Characterizing Metabolic Changes in Cancer Cells After Treatment with an Extract Created from Walnuts

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CHARACTERIZING METABOLIC CHANGES IN CANCER CELLS AFTER TREATMENT WITH AN EXTRACT CREATED FROM WALNUTS

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ABSTRACT

CHARACTERIZING METABOLIC CHANGES IN CANCER CELLS AFTER TREATMENT WITH AN EXTRACT CREATED FROM WALNUTS

by Codey Y. Huang

Cancer cells undergo metabolic reprogramming to meet the energy and metabolite demands of constant proliferation. Originally observed as the Warburg Effect, where cancer cells are more glycolytically active, recent research has shown that cancer cells can also utilize oxidative phosphorylation to meet these demands, better known as metabolic flux. Previous work from our lab showed that treatment with a Walnut Extract (WE) induced cell death in MDA-MB-231 and HeLa cells by destabilizing the mitochondrial outer membrane potential, suggesting that the extract may be able to impact oxidative phosphorylation. The aim of the current research was to further characterize the impact WE treatment had on both glycolytic and oxidative phosphorylation in cancer cells. Seahorse metabolic assays measuring the oxygen consumption and extracellular acidification of WE treated HeLa, MDA-MB-231, and MCF10a cells showed both a time- and dose-dependent reduction in oxidative and glycolytic rates in WE treated cells. The reduction in oxidative and glycolytic rates resulted in reduced total adenosine triphosphate (ATP) levels within treated cells when quantified. A permanent inhibition following washout of WE was also observed. The reduction in total ATP did not activate protein sensors of ATP and adenosine monophosphate (AMP) within the cell. The WE stimulated inhibition of oxidative and glycolytic activity that did not activate energy sensors within the cell suggests that WE treatment is inhibiting glycolysis and oxidative phosphorylation through an alternative mechanism.
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2-DG: 2-deoxy-D-glucose
ACC: Acetyl-CoA Carboxylase
AMPK: 5’ AMP Activated Protein Kinase
APS: Ammonium Persulfate
ATCC: American Type Culture Collection
ATP: Adenosine Triphosphate
ADP: Adenosine Diphosphate
AMP: Adenosine Monophosphate
CLB: Complete Lysis Buffer
CTG: CellTiter-Glo Assay
DMEM: Dulbecco’s Modified Eagle Medium
DTT: Dithiothreitol
ECAR: Extracellular Acidification Rate
ER: Estrogen Receptor
FBS: Fetal Bovine Serum
FCCP: Carbonyl Cyanide-p-trifluoromethoxyphenylhydrazone
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
glycoATP: Glycolytically Generated ATP Production Rate
glycoPER: Glycolytic Proton Efflux Rate
Her2: Human Epidermal Growth Factor Receptor 2
HIF-1: Hypoxia Induced Factor 1
HRP: Horseradish Peroxidase
mitoATP: Mitochondrially Generated ATP Production Rate
mTOR: Mammalian Target of Rapamycin
NAD+: Nicotinamide Adenine Dinucleotide
NEAA: Nonessential Amino Acids
OCR: Oxygen Consumption Rate
PFK2: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3
PMSF: Phenylmethylsulfonyl Fluoride
PVDF: Polyvinylidene Fluoride
PR: Progesterone Receptor
RAPTOR: Regulatory Associated Protein of mTOR
RICTOR: Rapamycin-insensitive Companion of mTOR
SDS: Sodium Dodecyl Sulfate
SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBS-T: Tris-buffered Saline with Tween-20
TEMED: Tetramethylethylenediamine
TNBC: Triple Negative Breast Cancer
UCP-1: Uncoupling Protein 1
WE: Walnut Extrac
Introduction

Cancer is the second leading cause of death in the United States. In 2018, over 1,700,000 new cases of cancer were diagnosed, and almost 600,000 patients died of cancer. Among women, breast cancer is the most commonly diagnosed, and is the second most fatal (Centers for Disease Control, 2021).

Breast cancer is characterized based on the expression of three receptors on the cell. Estrogen receptor- and progesterone receptor-positive breast cancers (ER and PR, respectively) are collectively known as luminal breast cancers. The expression of a third receptor, human epidermal receptor 2 (Her2), in combination with ER and PR expression determines the basoluminal breast cancer subtype. Breast cancers that express these receptors can be treated using targeted therapies specific to those receptors, in addition to the traditional therapies, such as radiation, chemotherapy, and surgery. However, a fourth type of breast cancer, triple-negative breast cancer (TNBC), is much harder to treat because it does not express ER, PR, or Her2. Because TNBC does not express any of the receptors, targeted TNBC therapies do not exist. As a result, TNBC patients have the lowest overall survival, as well as the highest rate of recurrence (Li et al., 2016). TNBC can only be treated with untargeted therapies, such as radiation, chemotherapy, or surgery, all of which have significant physical and mental side effects (Medina et al., 2020). Although receptor-specific targeted therapies do still have side effects, off-target effects on healthy tissue is generally avoided and are typically mild (Sodergren et al., 2016). However, conventional therapies often have off-target effects. Chemotherapy typically cause side effects such as hair loss, nausea, anemia, and cognitive decline (Kayl & Meyers, 2006). Radiotherapy also has
significant side effects. The close proximity of the breast tissue to the heart and lungs often results in damage to those organs during radiotherapy, leading to radiation pneumonitis and lung fibrosis, where tissue damaged by radiation is scarred over (Rutqvist et al., 2003; Senkus-Konefka & Jassem, 2006). Mastectomies are currently the gold standard in surgical removal of cancerous tissue in breast cancers. However, the removal of tissue often involves the entire breast to prevent cancer metastasis and recurrence, commonly resulting in mental side effects, such as body image issues, depression, and anxiety (Gottschalk & Hoigaard-Martin, 1986; Harcourt et al., 2003; Nozawa et al, 2015). Additionally, the removal of tissue is usually accompanied by chemotherapy to ensure full removal of the cancer, and therefore patients also experience the same side effects as chemotherapy-only patients as well. Thus, the search for a treatment that is as effective as current therapies while avoiding invasive procedures or significant side effects is becoming increasingly important.

One of the main hallmarks of cancer is constant cellular proliferation, where the cancer cells grow and divide unchecked and unregulated. The growth and division of cells is an energy and metabolite intensive process. As a result, the energy metabolism of a cancer cell is increased to meet these demands. This is known as metabolic reprogramming (Hanahan & Weinberg, 2000, 2011). Originally observed by Warburg (1956) as an increase in the glucose consumption and lactate production in tumors, this was attributed to an increase in glycolytic activity and became known as the Warburg Effect (Liberti & Locasale, 2016). However, recent research has shown that cancer cells also have metabolically active mitochondria, and are not limited to the cytosol bound glycolysis (Jose et al., 2011; Xu et al., 2015). Breast cancers are a type of cancer that can utilize mitochondria to produce energy (Lunetti et al.,
Thus, it is possible to target mitochondria and cancer cell energy metabolism in general as a potential therapy for TNBC.

Drugs and compounds isolated from bacteria and plants have been used as antibacterial, antiparasitic, and anticancer treatments (Katz & Baltz, 2016). Plants produce a class of compounds known as polyphenolic compounds. These polyphenolic compounds include flavonoids such as quercetin, which have been shown to have anticancer effects (Reyes-Farias & Carrasco-Pozo, 2019). Walnuts, in addition to being rich in mono- and polyunsaturated fatty acids and having antioxidant effects, are also rich in polyphenolic compounds, and specifically ellagitannins (Hayes et al., 2016; Le et al., 2014; Vinson & Cai, 2012). Mice with xenografted breast or prostate cancer cells fed with walnut-rich diets exhibited reduced growth of the resulting tumors, suggesting that walnuts can be used as an effective anti-cancer treatment (Davis et al., 2012; Hardman et al., 2011; 2014; 2019; Nagel et al., 2012; Reiter et al., 2013).

Additionally, previous studies in our lab showed that MDA-MB-231 breast cancer cells treated with WE had a dose-dependent effect on viability. The WE also appeared to affect the mitochondria in treated cells. Cells treated with WE had a destabilized mitochondrial membrane, resulting in decreased intracellular pH (Le et al., 2014). The reduction of intracellular pH has been linked to cell death via the activation of degrading enzymes such as proteases and nucleases (Morana et al., 1996; Zorova et al., 2018). Because the mitochondrial production of energy via the electron transport chain relies on a proton gradient across the inner mitochondrial membrane, it is possible that the WE destabilization of the mitochondria could also affect the energy production of the cell leading to the cell death previously
observed. Other previous studies in our lab have shown that treatment of cells with flavonoid plant compounds affects the ATP levels within the cell (Yadegarynia et al., 2012). Although the WE does not contain any flavonoids, it is possible that the natural ellagitannin compounds found in the WE could affect ATP levels as well via the mitochondrial effects seen previously.

The goal of the current research was to characterize the effects of the WE on cell energy metabolism in both cancer and non-cancer cells. Because TNBC and cancer in general has dysregulated energy production within the cell, targeting the energy production of these pathways with a WE could potentially result in a broad but targeted therapy for cancer. Characterizing the effects of WE and investigating the mechanism through which it exerts its effects on cancer cells could elevate WE into a potential therapy for TNBC and cancer as a whole.
Literature Review

**CELLULAR RESPIRATION IN NORMAL CELLS**

Cells constantly require energy to function. ATP is the main source of energy within the cell, and is produced by two main energy production pathways within the cell, glycolysis and oxidative phosphorylation. In normal, noncancerous cells, glucose is first converted into pyruvate via glycolysis. Pyruvate then feeds into the Krebs cycle via an intermediate enzyme, pyruvate dehydrogenase, that converts pyruvate into acetyl-CoA (Krebs & Johnson, 1937). During the process of glycolysis and Krebs cycle, a small amount of ATP is produced (Figure 1).

The main purpose of glycolysis and the Krebs cycle is to regenerate electron carriers for use in the electron transport chain. The electron transport chain is a series of four protein complexes in the inner mitochondrial membrane, terminating in a fifth complex, the ATP synthase (Figure 2). During glycolysis, nicotinamide adenine dinucleotide (NAD⁺) is reduced to NADH. In the Krebs cycle, NADH is also produced, along with another electron carrier, flavin adenine nucleotide (FAD²⁺). These regenerated electron carriers then provide electrons to flow through the electron transport chain, from a high energy state to a low energy state (Alfarouk et al., 2014). The complexes of the electron transport chain utilize the energy from the electrons to transport protons from the inner mitochondrial matrix to the intermembrane space, setting up a proton gradient where there are more protons in the intermembrane space and few protons in the inner mitochondrial matrix. This proton gradient also creates a mitochondrial membrane potential. These protons are then used to generate ATP using F₁-F₀ ATP synthase (Zhao et al., 2019). ATP synthase uses protons flowing down their gradient to
power the phosphorylation reaction to convert ADP and inorganic phosphate to ATP (Figure 2). The oxidative phosphorylation of ADP is highly efficient, producing a net 30-32 ATP per glucose. At the end of the electron transport chain, low energy electrons are then accepted by oxygen and combined with spare protons to form water.

**Figure 1**

*Cell Energy Production Pathways in Normal Cells*

Note. Glucose is taken into the cell via glucose transporters. In the cytoplasm (gray), it then undergoes glycolysis, where it is broken down into two pyruvate molecules. In the process, 2 ATP are produced, as well as regenerating 2 NADH electron carriers. The pyruvates are transported into the mitochondria (orange), where it is converted into acetyl-CoA, to be used in the Krebs cycle. In anaerobic conditions, pyruvate can be fermented into lactate instead of producing acetyl-CoA. The Krebs cycle produces 4 CO₂, as well as 2 ATP. The 6 NADH and 2 FADH₂ electron carriers, combined with the 2 NADH produced in glycolysis, feed into the electron transport chain, where they are used to produce 26-28 ATP via oxidative phosphorylation.
Note. NADH and FADH₂ produced in previous pathways enter the electron transport chain complexes at Complex I and II. High energy electrons from these electron carriers are used to transport protons from the mitochondrial matrix into the intermembrane space, forming a proton gradient. These protons then flow back into mitochondrial matrix via Complex V, ATP synthase, which uses the flow of protons to phosphorylate ADP, generating ATP.

**METABOLIC REPROGRAMMING OF CANCER CELLS**

Cancer cells are metabolically different compared to normal cells (Jang et al., 2013). Two of the main hallmarks of cancer cells are their constant proliferation, and reprogrammed metabolism to provide energy and metabolites to support that proliferation (Hanahan & Weinberg, 2000, 2011). This was originally observed as a disproportionate increase in consumption of glucose and lactate production by cancer cells despite being in normoxic conditions, leading to the hypothesis that cancer cells undergo glycolysis more than oxidative phosphorylation, better known as the Warburg effect (Warburg, 1956).

The shift towards glycolysis in cancer cells is mainly due to the hypoxic tumor...
environment resulting from insufficient blood flow and constant energy demand (Carmeliet et al., 1998). This hypoxic environment prevents tumor cells from producing ATP via oxidative phosphorylation, while promoting anaerobic energy production via glycolysis. As a result, in cancer cells, pyruvate does not mainly feed into the Krebs cycle (Figure 3). Instead, pyruvate is fermented into lactate via lactate dehydrogenase, bypassing the Krebs cycle and oxidative phosphorylation. The resulting lactate is then shuttled out of the cell, where it acidifies the surrounding environment (Brooks et al., 2009; Milosevic et al., 2004; Warburg et al., 1927).

**Figure 3**

*Cancer Cells Utilize Lactate Fermentation*

Note. In the hypoxic tumor environment, cancer cells do not utilize the oxidative phosphorylation pathway, unlike normal cells. Instead, pyruvate produced from glycolysis is fermented into lactate to produce ATP. The lactate is then transported out of the cell where it acidifies the surrounding extracellular environment.

One of the main effects of the hypoxic tumor environment is the upregulation of hypoxia induced factor 1 (HIF-1). HIF-1 is a transcription factor that is activated in hypoxic
conditions, and is involved in many downstream adaptation pathways (Nagao et al., 2019; Ziello et al., 2007), but most importantly, the upregulation of enzymes involved in glycolysis and downregulation of enzymes involved in mitochondrial respiration. Hypoxic activation of HIF-1 is involved in the recruitment of glucose transporters to the cell membrane, increasing glucose uptake into the cell (Ebert et al., 1995). This increase in glucose within the cell is then translated into an increase in glycolysis via HIF-1 induction (Iyer et al., 1998).

Glycolysis is advantageous to the cancer cell for multiple reasons – it not only avoids the apoptosis-involved mitochondria, but also provides many of the intermediate metabolites required for other cell proliferation processes (Lu et al., 2015; Sánchez-Aragó et al., 2010). Oxidative phosphorylation also requires oxygen, which is scarce in the hypoxic tumor environment. Additionally, HIF-1 is involved in the suppression of mitochondrial activity via downregulation of pyruvate dehydrogenase activity, shifting pyruvate produced in glycolysis towards lactate fermentation as opposed to the Krebs cycle (Golias et al., 2016, 2019).

Recent research has shown that not only are the mitochondria functional in cancer cells, but that they can also provide a significant amount of energy within the cell (Martin & McGee, 2019; Rogers et al., 2019). Breast cancers are an example of a cell type being metabolically flexible, meaning they are able to switch from producing ATP via glycolysis to oxidative phosphorylation (Lunetti et al., 2019). The increasing widespread use of instruments that can measure aspects of cellular metabolism accurately has allowed for the characterization of the metabolism of cancer cell types. This discovery of active and functioning mitochondria in cancer cells provides another therapeutic target for the treatment of cancer.
Historically, the measuring and quantifying cellular energy metabolism has occurred through the use of Clark electrodes and the microphysiometer (Clark et al., 1958; Owicki & Parce, 1992; Parce et al., 1989). The Clark electrode quantified the rate of oxidative phosphorylation in a solution by measuring the rate of oxygen consumption (OCR). When the electrode was placed in a solution with an oxygen consuming source, the voltage of the electrode would reduce as dissolved oxygen was consumed during cellular respiration. The microphysiometer could be used to quantify glycolytic rates by measuring the rate of extracellular acidification (ECAR) when lactate is shuttled out of the cell. However, because of the large size of both of these instruments, it was impossible to capture both OCR and ECAR data for the same sample without a large amount of sample. The instruments were also impeded by their large size, preventing an accurate local measurement of OCR and ECAR, allowing the local microenvironment to reequilibrate with the surrounding environment.

Advancements in technology have allowed for the simultaneous measurement of OCR and ECAR. By utilizing fiber optics and fluorescent probes, the measurement of OCR and ECAR on a local level in a microplate has become possible (Dmitriev et al., 2012; Gerencser et al., 2009; Wu et al., 2007). The Seahorse Analyzer is an instrument that utilizes these advantages to measure OCR and ECAR in real time. A sensor plate containing fluorescent probes is lowered onto a separate cell plate, creating a transient sealed microchamber where the local microenvironment can be accurately measured. After measuring is complete, the
sensor plate is raised, unsealing the microchamber and allowing the local microenvironment to reequilibrate to the rest of the cell plate environment (Gerencser et al., 2009). In addition to measuring OCR and ECAR, the Seahorse also has multiple injection ports in the sensor plate, allowing the instrument to inject and treat assayed cells with loaded compounds during the assay.

**THE SEAHORSE MITO STRESS TEST**

The Seahorse Analyzer analyzes mitochondrial and glycolytic capabilities of cells by injecting different compounds. The combination and injection order of these compounds allow different aspects of respiration to be assessed. For example, the Mito Stress Test is a test designed to measure the oxidative capacity of assayed cells (Figure 4A). To do this, multiple drugs are used to inhibit or affect specific parts of the electron transport chain (Agilent, 2019a).

As with all Seahorse assays, a basal measurement is first taken. This basal OCR measurement constitutes the oxygen consumption rate of assayed cells before any inhibitors are applied. Following the basal measurement, the mitochondrial toxin oligomycin is injected via an injection port (Figure 4B). Oligomycin is an inhibitor of ATP synthase (Huijing & Slater, 1961). This inhibition of ATP synthase not only halts ATP production, but also reduces the flow of protons back down the gradient into the inner mitochondrial matrix. The reduced flow of protons also reduces the electron transport chain demand to set up the proton gradient, leading to reduced oxygen consumption. The correspondent loss in OCR is known as ATP-linked respiration. However, there is still a residual amount of oxygen consumption following oligomycin treatment, referred to as proton leak. Despite the lack of ATP-linked
respiration, protons still flow through the inner mitochondrial membrane via proteins such as uncoupling protein 1 (UCP-1) (Nicholls & Locke, 1984; Stuart et al., 1999). This flow requires a minimal level of electron transport chain activity to maintain the proton gradient.

The next compound injected in the Mito Stress Test Assay is carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). FCCP is a protonophore that dissolves the proton gradient by allowing protons to freely flow through the inner mitochondrial membrane (Heytler & Prichard, 1962). This dissolution of the proton gradient causes the electron transport chain to work maximally in order to restore the gradient. This increase in oxygen consumption is referred to as maximal respiration. Additionally, the difference between the maximal respiration and the basal respiration is known as the spare respiratory capacity. The spare respiratory capacity is indicative of the cells’ ability to respire above basal conditions in response to changing cellular demands and extracellular environment (Hill et al., 2012; Marchetti et al., 2020; Nicholls, 2009).

The final compound injected in the Mito Stress Test is a mix of Rotenone and Antimycin A. Rotenone is an inhibitor of Complex I in the electron transport chain, while Antimycin A is an inhibitor of Complex III (Palmers et al., 1968; Slater et al., 1973). Inhibition of both Complex I and III results in the complete loss of mitochondrial oxygen consumption due to the inability of electrons to flow through the electron transport chain. This is seen as a reduction in OCR. The remaining consumption of oxygen seen is from non-mitochondrial sources, such as oxidases (Bedard & Krause, 2007).
The Seahorse Mito Stress Test Assesses Mitochondrial Capacity

![Figure 4](image)

**Note. (A):** Oxygen consumption rate trace of a Mito Stress Test for a typical cancer or normal cell type. Basal respiration is first measured. Following oligomycin injection and subsequent loss of ATP-linked respiration, a small amount of oxygen consumption is observed as a result of proton leak. FCCP is then injected and maximal respiration is measured, along with the spare respiratory capacity that is the difference between maximal and basal respiration. Following Rotenone/Antimycin A injection, electron transport chain contributed oxygen consumption is inhibited and only non-mitochondrial oxygen consumption remains. (B): The 3 injections of the Mito Stress Test target the electron transport chain. Oligomycin (red), the first injection, inhibits Complex V, ATP synthase. FCCP (green) is the next injection, and transports protons freely across the inner mitochondrial membrane, resulting in dissolution of the proton gradient. The final injection of Rotenone/Antimycin A (orange) inhibits Complexes I and III, resulting in the full shutdown of the electron transport chain.

**The Glycolytic Rate Assay**

The Seahorse Glycolytic Rate Assay is used to measure the glycolytic capacity of cells (Figure 5). Two injections are used to inhibit mitochondrial respiration and glycolysis (Agilent, 2019b). In the Glycolytic Rate Assay, raw ECAR readings are combined with a buffer factor and a carbon contribution factor to calculate glycolytically contributed proton.
efflux rate (glycoPER), a more accurate quantification of glycolytically contributed acidification (Agilent, 2018a, 2018b, 2020). The Glycolytic Rate Assay begins with a basal measurement of glycoPER. Following basal glycoPER measurement, the first injection of the Glycolytic Rate Assay is Rotenone/Antimycin A, which shuts down mitochondrial respiration. This shifts cellular metabolic demand entirely to glycolysis. As a result, the glycoPER increases, indicating the increased glycolytic demand. This compensatory glycolysis is analogous to the spare respiratory capacity of the Mito Stress Test, where it is indicative of the cells’ ability to increase glycolytic output in response to changing cellular demands and environment.

The second and final injection in the Glycolytic Rate assay is 2-deoxy-D-glucose (2-DG). 2-DG is an inhibitor of hexokinase, the first enzyme in glycolysis (Wick et al., 1957). Inhibition of hexokinase prevents the glycolytic pathway from occurring, since there is no longer any glucose-6-phosphate being produced for use in downstream glycolytic steps. This inhibition of glycolysis, combined with the inhibition of oxidative phosphorylation, results in complete metabolic shutdown of the cell, leaving behind a small amount of post 2-DG acidification.
Figure 5

The Seahorse Glycolytic Rate Assay Characterizes Glycolytic Capacity

Note. (A): glycoPER trace of a Glycolytic Rate Assay. Basal glycolysis is first measured, followed by injection of rotenone/antimycin A, which inhibits the electron transport chain and stops mitochondrial respiration. Glycolysis is increased to compensate for the reduced mitochondrial ATP production, indicated by an increased glycoPER. 2-DG is then injected to inhibit glycolysis, and glycoPER is then reduced to a minimal post 2-DG acidification. (B): The 2 injections of the Glycolytic Rate Assay target the electron transport chain and glycolysis respectively. The first injection of Rotenone/Antimycin A inhibits Complexes I and III of the electron transport chain, shutting off mitochondrial respiration. 2-DG, a competitive inhibitor of hexokinase, then shuts down glycolysis.

The measurement of OCR and ECAR on a local level has allowed for the accurate quantitation of glycolytic and oxidative activity (Divakaruni et al., 2014). This capability has further shown that the mitochondria in cancer cells are active and contribute to ATP production within the cell (Mookerjee et al., 2017). Furthermore, the characterization of the metabolic profile of cancer cells using the Seahorse technology has shown that not all cancer cells respiration similarly – some cancer cell lines are highly glycolytic, in line with the
Warburg Effect, while others are highly oxidative, deriving the majority of their ATP production from the mitochondria (Guha et al., 2018; Martin & McGee, 2019; Potter et al., 2016; Repas et al., 2022; Rogers et al., 2019).

**REGULATION OF ENERGY METABOLISM WITHIN THE CELL**

The cell requires a constant supply of energy to meet cellular demands. An imbalance in ATP, ADP, and AMP levels has been shown to cause cell death (Leist et al., 1997; Richter et al., 1996; Tsujimoto, 1997). Therefore, the concentration of ATP within the cell must remain stable and the balance between energy consumption and energy production must be highly regulated. The 5’AMP activated protein kinase (AMPK) is the main regulator of energy production within the cell (Herzig & Shaw, 2017).

AMPK is a protein kinase that serves as the main energy sensor within the cell. It is a heterotrimer, consisting of one of two catalytic α subunits, one of the two regulatory β subunits, and one of three regulatory γ subunits. The γ subunit contains multiple binding sites for ATP and AMP, although only AMP activates the enzyme (Kim et al., 2016; Xiao et al., 2011). The activation of AMPK occurs in two steps. First, AMPK is phosphorylated by upstream kinases such as liver kinase B or calmodulin-dependent protein kinase kinase at threonine 172 (Hawley et al., 1996; Hawley et al., 2005; Hurley et al., 2005; Shaw et al., 2004; Woods et al., 2003). These kinases phosphorylate AMPK in response to upstream signals, such as increased calcium concentration or growth signals, and activate the enzyme. Binding of AMP to the γ subunit then prevents the dephosphorylation of AMPK and further activates the enzyme by promoting phosphorylation (Davies et al., 1995; Hawley et al., 1995;
Gowans et al., 2013). This results in an increase in the kinase activity of the enzyme (Cheung et al., 2000; Mihaylova & Shaw, 2011).

The main purpose of phosphorylated AMPK is to reduce energy consumption in the cell while increasing energy production. pAMPK is involved in multiple downstream pathways, including lipid anabolism, glucose catabolism, and cell growth (Herzig & Shaw, 2017). A direct downstream target of pAMPK is Acetyl-CoA Carboxylase (ACC). The phosphorylation and subsequent inhibition of ACC at S79 prevents fatty acid synthesis from occurring, thus promoting fatty acid oxidation by preventing acetyl-CoA from being converted to malonyl-CoA (Angin et al., 2016; Saha & Ruderman, 2003). This increase in fatty acid oxidation promotes the production of ATP via the Krebs Cycle. pAMPK also promotes energy production directly by phosphorylating 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3 (PFK2). PFK2 is a dual purpose enzyme, responsible for both converting fructose-6-phosphate to fructose-2, 6-bisphosphate and back to fructose-6-phosphate (Marsin et al., 2002). Phosphorylated PFK2 promotes the production of fructose-2, 6-bisphosphate, a strong allosteric activator of phosphofructokinase 1 (PFK1). PFK1 converts fructose-6-phosphate to fructose-1, 6-bisphosphate, the first irreversible step of glycolysis. This increase in PFK1 activity directly increases glycolytic output and downstream energy production (Yalcin et al., 2009).

Although pAMPK plays a large role in meeting the ATP demands of the cell, it also serves as a regulator of energy consuming processes. A significant source of energy consumption in the cell is cell growth. The growth of the cell is regulated by the mammalian target of rapamycin (mTOR). mTOR is a protein kinase that is involved in cell growth
processes, including lipogenesis and mitochondrial biogenesis (Blanchard et al., 2012; Summer et al., 2019). In the cytoplasm, mTOR is normally associated with one of its two scaffolds, regulatory-associated protein of mTOR (Raptor) or rapamycin-insensitive companion of mTOR (Rictor). Although other proteins are also involved in these mTOR complexes, mTOR complexes are defined by which scaffolding protein mTOR is associated with. mTOR complex 1 (mTORC1) is formed when mTOR is associated with Raptor, and mTORC2 is formed when mTOR associates with Rictor (Wullschleger et al., 2006). Phosphorylated AMPK is a direct inhibitor of mTOR via Raptor (Gwinn et al., 2008). When pAMPK phosphorylates Raptor, mTOR is unable to phosphorylate downstream targets. This inhibition not only prevents downstream energy consumption from occurring but also prevents the cell from dividing (Gwinn et al., 2008; Jones et al., 2005), serving as a check against proceeding with energy intensive cell processes when there is not enough energy within the cell to meet the demand.

**Natural Products and Cancer**

The use of plants and other natural products to treat and cure diseases throughout history is well documented (Huang et al., 2021). Most notable of plant compounds are a group of secondary metabolites known as polyphenolic compounds. Although there are many classes of compounds under the umbrella of plant polyphenols, the most well known are the flavonoids, which exhibit anti-inflammatory, antioxidant, and anticancer effects (Ani & Naidu, 2008; Edwards et al., 2007; Yang et al., 2003;). In addition to the flavonoids, there are stilbenes, lignans, and phenolic acids (Manach et al., 2004; Pandey & Rizvi, 2009). Most plants are abundant with most types of polyphenolic compounds. However, the phenolic
acids and their tannin multimers are only found in certain foods, such as walnuts (Clifford & Stalbert, 2000; Manach et al., 2004; Scalbert & Williamson, 2000).

**POLYPHENOLIC MAKEUP OF WALNUTS**

Walnuts are the fruit of the *Juglans regia* tree. Although already well-known as a rich source of mono- and polyunsaturated fatty acids and for their antioxidant effects, walnuts also have other positive health effects. Walnuts are also a rich source of polyphenolic compounds. A previous study investigating the polyphenolic abundance in foods found that walnuts are highly abundant in polyphenolic compounds and contain the most polyphenolic compounds amongst nuts (Abe et al., 2010; Vinson & Cai, 2012). Additionally, the most abundant class of polyphenols in walnuts are ellagitannins (Hayes et al., 2016; Le et al., 2014; Regueiro et al., 2014).

Ellagitannins are tannins made up from ellagic acid subunits (Lipińska et al., 2014; Sharifi-Rad et al., 2022). Recent research has shown that ellagic acid and ellagitannins are anti-inflammatory, antioxidant, antiproliferative, and anticancer, among other effects. The consumption of walnut enriched diets reduced tumor growth in xenografted mice (Hardman, 2014; Hardman & Ion, 2008; Hardman et al., 2011). In humans, the consumption of walnuts has been linked to a reduction in blood pressure, blood sugar, and cardiovascular disease risk (Ani & Naidu, 2008; Tindall et al., 2019). Additionally, treatment of Caco-2 colon cancer epithelial cells with punicalagin, an ellagitannin found in pomegranates, induced apoptosis via the mitochondrially involved intrinsic pathway, suggesting that the mitochondria are affected by ellagitannin treatment. Furthermore, punicalagin was found to be spontaneously hydrolyzable, with punicalagin levels nearly disappearing after 2 hours in cell culture
medium with a concordant increase in free ellagic acid, suggesting that the punicalagin was hydrolyzed into its ellagic acid subunits, which activated the intrinsic apoptotic pathway, inducing cell death (Larrosa et al., 2006).

Previously in our lab, a WE was created as previously described (Le et al., 2014). In short, walnut kernels were crushed and extracted in methanol twice. The crude methanol extracts were then concentrated using a rotovap before purifying out lipids by washing with a 10% methanol/hexanol mixture thrice. The final, delipidated extract was lyophilized before storing at -20°C.

In our lab, a destabilization of the mitochondrial membrane potential resulting in cell death via apoptosis was seen in MDA-MB-231 cells following WE treatment (Le et al., 2014). A decrease in intracellular pH was also observed. This suggested that the WE could impact the energy production of the cell via the mitochondria in some way. My goal is to determine the effects of WE on the processes through which the cell produces energy.
Methods

MATERIALS

Walnut Extract (WE) used in all studies was prepared as previously described (Le et al., 2014), dissolved in DMSO (Thermo, #85190) to a final concentration of 100mg/mL, and stored at -20°C. FCCP was purchased from Tocris Biosciences (#0453) and resuspended in DMSO for a final concentration of 5mM. 100µL aliquots were made and stored in -80°C. Hoescht 33342 used in cell staining was purchased from Thermo (#3570). Ammonium persulfate (APS) and tetramethylethlenediamine (TEMED) was purchased from Fisher (#BP179-100 and #BP150, respectively).

The Pierce BCA Protein Assay Kit was purchased from Thermo (#23227). Seahorse ATP Rate and Glycolytic Rate Assay Kits were purchased from Agilent (#103592-100 and #103344-100 respectively). Powdered Dulbecco’s Modified Eagle’s Medium (DMEM) for use in Seahorse Assays was purchased from Millipore (#D5030) and supplemented with 1M Seahorse glucose, 200mM glutamine, 100mM pyruvate (Agilent #103577-100, #103578-100, #103579-100, respectively), 1X nonessential amino acids (NEAA) (Gibco, #11140050) and HEPES (Gibco, #15630-080).

Primary antibody to glyceraldehyde-3-phosphate dehydrogenase, anti-GAPDH, was purchased from Santa Cruz Biotechnology (sc-25778). Primary antibodies to AMPKα, phospho-AMPKα (Thr172), mTOR, phospho-mTOR (Ser2448), phospho-Acetyl-CoA Carboxylase (Ser79), and Akt pathway kit were purchased from Cell Signaling Technologies (#2532, #2535, #2983, #5536, #1181, #9916). HRP-conjugated secondary antibodies for anti-
mouse IgG and anti-rabbit IgG were purchased from Santa Cruz Biotechnology (sc-2748) and Thermo respectively (# 32460).

**CELL CULTURE AND TREATMENT**

HeLa, MDA-MB-231, and MCF10a cells were grown and maintained in the media described above in a 37°C incubator in a 5% CO₂, humidified atmosphere. HeLa cells were obtained from the American Type Culture Collection (ATCC), and cultured in DMEM purchased from Corning (#10-013-CM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin (PenStrep), purchased from Sigma (#F2442) and Gibco (#15140122) respectively. MDA-MB-231 cells were acquired from ATCC and cultured in DMEM supplemented with 10% FBS, 1% PenStrep, and 1X NEAA. The MCF10a cell line was obtained from ATCC and cultured in DMEM-F12 (Gibco, #11320-032), supplemented with 5% Horse Serum (Gibco, #16050130), 100ng/mL cholera toxin (Sigma #C8052), Bovine Pituitary Extract, hEGF, insulin, and hydrocortisone (Lonza, #CC-4136).

To split cells, 0.25% Trypsin-EDTA (Corning, #25-053-CI) was pipetted gently onto cells and incubated in the 37°C, 5% CO₂ incubator until cells released from the plate. Cells were then collected by washing with growth media and pelleted at 500 x g. Cells were allowed to grow to a confluency of 80-90% before splitting. HeLa cells were reseeded at a ratio of 1:8, or a density of 2x10⁶ cells in a 10cm dish. MDA-MB-231 cells were seeded at a ratio of 1:3, or a density of 3x10⁶ cells in a 10cm dish. To seed into a T175, 4x10⁶ cells were used. MCF10a cells were reseeded at a ratio of 1:4, or a density of 3x10⁶ cells. On a T175 flask, 5x10⁶ cells were seeded. Media was replaced every 3 days.
SEAHORSE ASSAYS

A comparable Sigma media was used for the treatment of cells and resuspension of assay compounds in the Seahorse assays. Sigma media was made using powdered DMEM described above. Powdered DMEM was resuspended in 1L of sterilized MilliQ water and supplemented with 1X NEAA and 5mM HEPES. Sigma media was then pH adjusted using NaOH to a final pH of 7.2. The pH adjusted Sigma media was then 0.22µm filtered into a sterile bottle and refrigerated at 4°C protected from light until use. Complete assay medium was made by combining 97mL of Sigma media and 1mL each of 1M Seahorse glucose, 200mM glutamine, and 100mM pyruvate in a sterile bottle.

For each Seahorse assay, 1x10⁴ cells per well were plated in 100µL of growth medium in a Seahorse XFe96 cell plate included in a Seahorse FluxPak (Agilent, #102416-100). Care was taken not to plate cells in the background wells A1, H1, A12, and H12. Instead, 100µL of growth medium was added to each of the background wells. After plating the cells, the Seahorse cell plate was incubated at room temperature in the cell culture hood for 1 hour before placing into the rear of the humidified 37°C, 5% CO₂ incubator.

To treat cells, complete assay medium was prepared as described above and prewarmed to 37°C. DMSO and WE treatments were then prepared at 2X concentration in complete assay medium. DMSO was diluted to a final concentration of 1%, and WE was diluted to 0.5mg/mL, then serially diluted with complete medium 1:2 to 0.0625mg/mL. 50µL growth medium in each well was replaced with 50µL of the treated complete assay medium, for a final concentration of 0.5% DMSO, or 0.25, 0.125, 0.0625, and 0.03125mg/mL WE in a final volume of 100µL.
The treatment schema for each experiment was different (Figure 6). Dose response experiments were treated simultaneously 24h before assay time. Time course assays had treatments immediately prior to assay, where 24h timepoints were treated 24h before assay time, while 1h timepoints were treated 1h immediately prior to assay. Washout assays were treated simultaneously 24h before assay time, but was washed out after specified treatment times with untreated growth medium. For example, a 1h washout means that the cells were treated for 1h then treatment media removed and replaced with fresh media for 23h before the assay was run. After treatment, the cell plate was placed back into the humidified 37°C, 5% CO₂ incubator. The complete medium was then placed back in the 4°C refrigerator protected from light.

Additionally, a Seahorse sensor cartridge included in the FluxPak was hydrated by adding 200µL of autoclaved and 0.22µm sterile filtered MilliQ water into each well of the utility plate in the sensor cartridge. The sensor plate was then carefully placed over the utility plate, taking care to avoid creating air bubbles under the sensor pads to prevent uneven hydration of the sensors. A 22mL aliquot of Seahorse Calibrant (Agilent, #100840-000) was also made, and both the sensor cartridge and the aliquot of calibrant was placed in a 37°C, humidified, non-CO₂ incubator overnight.

On the day of the assay, the complete assay medium was prewarmed to 37°C in a water bath. The sensor cartridge was taken out of the non-CO₂ incubator and the water was replaced with 200µL of the prewarmed Seahorse Calibrant 2 hours before the start of the assay. 24h after treating and one hour before the assay time, the cell plate was taken out of
the humidified 37°C, 5% CO₂ incubator and washed with the prewarmed complete assay medium according to the manufacturer protocol.

**Figure 6.**

*Seahorse Treatment Schema*

The cell plate was then placed in the humidified 37°C, non-CO₂ incubator for 1 hour. The experimental setup was input into the Wave 2.6 software following Agilent recommendations and the assay compounds for the Seahorse assay were then prepared. In the Wave 2.6 software, basal readings were set to 4 cycles of 3min mix, 3min measure. All injection readings consisted of 3 cycles of 3min mix, 3min measure. 30 minutes before assay time, the
sensor cartridge was taken out of the non-CO₂ incubator and the assay compounds were then pipetted onto the sensor plate using the FluxPak provided injection well guides.

For Seahorse Mito Stress Tests, Oligomycin (Oligo) and Rotenone/Antimycin A (Rot/AA) were sourced from Agilent ATP Rate Assay Kits. FCCP was sourced from previously frozen 5mM aliquots prepared as described above. Oligo was resuspended in 630µL complete assay medium and Rot/AA was resuspended in 540µL for working concentrations of 100µM and 50µM respectively. The 5mM FCCP stock was thawed from -80°C and 12µL was diluted into 588µL of complete assay medium to a working concentration of 100µM. 300µL each of Oligo, FCCP, and Rot/AA were then diluted according to the manufacturer protocol to make injection concentrations of 10µM, 10µM, and 5µM respectively. 20µL of 10µM Oligo was then pipetted into each Injection Well A. 22µL of 10µM FCCP was pipetted into each Injection Well B, and 25µL of 5µM Rot/AA was pipetted into each Injection Well C.

For Seahorse Glycolytic Rate Assays, Rot/AA and 2-deoxy-D-glucose (2-DG) were sourced from the Agilent Glycolytic Rate Assay Kit. Rot/AA and 2-DG were resuspended and diluted according to manufacturer protocol using complete assay medium. 20µL of 5µM Rot/AA was pipetted into each Injection Well A and 22µL of 500mM 2-DG was pipetted into each Injection Well B.

Additionally, a Buffer Factor assay was run to measure the Buffer Factor (BF) of the complete assay medium for the accurate calculation of Proton Efflux Rate (Agilent, 2018a; Agilent, 2018b). To do this, 12 wells on the cell plate were excluded from cell plating in addition to the 4 background wells, for a total of 16 empty wells on the cell plate. Only
100µL of growth medium was added in each well. On the day of the assay, the wells were washed as normal and replaced with complete assay medium. Instead of adding 160µL of assay medium in the last wash step, 155µL was added, for a final volume of 175µL in the BF assay wells on the cell plate. 6 of the 12 BF assay wells on the sensor cartridge were loaded with 25µL of 5.0mM HCl in Injection Wells A, B, and C. The other 6 BF assay wells were loaded with 25µL of the complete assay medium in Injection Wells A, B, and C. After the Seahorse assay, the HCl and Media Only well data were then input into the Seahorse Buffer Factor Assay Report Generator (Agilent) for calculation of the Buffer Factor.

ATP Rate Assays were run using the Agilent kit. Oligo and Rot/AA were resuspended and diluted according to manufacturer protocol in complete assay medium. 20µL of 15µM Oligo was loaded into Injection Well A, and 22µL of 5µM Rot/AA was loaded into Injection Well B.

The compound-loaded sensor cartridge was placed into the Seahorse XFe96 Cell Analyzer (Agilent) 20 minutes before assay time to allow the sensor cartridge to equilibrate and calibrate before the assay starts. Immediately after the sensor cartridge completed calibration, the cell plate was loaded into the Seahorse Analyzer and the assay proceeded with the first basal reading after a 15 minute equilibration period.

After the assay completed, the Seahorse cell plate was stained with Hoescht 33342. The cell plate was removed from the Seahorse Analyzer and the sensor plate was removed. Media in the cell plate wells were gently shaken out and replaced with 200µL of 5µg/mL Hoescht 33342 prepared using complete assay medium. Stained cells were incubated for 30m in the humidified 37°C, 5% CO₂ incubator before reading fluorescence (Ex 352/Em 454) on a
SpectraMax M3 plate reader (Molecular Devices). The fluorescence values were then input into the Seahorse Wave software for normalization, with a scale factor of 1000.

**CELLTITER-GLO ASSAY**

CellTiter-Glo (CTG) assays were performed using the CTG kit from Promega (#G7572). CTG reagent was made up according to manufacturer protocol. CTG reagent was frozen at -20°C in 11mL aliquots. Cells were plated at a concentration of 1x10⁴ cells per well in 100µL of growth medium in a Costar black plate with clear bottoms (#3903). A total of 4 wells per treatment condition was plated.

24h after plating, DMSO and WE treatments were prepared at 2X concentration in growth medium. DMSO was diluted to a final concentration of 1%, and WE was diluted to 0.5mg/mL, then serially diluted 1:2 to 0.0625mg/mL. Cells were then treated by replacing 50µL of well volume with 50µL of the 2X treated growth medium. The final concentration of DMSO was 0.5%, and the WE had a final concentration of 0.25, 0.125, 0.0625, or 0.03125mg/mL in 100µL well volume. After treatment, the cell plate was placed back into the humidified 37°C, 5% CO₂ incubator.

To normalize cell amount within each well, cells were stained with a 5µg/mL Hoescht 33342 solution was prepared with 1X sterile PBS 24h after treating. Cells were washed once with 100µL of prewarmed PBS, then stained with 100µL of the staining solution. The stained cells were incubated for 30m in the humidified 37°C 5% CO₂ incubator before reading fluorescence on the SpectraMax M3 plate reader.

After fluorescence readings were taken, 100µL of thawed CTG reagent was pipetted into each well, mixing by pipetting up and down. The plate was then incubated on an orbital
shaker at room temperature for 15m at 225rpm. During this time, an rATP standard curve
consisting of 1000, 100, 10, 1, and 0nM ATP was prepared using 10mM stock rATP
(Promega, #P1132) diluted in sterile PBS. After the 15m incubation on the orbital shaker,
100µL per well of each rATP standard was pipetted into 4 wells. 100µL of CTG reagent was
then immediately pipetted into each rATP standard well, and the plate was then recorded on a
Turner Biosystems Veritas luminometer. Integration time was set at 5 seconds, for 10 runs,
with no delay between runs.

Because cell death could reduce quantified ATP, collected Hoescht data was background
subtracted and a normalization factor was calculated by dividing background subtracted data
by the average background subtracted Hoescht reading of DMSO-treated cells. Raw
luminescence data was then converted into an uncorrected ATP concentration using a
“pooled” standard curve. Data from all ATP standard curves performed were combined and
calculated to form “pooled” standard curve. The uncorrected ATP concentrations were then
corrected by multiplying by the normalization factor.

**PREPARATION OF CELL PROTEIN EXTRACTS FOR WESTERN
BLOTTING**

1x10^6 HeLa, 2x10^6 MDA-MB-231, or 2x10^6 MCF10a cells were plated on a 10cm dish
before being grown overnight. The cells were then washed with prewarmed PBS before
treating with 0.5% DMSO or 0.25mg/mL WE in growth medium. Cell protein extracts were
then collected 24h after treatment.

To create the cell protein extracts, a complete lysis buffer (CLB) was made by
supplementing radioimmunoprecipitation assay (RIPA) buffer (150mM NaCl, 50mM Tris pH
7.4, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% Nonidet P-40) with Halt protease inhibitor cocktail (Thermo, #78438), 1mM phenylmethylsulfonyl fluoride, 9.5mM sodium fluoride, 1mM sodium orthovanadate, and 5mM tetrasodium pyrophosphate. Media was aspirated from each dish before washing 2x with ice-cold PBS. Cells were then lysed with 300µL of CLB and placed at -80°C overnight. Cells were then thawed on ice, and the crude extract was scraped into a 1.5mL tube. The crude lysate was then sonicated 3x for 3s each at 25% power using a Branson sonicator using the 1/8” tip. The sonicated lysate was spun at 4°C 25,000xg for 15m. The supernatant was then transferred to a new tube before quantitating using the BCA Assay.

Protein concentration was measured using the Pierce BCA Protein Assay Kit following the manufacturer’s protocol for the 96 well plate format. Two dilutions of each protein sample were prepared by adding 2µL or 5µL of protein to MilliQ water for a final volume of 25µL before adding BCA reagent. A standard curve ranging from 1000 to 15.625µg/mL BSA was prepared by serially diluting BSA (Thermo, #23209) in MilliQ water. Additionally, a blank of MilliQ water was also added to the 96 well plate. The plate was incubated at room temperature for 2m on an orbital shaker set at 225rpm before incubating in the humidified 37°C non-CO2 incubator for 30m. Absorbance was read at 562nm using the SpectraMax M3 plate reader (Molecular Devices).

**SDS-PAGE and Western Blot Analysis**

SDS-PAGE gels were prepared in-house. A 1.5mm 8% resolving gel (8% acrylamide w/v) was prepared by combining 5.5mL MilliQ water, 2.5mL 4X lower buffer (1.5M Tris-Cl pH 8.8, 0.2% SDS), 2mL 40% 37.5:1 acrylamide (Alfa, #J60868), 60µL 10% APS, and 15µL 30
TEMED. The resolving gel was poured into a cleaned Bio-Rad Mini-Protean glass plate assembly, topped with MilliQ water, and allowed to solidify before draining the water from the plate.

A 4.2% stacking gel was prepared with 3.225mL MilliQ water, 1.25mL 4X upper buffer (0.5M Tris-Cl pH 6.8, 0.2% SDS), 525µL 40% 37.5:1 acrylamide, 25µL 10% APS, and 6µL TEMED. Stacking gel was poured into the drained plate assembly on top of the resolving gel, and a 10-well comb was placed into the plate assembly. After fully solidifying, the plate assembly was loaded into a Bio-Rad Mini-Protean electrophoresis tank and filled with running buffer (25mM Tris, 192mM glycine, 0.1% SDS). The comb was removed and each well was flushed with 200µL of running buffer.

To run the samples on SDS-PAGE, 30µg of total protein was prepared in 1X Laemmli buffer (62.5mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) prepared as described previously (Laemmli, 1970) supplemented with 100mM dithiothreitol (DTT). Prepared samples were boiled for 5m in a dry heat block, before cooling on ice for 5 more minutes. After cooling, sample was collected at the bottom of the tube using the desktop microfuge before loading on the prepared gel. 15µL of Bio-Rad Precision Plus Protein ladder (#1610363) was also loaded in a separate lane. The gel was run at 100V for 10m, before running at 200V until the dye ran off the gel.

The gel was then removed and stained for 15m in a 1.5% chloroform and MilliQ water solution as previously described (Kazmin et al., 2002; Ladner et al., 2004) before imaging on the GelDoc EZ imager (Bio-Rad). The green stain-free tray was used with a 5m activation before imaging the gel.
After imaging, the gel was assembled into a transfer sandwich for transfer onto a methanol-hydrated 0.2µm polyvinylidene fluoride (PVDF) membrane (Millipore, #ISEQ00010). The sandwich was then placed in a transfer apparatus with ice-cold 1X transfer buffer (25mM Tris, 192mM glycine, 20% methanol) and an ice pack. The assembled transfer apparatus was then placed in a 4°C cold room to transfer overnight at a fixed 45V (60mA) with stirring. The next day, the amperage was checked. If the increase was minimal and was still <100mA, the ice pack was switched out for a new one and the power supply was switched to fixed amperage mode and set at 270mA for 2h.

After transfer was complete, the transfer sandwich was disassembled and transfer efficiency was assessed by imaging the gel and membrane on the GelDoc with no activation with the same exposure time. The membrane was then blocked for 1h at room temperature with 5% milk in Tris-buffered saline supplemented with Tween-20 (TBS-T) (20mM Tris-Cl, pH 7.5, 500mM NaCl, 0.1% Tween-20). The membrane was then washed 3x for 10m with TBS-T. The membrane was then dissected and each section was placed in a sealed plastic pouch. Within each pouch, 4mL of the appropriate primary antibody. The anti-GAPDH antibody, and all other antibodies that were not phospho- specific were prepared in 5% milk/TBS-T. The phospho- specific antibodies were prepared in 5% BSA/TBS-T. All primary antibodies were diluted 1:1000. The blots were then incubated overnight in the 4°C cold room on the nutator.

The next day, the membranes were removed from their pouches and washed 3x for 15m with TBS-T. They were then separated based on primary antibody host species and probed with the appropriate HRP-conjugated secondary diluted 1:5000 in 5% milk/TBS-T for 1h at
room temperature with rocking. The membranes were then washed 3x for 10m with TBS-T and developed with the Pierce SuperSignal Pico PLUS (Thermo, #34580) before imaging with the ImageQuant LAS4000 Imager (GE Healthcare). All recorded images of gels and blots were quantitated using the Bio-Rad ImageLab Software.

**Statistical Analysis**

Analysis of Seahorse data consisted of analyzing all 16 wells across 2 independently plated and treated Seahorse experiments. Seahorse data was exported from the Seahorse Analytics online software (Agilent) into Microsoft Excel and analyzed using both one-way and two-way ANOVA to determine effects of cell line and WE dosage on measured parameters. The R statistical software was used to calculate ANOVA results. Two-way ANOVA results were then followed up with a Tukey’s HSD post-hoc test. A significance level was set at p<0.05. CellTiter-Glo data was analyzed in Microsoft Excel using a two-tailed Student’s t-test for equal variance. A p<0.05 was regarded as statistically significant.

Western blots were analyzed using the Bio-Rad ImageLab Software and Microsoft Excel. Gel images taken from the LAS4000 imager was imported into the ImageLab software. Bands were then selected and quantitated using the rolling-ball method. Raw quantitation numbers were exported to Excel and analyzed using a two-tailed Student’s t-test for equal variance. A p<0.05 was regarded as statistically significant.
Results

DOSE DEPENDENT REDUCTION IN OCR FOLLOWING TREATMENT OF CELLS WITH WE

Previous studies in the laboratory have demonstrated that treatment with WE destabilized the mitochondrial membrane potential. A dose dependent cell death response was also observed (Le et al., 2014). Because the mitochondria are involved in the production of energy within the cell, which is dependent on the maintenance of a proton gradient, the destabilization of the mitochondrial membrane potential previously observed suggests that WE treated cells could potentially have reduced ATP production. To test this, the oxygen consumption rate (OCR) of MDA-MB-231 breast cancer cells were initially tested using the Seahorse Analyzer and the Mito Stress Test.

MDA-MB-231 cells were treated with DMSO (0.5%) or WE (0.25, 0.125, 0.0625, and 0.03125mg/mL) to determine if a dose dependent effect could be observed compared to DMSO treated. 24h after treating, cells were assayed in the Seahorse Analyzer (Figure 7A). The basal OCR trace for WE treated MDA-MB-231 cells showed a dose dependent response to the WE treatment. High dosages of WE treatment exhibited the greatest reduction in basal OCR, while the lowest dose showed the smallest reduction (Figure 7B). One-way ANOVA analysis showed a statistically significant ($F_{\text{Dose}(4,75)} = p<0.001$) decrease in the basal OCR for all doses compared to the DMSO control. A Tukey’s post-hoc test performed on the one-way ANOVA results found significance at all dosages ($p<0.001$) of WE treatment compared to the DMSO treated cells.
Figure 7.

Walnut Extract Effects on OCR in MDA-MB-231 Cancer Cells

Note. (A): A representative Seahorse Analyzer trace of the OCR of WE treated MDA-MB-231 cells in the Mito Stress Test. HeLa and MCF10a cells exhibited a similar dose dependent reduction in OCR and response to the different drug treatments. (B): Basal OCR of MDA-MB-231 cells is reduced in a dose dependent manner when treated with WE. (C): MDA-MB-231 cells treated with WE have reduced SRC in comparison to DMSO treated cells. Error bars represent mean ± standard deviation of 16 technical replicates across 2 biologically independent experiments. Asterisks represent a statistically significant difference (p<0.01) compared to DMSO as assessed by one-way ANOVA with a Tukey’s post-hoc test.

Additionally, the spare respiratory capacity of the WE treated MDA-MB-231 cells were also evaluated (Figure 7C). The spare respiratory capacity of a cell (SRC) is the extra
capacity of a cell to respire in response to changing cellular demands and environment. The SRC is represented as a percentage ability to respire above the basal OCR. A low SRC is indicative of a cell that is basally respiring near capacity or is unable to adapt to changing cellular demands. In WE treated MDA-MB-231 cells, the spare respiratory capacity of WE treated cells was reduced significantly (F_{Dose(4,75)}=14.25, p<0.001) compared to DMSO treated cells using a one-way ANOVA. Adjustment of p-values following a Tukey’s post-hoc test showed significant decreases in the spare respiratory capacities at all dosages (p<0.001).

Following the observations of WE impact on oxidative phosphorylation in MDA-MB-231 cells, two other cell lines, HeLa cervical cancer epithelial cells and MCF10a normal breast epithelial cells were also treated with WE and their basal oxidative capabilities assessed (Figure 8) for comparison purposes. Both cell lines showed similar dose-dependent decreases in basal OCR. One-way ANOVA of basal HeLa OCR showed a significant reduction of WE treated cells compared to DMSO treated cells (F_{Dose(2, 73)}=169.3, p<0.001). A Tukey’s post-hoc test confirmed findings, with all dosages of WE treatment showing a significant decrease in basal OCR (p<0.001).

MCF10a cells exhibited a similar reduction in basal OCR (F_{Dose(4,75)}=14.44, p<0.001). Yet, further analysis of one-way ANOVA results using a Tukey’s post-hoc test showed significance at the highest WE dosage only (p_{0.25-0}<0.001), with all other WE treatment dosages insignificant statistically (0.125 and 0.0625). This return to an DMSO-treated-like basal OCR suggested that the lowest doses of WE treatment did not affect the basal OCR in MCF10a cells while the cancer cell lines were affected.
Figure 8.

Walnut Extract Treatment Reduces Basal OCR in Multiple Cell Lines

Note. MCF10a, MDA-MB-231, and HeLa cells treated with varying doses of WE for 24h exhibit reduced OCR in a dose dependent pattern. MCF10a cells recover at the lowest 3 dosages while MDA-MB-231 cells recover only at the lowest dosage. HeLa cells are significantly affected even at the lowest dosage of WE treatment. Error bars represent mean ± standard deviation of 16 technical replicates across 2 biologically independent experiments. Asterisks represent a statistically significant difference (p<0.001) as assessed by a two-way ANOVA followed by a Tukey’s post-hoc test.

A two-way ANOVA was utilized to analyze the basal OCR of all 3 cell lines and all treatments (Figure 8). The ANOVA showed statistically significant differences between the normal cell line and the cancer cell lines ($F_{\text{Cell}(2,223)}=75.79$, p<0.001), while showing no significant difference between the two cancer cell lines. Additionally, a significant dose effect was also observed ($F_{\text{Dose}(4,223)}=105.49$, p<0.001), as well as a significant cell line-dose interaction ($F_{\text{Cell*Dose}(8,223)}=12.46$, p<0.001). Analysis of individual differences using a Tukey’s post-hoc test showed that MCF10a, HeLa, and MDA-MB-231 cells had similar basal OCRs at the highest WE treatment dosage and untreated. Significant differences were
found between the MCF10a cells and both cancer cell lines at the 0.125, 0.0625, and 0.03125 mg/mL dosages ($p_{\text{MCF10a}*\text{HeLa}}<0.001$, $p_{\text{MCF10a}*\text{MDA-MB-231}}<0.001$ at all dosages). No significant difference was found between the HeLa and MDA-MB-231 cells at any dosage.

A two-way ANOVA analysis of the spare respiratory capacity of all three cell lines showed differences between normal and cancer cells (Figure 9). A dose effect ($F_{\text{Dose}(4, 223)}=33.54, p<0.001$) and a cell effect ($F_{\text{Cell}(4, 223)}=1641.37, p<0.001$) was observed, along with a cell-dose interaction ($F_{\text{Cell}*\text{Dose}(8, 223)}=29.17, p<0.001$). Individual $p$ values were calculated and adjusted using a Tukey’s post-hoc test. Untreated MCF10a, MDA-MB-231, and HeLa cell lines had significantly different spare respiratory capacities ($p_{\text{MCF10a}*\text{HeLa}}<0.001$, $p_{\text{MCF10a}*\text{MDA-MB-231}}<0.001$, $p_{\text{HeLa}*\text{MDA-MB-231}}<0.001$). The spare respiratory capacity of WE treated HeLa cells remained insignificantly different from the untreated cells at all dosages. However, MDA-MB-231 cells exhibited significant reductions in spare respiratory capacity at all dosages except for the lowest ($p_{0.25}*0<0.001$, $p_{0.125}*0<0.001$, $p_{0.0625}*0<0.01$). MCF10a cells were only significantly reduced in spare respiratory capacity at the highest two dosages ($p_{0.25}*0<0.001$, $p_{0.125}*0<0.001$), but had much a much higher spare respiratory capacity when compared to the cancer cell lines at all dosages.

Comparison between cell lines using a two-way ANOVA with a Tukey’s post-hoc test showed significant differences between MCF10a cells and the two cancer cell lines at all dosages. Cancer cells remained significantly decreased compared to MCF10a cells even at the lowest dosage of WE treatment ($p_{\text{MCF10a}*\text{HeLa}}<0.001$, $p_{\text{MCF10a}*\text{MDA-MB-231}}<0.001$ at all dosages).
A TIME DEPENDENT REDUCTION OF OCR IN WE TREATED CELLS

The observation of a dose dependent reduction in the OCR of WE treated cells suggested that there may also be a time dependent effect as well. To determine the earliest timepoint at which a significant effect on OCR could be observed, a time course assay was performed. MCF10a, MDA-MB-231, and HeLa cells were treated with 0.25mg/mL of WE at different timepoints immediately prior to assay (Figure 10). The dosage was chosen because in previous dose-response assays, a significant effect was observed while also avoiding the most severe cell death effects. Two-way ANOVA analysis showed both significant time
(F_{Time(4,225)}=40.500, \ p<0.001) \ and \ cell \ line \ (F_{Cell(2, 225)}=94.546, \ p<0.001) \ differences, \ as \ well
as \ a \ significant \ interaction \ between \ cell \ line \ and \ dose \ (F_{Cell*Time(8, 225)}=3.049, \ p<0.01). \n
Following \ a \ Tukey’s \ post-hoc \ test, \ individual \ significance \ values \ were \ determined \ (Figure
10). \ MCF10a \ cells \ had \ a \ significantly \ decreased \ basal \ OCR \ at \ the \ longest \ treatment \ time \ of
24h \ compared \ to \ untreated \ cells \ (p_{24h*0h}<0.001), \ but \ was \ not \ significantly \ different \ at \ the \ 3
lowest \ timepoints, \ suggesting \ a \ potential \ time \ dependent \ effect \ of \ WE \ on \ basal \ OCR. \ HeLa
cells \ exhibited \ the \ same \ characteristic, \ with \ 24h \ WE \ treatment \ exhibiting \ a \ significant
decrease \ (p_{24h*0h}<0.001), \ while \ the \ shorter \ treatment \ timepoints \ did \ not. \ MDA-MB-231 \ cells
did \ not \ have \ a \ significant \ difference \ in \ basal \ OCR \ at \ any \ timepoint.

When \ comparing \ treatment \ effects \ between \ cell \ lines, \ all \ cells \ showed \ a \ similar \ decrease
in \ basal \ OCR \ at \ the \ longest \ treatment \ time \ of \ 24h. \ However, \ certain \ cell \ lines \ were \ affected
more \ than \ other \ cell \ lines. \ MCF10a \ and \ HeLa \ cells \ exhibited \ significantly \ decreased \ basal
OCR \ at \ the \ longest \ treatment \ time \ of \ 24h \ (p_{24h*0h}<0.001, \ p_{24h*0h}<0.001, \ respectively).
Additionally, \ there \ were \ significant \ cell \ line \ differences \ in \ basal \ OCR \ response \ to \ WE
treatment. \ MCF10a \ cells \ had \ significant \ differences \ from \ MDA-MB-231 \ cells \ at \ the \ three
lowest \ treatment \ times \ of \ 6h, \ 3h, \ and \ 1h \ (p_{MCF10a*MDA-MB-231}<0.001 \ for \ all \ three \ timepoints).
HeLa \ cells \ also \ had \ significantly \ higher \ basal \ OCR \ at \ the \ two \ lowest \ timepoints
(p_{HeLa:3h*MDA-MB-231:3h}<0.001, \ p_{HeLa:1h*MDA-MB-231:1h}<0.001). \ However, \ MDA-MB-231 \ cells \ did
not \ exhibit \ a \ significant \ reduction \ in \ basal \ OCR \ at \ any \ timepoint.
Because both a dose- and time-dependent response to WE were observed, a washout assay was performed to determine whether the inhibition of oxidative phosphorylation could recover following removal of WE and replacement with untreated growth medium. For the washout assay, HeLa and MDA-MB-231 cells were treated with 0.25mg/mL WE 24h prior to assay time (Figure 6). After a specified treatment time (1, 3, 6 h), cells were then washed and the media within the wells were replaced with untreated growth medium. The washed-out cells were then incubated for a total of 24h after the treatment time had elapsed. The washout
cells were then compared to the time course treated non-washout cells to determine the type of inhibition following WE treatment.

Washout of the WE from treated cells revealed that basal OCR remained inhibited, despite removal of WE from the cell environment. A comparison of the non-washout and washout data using a two-way ANOVA found both a significant cell effect (FCell (1, 268)=252.33, p<0.001) and treatment effect (FTreatment (9, 268)=86.16, p<0.001) on basal OCR. The ANOVA also found a significant cell-treatment interaction (FCell*Treatment (9, 268)=19.57, p<0.001).

Individual comparisons of washout and non-washout treated HeLa cells with a Tukey’s post-hoc test showed significant differences at the 6h (pWashout*Nonwashout<0.001), 3h (pWashout*Nonwashout<0.001), and 1h (pWashout*Nonwashout<0.001) timepoints. No significant difference was found between 24h treated washout and nonwashout HeLa cells (Figure 11A). Comparisons of washout treated cells to the non-washout 24h treated cells showed similar inhibition of basal OCR at all treatment times, showing that the OCR of treated HeLa cells do not recover even after removal of WE from the growth media. A further comparison of 6h, 3h, and 1h non-washout treated cells to the washout 24h treated cells showed that a significant decrease in OCR was found at all timepoints (pWashout 24h*Nonwashout 6h<0.05, pWashout 24h*Nonwashout 3h<0.001, pWashout 24h*Nonwashout 1h<0.001), mirroring the same timepoint treatments compared to the non-washout 24h cells (pNonwashout 24h*Nonwashout 6h<0.001, pNonwashout 24h*Nonwashout 3h<0.001, pNonwashout 24h*Nonwashout 1h<0.001).

MDA-MB-231 cells exhibited a similar inhibition pattern. No significant difference in the basal OCR of 24h treated washout or non-washout cells was observed (Figure 11B).
Comparisons of washout and non-washout cells at each timepoint showed significant differences except for the 24h treated cells ($p_{\text{Washout 6h}*\text{Nonwashout 6h}} < 0.05$, $p_{\text{Washout 3h}*\text{Nonwashout 3h}} < 0.001$, $p_{\text{Washout 1h}*\text{Nonwashout 1h}} < 0.001$). When comparing non-washout treated cells to the non-washout 24h treated cells, significance was observed at all three timepoints ($p_{\text{Nonwashout 24h}*\text{Nonwashout 6h}} < 0.05$, $p_{\text{Nonwashout 24h}*\text{Nonwashout 3h}} < 0.001$, $p_{\text{Nonwashout 24h}*\text{Nonwashout 1h}} < 0.001$). A comparison of non-washout treated cells to the washout treated 24h cells also showed similar decreases in OCR ($p_{\text{Washout 24h}*\text{Nonwashout 3h}} < 0.001$, $p_{\text{Washout 24h}*\text{Nonwashout 1h}} < 0.001$).

**Figure 11.**

*Washout of WE from Treated Cells Does Not Recover Basal OCR*

Note. (A): HeLa cells were treated with 0.25mg/mL WE or 0.5% DMSO 24h before assay time. WE was then washed out with growth medium at 4 different treatment times prior to assay time, for a total of 24h from...
WE treated washout cells showed significant reduction in basal OCR compared to DMSO treated cells, even at the lowest treatment time of 1 hour. No significant difference was found between the 24h treated washout and non-washout cells, nor were significant differences found between all timepoints of the washout cells compared to the 24h washout or non-washout cells, suggesting that the WE treatment had a permanent reduction of OCR that could not be recovered even with a return to normal growth medium.

**WE Treatment Reduces Glycolytic Capacity**

In addition to the OCR measurements, the Seahorse Analyzer captures ECAR simultaneously allowing both oxidative phosphorylation and glycolysis to be measured. In a previous Mito Stress Test of WE treated cells, a similar dose dependent reduction in ECAR was observed (Figure 12A). Therefore, the glycolytic rate of WE treated cells were then assayed directly using the Glycolytic Rate Assay.

Similar to the Mito Stress Test, which challenges the mitochondria and determines the spare respiratory capacity of the cell, the Glycolytic Rate Assay is used to measure the cell’s ability to respond to increased glycolytic demand. The Glycolytic Rate Assay measures glycolysis using glycoPER.
Analysis of the basal glycoPER using a two-way ANOVA showed that MCF10a, HeLa, and MDA-MB-231 cells treated with WE at various dosages exhibited a significant reduction in the basal glycoPER (Figure 12B). Both a cell (F_{Cell}(2, 225)=50.97, p<0.001) and a dose (F_{Dose}(4, 225)=33.339, p<0.001) effect was observed, as well as a significant cell and dose interaction (F_{Cell*Dose}(8, 225)=7.733, p<0.001). Further analysis of the basal glycoPER with a Tukey’s post-hoc test showed that MCF10a cells have a dose-dependent reduction in basal glycoPER at all WE treatment dosages except for the lowest when compared to untreated cells (p_{0.25}<0.001, p_{0.125}<0.001, p_{0.0625}<0.05). The MCF10a cells also showed a dose dependent response to WE treatment, where the basal glycoPER increased as WE treatment dosage decreased.

In a similar pattern, MDA-MB-231 cells also exhibit a reduction in glycoPER. Compared to the untreated cells, the two highest WE treated cells were significantly reduced in glycoPER (p_{0.25}<0.001, p_{0.125}<0.001). At the lowest two dosages, however, no significant difference in basal glycoPER was observed between untreated and treated cells. A dose dependent recovery of glycoPER was also seen.

The glycoPER of HeLa cells were significantly decreased at all WE dosages except for the highest (p_{0.25}<0.001, p_{0.125}<0.001, p_{0.25}<0.01). Unlike the other cell lines, the HeLa cells did not exhibit a dose response. Although glycoPER was reduced in response to WE treatment, a dose dependent response was not observed, as there was not an increase of glycoPER in response to a reduction in WE treatment dosage.

The post-hoc analysis of all three cell lines also showed a cell line effect. A significant difference between the MCF10a cells and both HeLa and MDA-MB-231 cancer cell lines

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was observed in untreated cells, as well as cells treated with the two highest dosages of WE. The impact of WE treatment on MCF10a cells appeared to be greater than that of the cancer cells. The highest 0.25mg/mL dose of WE treatment induced a 96% reduction in basal glycoPER from untreated MCF10a cells, compared to a 56% reduction in MDA-MB-231 cells. At the second highest 0.125mg/mL WE treatment, MCF10a cells had a 86% decrease whereas MDA-MB-231 cells were only reduced by 48%. This increased reduction was also observed at the second lowest dosage of 0.0625mg/mL, where the glycoPER of MCF10a cells was reduced 55% and MDA-MB-231 cells was reduced 26%.

An analysis of the compensatory glycolysis showed a similar trend (Figure 13). Two-way ANOVA analysis of the compensatory glycolysis of WE treated cells showed a significant cell (F_{Cell (2, 225)}=59.51, p<0.001) and dose (F_{Dose (4, 225)}=86.2, p<0.001) effect, as well as a significant cell-dose interaction (F_{Cell*Dose (8, 225)}=10.07, p<0.001). A Tukey’s post-hoc test was performed to evaluate individual comparisons of cell lines and treatment dosages.

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MCF10a cells showed a significant reduction in compensatory glycolysis at the two highest dosages of WE treatment (p_{0.25}<0.001, p_{0.125}<0.001). At the lower two dosages of WE treatment, no significant effect was observed. Additionally, a dose dependent recovery of compensatory glycoPER was observed as the WE treatment dosage was reduced.
Figure 12

Glycolytic Effects of WE Treatment

Note. Example of a single run of WE treated MDA-MB-231 cells exhibiting a dose dependent reduction of ECAR, suggesting that the WE also affects the glycolytic capacity of cancer cells. WE treated MCF10a and HeLa cells exhibit a similar response in ECAR. (B): MCF10a, HeLa, and MDA-MB-231 cells treated with different dosages of WE were assayed using the glycolytic rate assay. Basal glycoPER is significantly reduced in MCF10a cells at all dosages except for the lowest, while HeLa glycoPER is significantly reduced at all dosages. MDA-MB-231 cells recover at the lowest 2 dosages. Error bars represent mean ± standard deviation of 16 technical replicates across 2 independent experiments. Asterisks represent a statistically significant difference (p<0.05).
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MCF10a cells showed a significant reduction in compensatory glycolysis at the two highest dosages of WE treatment (p_{0.25}<0.001, p_{0.125}<0.001). At the lower two dosages of WE treatment, no significant effect was observed. Additionally, a dose dependent recovery of compensatory glycoPER was observed as the WE treatment dosage was reduced.

WE treated HeLa cells also exhibited a significant decrease in compensatory glycolysis. All four treatment dosages of HeLa cells were significantly different from the untreated cells (p_{0.25}<0.001, p_{0.125}<0.001, p_{0.0625}<0.001, p_{0.03125}<0.001). Similar to the basal glycoPER, a dose dependent increase in compensatory glycoPER was not observed, and glycoPER remained equally reduced at all treatment levels.

The compensatory glycoPER of WE treated MDA-MB-231 cells was significantly reduced at the three highest treatment dosages (p_{0.25}<0.001, p_{0.125}<0.001, p_{0.0625}<0.05) compared to untreated cells. A dose dependent response to a reduction in WE treatment dosage was also observed, and the MDA-MB-231 cells were observed to recover at the lowest treatment dosage of WE. The compensatory glycoPER of the lowest WE dosage was not significantly different from untreated cells.
Figure 13

Compensatory Glycolysis in WE Treated Cells

Note. MCF10a, HeLa, and MDA-MB231 cells treated with WE and assayed with the Glycolytic Rate Assay exhibit reductions in compensatory glycolysis when the mitochondria were prevented from producing ATP. Error bars represent mean ± standard deviation of 16 technical replicates across 2 independent experiments. Asterisks represent a statistically significant difference (p<0.05).

Further analysis of the cell line effects in all three cell lines with a Tukey’s post-hoc test showed that there was a significant cell line difference in compensatory glycoPER impact. At the highest 0.25mg/mL dosage of WE treatment, MCF10a cells exhibited a 98% reduction on compensatory glycoPER, compared to a 56% reduction in MDA-MB-231 cells. This disparate impact was observed at the second highest dosage of 0.125mg/mL WE as well, with the MCF10a cells reduced by 91% and MDA-MB-231 cells reduced 50%. MCF10a
cells recovered by the second lowest dosage of WE treatment, with a reduction of only 10% compared to a 30% reduction in MDA-MB-231 cells.

**ATP Production Rate of WE Treated Cells**

After assaying WE treated cells and determining that both glycolysis and oxidative phosphorylation is reduced, the ATP production rate was measured directly on the Seahorse Analyzer using the ATP Rate Assay. The ATP Rate Assay mathematically derives ATP production rates contributed by oxidative phosphorylation (mitoATP) or glycolysis (glycoATP) from raw OCR and ECAR readings (Agilent, 2018c; Romero et al., 2018). Plated cells were treated with a range of doses of WE before assaying 24h after treatment.

Not surprisingly, treatment of the three cell lines with WE greatly reduced both the glycoATP and mitoATP rate (Figure 14). glycoATP and mitoATP production data was analyzed using a two-way ANOVA. A significant glycoATP difference between cell line ($F_{\text{Cell}(2, 225)}=67.74$, $p<0.001$) and dose ($F_{\text{Dose}(4, 225)}=43.67$, $p<0.001$) was found. A cell-dose interaction was also observed ($F_{\text{Cell}*Dose(8, 225)}=38.31$, $p<0.001$). A similar difference was also observed in the mitoATP ($F_{\text{Cell}(2,225)}=25.76$, $p<0.001$; $F_{\text{Dose}(4, 225)}=156.72$, $p<0.001$; $F_{\text{Cell}*Dose(8, 225)}=30.32$, $p<0.001$).

Analysis of the glycoATP production rate of individual cell and dosage treatments with a Tukey’s post-hoc test showed that the ATP production of the three cell lines are not affected in the same way (Figure 14A). The glycoATP production of MCF10a cells exhibited a dose response reduction. The highest two dosages of WE treatment remained significantly reduced ($p_{0.25}<0.001$, $p_{0.125}<0.001$) compared to untreated cells. The glycoATP of the 0.0625mg/mL
dosage was not significantly different, while the 0.03125mg/mL dosage glycoATP was significantly elevated as compared to untreated cells (p_{0.03125}<0.001).

Similarly, MDA-MB-231 cells had a dose dependent reduction in glycoATP production. The two highest dosages of WE treatment had significantly reduced glycoATP production (p_{0.25}<0.001, p_{0.125}<0.001). However, the glycoATP production increased as WE treatment dosage decreased, and the treated cells recovered by the second-lowest dosage of 0.0625mg/mL WE, where no significant difference was found between treated cells and untreated cells. Just like the MCF10a cells, the lowest dosage of WE treatment had a significantly elevated glycoATP in MDA-MB-231 cells (p_{0.03125}<0.05).

The glycoATP of HeLa cells were minimally affected. At the two highest dosages of WE treatment, glycoATP was elevated significantly (p_{0.25}<0.001, p_{0.125}<0.05). The two lowest dosages of WE treatment did not have a significant difference from the untreated cells. A comparison between cell types also showed a difference in how the WE treatment affected glycoATP production. MCF10a cells appeared to be much more impacted compared to the MDA-MB-231 cells. At the highest WE treatment dosage, MCF10a cells exhibited a 95% reduction in glycoATP production rate, compared to a 42% reduction in MDA-MB-231 cells. At the second lowest dosage of WE treatment, an 81% reduction was observed in MCF10a cells, a much larger reduction compared to the 33% reduction seen in MDA-MB-231 cells treated with the same dosage.
Figure 14

*ATP Production from Glycolysis and Oxidative Phosphorylation in Cells Treated with WE*

Note. MCF10a, HeLa, and MDA-MB231 cells treated with WE and assayed with the ATP Rate Assay on the Seahorse Analyzer. (A): ATP production from glycolysis is reduced at the two highest dosages for both MCF10a and MDA-MB-231 cells, with recovery observed by the second-lowest dosage. HeLa cells glycoATP production do not appear to be affected by WE treatment. (B): Mitochondrially generated ATP is significantly reduced in all cells. Similar to the glycoATP, MCF10a and MDA-MB-231 cells recover by the second-lowest dosage, while HeLa mitoATP remains significantly reduced even at the lowest WE dosage. (C): Combined ATP production rates for DMSO treated cells. HeLa cells are highly oxidative, while MCF10a and MDA-MB-231 cells have a more balanced contribution from both the oxidative phosphorylation and glycolysis. Error bars represent mean ± standard deviation of 16 technical replicates across 2 independent experiments. Asterisks represent a statistically significant difference (p<0.05) when analyzed with a two-way ANOVA followed by a Tukey’s post-hoc test.
The mitoATP production of the cells was also impacted following WE treatment (Figure 14B). MCF10a cells exhibited a dose dependent reduction in mitoATP, with the highest two concentrations of WE treatment significantly reduced compared to the untreated cells (p_{0.25}<0.001, p_{0.125}<0.001). The mitoATP production increased as WE treatment concentration decreased, and no significant difference was found at the two lowest concentrations of WE treatment. MDA-MB-231 cells had a similar response to the MCF10a cells. The two highest dosages of treatment had a significant decrease in mitoATP (p_{0.25}<0.01, p_{0.125}<0.05), and the two lowest dosages recovered and exhibited no significant difference to untreated cells. HeLa cells were affected in a dose dependent manner as well. All four dosages of WE treatment exhibited significant reductions in mitoATP compared to DMSO treated (p_{0.25}<0.001, p_{0.125}<0.001, p_{0.0625}<0.001, p_{0.03125}<0.001).

Comparison of the mitoATP of the three different cell lines also revealed that the WE affected cell lines differently. The mitoATP of untreated MCF10a cells were significantly different from that of untreated HeLa cells (p_{MCF10a*HeLa}<0.001), and the same significance was found between HeLa and MDA-MB-231 cells (p_{HeLa*MDA-MB-231}<0.001). MCF10a cells were significantly different from MDA-MB-231 cells at the 0.125mg/mL treatment concentration (p_{MCF10a*MDA-MB-231}<0.05), and significant differences between MCF10a and HeLa cells were found at the two lowest concentrations of WE treatment (p_{MCF10a 0.0625*HeLa 0.0625}=0.001, p_{MCF10a 0.03125*HeLa 0.03125}<0.001).

The mitoATP response of all 3 cell lines to WE treatment was different. MCF10a cells had a 96%, 95%, and a 20% reduction in mitoATP at the 0.25, 0.125, and 0.0625mg/mL treatment concentrations respectively. MDA-MB-231 cells exhibited less impact on the
mitoATP, with a 53%, 46%, and 21% reduction at the three doses mentioned above. The WE treatment had a strong impact on HeLa mitoATP production, with reductions of 91%, 88%, and 82% at the three highest dosage. The lowest dosage of 0.03125mg/mL had a reduction of 33% in HeLa cells.

When put together, the total ATP production and the individual contributions from glycolysis or oxidative phosphorylation can be seen (Figure 14C). DMSO-treated HeLa cells have a high mitoATP production rate and a low glycoATP rate, suggesting that the mitochondria produce more ATP compared to the glycolytic contribution within the cell. In contrast, MDA-MB-231 and MCF10a cells exhibited a more balanced ATP production phenotype, with nearly equal contributions from both oxidative and glycolytic pathways. Of note, MCF10a cells were slightly more oxidative while MDA-MB-231s were slightly more glycolytic.

**ATP LEVELS IN THE CELL ARE REDUCED FOLLOWING TREATMENT WITH WE**

Following the observation of reduced OCR, glycoPER, mitoATP, and glycoATP production rates from the Seahorse Analyzer, a direct quantitation of ATP levels within the cell was done to confirm if the inhibition correlated with a loss of ATP levels.

WE treated cells exhibited a reduction in total ATP in all 3 cell lines. Analysis of the data using a Student’s t-test found significant differences in WE treatment dosages (Figure 15). The ATP concentration of MDA-MB-231 cells was significantly reduced at the highest two dosages ($p_{0.25}<0.01$, $p_{0.125}<0.01$). At the lower two dosages, no significant difference between the treated and untreated cells was found. In HeLa cells, a similar pattern was observed. The
The highest two dosages of WE treatment were significantly reduced ($p_{0.25}<0.01, p_{0.125}<0.01$), whereas the lowest two dosages did not have a significant reduction. Despite the lack of significance found, all cell lines exhibited a pattern similar to a dose dependent reaction to WE treatment. As treatment dosage decreased, the ATP concentration of the cells increased.

**Figure 15**

*Concentration of ATP in WE Treated Cells*

Note. MCF10a, HeLa, and MDA-MB231 cells were treated with WE and endogenous ATP was quantified using the CellTiter-Glo assay. Prior to performing the CTG assay, a Hoescht stain was read to normalize cell amounts within each well. All 3 cell lines exhibit a pattern of dose dependent decrease in total ATP. The MCF10a data represents 2 independent experiments. HeLa and MDA-MB-231 data represents 4 independent experiments. Error bars represent mean ± standard deviation. Asterisks represent a statistically significant difference ($p<0.01$) as calculated by a Student’s paired t-test.

**WESTERN BLOT ANALYSIS OF ENERGY REGULATORS IN THE CELL**

The WE induced reduction in glycolysis and oxidative phosphorylation translated into a reduction in total ATP within the cell. This reduction in ATP should also have a subsequent
increase in ADP and AMP. Energy production pathways within are upregulated while energy consuming pathways are downregulated to return ATP and AMP levels to normal. The main energy sensor within the cell, AMPK, is allosterically activated by high levels of AMP and is phosphorylated (pAMPK) to activate glycolysis, producing energy.

Figure 16

*WE Effects on Phosphorylation of Protein Regulators of Metabolism*

Note. Cell protein extracts prepared from WE treated HeLa and MDA-MB231 cells were run on an SDS-PAGE and probed for multiple proteins involved in energy sensing and metabolic regulation. (A): No increase in pAMPK is detected in response to the reduced levels of ATP within the cell. Nonphosphorylated AMPK levels remain the same. (B): Phosphorylation of mTOR is also not affected by WE treatment. (C): In response to WE treatment, Akt levels remain constant and unphosphorylated. (D): Quantification of pmTOR/mTOR levels show no significant difference between DMSO and WE treated cells. HeLa DMSO and WE data, as well as MDA-MB-231 WE data shown represent 3 independent experiments. Error bars are 1 standard deviation above and below the mean. Asterisks represent a statistically significant difference (p<0.05).
Conversely, AMPK also serves to inhibit energy consuming pathways in the cell via its kinase activity. One of the most notable targets of AMPK is mTOR. The mTOR pathway regulates cell proliferation and protein synthesis when phosphorylated and activated (pmTOR), both of which are energy consuming pathways. The two proteins are also involved and interact with multiple other proteins, including Protein Kinase B (Akt).

Quantitation of protein was normalized to the total protein of each lane. Western blot of WE treated HeLa and MDA-MB-231 cells showed no stimulation or change in the levels of pAMPK (Figure 16A). Interestingly, no pAMPK was detected at all in either HeLa or MDA-MB-231 cells, even in untreated cells. WE treatment also had little effect on pmTOR levels within the cell (Figure 16B, 16D). A Student’s t-test of three independent sets of WE treated HeLa cells showed no significant difference between DMSO and WE treated ratios of pmTOR/mTOR (p=0.958). Western blot of a direct activator of mTOR, Akt, also show no increase in phosphorylated Akt levels. No change was observed in unphosphorylated Akt (Figure 16C).
Discussion

The treatment of MDA-MB-231 cells with WE has been shown to destabilize the mitochondrial membrane potential, thus decreasing intracellular pH and inducing cell death (Le et al., 2014). Additionally, the polyphenolic compounds found in plants have previously been shown to affect the ATP levels within the cell (Yadegarynia et al., 2012). Although the WE used in my research do not contain the flavonoids previously studied, it was shown that plant natural products can affect ATP levels. This, combined with the WE effects on the mitochondria, suggest that the energy production of the cell could be affected. The findings in this study show that not only does WE treatment induce a reduction in the oxidative capacity of cells, but that glycolysis is also affected as well. This reduction in oxidative phosphorylation and glycolysis translated into a reduction in total ATP within the cell. However, even though ATP levels were significantly lower following WE treatment, AMPK was not activated, nor was pmTOR reduced.

WE INDUCED CANCER CELL SPECIFIC REDUCTIONS IN OXIDATIVE PHOSPHORYLATION AND GLYCOLYSIS

Because previous work in our lab showed a direct impact on the mitochondria following WE treatment, oxidative phosphorylation was first assayed using the Seahorse Analyzer and the Mito Stress Test. HeLa and MDA-MB-231 cells were chosen because they were the cell lines previously tested that exhibited WE impacts on mitochondria. MCF10a cells, an immortalized but non-transformed breast epithelial cell line, were tested to compare to the two aforementioned cancer cell lines.
At the highest dosage of WE treatment, basal oxidative phosphorylation was significantly reduced in all cell lines. The OCR of DMSO-treated cells was in line with previous studies in the literature (Martin & McGee, 2019; Repas et al., 2022; Rogers et al., 2019). However, a difference in OCR impact of WE-treated cells was observed between cell lines. The OCR of WE-treated MCF10a cells returned to the DMSO-treated levels by the second highest dosage and exhibited an oxidative rate similar to untreated cells whereas the basal OCR of MDA-MB-231 cells remained significantly reduced at all dosages except for the lowest. HeLa cells remained significantly reduced even at the lowest dosage. This difference in the reduction of basal OCR was also observed in the spare respiratory capacity of treated cells. MCF10a cells were significantly impacted following WE treatment at the two highest dosages, while MDA-MB-231 cells were impacted at the three highest dosages. Both cell lines recovered to a spare respiratory capacity similar to the untreated cells in a dose dependent manner, where basal OCR and spare respiratory capacity increased as WE dosage decreased. Most interestingly, the spare respiratory capacity of HeLa cells did not follow the dose response pattern observed in the basal OCR. HeLa cells treated with WE did not have an impacted spare respiratory capacity. The disparity in the impact of WE on different cell lines suggested that WE had a different effect on different cell lines.

Further analysis of the oxidative characteristics of WE treated cells showed that the spare respiratory capacity of MDA-MB-231 cells was also significantly reduced, indicating that the cells’ ability to ramp up mitochondrially generated ATP production was impaired. The spare respiratory capacity of HeLa cells was unaffected by WE treatment, while the MCF10a cells had a significant decrease in spare respiratory capacity at the highest 2 dosages of WE.
Together, this suggested that there was a cell line difference in how the WE impacted cellular metabolism. WE affected the oxidative activity of the MCF10a cells less than the cancer cell lines, although it affected all cell lines similarly glycolytically. However, this difference still suggests a cancer cell-specific effect, and therefore the WE could potentially be advantageous as a therapy for cancer.

Assessment of WE impacts on glycolysis further showed a cell line difference. MCF10a cells had drastically reduced glycolytic rates compared to the 2 cancer cell lines. MDA-MB-231 cells also had a significant reduction in glycolytic rate, but recovered by the second lowest dosage of WE. Both MCF10a and MDA-MB-231 cells exhibited a dose dependent reduction in glycolytic rate, differing from WE treated HeLa cells, which had significant reductions in glycolytic rate but did not show a recovery as the dose of WE decreased.

In addition to the basal glycolytic rate, the compensatory glycolytic rate of the 3 cell lines were also impacted. MCF10a cells had significant reductions in compensatory glycolytic rate, but again recovered by the second lowest dose. A similar pattern was observed in MDA-MB-231 cells, where a dose dependent reduction that recovered by the lowest dosage of WE was seen. HeLa cell compensatory glycolytic rate remained significantly reduced at all dosages of WE treatment, and did not recover. Altogether, this showed that a WE treatment at low doses was able to impair glycolysis in some cancer cells without affecting glycolysis in non-cancer cells.
WE TREATMENT EXHIBIT A TIME DEPENDENT AND PERMANENT INHIBITION

Following the observation of a dose dependent response to WE treatment, both a time course and a washout assay was performed to further elucidate the pattern of WE effect on cellular metabolism. Cells treated with WE exhibited a time dependent reduction in basal oxidative rate. MDA-MB-231 cells appeared to be the least affected with no significant differences found between the untreated cells and any timepoint. However, HeLa and MCF10a cells both had significantly reduced basal oxidation at the longest treatment time of 24h.

Because a time-dependent response was observed in the time course assay, the pattern of inhibition was assessed with a washout assay. HeLa cells treated for 1, 3, or 6h before washout for 23, 21, or 18h respectively exhibited a similar reduction in basal oxidation as cells that had been treated for 24h. This, combined with the time course data showing no difference between 1h treatment and untreated cells, indicated that the inhibition of oxidation was permanent. A similar pattern was seen in MDA-MB-231 cells, where washout cells treated at all timepoints had the same reduction in basal oxidation compared to 24h treated cells. Together, this suggested that the effect of WE treatment on cancer cell energy metabolism was a permanent inhibition. Although normal MCF10a cells were not tested, it is also possible that the WE treatment is permanent in MCF10a cells as well. However, this has yet to be experimentally determined.
ATP PRODUCTION AND CONCENTRATION IN WE TREATED CELLS

To further confirm the previous findings, the ATP production rate was measured. In line with the glycolytic and oxidative reductions observed in previous assays, MCF10a cells exhibited a dose dependent reduction in both glyco- and mitoATP production rates that recovered by the second lowest dosage. Similarly, MDA-MB-231 cells had a glycoATP production rate that recovered at the second lowest dosage, mirroring glycolytic rate data. The mitoATP production rate also recovered at the lowest dosage, though oxidative rate data recovered by the second lowest dosage. Just as the glycolytic rate data, HeLa cells did not have exhibit a dose dependent reduction in glycoATP production rate in response to WE treatment, but did show one for mitoATP production rate, in line with the oxidative rate data previously generated.

Quantification of the total ATP production rate showed that HeLa cells were highly active mitochondrially, in line with previous publications (Rogers et al., 2019). MCF10a and MDA-MB-231 cells exhibited relatively equal rates of glyco- and mitoATP production, with MCF10a cells slightly more mitochondrially active. This is also in line with previous publications on ATP production rates in these cells (Repas et al., 2022; Rogers et al., 2019).

Following the observations of reduced oxidative and glycolytic rates in response to WE treatment in combination with the reduction in glyco- and mitoATP production rates, the concentration of ATP within WE treated cells was directly quantified. Significant reductions in ATP concentration were found at the highest 2 dosages of WE treatment in MCF10a, HeLa, and MDA-MB-231 cells. This further confirmed that WE treatment had significant
negative impacts on ATP concentration within the cell as a result of inhibition and reduction of oxidative and glycolytic ATP production.

This loss of ATP within the cell could potentially be from multiple causes. WE treatment has been shown to inhibit Sp1 transcription factor activity, and subsequently, Cyclin B1 transcription and protein levels in cancer cells (Larrosa et al., 2016; Shah, 2016). In addition to its role in the cell cycle, Cyclin B1 has also been previously shown to localize in the mitochondria to phosphorylate and increase activity of electron transport chain proteins (Wang et al., 2014; Xie et al., 2019). This reduction of Cyclin B1 levels within the cell could contribute to the reduction in oxidative activity observed.

**Lowered ATP Concentration Does Not Affect Energy Sensors in WE Treated Cells**

The reduction in ATP concentration within WE treated cells should have resulted in an imbalance of the ratios of ATP:ADP:AMP. This imbalance is detected by AMPK, which is phosphorylated and activated at T172 to increase glycolytic energy production via PFKFB3 (Marsin et al., 2002). Additionally, pAMPK inhibits mTOR to prevent further energy consumption (Gwinn et al., 2008). Akt, another regulatory kinase, is upstream of mTOR and AMPK, and phosphorylates mTOR when activated. It is also expected that due to the imbalance of ATP within the cell, pAkt levels are not increased.

Western blot analysis of MDA-MB-231 and HeLa cells showed a lack of pAMPK following WE treatment. To further confirm this, the ratio of pmTOR/mTOR remained unchanged. Additionally, no pAkt was detected. Taken together, this suggested that the cell was not detecting the energy imbalance created by the WE.
Walnut extract is an extract made up of a multitude of ellagitannins, including pedunculagin and ellagic acid (Le et al., 2014). This combination of compounds is possibly what elicits the widespread metabolic effects observed. Previous studies have shown that other ellagitannins, such as punicalagin from pomegranates, along with ellagic acid, can inhibit mTOR (Banerjee et al., 2013; Cheng et al., 2016; Vanella et al., 2013). Additionally, ellagic acid has been shown to inhibit AMPK activity (Martin et al., 2015). Although these are only two of the multitude of compounds within walnut extract, there are many more that have not been fully characterized and could contribute to the activity of WE that we observed.

**CONCLUSIONS**

In this study, I have shown that the treatment of cells with WE has major impacts on glycolysis and oxidative phosphorylation, leading to a reduction in the total ATP concentration within the cell. Through some unknown mechanism, WE is also inhibiting the cells' ability to sense the energy imbalance. The loss of glycolytic and oxidative activity resulted in a loss of ATP, which was not detected by the cell.

Because current treatments for breast cancer are invasive and can have devastating physical and mental side effects, the search for more targeted therapies that avoid these off-target effects is of importance. The use of natural products as anticancer therapies is well documented (Huang et al., 2021). Because of its ability to target the energy metabolism of cancer cells more effectively than normal cells, it is possible that WE could be used in combination with other current chemotherapies to treat breast cancer.
References


Ziello, J. E., Jovin, I. S., & Huang, Y. (2007). Hypoxia-inducible factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia. *Yale Journal of Biology and Medicine, 80*(2), 51. /pmc/articles/PMC2140184/