributes to an increase in cytoplasmic p27. Cytoplasmic mislocalization of p27 can also occur from oncogenically activated Akt, which phosphorylates

p27 on its T157 residue (Liu et al. 2000). p27 can also be inactivated in human tumours through sequestration by Cyclin D-CDK4/6 (Baldassarre et al. 1999). The two previously mentioned pathways, Ras/Raf/Mek1 and PI3K/Akt, directly affect Cyclin D levels. This interaction between the two pathways and Cyclin D may impact p27 localization indirectly. Cyclin D1 is upregulated by MAPK activation, and its degradation is inhibited by Akt-dependent phosphorylation of GSK3-β. These mechanisms further increase the inhibition of p27 of downstream targets through ensuing sequestration (Pruitt and Der, 2001) (Figure 4). p27 is also phosphorylated by Akt on two of its threonine residues (T157 and T198). The PKB/Akt pathway is responsible for impairing nuclear import and the action of p27, which contributes to resistance to antiproliferative signals and breast cancer progression (Figure 4). Protein Kinase B (PKB/Akt) indirectly reduces $p27^{kip1}$ by triggering a pathway that inhibits Forkhead family transcription factors and tumour suppressor tuberin (TSC2) (Liang and Slingerland, 2003). Studies on Akt have demonstrated that p27 phosphorylation by the oncogenically activated Akt/PKB prevents the capacity of p27 to localize in the nucleus. Since p27 is a direct inhibitor of CDK2 and cell cycle arrest, it provides an advantage to tumour growth and contributes to genome instability. Akt-phosphorylated p27 correlate with tumour aggressiveness the same way a decrease in p27 expression would (Blain and Massagué). Activated Akt can play an important role in breast cancer. In normal breast tissue the majority of p27 is nuclear with activated Akt being undetectable. While in many tumours p27 expression is low, there are a significant portion of tumours that express “normal” or greater than normal levels of p27. However, multiple studies have found that in 40% of p27-positive breast carcinomas, cytoplasmic p27 occurs with correlated activated Akt (Viglietto et al. 2002). This highlights targeting Akt as a viable therapeutic avenue.

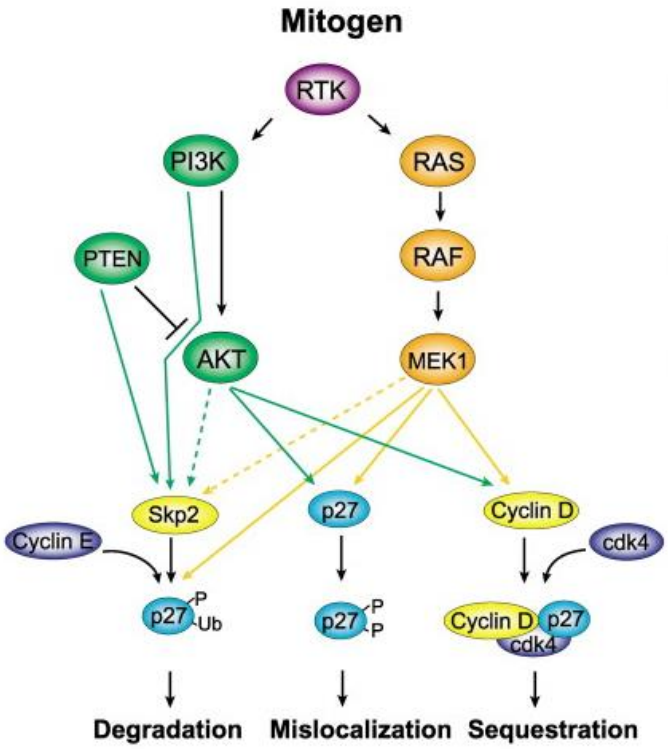

Figure 4: p27 is targeted by multiple oncogenic stimuli including the PI3K/Akt and Ras pathway. These all work to reduce the inhibitory effect caused by p27 on the cell cycle. Adapted from Blain et al. 2003. *Cancer Cell.*

This notion of higher p27 levels and its negative impact has proven to be significant as a prognostic factor. Women with breast cancer and higher nuclear p27 expression in their tumours showed an increase in survival and longer relapse period relative to women with low nuclear p27 expression in their tumours. In the same study (Gillett et al. 1999) 80% of patients with tumours high in p27 expression survived compared to only 50% of patients with tumours low in p27 expression. p27 is clearly a strong predictor of tumour aggressiveness and survival in cancer

patients, specifically in breast carcinomas. Its regulation, and thus expression should be carefully looked at in order to maintain proper p27 function and prevent breast cancer progression.

1.2 – Obesity:

Obesity is important on a global scale. It has many public health implications, especially in North America. Obesity is defined as "a condition of abnormal or excessive fat accumulation in adipose tissue, to the extent that health is impaired" (Drewnowski, 1989). It is associated with premature death from cardiovascular diseases (CVD) including hypertension, stroke, and coronary heart disease. It is also associated with disability or premature death from type 2 diabetes mellitus (T2DM), gall bladder disease, and cancer (colon, breast, endometrial, prostate). The classification of obesity is typically done with a person's BMI (body mass index). This represents their weight as a distribution of their height, and the range of BMI is used to assign body class. While BMI is limited in its application and only a crude measure of body fat percentage, it is a fair and easily obtained estimate in obesity classes. It is calculated by the weight of an individual in kilograms divided by the square of their height in metres (kg/m^2). By the current World Health Organisation (WHO) criteria, a BMI $<18.5 kg/m^2$ is considered underweight, 18.5–24.9 kg/m^2 ideal weight and 25–29.9kg/m^2 overweight or pre-obese. The obese category is sub-divided into obese class I (30–34.9kg/m^2), obese class II (35–39.9kg/m^2) and obese class III ($\geq$40kg/m^2; WHO, 2000). Having a BMI considered overweight or pre-obese is associated with a three to four-fold greater risk of morbidity in adults due to T2DM and CVDs than the average person (Van Itallie, 1995).

Obesity rates are at an all-time high and have grown significantly in the past few decades. From 1995 to 2000, the number of obese adults worldwide grew from 200 to 300 million. This

number has tripled since 1975, and by 2016 more than 1.9 billion adults, 18 years and older, are overweight. Out of this large group, over 650 million adults are considered obese (WHO, 2016). Obesity appears to be a global issue and not just a first world problem, with 115 million people suffering from obesity-related illnesses in developing countries. All of this is accompanied with an increase in childhood obesity and issues stemming from younger generations (Ong et al. 2000). As of 2015, over a third of the world's population is considered overweight or obese. This number is projected to increase to 58% of the world's population. Specifically, in the USA the projections are over 85% of adults being overweight or obese by 2030 (Hruby and Hu, 2015). What is most important to note is that gender also plays a large role in obesity. Women are more affected by extreme obesity than men (classes 2–3, BMI $\geq$35 kg/m^2), and this is independent of age and race/ethnicity (Ogden et al. 2014). Of the more recent numbers claiming that 1.5 billion adults are overweight, nearly 300 million women are considered clinically obese. Obesity in women can lead to polycystic ovarian syndrome (PCOS), insulin resistance, and breast cancer (Templeton, 2014). Obesity prevalence in women in Canada increase as age increases, which presents further concern for cancer risk. Obesity might be contributing to the earlier onset of cancers. By the year 2000, obesity in Canada was responsible for $2.0 billion in direct health care expenditures (Brian and Walsh, 2004).

1.2.1 – Carbohydrate Intake & Obesity:

In the United States alone, consumers annually spend more than $30 billion dollars on weight loss products and services (Serdula et al., 1999) along with $750 billion dollars on pharmaceuticals. While high levels of physical activity as a weight loss intervention are not always implemented and pursued with consistency in the North American lifestyle, drugs have

often been considered as a substitute treatment plan. These drugs can often times cause harmful side effects that are worse than those they intended to treat in the first place (Padwal and Majumdar, 2007). Nutritional interventions for treating obesity should be considered. One simple method of treating obesity is through weight loss. Weight loss is associated with significant health and economic benefits (Ofei, 2005). This is most often done through dietary restriction to create a net caloric deficit.

The Standard American Diet (SAD), also known as a Western Diet, is made up of a high carbohydrate intake, that includes processed sugars and refined carbohydrates. The prevalence of obesity has increased with carbohydrate intake (Figure 5). Studies done on high carbohydrate intake have been associated with higher risk of diabetes in obese patients (Sakurai et al. 2016). High carbohydrate and sugar intake have also been linked to high blood pressure, adipose tissue, insulin resistance, LDL cholesterol, and fasting blood triglycerides (Mirmiran et al. 2016). Sugar intake is the most dangerous source of dietary carbohydrates and thus contributor to obesity. Excess sugar consumption promotes the development of cardiovascular diseases (CVD) and type 2 diabetes (T2DM). High fructose corn syrup (HFCS) found in sugary beverages is a harmful sugar and its metabolism leads to fatty liver, dyslipidemia, reduced insulin sensitivity, and an increase in uric acid levels (Rippe and Angelopolous, 2013, Melanson et al. 2007). Epidemiological data suggests that these direct effects of sugar are independent of body weight gain, which presents a two-fold risk for obese patients (Stanhope, 2016). The risk from adipose tissue to CVD and T2DM combined with further added risk from sugar in the diet.

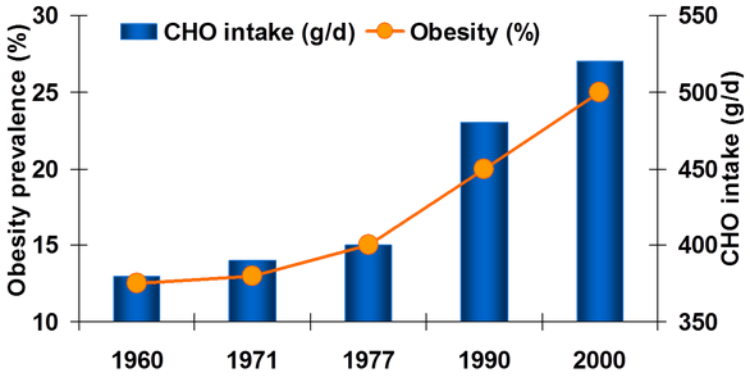

Figure 5: Obesity prevalence and increased carbohydrate intake from 1960 to 2000. Adapted from Gross et al. 2004. *The American Journal of Clinical Nutrition.*

1.2.2 – Obesity & Breast Cancer:

The obesity epidemic has resulted in an increase of morbidity and mortality and the association of cancer and age-related diseases are increased with obesity. Obesity has been linked strongly to colon, female breast (postmenopausal), endometrium, kidney (renal cell), and esophagus (adenocarcinoma) cancers. With the high amount of refined sugar in the average diet, it is important to know that cancer cells show an increase in glucose uptake, higher rates of glycolysis, and an increased level of lactate secretion even in the presence of oxygen. This phenomenon is known as the Warburg effect (Warburg et al. 1927). An increase in glycolytic flux in cancer cells is associated with activated oncogenes and mutant tumour suppressors (Hanahan and Weinberg, 2011). Data has suggested that overeating may be the most avoidable risk to cancer in non-smokers (Calle and Thun, 2004) with weight gain and obesity accounting for roughly 20% of all cancer cases (Wolin et al., 2010). Work done previously by Doll and Peto suggested that simply over nutrition from diet (causing overweight) accounted for 35% of all

cancer. More recent work done by the Center or Disease Control and Prevention showed that obesity and overweight accounted for over 40% of all cancers diagnosed in the United States in 2014. More importantly, a staggering 55 percent of all cancers diagnosed in women are associated with overweight and obesity (CDC, 2016).

In women, breast cancer is the most commonly diagnosed cancer, representing 25% of all cancer types, leading it to become the fifth most common cause of death in women (Rausch et al. 2017). Obesity and weight gain are considered risk factors for postmenopausal breast cancer in women. Research has shown that the environment of cancer cells can affect proliferation or tumour development. Leptin, estradiol, and high glucose, factors high in obese patients, have been shown to stimulate cell proliferation in MCF7 human breast cancer cells (La and Giammanco, 2001). Obesity is correlated with hormonal profiles that are likely to stimulate breast cancer growth (Chlebowski et al., 2002). Obesity also increases the risk of aggressive breast cancer regardless of menopausal status (Stephenson and Rose, 2003). Leptin is a hormone that is made by adipose cells. Work done in our lab (Theriau) has demonstrated that a decrease in the adiponectin:leptin ratio as a result of increases in visceral adipose induced cell cycle entry in MCF7 cells and repressed p27 expression (Theriau et al. 2016). Leptin is involved in regulating the energy balance of the body by regulating, and inhibiting hunger. In obese patients, leptin is elevated in the blood and the body becomes desensitized to the hormone. Studies done on ketogenic diets (KD) have shown significant decreases in leptin in as short as 8 weeks and have shown inverse relationships with hydroxybutyrate (Sumithran et al., 2013). Leptin has also been shown to be stimulated by factors present in overweight post-menopausal women, including insulin, IGF, and estradiol (Garofalo et al., 2006). Estradiol is the primary female hormone, some of which can be produced by adipose tissue. In post-menopausal women specifically, obesity is

related to levels of circulating estradiol which itself has been linked to breast cancer risk. Theriau et al. (2016) also demonstrated a correlation between circulating estradiol and obesity. By implementing the consumption of KD in obese women, the risk of breast cancer is reduced due to a subsequent decrease in adipocytes and thus circulating estradiol (Key et al., 2003). Obesity interventions should be the first line of defence to reduce risk of breast cancer in women.

1.2.3 – Insulin & Breast Cancer:

The role of insulin in breast cancer is also being carefully studied. Insulin is an anabolic hormone that has signaling pathways in hepatic, adipose, and muscle tissues. While it is mainly involved in glucose metabolism, it has also shown proliferative effects on cancer cells. Fasting and post-prandial hyperinsulinemia is seen in obesity-induced insulin resistance as a compensation method (Orgen and Mittelman, 2013). Recent studies in animal and human models have shown that hyperinsulinemia, high levels of circulating IGF-1, and type 2 diabetes can increase breast cancer development and progression (Lann and LeRoith, 2008). Type 2 diabetes, which can arise from a diet high in carbohydrates, has been seen in up to 16% of breast cancer patients. Old age and obesity are two major risk factors for type 2 diabetes and are also associated with increased breast cancer risk (Wolf et al., 2005). A high carbohydrate diet is especially harmful for obese diabetic patients, with increases in level of blood glucose, insulin, and serum triglycerides in patients with insulin resistance following a large carbohydrate meal. A higher carb diet also raises triglyceride levels and reduces HDL-cholesterol while promoting insulin resistance (Zammit et al. 2001). Research done on post-menopausal women showed that high fasting insulin levels leading to insulin resistance was associated with a 2.4-fold risk of developing breast cancer (Gunter et al. 2009). Other research has demonstrated that there is a

27% increase in the risk of breast cancer in women with type 2 diabetes (Boyle et al. 2012). This suggests that hyperinsulinemia is an independent risk factor for breast cancer and is helpful in explaining the obesity-breast cancer relationship. Thus, a decrease in obesity and its associated morbidities remains as an option for decreased breast cancer risk.

The insulin receptor has two tyrosine kinase isoforms: IR-A and IR-B. The IR-A isoform is expressed in cancer cells, which is involved in proliferation and cell survival signaling through the Ras-MAPK (mitogen-activated protein kinase) pathway (Belfiore and Malaguarnera, 2011). Studies done on blocked insulin signaling with hydrolyzed products (e.g. Indole-3-Carbinol) arrest breast cancer cell proliferation by decreasing expression of IGF1 receptor and IRS1 (Marconett et al. 2012). Inhibition of these two receptors has been shown to decrease PI3K/Akt signaling, while suppressing MCF7 xenograft growth in mice (Fox et al. 2011). The importance of the PI3k pathway in cancer has been demonstrated through proliferation of breast cancer by insulin (Rose and Vona-Davis, 2012). These same effects were reversed by an Akt inhibitor. In the same study, diabetic levels of glucose in vitro led to an increase in Akt expression, proliferation rate, and migration activity of breast cancer cells (Tomas et al. 2012).

The interaction of a higher carbohydrate diet leads to excess glucose, and insulin resistance over time. It is clear that insulin/IGF/IRS play a large role in cancer growth and pose a large risk for obese patients. A lower carbohydrate diet would be one avenue of prevention.

1.3 - The Ketogenic Diet:

Low carbohydrate diets have been shown to reduce body weight, body mass index (BMI), abdominal circumference, systolic and diastolic blood pressure, and fasting blood triglycerides (Santos et al. 2012). They have also been shown to reduce fasting glucose, glycated

haemoglobin, plasma insulin, plasma C reactive protein, and increase high-density lipoprotein (Sartorius et al. 2016). Because dietary carbohydrates are positively associated with weight gain and obesity (Ma et al. 2005), this presents an opportunity for a low carbohydrate diet to reduce caloric intake and induce weight loss. Besides a basic reduction in calories consumed, the reduction in insulin concentrations would promote free fatty acid mobilisation from body fat storage (Krieger et al. 2006). The ketogenic diet is quite simple – cut out all refined carbohydrates, and increase dietary fat (Figure 5).

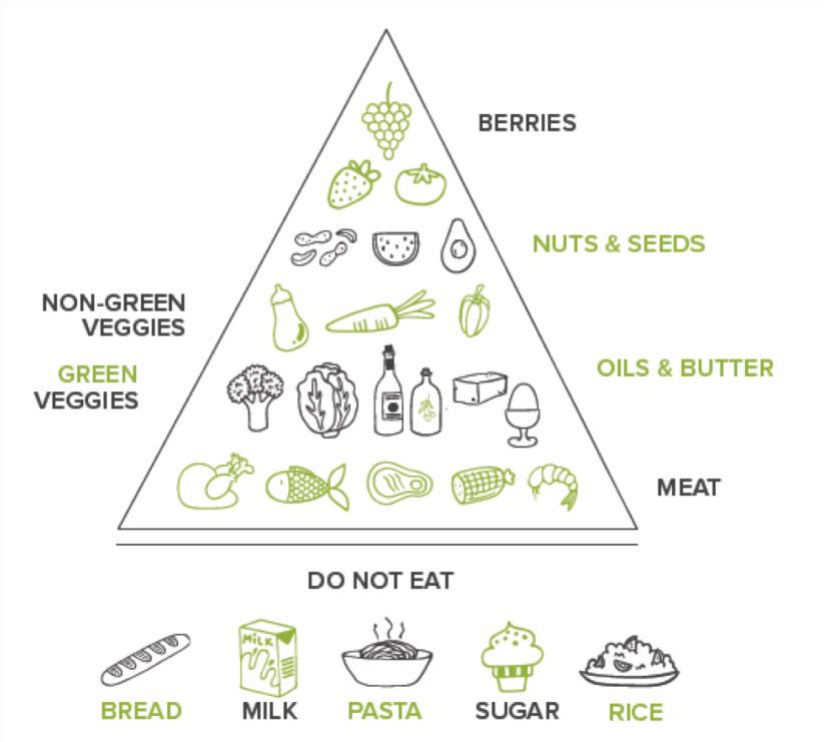

Figure 6: Ketogenic diet food pyramid. Note removal of refined carbohydrates and foundation of meat and fat sources.

1.3.1 - The Ketogenic Diet & Obesity:

The ketogenic diet (KD) or a low-carb high-fat diet (LCHF) is a low carbohydrate, moderate protein, and high fat approach to eating (Figure 6). It is a natural way of eating that is

similar to how our ancestors ate, as carbohydrates were rare and opportunistic (Krilanovich, 2007). The ketogenic diet triggers the breakdown of fat in the liver by decreasing glucose and ultimately producing ketone bodies. Ketone bodies are an efficient fuel source for the human brain, heart, and skeletal muscle (Mitchell et al. 1995). These are metabolic adaptations by the body that are made when the insulin response is reduced and glycogen stores are depleted in muscle and liver tissue (Dashti et al. 2004).

Ketosis is the process of producing ketones from fat in the liver. Ketone production occurs after a few days of fasting, or when dietary changes are made from the form of diet and carbohydrate intake is reduced to roughly 20g per day (Felig et al. 1969). At this point the body does not have enough glucose reserves to produce oxaloacetate which supplies normal fat oxidation to the Krebs cycle. It also has an insufficient amount of glucose to supply the central nervous system (CNS) (Owen, 2005). In order to make up for the lack of energy supply, the body overproduces acetyl-CoA in order to produce three distinct ketone bodies (KB): acetoacetate (AcAc), β-hydroxybutyrate (βHB), and acetone. The primary circulating ketone body is β-hydroxybutyrate (βHB). The process of producing ketone bodies for fuel is called ketogenesis and it occurs in the mitochondrial matrix of the liver (Fukao et al. 2004).

A reduction in caloric and fat intake combined with exercise has shown poor weight reduction long-term (Kramer et al. 1998). As previously mentioned, low-carb diets like the ketogenic diet are beneficial for reducing cardiovascular markers of heart disease in obese patients. They are also extremely effective at weight loss in obese patients (Dashti et al. 2003). Further studies done on obese diabetic patients (T2DM) showed a significant decrease in body weight, BMI, blood glucose, total cholesterol, LDL-cholesterol, triglycerides and urea from week 1 to week 56. This was matched with a significant increase in HDL-cholesterol, with a higher

significance in patients with high blood glucose levels versus those with normal blood glucose levels (Dashti et al. 2007). Multiple research papers have shown similar results with improvements in cardiovascular markers and a reduction in body weight versus other diets (Dashti et al. 2003, Rabast et al. 1979, Dashti et al. 2004, Dashti et al. 2006, Yancy et al. 2004, Westman et al. 2003, Kwiterovich et al. 2003, Sharman et al. 2002, Lofgren et al. 2005, Boden et al. 2005, Ezenwaka and Kalloo, 2005).

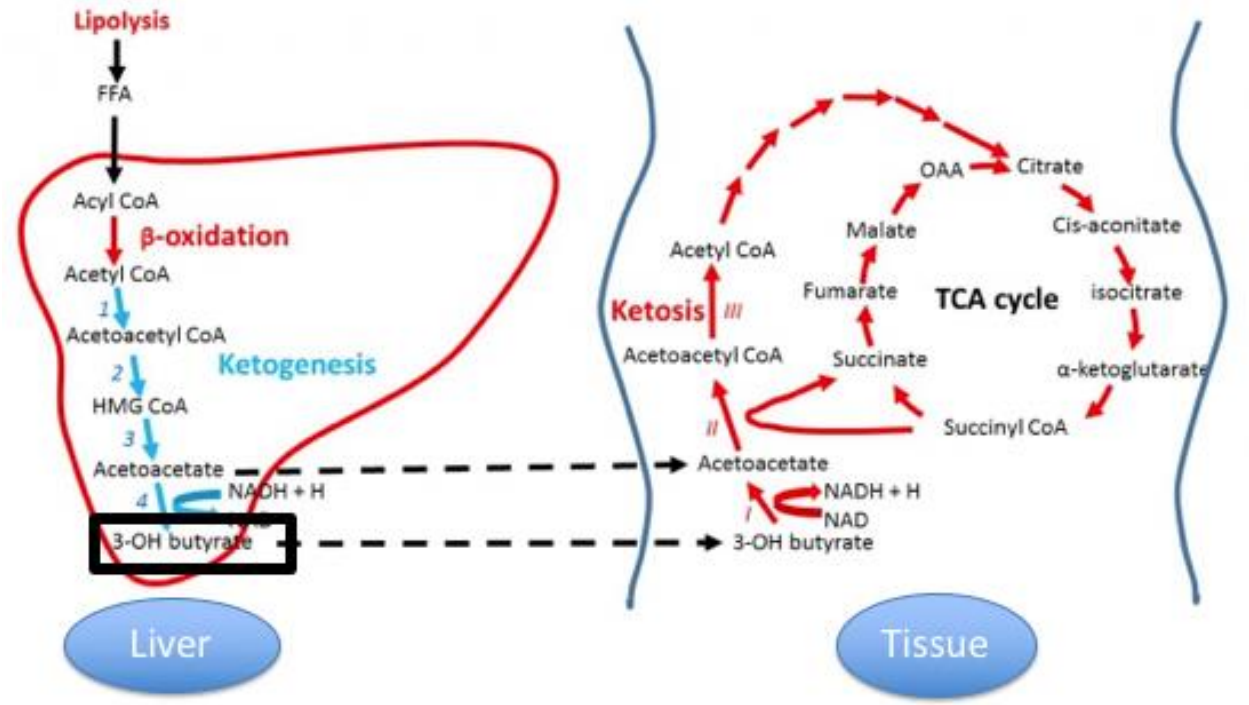

Figure 7: ATP production via ketones produced on a ketogenic diet

The mechanism behind ketone production is responsible for many of the health benefits of a ketogenic diet. During fasting or ketosis in humans, ketones are is produced via dietary fat and endogenous adipose tissue. Free fatty acids are converted in the liver to ketone bodies which can provide energy to the mitochondria of cells. ATP is then produced by the conversion of

ketone bodies to acetyl CoA via the tricarboxylic acid cycle and oxidative phosphorylation (Figure 7). This allows protein sparing from lean tissue, while serving glucose-sparing effects as ketone bodies become the preferred energy substrate of the heart and muscle. To achieve a state of ketosis, it is typically recommended for males to consume below 30-50g of carbohydrates a day, and for women to consume below 20-30g of carbohydrates a day. The fat intake will represent 60-70% of calories, with protein combining for the remaining 30-40%, and carbohydrates around 5% (Westman et al. 2003). By tapping into adipose tissue, improving cardiovascular risk factors, and providing an effective method of weight loss, the ketogenic diet can directly and indirectly benefit obese patients.

1.3.2 – Ketogenic Diet & Breast Cancer:

The ketogenic diet could have implications for indirectly and directly slowing down cancer cell growth. Through the reduction in adipose tissue, the reduction of insulin, glucose (and other factors associated with cancer growth), along with the theory of mitochondrial dysfunction present in cancer cells. Mitochondrial dysfunction in cancer cells is thought to be one reason that ketone bodies can slow down cellular growth. Ketone bodies, mainly beta-hydroxybutyrate (β-OHB), are converted back to acetyl-CoA inside the cell to eventually create energy. The expression of two key mitochondrial enzymes β-OHB dehydrogenase (β-OHBD) and succinyl-CoA: 3-ketoacid CoA transferase (SCOT) are responsible for this conversion (Chriett and Pirola, 2015). While ketone bodies are an efficient fuel source for regular human tissue, certain cancer cells show an impairment in their abilities to metabolize ketone bodies (Chia-Chi Hsu et al. 2016).

Early in 1927, Otto Warburg recognized that nearly all cancer calls share a common metabolic phenotype. They can be grouped together by their shift in metabolism from aerobic respiration towards glycolysis that is independent of oxygen availability, allowing them to survive in hypoxic conditions (Warburg et al. 1927). Typically, normal cells that have functional mitochondria can exercise their mitochondrial oxidative metabolism by shuttling pyruvate generated via glycolysis to the tricarboxylic acid (TCA) cycle. Unlike normal cells, cancer cells primarily use pyruvate in the lactic acid fermentation pathway (Branco Ana F. et al. 2016). This metabolic phenotype offers cancer cells three select advantages for survival. First, it is advantageous for proliferation as it allows more an efficient generation of carbon equivalents for macromolecular synthesis when compared with oxidative phosphorylation (OXPHOS) (Vander Heiden et al. 2009). Secondly, it prevents production of reactive oxygen species by bypassing mitochondrial oxidative metabolism. This is advantageous to cancer cells, as they have higher steady-state levels of oxidative stress relative to normal cells. This would improve survival as they would be more sensitive to ROS-mediated apoptotic stimuli (Aykin-Burns et al. 2009). Lastly, an increase in acidification of the tumour site, as a result of elevated glycolytic flux, can facilitate tumour invasion and progression (Peppicelli et al. 2014).

There are a few factors that can contribute to the shift towards a glycolytic phenotype. Metabolic transformations that occur can be caused by mutations in genes coding for certain pathway proteins, or due to factors in the cellular microenvironment. Overactivation of insulin/IGF-1-dependent phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) system is one source of metabolic transformation (Baserga, 2000, DeBerardinis et al. 2008, Robey and Hay, 2009). Mutations in genes that code for proteins in pathways like PI3K-Akt are possible sources of metabolic transformation (Engelman, 2009).

Factors in the tumour microenvironment that are hallmarks of cancer, like hyperglycaemia and hyperinsulinemia, could also be sources of this shift in cellular metabolism (Klement and Kämmerer, 2011). Upregulation and membrane translocation of glucose transporters (GLUT) leads to an increase in glucose uptake and retention via activation of PI3K/Akt/mTOR pathway (Makinoshima et al. 2015). Downstream effectors of mTOR (c-Myc and HIF-1α) upregulate important glycolytic enzymes to reinforce glycolysis (Parajuli et al. 2015). Meanwhile, the downregulation of carnitine palmitoyl transferase 1A (CPT1A) via PI3K/Akt inhibits β-Oxidation (Schlaepfer, 2014). Uptake of glucose, along with increased glucose levels are associated with poor prognosis in cancer patients (Kunkel et al. 2003).

These data further support the notion that the ketogenic diet, and the state of nutritional ketosis, should interfere with the dependence of cancer cells on glucose to grow, produce energy, and thus proliferate. The metabolism and genome of cancer cells allows for the potential to selectively starve them of glucose while providing energy via ketones to normal cells (Seyfried et al. 2011). Research has shown that non-hepatic tumours may be unable to metabolize ketone bodies (Tisdale and Brennan, 1983) and that gene expression for mitochondrial enzymes, beta-hydroxybutyrate dehydrogenase and succinyl-CoA: 3-ketoacid CoA transferase was lower in tumours versus normal tissue (Zhou et al. 2007). Structural defects that lead to mitochondrial abnormalities in cancer cells are another source of inefficient ketone body metabolism (Seyfried et al. 2012). Another benefit of a ketogenic diet is that the reduction in circulating glucose will lower insulin and IGF-1 levels, resulting in a decrease of activation in the PI3K/Akt/mTOR pathway (Figure 8). By limiting or inhibiting insulin secretion there is an additional method that is consistent with published mechanisms to downregulate this pathway in patients with advanced cancer (Fine et al. 2012).

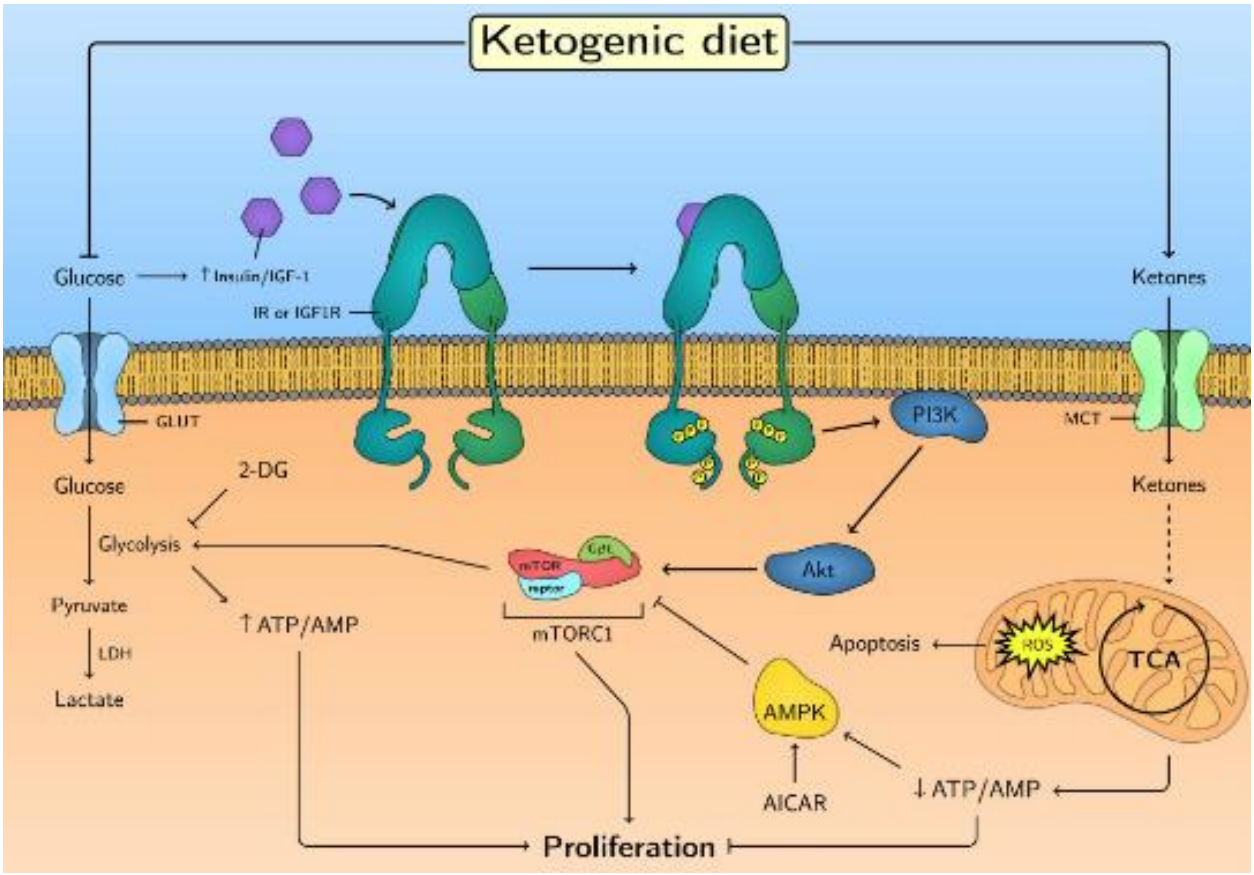

Figure 8: Reduction in glucose and insulin downregulates the PI3K/Akt/mTOR pathway. Tumours are unable to efficiently metabolize ketone bodies.

Adapted from Branco et al. 2016. *European Journal of Clinical Investigation.*

In vitro, there were inhibitory effect of ketone bodies (β-hydroxybutyrate and acetoacetate) on tumour cell growth in breast cancer cell lines (Fine at al. 2009). Patients with breast cancer have shown positive results represented by a novel tumour marker, transketolase-like-1 (TKTL1), associated with aerobic glycolysis of tumour cells (Jansen and Walach, 2016). Further studies are being done in clinical trials with patients suffering from breast cancer (NCT02092753). The ketogenic diet, through the depletion of glucose and the production of ketone bodies, warrants further research as an adjuvant to cancer therapy.

2.0 – Study Rationale:

There are clear effects of alterations in the tumour growth microenvironment that adipose in obese patients imparts on accelerating cancer proliferation (Theriau and Connor, 2017). Thus, reductions in adiposity by any means may serve to improve patient prognosis in obese patients. Ketogenic diet (KD) reduces adiposity as a secondary effect, but also increase ketones, decreases glucose, and decreases insulin directly. This thesis was designed to determine if these primary effects of KD can affect breast cancer growth and metabolism in the absence of the secondary effects of KD. Reductions in adiposity typically takes weeks, however KD-induced changes in glucose and insulin can occur within a matter of days. Thus, this thesis looks at primary and immediate effects seen in the tumor microenvironment.

3.0 – Hypotheses:

Based on the literature described in Section 1, I hypothesize that:

1. The changes in ketones (βHB), insulin and glucose that accompany KD will affect metabolism of MCF7 cells.
2. In addition, the increase in ketones, decrease in glucose and decrease in insulin will promote an increase in p27.

4.0 – Manuscript:

Changes in βHB, Glucose, and Insulin That Mimic Ketogenic Diet-Induced Changes May Affect MCF7 Growth and Metabolism

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4.1 – Abstract:

Adipose tissue in lean cancer patients acts as an endocrine gland that secretes an adipokine profile which creates a tumour microenvironment that impairs tumour growth, while the profile secreted by obese adipose tissue promotes tumour growth. Dietary alteration is a powerful intervention to reduce body fatness, and one such alteration is the ketogenic diet (KD). In addition to a decrease in adiposity, KD decreases glucose and insulin while increasing ketone bodies. We set out to determine whether these individual alterations alone, or in concert, would affect MCF7 breast cancer cells, promoting cell cycle arrest. Experiments showed that 24 hour and 48 hour treatment with βHB in a standard high glucose, high insulin conditions caused changes in the protein expression of $pAkt^{T308}$ and $p27^{T198}$. In addition, βHB treatment appeared to elicit a 2-fold increase in cytochrome c oxidase subunit IV (COX IV) protein levels. When glucose was decreased to post-prandial physiological glucose levels we saw βHB dose-dependent increases of COX IV and total p27 protein with a decline as βHB approached the upper end of physiological βHB levels (14mM). At the lowest glucose concentration there was an observed increase in AMPK phosphorylated at T172 and a decrease in both $pAkt^{S473}$ and $pAkt^{T308}$. Thus, the primary changes induced by a KD may themselves induce growth inhibitory effects on breast cancer cells. When combined with the established effects of decreasing adiposity on breast cancer proliferation, KD may change the tumour growth microenvironment in multiple ways to effectively slow tumour growth in breast cancer patients.

4.2 – Introduction:

The ketogenic diet (KD) is a low carbohydrate, moderate protein and high fat diet that was initially used in the 1920s to treat epilepsy (Wilder and Winter, 1922). Since then, this diet has also been used in the treatment of pancreatic cancer, malignant brain cancer, Alzheimer's disease, and traumatic brain injury (Gasior et al., 2006). During times of decreased carbohydrate availability, such fasting or prolonged exercise, ketone bodies are produced from body fat stores and circulated as an accessible source of energy. (R)-3-hydroxybutyrate (βHB) is the most abundant and stable ketone body produced in humans (Newman and Virdin, 2014). It is a physiological alternative to glucose and fatty acids as an energy producing substrate via its conversion to Acetyl-CoA. During prolonged fasting, or controlled consumption of a KD, these ketone bodies rise to levels between 3-7mM in the blood. This is considered a safe and nutritional level of ketosis (Courchesne-Loyer et al. 2013). When glycogen stores in the liver and muscle are depleted, as occurs during the consumption of KD, fatty acids in the adipocytes are released in to the circulation. These fatty acids can subsequently be broken down and converted into ketone bodies in the liver, and then released into the systemic circulation. Metabolically active tissues like muscle and the brain are able to convert ketones into Acetyl-CoA, which enters the citric acid cycle in the mitochondrion and is converted into ATP in the presence of O_2. Importantly, ketosis allows for glucose sparing by circumventing glycolysis (Berg et al., 2002). This shift in metabolism to ketone bodies as a fuel source increases high-density lipoprotein-cholesterol, decreases fasting circulating levels of glucose, decreases low-density lipoprotein-cholesterol and decreases triglycerides in the circulation (Paoli et al., 2013).

During normal energy status in metabolically active tissue, most cellular energy is produced through the breakdown of glucose, via glycolysis, into pyruvate which is shuttled into

the mitochondrion to produce ATP via oxidative respiration. In cancer cells this pattern is altered, whereby there is a much less reliance on aerobic means for energy production. This shift, termed the Warburg effect (Warburg et al., 1927), allows cancer cells to thrive in a hypoxic environment and represents one of the hallmarks of cancer (Hannahan and Weinberg, 2011). This leads to glycolytic breakdown of glucose in cancer cells with a reduced contribution of aerobic energy production through oxidative phosphorylation, giving cancer cells a growth advantage as they exist in hypoxic environments. Since KD decreases blood glucose, cancer cells may be starved of their primary fuel source in patients consuming a KD. While normal cells can use ketone bodies for aerobic respiration, cancer cells may struggle in this regard due to their inherent impairments in oxidative phosphorylation in the mitochondria, as evidenced when tumours in mice fed a KD decreased in size by 65% (Gluschnaider et al., 2014).

Through the alterations in IGF, insulin, estradiol, and many of the over 400 hormones produced by adipose tissue, the metabolism of an obese person creates a tumour growth microenvironment that promotes cancer proliferation (Sartorius et al., 2016). One way to reverse this microenvironment in obese people may be by introducing a high fat, low carbohydrate diet instead. This induces nutritional ketosis which may affect breast cancer growth by decreasing adiposity (Veech, 2014). These effects of obesity can be reversed by altering the secretory function of adipose tissue via diet, exercise and nutritional supplementation (Connor and Theriau, 2013, Theriau et al. 2016, Theriau and Connor, 2017). Thus, KD may induce primary and secondary effects of KD consumption and highlight the possibility of implementing a KD as an adjuvant therapy in obese cancer patients.

4.3 - Materials and Methods:

Cell Culture

MCF7 cells (ATCC, Manassas, VA) were cultured in flasks (T75 Falcon) at 37^{o}C and 5% CO_2, in alpha's modification of eagle's medium (AMEM; Sigma, St. Louis, Missouri), 5% fetal bovine serum (FBS; Wisent, St. Bruno, QC), 5% non-essential amino acids (Sigma), 5% sodium pyruvate (Sigma) 500µl of insulin (10µg/ml, Sigma), 3% antimycotic/antibiotic (Wisent) and media was replenished every 48 hours.

Treatment

Following trypsinization, MCF7 cells were seeded in 6-well plates. To test the effect of insulin on growth, cells were grown in varying levels of insulin (0–10µg/ml). Manipulation of the dose of insulin in the growth media was done since circulating insulin levels decrease during KD consumption. MCF7 cells were plated at 80% confluence and starved of insulin for 24 hours in AMEM. Insulin was added back for 24 hours and cells were collected. To test the effects of ketone bodies, MCF7 cells were treated with β-hydroxybutyrate (BHB, 3.5-14mM), a range of ketone bodies found in the circulation during nutritional ketosis, for 24 and 48 hours. We also examined the effect of altering glucose levels (0-10mM). To assess the cumulative effects of insulin, glucose and βHB, glucose-free AMEM (Sigma) was supplemented with 5µg/ml of insulin and treated with various combinations of D-glucose (Sigma) and βHB. Prior to βHB/glucose treatment, a 6 hour glucose depletion time was implemented. Following depletion, glucose was added back to the media and cells were collected 18 hours later for a total treatment time of 24 hours.

Cell Harvesting

Following all treatments, media was suctioned off, and cells were washed twice with cold phosphate buffered saline (PBS). Cells were then scraped, washed twice with cold PBS and the remaining pellet was resuspended in 0.2% TENT buffer solution (TRIS, EDTA, NaCl, 0.2% Triton x-100) supplemented with 1% protease inhibitor (Sigma, Oakville, ON), and 1% phosphatase inhibitor (Roche Diagnostics, Indianapolis, IN). The cells were then sonicated on ice and centrifuged at 16,100 xg at 4^{o}C. The supernatant was then separated from the pellet and the protein lysate stored at -84^{o}C.

Immunoblotting

Protein concentrations of experimental lysates were determined using a standard Bradford assay (Bio-Rad). To assess effects of treatments 25μg of protein was loaded onto a 12% SDS-PAGE gel and run at 120V for 90-120 minutes. Separated proteins from the gel were then transferred onto a PVDF membrane (Bio-Rad, Mississauga, ON) overnight at 40V at 4^{o}C. These membranes were stained in amido black the next day to determine the efficacy of transfer and blocked in 10% skim milk with shaking at room temperature. Subsequently, membranes were incubated with primary antibodies overnight at 4^{o}C for: Cyclin E, AMPK, pAMPK^{T172}, Akt, pAkt^{T308}, pAkt^{S473} (Cell Signaling, Pickering, ON), p27 (BD Biosciences, Mississauga, ON), p27^{T198} (R&D Systems, Minneapolis, CA), Cox IV (Abcam, Cambridge, MA), AdipoR1 (Santa Cruz Biotech, Santa Cruz, CA) and β-Actin (Abcam, Cambridge, MA). Membranes were subsequently washed with Tris-Buffered Saline with 0.05% Tween (TBST) and then incubated with the appropriate HRP-linked secondary antibody (Cell Signaling) in 5% milk for 1 hour with shaking at room temperature. Proteins were visualized using an enhanced chemiluminescence substrate (Millipore, Whitby, ON) and imaged on a Kodak In Vivo FX Pro Imager (Marketlink Scientific,

Burlington, ON) and quantified using Carestream software. Protein loading was corrected to β-Actin levels.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 5 software. One-way ANOVAs were conducted to determine the effects of insulin, βHB, and glucose. Differences in means were considered statistically significant when $p \leq 0.05$ and Tukey's post-hoc test was performed to determine if there was any statistical significance between groups.

4.4 – Results:

Insulin regulates pAkt pathway and can cause insulin resistance in MCF7 cells

Treatment of MCF7 cells without insulin resulted in low levels of phosphorylation of Akt on the T308 and S473 residue, while total Akt was unaffected. With a 5 μg/ml dose of insulin, half of the standard amount supplemented in normal MCF7 growth media, we saw increases in $pAkt^{T308}$ and $pAkt^{S473}$ in protein expression, while total Akt was unaffected (Figure 9A). When insulin was further increased to 10 μg/ml of insulin, there were observed reductions in $pAkt^{T308}$ and $pAkt^{S473}$ protein levels (Figure 9B,C).

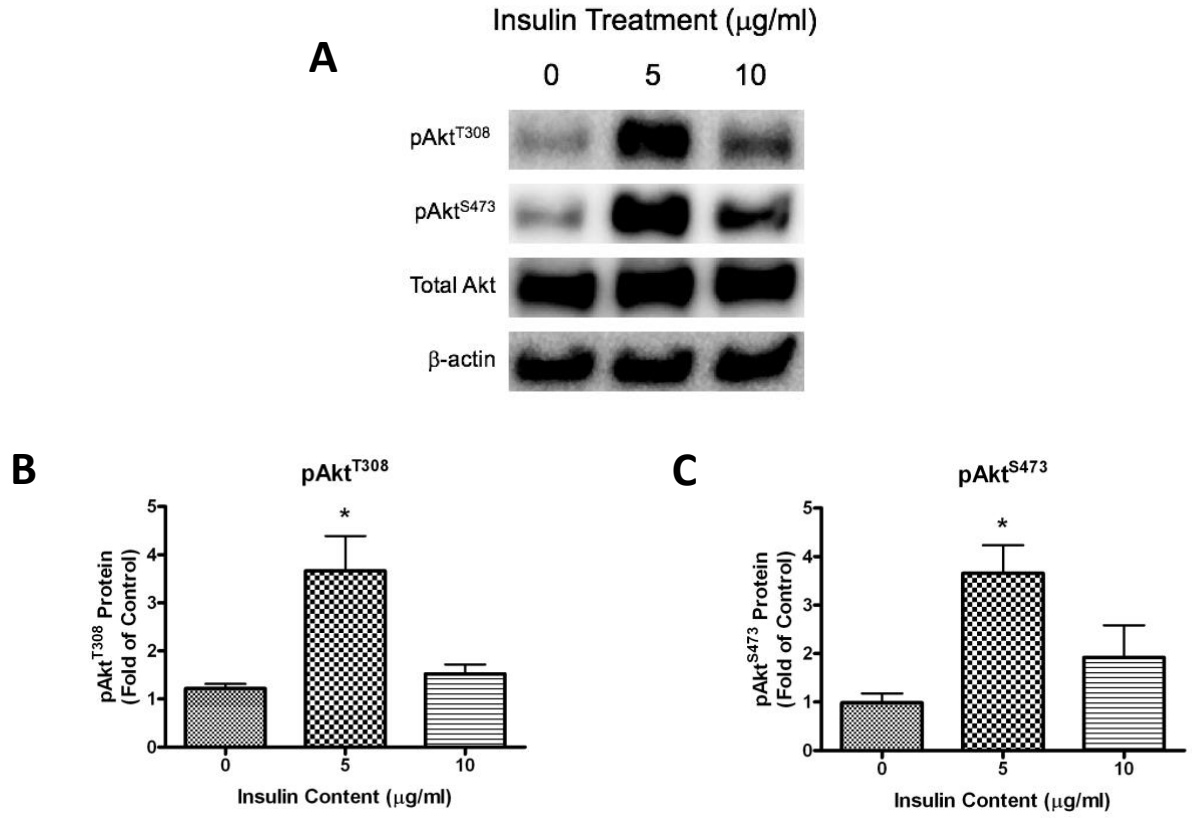

Figure 9:
Insulin dose in high glucose medium affects Akt activation in MCF7 cells. $pAkt^{T308}$ (A,B) and $pAkt^{S473}$ (A,C) saw significant increases in protein expression with a 5μg/ml dose of insulin. This is roughly 5x physiological levels of post-prandial insulin. All values are mean ± S.E.M.; * $p<0.05$ compared to control (n=6).

βHB affects mitochondrial protein expression

Treatment of ketone bodies caused a dose dependent increase in protein expression of COXIV. This protein is a marker of mitochondrial content, found in Complex IV of the electron transport chain. In both 24 hour (Figure 10A) and 48 hour (Figure 10B) treatments, we saw a step-wise increase in COX IV.

Total AMPK protein expression was unaffected in the 24 hour treatment (Figure 10A). However, Total AMPK protein expression was reduced by βHB with significant differences observed between 0 and 5mM of βHB in the 48 hour treatment (Figure 10B).

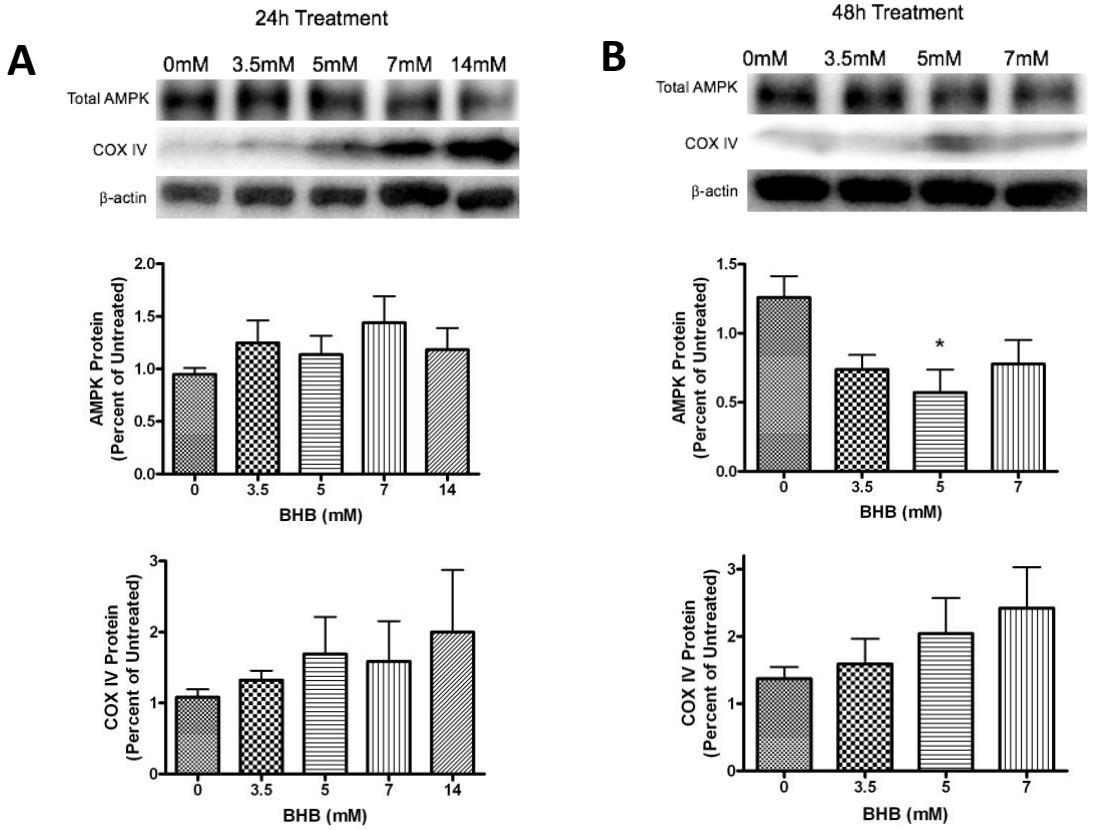

Figure 10: βHB treatments in high glucose medium with 10μg/ml of insulin lead to a step-wise increase in COX IV for 24 hour and 48 hour treatments and lowered total AMPK in the 48 hour time course **(B).** Significant decrease from 0-5mM of βHB in total AMPK for 48 hour treatment. Increases in COX IV protein for 24 hour and 48 hour treatments of βHB (0, 3.5, 5, 7, and 14mM). All values are mean ± S.E.M.; * $p<0.05$ compared to control (n=4-8).

βHB in high glucose medium does not affect p27

Total p27 protein was unchanged in response to βHB treatment in high glucose AMEM, with both a 24 hour and 48 hour treatment (Figure 11A,B). However, $p27^{T198}$ was significantly reduced following a 48 hour treatment of βHB (Figure 11B). While no changes were evident after a 24 hour treatment of βHB exposure, after a 48 hour treatment of βHB there was a reduction in $p27^{T198}$ when expressed as a percentage of total p27 (Figure 11A,B).

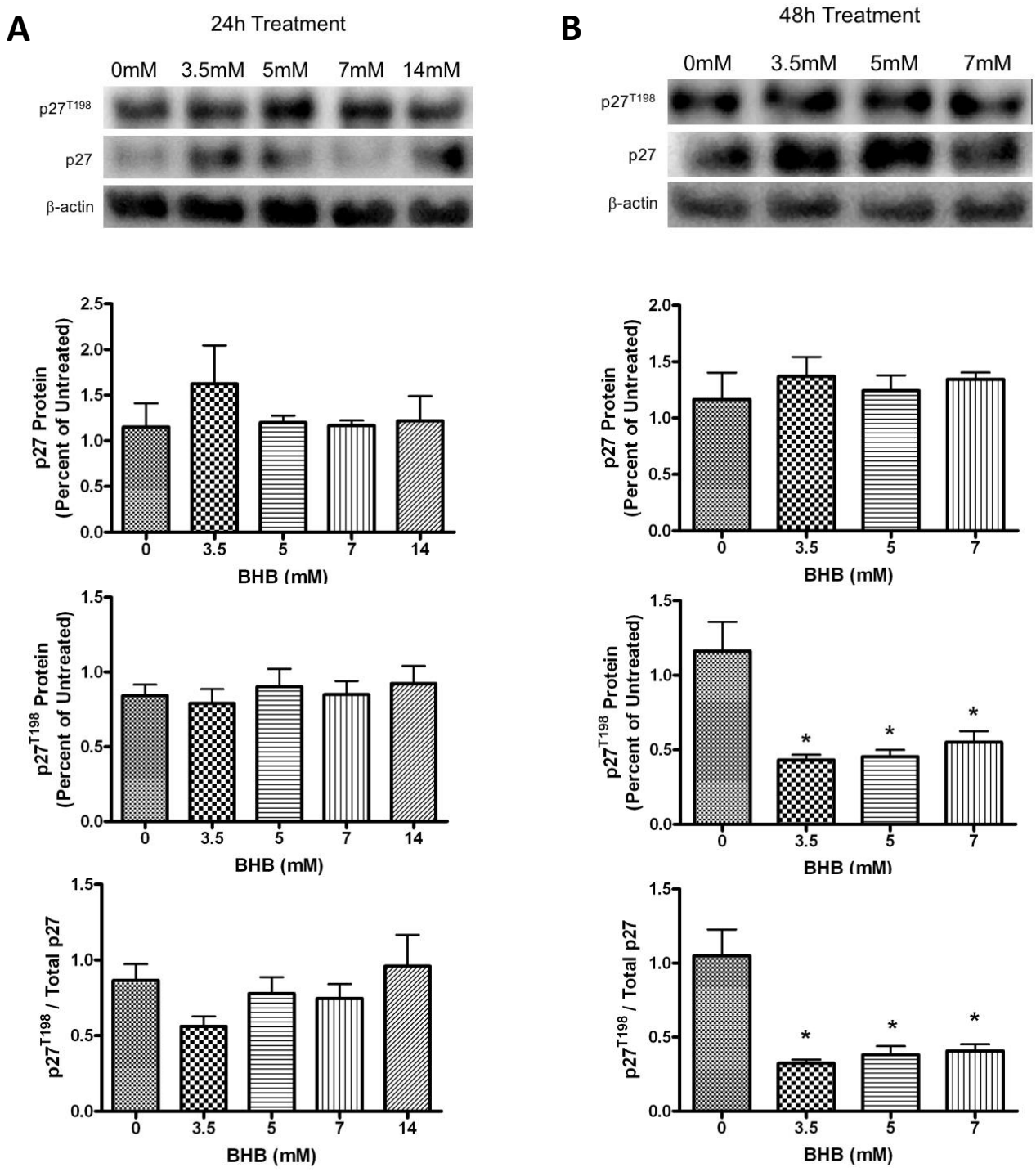

Figure 11:

βHB treatment in high glucose medium with 10μg/ml of insulin did not change p27 protein but destabilized the phosphorylated p27 on the T198 residue with 48 hour treatment **(A,B).** No changes occurred in total p27 protein expression between control and 3.5mM of βHB for 24 hour and 48 hour treatments **(B).** Significant decrease was seen in $p27^{T198}$ between control and 3.5, 5, and 7 mM in 48 hour treatments. Significant decrease was seen in $p27^{T198}$ as a percentage of total p27 protein between control and 3.5, 5, and 7 mM in 48 hour treatments. All values are mean ± S.E.M.; * $p<0.05$ compared to control (n=4-8).

Ketone treatment downregulates the pAkt pathway

No effects were seen after a 24 hour treatment of βHB (Figure 12A) but increasing βHB in the presence of high glucose AMEM at a 48 hour treatment decreased $pAkt^{T308}$ with significant decreases observed at 5mM βHB. (Figure 12B). Similarly, protein expression of $pAkt^{T308}$ as a percentage of total Akt was significantly unchanged after 24 hour treatment (Figure 12A) but was reduced from control to 5mM of βHB in a 48 hour treatment condition (Figure 12B).

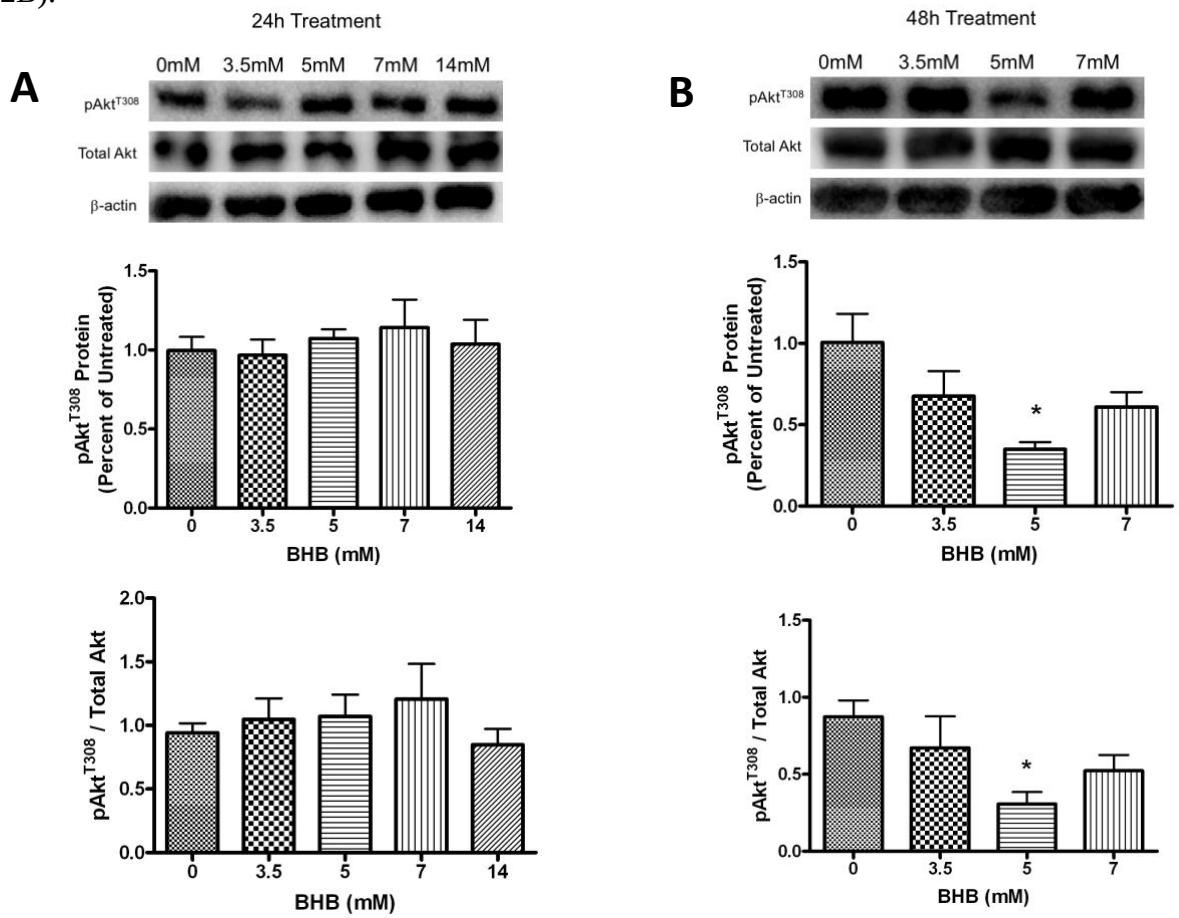

Figure 12:
βHB treatment in high glucose medium with 10μg/ml of insulin. $pAkt^{T308}$ protein expression saw significant changes in the 48 hour time course with total Akt staying fairly consistent across both time courses **(B).** $pAkt^{T308}$ decreased in a step-wise manner between control, 3.5mM and 5mM βHB with 48 hour treatment. $pAkt^{T308}$ as a percentage of total Akt decreased in a step-wise manner between control, 3.5mM and 5mM βHB with a 48 hour treatment. All values are mean ± S.E.M.; * $p<0.05$ compared to control (n=4-8).

βHB increases mitochondrial content and p27

The shift from high glucose AMEM to a glucose controlled DMEM treatment media was intended to test the effects of decreasing glucose and ketones to physiological levels observed during nutritional ketosis. Treatment of βHB with a range of physiological levels of glucose (0, 1, 2, and 5mM) increased the protein expression of the mitochondrial marker COX IV and also increased expression of total p27 protein (Figure 13A-D). This consistently appears as a step-wise increase with a decrease, seen at high levels of βHB (14mM) (Figure 13A-D). At high levels of glucose (10mM) we don't see changes in protein expression across βHB treatments relative to control (3.5, 5, 7, and 14mM) (Figure 13E). Clearly, the biggest effects are seen at physiological levels of βHB and glucose (1-2mM). As glucose climbs to 10mM, these effects are no longer evident (Figure 13A,E).

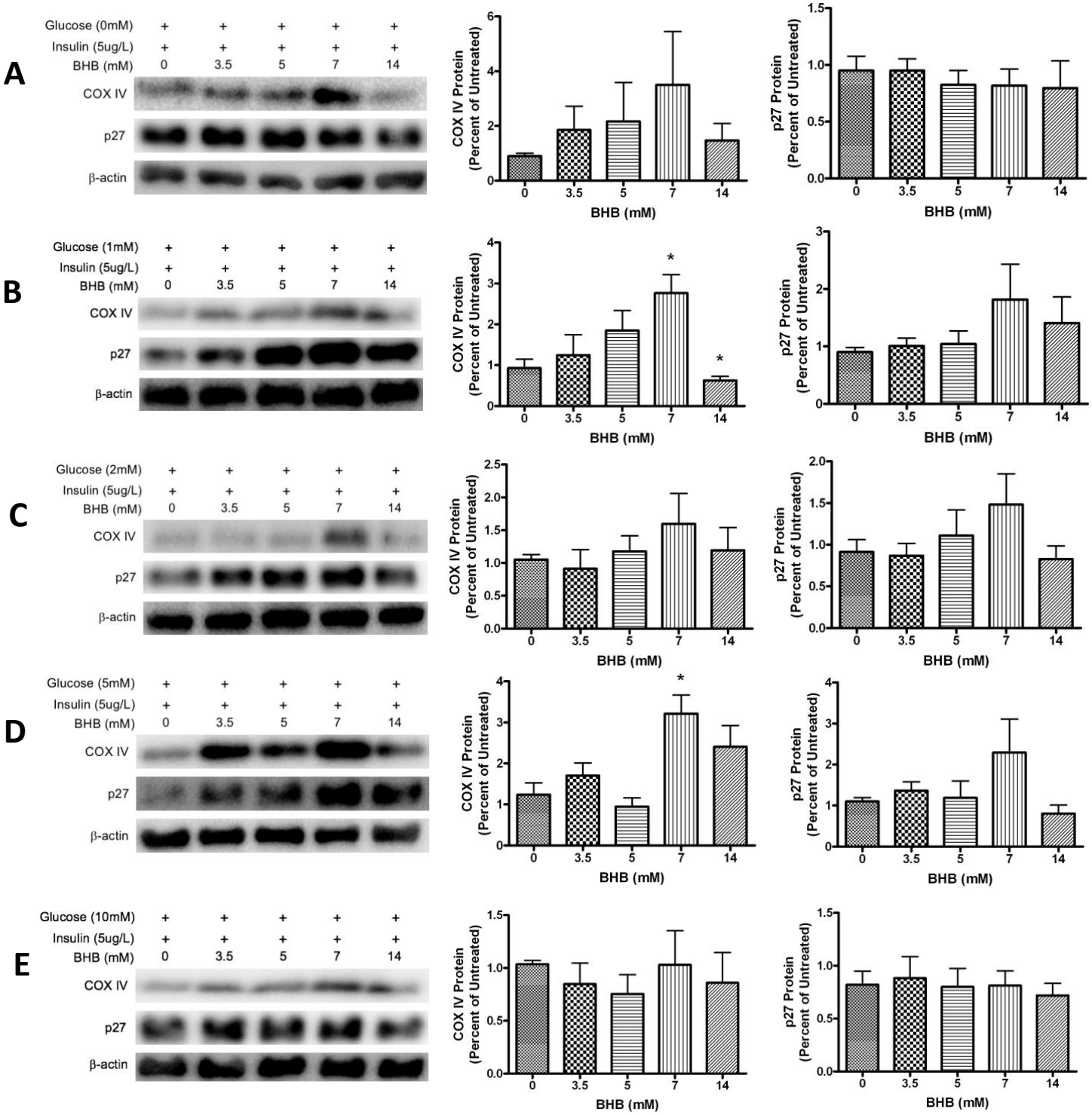

Figure 13:
(A-D). βHB treatment with 5μg/ml insulin across glucose (mM) treatments 0, 1, 2, and 5 lead to dose-dependent increase in protein expression of p27 and COX IV **(E).** Plateau effects of βHB treatment were seen in 10mM glucose for p27 and COX IV protein levels. All values are mean ± S.E.M.; * $p<0.05$ compared to control (n=6).

AdipoR1 levels stay constant with βHB treatment

AdipoR1 protein expression was unchanged in treatments with βHB independent of glucose levels (Figure 14A-B). No changes were seen with βHB treatment across all glucose conditions (0, 1, 2, 5, 10mM) similar to the effects in 2mM glucose shown in Figure 14. AdipoR1 was not necessarily expected to change, but was examined because its protein expression is increased in breast cancer cells following weight loss and slows cancer growth.

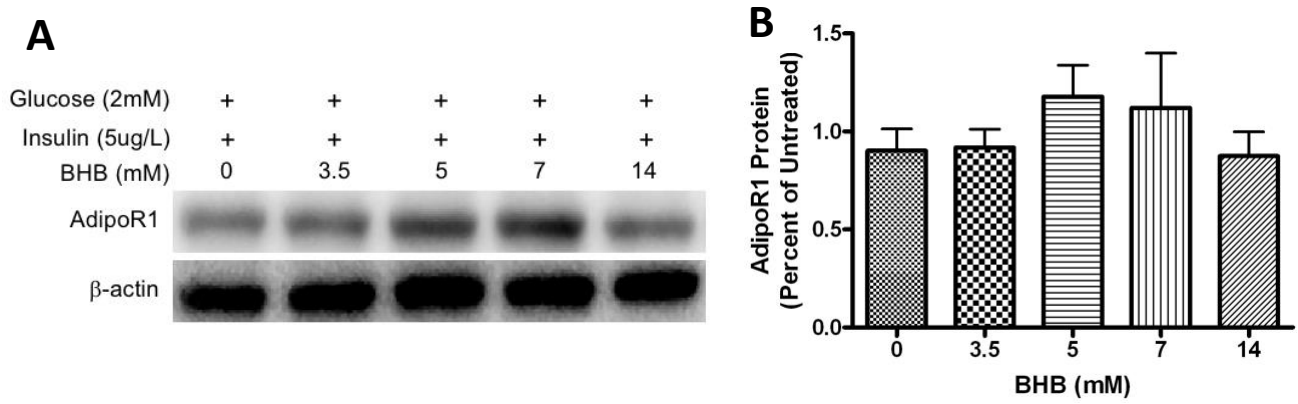

Figure 14:
βHB treatment with 5μg/ml across all glucose (mM) treatments 0, 1, 2, 5, 10 lead to consistent AdipoR1 protein expression **(B).** AdipoR1 expression gradually increases at 5mM and 7mM βHB treatments, however relatively unchanged across all experiments. All values are mean ± S.E.M.; * $p<0.05$ compared to control (n=6).

AMPK phosphorylation increases with βHB treatment under low glucose conditions

Treatment of MCF7 cells with βHB in a low glucose environment (1mM) lead to increased levels of pAMPK^{T172} in a dose-dependent manner with a potential decrease at 14mM (Figure 15B). When normalized to Total AMPK, pAMPK^{T172} increased in a dose-dependent manner (Figure 15C).

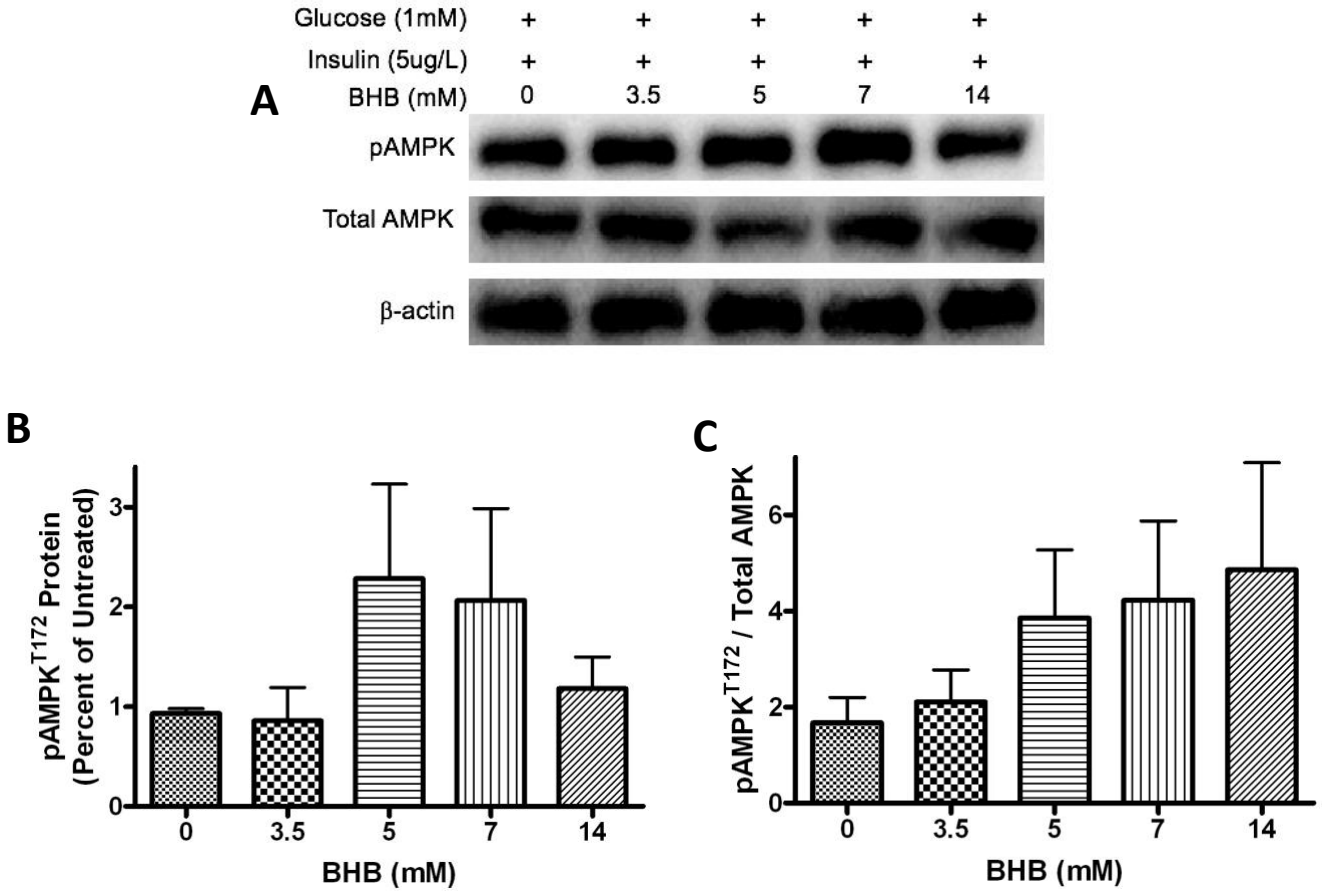

Figure 15: βHB treatment with 5μg/ml insulin and 1mM glucose upregulated the pAMPK pathway **(B).** pAMPK^{T1728} is upregulated in a dose-dependent manner between 0, 3.5 and 5mM of βHB, with a plateau between 5, 7 and 14mM of βHB **(C).** pAMPK^{T172} as a percentage of total AMPK is upregulated in a dose-dependent manner between 0, 3.5, 5, 7, and 14mM of βHB. All values are mean ± S.E.M.; * $p<0.05$ compared to control (n=6).

βHB treatment under a low glucose condition decreases activation of the pAkt pathway

The largest effects on pAkt were evident at 2mM glucose. $pAkt^{S473}$ was downregulated significantly between control and 5mM and 14mM of βHB, with a similar downregulation when expressing $pAkt^{S473}$ as a percentage of total Akt (Figure 16B). Similarly, $pAkt^{T308}$ was downregulated significantly between control and 5mM of βHB, with a similar downregulation when expressing $pAkt^{T308}$ as a percentage of total Akt (Figure 16C).

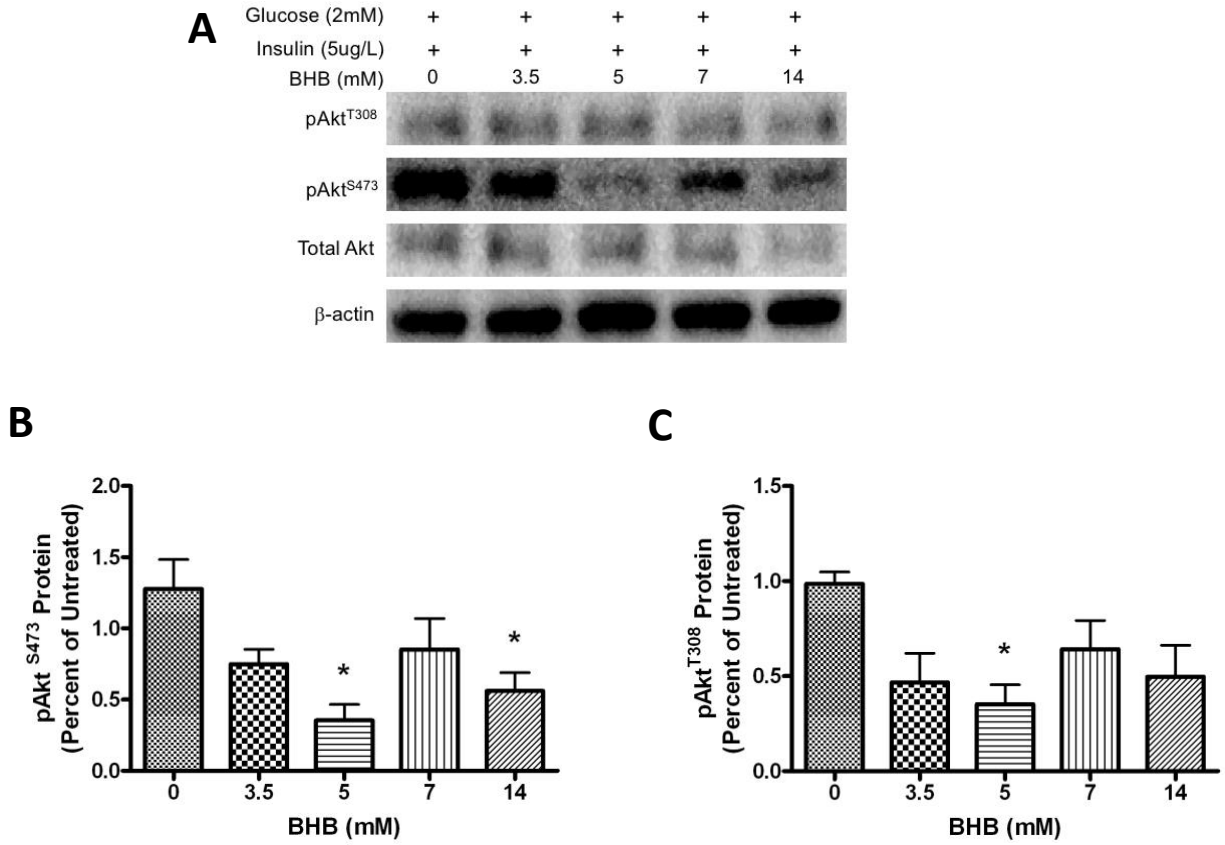

Figure 16: βHB treatment with 5μg/ml insulin and 2mM glucose downregulated the pAkt pathway **(B).** $pAkt^{S473}$ is downregulated in a dose-dependent manner between 0, 3.5 and 5mM of βHB **(C).** $pAkt^{T308}$ is downregulated in a dose-dependent manner between 0, 3.5 and 5mM of βHB. All values are mean ± S.E.M.; * $p<0.05$ compared to control (n=6).

4.5 – Discussion:

A reduction in obesity is known to decrease the growth of breast cancer (MCF7) cells. One way to decrease obesity is via a high fat, low carbohydrate ketogenic diet (KD). KD also increases the circulating levels of βHB, which can be used as a fuel source in metabolically active tissues. We used a cell culture model to try to tease out the effects of the changing conditions that occur in the circulation following the implementation of a KD. Other established effects of KD include, but are not limited to, a reduction in insulin and glucose and an increase in ketone bodies produced by the mobilization/catabolism of FFA. Srivastava et al. showed that animals fed a KD significantly lowered blood glucose levels (<5mM) while β-hydroxybutyrate (βHB) levels increased compared to a chow fed group. Furthermore, insulin concentrations in the plasma for the KD group were about 60% lower than the chow group after a month of being on a ketogenic diet (Srivastava et al. 2012). Each of these changes represents an alteration in the tumour growth microenvironment. Thus, we evaluated the effects of each of these variable ranges across physiological ranges on MCF7 cell regulation. Our results suggest that physiological doses of ketone bodies (βHB) are most effective at lower levels of glucose, which may be beneficial since KD is effective at reducing post-prandial glucose levels.

Cytochrome c oxidase subunit 4 (COX IV) is one of 13 COX subunits and is often used as an indicator of cellular mitochondrial content. COX IV protein levels were upregulated in a dose dependent manner in response to increasing βHB in both low and high glucose treatments. This increase in COX IV suggests that βHB is directly leading to an increase in mitochondrial content, increasing the cell reliance on oxidative phosphorylation and possibly reversing the Warburg effect (Kogot-Levin et al. 2016). As the last enzyme of the mitochondrial respiratory chain, it is responsible for the catalytic transfer of electrons to molecular oxygen from reduced

cytochrome *c*. This reaction is paired with proton pumping across the inner mitochondrial membrane to establish an electrochemical gradient in the mitochondria (Calhoun et al, 1994). By removing glucose from treatment media, or adding βHB, the metabolism of MCF7 cancer cells may have begun to shift from glycolysis towards oxidative phosphorylation in the mitochondria. MCF7 cells have impaired mitochondrial function and are sensitive to glycolytic inhibition (Pelicano et al. 2014). This increase in COX IV protein expression suggested that a decrease in glucose may act as a metabolic intervention to shift cancer cell metabolism that may make them less able to thrive in hypoxic conditions and confer a greater susceptibility to adjuvant therapies.

AdipoR1 is affected by obesity-dependent changes in tumour growth microenvironment. Obese subjects typically show a decrease in circulating adiponectin and cellular AdipoR1 receptor content, with a correlated increase in plasma leptin concentration (Ruhl and Everhart, 2001). The binding of adiponectin to AdipoR1 typically upregulates AMPK, which plays a role in cellular metabolism and cell quiescence (Martha Daniela Mociño-Rodríguez et al. 2017). Our goal was to test AdipoR1 protein expression in order to see if βHB induced any effects on AdipoR1 signaling and, as expected, AdipoR1 protein expression remained constant through all experimental conditions. Since we did not alter adipokines in our experiments, we hypothesized there would be no effect of βHB on AdipoR1 protein levels. Despite increases in pAMPK, there were no changes in AdipoR1, suggesting primary effects of βHB are independent of AdipoR1 signaling (Figure 14).

Insulin is an activator of the Akt pathway and promotes glucose uptake in fat and muscle tissue which are insulin-dependent (Ward and Thompson, 2012). βHB treatment of MCF7 cells in a low glucose environment deactivated Akt signaling ($pAkt^{T308}$ and $pAkt^{S473}$; Figure 16A,B), potentially indicative of a reduction in reliance on glycolysis, as Akt activation is known to

increase glycolytic rates and lactate output in cells (Elstrom et al. 2004). This suggests that increases in βHB, decreases in insulin and glucose, all of which occur following consumption of a KD, may render cells less reliant on glucose and anaerobic glycolysis as a means of energy production. In support of this, KD consumption in rats has been shown to downregulate mTOR, a downstream target of Akt (McDaniel et al. 2011). Akt is overexpressed in many breast cancers (Shi et al. 2005) and unfortunately Akt pharmaceutical inhibitors have shown little promise and are limited in their application due to their toxicity (Nitulescu et al. 2005). Given that KD consumption increases βHB and decreases glucose, which we show decreases Akt activation, KD could play a role as an intervention for reducing breast cancer cell growth directly by naturally inhibiting Akt signaling. Fasting glucose levels are typically ≤ 2.5 mmol after ketogenic adaptation (Wiesli et al. 2012). Observations that at 2mmol glucose βHB is most effective gives physiological and clinical relevance to our results (Figure 16B-C).

In addition to decreasing Akt signaling, βHB treatment at low glucose concentrations increased pAMPK signaling (Figure 15B). Based on previous work (Theriau et al. 2016), AMPK antagonizes Akt signaling and is beneficial in reducing MCF7 growth. This antagonization of Akt is also evident with βHB treatment at low glucose concentrations. Previous work has demonstrated that KD increased AMPK expression (Kennedy et al. 2007) and AMPK phosphorylation in the liver (McDaniel et al. 2011) which agrees with the results herein. AMPK is widely acknowledged to be activated in calorie deficient conditions and inhibited when energy is in excess (Cantó and Auwerx, 2011). Ketosis should drive phosphorylation of AMPK when glucose and insulin are low in MCF7 cells (Klein and Wolfe, 1992, Horman et al. 2006). The increase in pAMPK at 1mM of glucose gives us further insight into the possibility that total energy available to MCF7 cells is potentially reduced under a ketogenic environment.

Increased AMPK in MCF7 cells has been shown to increase p27 and induce G1 arrest (Theriau and Connor, 2017). Treatment with physiological levels of βHB indicated cell cycle arrest in some conditions due to the dose-dependent increase in total p27 protein when glucose levels are low (Figure 13B-D). βHB did not affect p27 until glucose and insulin levels were low, conditions observed during KD consumption. $p27^{kip1}$ is a powerful inhibitor of the cell cycle that is de-regulated in many types of cancer, including breast cancer (Slingerland and Pagano, 2000). p27 is also known to be increased during caloric restriction in tumours, further pointing to KD-induced increases in βHB being beneficial to creating an unfavourable growth environment for breast cancers (Zhu et al. 1999). The majority of studies looking at ketogenic diets and tumours show glucose levels between 3-10mM, with glucose levels dropping as ketone levels increased over time (Meidenbauer et al. 2005). This agrees with our results where we saw significant changes at 1, 2, and 5mM of glucose, levels that are the closest to physiological post-prandial glucose levels during KD consumption. It is important to note that all changes in protein expression were negated at glucose levels of 10mM. This level is considered a risk factor as a diabetic range of post-prandial glucose by the International Diabetes Federation suggesting that ketones alone are not effective as a counter measure to obesity dependent breast cancer risk/progression (5.6–11.0 mmol; Bowen et al. 2015). While changes in p27 appear physiologically significant, it still remains to be determined whether these changes lead to actual cell cycle arrest in response to βHB when glucose and insulin levels are low. By limiting glucose, the preferred fuel source of breast cancer cells, and adding ketones we may be able to effectively reverse the Warburg effect, thereby eliminating one of the hallmark changes in cancer cells that allows for their survival in less than ideal (i.e. hypoxic) conditions.

Our results indicate that changes in the expression of proteins involved in metabolism and the cell cycle are affected by βHB in low physiological glucose conditions. Our results also suggest that the implementation of a KD to increase ketones and reduce glucose and insulin would have similar effects on breast cancer cells *in vivo*. The Warburg effect is a hallmark of cancer that leads to a growth advantage for cancer cells and our results highlight the significance of ketone bodies in low glucose environments and the need for further research in the field of ketogenic diets on obesity and breast cancer. The importance of prevention in obese patients is highlighted by our experimental model, represented by the primary and secondary effects of KD in reducing glucose, insulin and adiposity.

5.0 - Overall Conclusions:

The obesity epidemic has resulted in an increase of morbidity and mortality worldwide. In the United States alone, consumers annually spend more than $30 billion dollars on weight loss products and services (Serdula et al., 1999) along with $750 billion dollars on pharmaceuticals. Work done previously by Doll and Peto suggested that over nutrition from diet alone (causing overweight) accounted for 35% of all cancers (Doll and Peto, 1981). More recently, the Center for Disease Control and Prevention found that 55 percent of all cancers diagnosed in women are associated with overweight or obesity (CDC, 2016). My thesis aimed to show that the effects of KD, including reduced insulin and reduced glucose with an increase in circulating ketones, would force cancer cells to rely on a dysfunctional oxidative phosphorylation cycle for energy needs and potentially induce cell cycle arrest or death. Research suggests that ketone bodies may reduce tumour growth and altering ketones, insulin, and glucose levels within the tumour growth microenvironment may prove beneficial in cancer therapy.

It is becoming more evident that there are benefits to the ketogenic diet and that the co-consumption of carbohydrates leading to higher levels of insulin may enhance signaling cascades to fuel tumour growth (Bonuccelli et al. 2010). Our experiments demonstrated that βHB effects were most evident during low glucose conditions. It is realized that it is a tricky undertaking to recapitulate the effects of KD using a cell culture model given that the human body, organs, tissues, and surrounding hormones are incredibly complex.

My research was most importantly able to show increases in COX IV in response to βHB treatment under low glucose conditions (Figure 13B-D). Cytochrome oxidase c is a protein that can be used as a marker for the OXPHOS (oxidative phosphorylation) system (Kogot-Levin et

al. 2016). Studies done on ketogenic diets have shown an increase in COX IV protein expression, along with markers of mitochondrial biogenesis (Srivastava et al. 2012). The increase in COX IV expression in our research was important because literature shows that some types of cancer have dysfunction in the mitochondria (Solaini et al. 2011). Another key finding is that ROS production from the OXPHOS system can cause cell cycle arrest and potentially slow down cancer cell growth by causing the cell to arrest during G1 (Yang et al. 2017).

According to the Crabtree effect, there is a down-regulation of the catalytic subunit of the mitochondrial ATP synthase subunit, where expression level of β-F1-ATPase protein is inversely correlated with the rate of aerobic glycolysis (Diaz-Ruiz et al. 2011). One study done by the Fantin group showed that OXPHOS was enhanced when a glycolytic enzyme (LDH-A) was suppressed in cancer cells, in order to compensate for the reduction in ATP by glycolytic inhibition. Most importantly, they demonstrated that the shift to OXPHOS showed inhibition of proliferation and tumorigenicity of cancer cells which suggests impairment of OXPHOS to provide a sufficient amount of energy for cancer growth (Fantin et al. 2006). Based on the literature, stimulating ROS production in cells via glucose deprivation directly causes inhibition of cell proliferation (Aulwurm and Brand, 2000). Likewise, when glycolysis was enhanced, it reportedly protected cells from oxidative stress. Our findings of pushing MCF7 cells towards OXPHOS is also important due to the Warburg effect, where cancer cells typically show increase in glucose uptake and higher rates of glycolysis (Gogvadze et al. 2010). Our findings from the step-wise increase in COX IV and p27 protein under low glucose conditions, with a consequent decrease at toxic βHB levels (14mM), further supports the notion that shutting off the glycolytic pathway and driving OXPHOS can promote cell cycle arrest at G1.

$p27^{kip1}$ is arguably one of the most important 'brakes' in the cell cycle. With its role in late G1/early S phase, it has been implicated in many studies done on cancer (Lloyd et al. 1999, Blain et al. 2003). A more recent study helps us tie the link between similar dose-dependent increase of COX IV and p27 protein expression in low glucose environments when treated with physiological levels of βHB. Bachs et al. demonstrated that at least 18 genes that are involved in oxidative phosphorylation within the respiratory chain are downregulated in $p27^{kip1}$ knock-out cells, which helps draw a bridge between the results that we observed (Bachs et al. 2018). Since p27 levels are low in breast cancer cells, we would expect to see a decrease in oxidative phosphorylation. The decrease in respiration leads to aerobic glycolysis and fuels tumour cells (Lunt and Heiden, 2011). Our results agree with the literature in that mitochondrial activity and p27 protein expression are upregulated in a similar manner (Figure 13B-D). This supports our hypothesis regarding the impairment of oxidative phosphorylation while activating markers of cell cycle arrest. Further research is needed to distinguish if they are related and if the effects seen on p27 are secondary.

Although our results did not show statistical significance likely due to variability and lower experimental numbers, we see a clear trend in our p27 protein expression. Based on the extensive literature done by the Lloyd group demonstrating that p27 expression decreases as tumours progress (Lloyd et al. 1999), this advanced our hypothesis that the reduction of glucose and the presence of ketone bodies promotes cell cycle arrest. Without FACS analysis, we are not able to clearly show cell cycle arrest during late G1. However, the pattern seen in p27 protein expression did provide some support to our hypothesis (Figure 13B-D). Diving deeper into MCF7 cells and breast cancer specifically, the literature in conjunction with the results we saw supports our hypothesis even further as p27 causes cell cycle arrest in G1 (Toyoshima and

Hunter, 1994). Further studies show that p27 expression is lower in more aggressive tumours (Catzavelos et al. 1997, Porter et al. 1997, Tan et al. 1997). The Tan group specifically found that decreased p27 in small invasive breast carcinomas were at a greater risk for tumour progression, and should consider an adjuvant therapy. The ketogenic diet in our case presents itself as an opportunity for a non-toxic, and non-invasive adjuvant to present cancer therapies to drive p27 expression in cancer cells and promote cell cycle arrest.

Activation of the Akt/PKB pathway, similar to p27, is a marker of poor health in breast cancer (Veeriah et al. 2014). Activation of the Akt/PKB protein kinase family is also known to trigger glycolytic activity (Mackenzie and Elliot, 2016). More recently, this family of proteins has emerged as the most frequently activated protein kinases in human cancer. Upon activation, Akt is able to phosphorylate downstream target proteins that are involved in glucose uptake and metabolism. It is extremely important to note that during prolonged fasting, which has similar effects on metabolism as a ketogenic diet, insulin levels approach zero. Mild ketosis can compensate for the metabolic effects of insulin in its absence (Veech, 2004). Thus, we could have a therapeutic approach to downregulating the Akt/PKB pathway without losing critical metabolic effects that insulin is responsible for.

Our research on ketone treatment in both low and high glucose conditions showed a downregulation in Akt pathway activation. Our studies on the doses of glucose were done with treatment media supplemented at 5mg/ml of insulin, where we saw the highest protein expression of both $pAkt^{T308}$ and $pAkt^{S473}$ in our initial insulin experiments. Insulin comes into play when we consider that IGF-1 drives Akt phosphorylation to promote the cell cycle and fuel tumour growth (Chin and Toker, 2009). We were able to see significant decreases between control and 5mM of βHB treatment in our low glucose condition, thus supporting the hypothesis

that the reduction of insulin and the presence of ketone bodies in a ketogenic diet will drive markers of cell cycle arrest.

The KD produces ketone bodies as an alternative fuel source in the absence of glucose. This is the principle behind driving OXPHOS and attempting to reverse the Warburg effect. In order to slow down cancer growth, the brakes of the cell cycle (p27, AMPK) need to be activated while the gas pedals of the cell cycle need to be downregulated (Akt, glycolysis). The Akt downregulation and concurrent AMPK upregulation during low glucose conditions agrees with our working model (Figure 17B). AMPK also upregulates mitochondrial content, as evidenced by COX IV, through activation of PGC-1α. The dose-dependent increases of p27 and COX IV at low glucose conditions further support this model. We begin to see a system where low glucose conditions and the production of βHB via KD will lower insulin and repress glycolysis and proliferation through a complex interaction between Akt, AMPK, COX IV, and p27 (Figure 17A,B). Based on the literature provided and changes in protein expression seen in our research, we were able to gain more insight into the topic of diet and cancer. The data suggested that mitochondrial dysfunction could occur in cancer cells and coincide with markers of cell cycle arrest. Thus, our results warrant further research on ketogenic diets as an intervention for obesity and breast cancer.

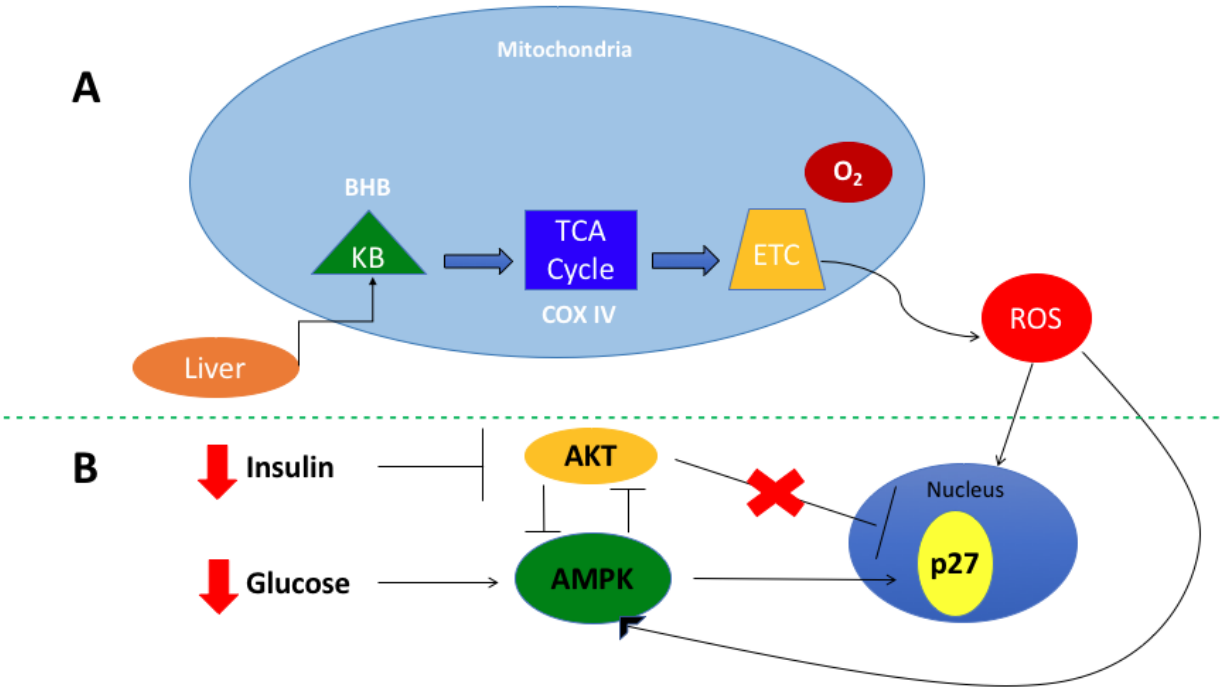

Figure 17: Current working model based on data and observations collected. Ketone body treatment in a low glucose and low insulin environment promotes a marker of cell cycle arrest and increases mitochondrial content in MCF7 breast cancer cells.

6.0 Limitations and Future Directions:

6.1 Limitations:

As with all experimental models, our research model poses a few limitations to consider. The most important limitation is on the complexity of the human body and the cancer microenvironment. With a cell culture model, there is an oversimplification of the interactions between cancer cells and the pool of media present (tumour microenvironment). While work is being done in animal models on ketogenic diets and cancer, it is very rare in the scientific field to study this *in-vitro*, which allows us to control for variables like glucose and βHB to study dose-dependent relationships. This is an important research area that should be looked at further, but can be oversimplified when adapting the results to a human body.

Another potential limitation is the dose of insulin used in the treatment media. When we reduced insulin closer to physiological levels, we shut off the Akt pathway and struggled to capture protein expression during immunoblotting. The insulin levels used provided enough protein for imaging, however they were five-fold physiological levels at 5μg/ml and could have masked changes in protein expression that would be induced by a low-glucose and high-ketone environment. It was also difficult to control for contributions of insulin and glucose from Fetal Bovine Serum (FBS) added to the growth media. However, contributions from FBS were likely limited, and the content was not drastically different from batch to batch.

Lastly, the treatment time was 24 hours and may be shorter than what is needed to see changes in protein expression. The ketogenic adaptation stage can take 2-3 days in humans, and has not been solidified in cell culture. The changes we were looking for in specific proteins may have not occurred we collected and analyzed lysates, or there may not have been enough time for enzymatic adaptations to take place, evidenced by 48 hour treatments showing increased effects.

6.2 - Future Directions:

This field of study is fairly new and is beginning to gain recognition over the past few years. Our research was able to look at the ketogenic diet (KD) as an intervention for obesity and breast cancer on a superficial level.

6.2.1: Insulin

Future work *in-vitro* should take into consideration the importance of insulin levels and the role they play in the cell cycle. Arguably the most important component of this field of research, the next step would be to isolate the most physiological glucose and ketone level as the dependent variable, and test a dose-dependent treatment of physiological insulin as the independent variable. This would be a more accurate cell culture model to test the effects of a higher carbohydrate diet versus a ketogenic diet on breast cancer growth.

6.2.2: Treatment Time

Work done on KD and cancer should consider a time-course treatment protocol. We know that ketogenic diets can reduce fasting insulin levels after just a few days. It would be interesting to see a time course less than, and greater than our treatment protocol using a defined set of variables. The same variables can be used for a 3, 5, and 7-day protocol to assess both short and long-term changes in protein expression.

6.2.3: Animal Model

The most important literature in the field of ketogenic diets and cancer has been done in human and animal models. Xenograft models offer an advantage to studying tumour growth in response to a KD.

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