The Relationship Between Insulin Resistance and Hyperinsulinemia on Mammary Cancer Growth and Development

by

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Abstract:

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Insulin resistance associated with obesity has been suggested to contribute to an increased risk and poor prognosis for breast cancer. In this study, a HER2/Neu transgenic mouse model of breast cancer was used to assess how obesity-induced insulin resistance and hyperinsulinemia can influence the development and progression of breast cancer. We investigated the effect of a high-fat diet and found a tumor-promoting effect in the absence of overt insulin resistance. In contrast, a high-fat combined with fructose diet induced significant hyperinsulinemia but no tumor promoting or growth effect was observed. Treatment with the anti-diabetic, insulin-lowering agent metformin led to a delay in tumor onset in mice on control diet, but this effect was abrogated by the high-fat fructose diet. These data indicate that the effects and potential interactions of insulin, nutrition and drugs on breast cancer development and progression are complex and require further study.

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List of Abbreviations:

4E-BP1	Eukaryotic initiation factor 4E Binding Protein 1
ACC	Acetyl CoA-Carboxylase
Akt/PKB	Protein Kinase B
AMP	Adenosine Monophosphate
AMPK	AMP-activated Protein Kinase
AMPKK	AMPK Kinase
ATP	Adenosine Triphosphate
BAD	Bcl-2 associated death promoter
BMI	Body Mass Index
BRCA1	Breast Cancer susceptibility gene 1
BRCA2	Breast Cancer susceptibility gene 2
BSA	Bovine Serum Albumin
CDK	Cyclin-Dependant Kinase
DAPI	4,6-diamidino-2-phenylindole
DMBA	7,12-dimethylbenz[a]anthracene
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epithelial Mesenchymal Transition
ER	Estrogen Receptor
ERK	Extracellular signal-Regulated Kinase
FA	Fatty Acid
FASN	Fatty Acid Synthase
FFA	Free Fatty Acid
FOXO1A	Forkhead Box O1A
FTase	Farnesyl Transferase
GAP	GTPase Activating Protein
GGTase	Geranyl Geranyl Transferase
GSK3	Glycogen Synthase Kinase-3
GTP	Guanosine Triphosphate
HER2/Neu	Human Epidermal growth factor Receptor 2
HFD	High-Fat Diet

HFF	High-Fat Fructose
HIF-1	Hypoxia Inducible Factor-1
i.p. GTT	intra-peritoneal Glucose Tolerance Test
i.p. ITT	intra-peritoneal Insulin Tolerance Test
IGFBP	Insulin-like Growth Factor Binding Protein
IGF-I	Insulin-like Growth Factor-I
IGF-II	Insulin-like Growth Factor-II
IGF-IR	Insulin-like Growth Factor-I Receptor
IR	Insulin Receptor
IR-A	Insulin Receptor isoform A
IR-B	Insulin Receptor isoform B
IRS-1	Insulin Receptor Substrate-1
IRS-2	Insulin Receptor Substrate-2
JAK	Janus Kinase
LFD	Low-Fat Diet
MAPK	Mitogen-Activated Protein Kinase
MMTV	Mouse Mammary Tumor Virus
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribosomal Nucleic Acid
mTOR	mammalian Target of Rapamycin
NF-kappa B	Nuclear Factor kappa B
OA	Oleic Acid
$p70^{S6K}$	Protein 70 S6 Kinase
PBS	Phosphate Buffered Saline
PDK-1	Phosphoinositide-Dependent Kinase-1
PgR	Progesterone Receptor
PI	Phosphatidylinositol
$PI(3,4)P_2$	Phosphatidylinositol-3,4-bisphosphate
$PI(3,4,5)P_3$	Phosphatidylinositol-3,4,5-triphosphate
PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PMSF	Phenylmethylsulphonyl Fluoride
PTEN	Phosphatase and Tensin homologue deleted on chromosome 10
Rheb	Ras homology enriched in brain
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH	Src Homology

SHBG	Sex Hormone Binding Globulin
STAT	Signal Transducers and Activators of Transcription
TGF-α	Transforming Growth Factor alpha
TSC1/2	Tuberosclerosis Complex 1/2
TTNS	Tris-Tween Normal Saline
VEGF-A	Vascular Endothelial Growth Factor-A

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CHAPTER 1:

General Introduction

1.1 Breast Cancer

1.1.1 Classification and statistics

Breast cancer is the most prevalent form of cancer in women, and the second leading cause of death from cancer in Canadian women. According to the Canadian Cancer Society Statistics for the year 2008, it is estimated that 22,400 women will be diagnosed with breast cancer, and 5300 will die from the disease [1]. This translates into one in every nine women expected to develop the disease at some point during her lifetime. Breast cancer begins in the breast tissue, and is a malignant tumor that has developed from the cells that comprise the breast tissue. Over time, the cancer cells can invade the nearby healthy breast tissue and make their way into the nearby axillary lymph nodes and blood vessels, which can act as the pathways in which these cancerous cells can spread to other parts of the body.

1.1.2 Pathophysiology of breast cancer

Most breast cancers are adenocarcinomas that begin in the cells lining the ducts and lobules in the breast tissue. Adenocarcinomas of the breast may be either *in situ* or invasive. *In situ* breast cancers may be further classified into either localized or extensive in nature, and invasive breast cancers usually fall under localized, regional, or metastatic. Invasive breast cancer occurs when the malignant cells begin to break through the basement membrane and reach the underlying fatty tissue, and then spread further to the lymphatic system surrounding the mammary ducts and lobules, and/or the bloodstream. Approximately 90% of all breast cancers have no known etiology, while the other 5-10% are attributed to known hereditary factors (e.g. women who inherit mutations of the breast cancer susceptibility genes BRCA1 and BRCA2 are at a 30-80% increased risk of developing breast cancer due to the loss of function of this tumor suppressor) [2]. The first case-controlled study on breast cancer epidemiology was performed by British Physician Dr. Janet Lane-Claypon, a pioneer of the so-called cohort studies. In 1926, she published a comparative study of 500 breast cancer cases with 500 control patients of the same background and

lifestyle for the British Ministry of Health [3, 4]. This pioneering study was the first to examine the exact etiology of breast cancer. Presently, breast cancer, like many other cancer types is considered to be the final outcome of numerous hereditary and environmental factors.

1.1.3 Risk factors

Although a number of epidemiological risk factors have been identified, the exact cause of any individual breast cancer is not known. However, there are numerous primary risk factors that have been identified for breast cancer. They include: age [5, 6], being female [6, 7], race (especially Caucasian and African American women) [6, 8], a family history of the disease [6, 9], genetics (germline mutations in the BRCA1 and BRCA2 or phosphatase and tensin homologue deleted on chromosome 10 (PTEN)) [2, 6], a long uninterrupted menstrual history [6], an above average exposure to the hormone estrogen [10, 11], obesity [6], excessive alcohol intake [12], environmental carcinogens such as tobacco or radiation [13] among others **(Figure 1)**. The majority of the hereditary forms of breast cancer occur in premenopausal women and the majority of sporadic cases occur in postmenopausal women. Of particular interest is the role that obesity plays in the development and progression of breast cancer. The high incidence of breast cancer among Canadian women, and women across North America in general has been correlated to a major environmental influence, and that is the suggested "western lifestyle" which is a combination of poor dietary habits, along with a lack of exercise [14], which manifests itself as obesity. It has been previously noted that obesity is a risk factor for both the development, and the poorer prognosis of postmenopausal breast cancer [15-19].

Obesity is a condition in which excess body fat has accumulated to such an extent that the overall health of an individual begins to become compromised [20]. Body mass index (BMI) is a calculated measurement which takes into account an individuals' weight and height, and obesity is defined as a BMI of 30 kg/m^2 or higher [20]. It is important to note that it is not an actual measurement of the percentage of body fat, but is a useful tool to estimate a healthy body weight based on the height of an individual. Excessive body weight has been associated with a number of diseases including cardiovascular disease [21, 22] and

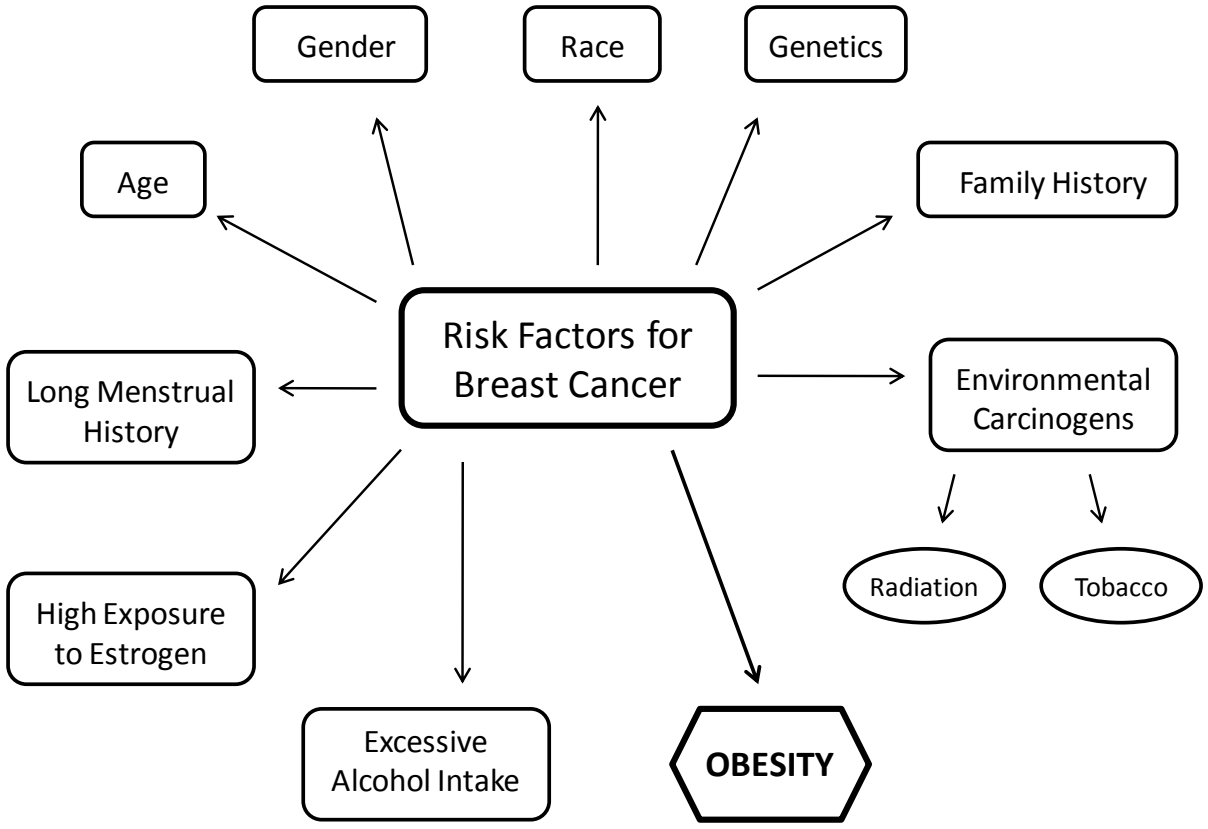


Figure 1: Risk Factors for the Development of Breast Cancer

A representation of the major primary risk factors for the development of breast cancer, which include both genetic, as well as environmental factors. Of particular interest in this study, is the role that obesity plays, and its relationship to the metabolic syndrome and insulin resistance, and the correlation to breast cancer.

Type 2 Diabetes Mellitus [20]. In addition, obesity is also considered a major health concern because of its association with various types of cancer [18]. Obesity has been shown to be an independent risk factor for breast cancer, especially in postmenopausal populations [19]. In addition, regardless of the menopausal status, obese women are more likely to have metastatic breast cancer when they are first diagnosed and have a worse final outcome [23, 24]. Obesity is also a known risk factor for the development of the metabolic syndrome. This syndrome is common in North America where the "western lifestyle" is very prevalent, and has been considered as a potential mediator of the increased risk of breast cancer development and progression [25-30].

1.2 The Metabolic Syndrome & Insulin Resistance

The metabolic syndrome, also known as the insulin resistance syndrome, is a combination of medical disorders that increase the risk of developing cardiovascular disease [31, 32] and Type 2 Diabetes [33, 34]. The characteristics of the metabolic syndrome include insulin resistance associated with glucose intolerance, dyslipidemia, hyperinsulinemia, central obesity, hypertension, and elevated triglycerides [35, 36]. Insulin resistance is the condition in which normal amounts of the hormone insulin are inadequate to produce a normal insulin response from muscle, liver and fat, the classical metabolic target tissues of insulin. Because the circulating levels of insulin in the insulin resistant state cannot act properly on these target tissues, the beta cells augment secretion so that it leads to an overall increase in the circulating levels of plasma insulin, thus leading to a state of hyperinsulinemia.

1.2.1 Insulin and Insulin-like Growth Factor I (IGF-I)

Insulin is an anabolic hormone that is synthesized within the pancreas by the β-cells of the islets of Langerhans. It is released in conjunction with various stimuli which include certain amino acids and glucose in the blood as a result of ingesting protein and carbohydrates. Insulin then elicits a signal transduction response that increases the uptake and storage of glucose in the various target tissues. In addition to its primary involvement in glucose metabolism, insulin has also been shown to exhibit proliferative, anti-apoptotic, and mitogenic effects [37, 38]. There have been a number of epidemiological studies that link

breast cancer with conditions associated with hyperinsulinemia and insulin resistance [39], but the exact role of insulin in breast cancer remains to be elucidated.

In addition to insulin, it is common to see an elevation in the circulating levels of the insulin-like growth factor-I (IGF-I) in cases of the metabolic syndrome caused by obesity [30, 40-42]. IGF-I is a polypeptide that possesses characteristics of both a circulating hormone and a tissue growth factor. Most circulating or endocrine IGF-I is produced by the liver, which is regulated by the growth hormone secreted by the anterior pituitary gland, and also by insulin itself, along with nutritional factors [40, 41, 43]. It has also been shown that IGF-I is produced directly at the tissue level, whereby it possesses paracrine and/or autocrine function [41, 43].

Previous results from epidemiological studies and *in vivo* carcinogenesis models indicate that high levels of circulating IGF-I are associated with increased risk of several common cancers [42]. A recent case-cohort study of association between incident breast cancer and fasting levels of insulin, total and free IGF-I and estradiol among non-diabetic women who were enrolled in the Women's Health Initiative Observational Study (WHI-OS), a prospective cohort of postmenopausal women, showed that hyperinsulinemia is an independent risk factor for breast cancer, and may play a crucial role in explaining the obesity-breast cancer relationship [44]. However, a comprehensive examination of the association between common genetic variations in the IGF-I genes and the binding proteins in relation to circulating levels of IGF-I and breast cancer risk demonstrated that genetic variations of IGF-I, and its major binding protein, insulin-like growth factor I binding protein-3 (IGFBP-3), do not appear to substantially influence breast cancer risk in postmenopausal women [45]. In previously published studies primarily in premenopausal women, there was an association of elevated levels of IGF-I and in some cases IGFBP-3 with an increased risk for the development of breast cancer [46-50]. A similar result was observed from the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, which supported the same trend of IGF-I in postmenopausal women [51]. However, more recent studies have proved somewhat controversial. In the Nurses' Health Study II, there was no significant association between serum IGF-I and IGFBP-3 and breast cancer risk in a large cohort of premenopausal women [52]. A possible explanation for these differences in

findings was suggested to be the study design variations and a lack of standardized assays within these studies [53]. In addition, there have been a number of systematic reviews and meta-analyses on breast cancer and IGF-I and IGFBP-3, and these showed no overall association between total IGF-I and IGFBP-3 levels and the risk of breast cancer [46-48, 54, 55]. Although there are many studies that have examined the role of IGF's and their related binding proteins in breast cancer, the clinical evidence that these factors are important is both prognostically weak and inconsistent, and more research is needed to explore the role of these factors in breast cancer and their relationship to insulin.

In North America, the prevalence of obesity and the metabolic syndrome is becoming increasingly common and the incidence of breast cancer is also on the rise. It raises the question of the role of insulin resistance and the associated hyperinsulinemia in breast cancer development and progression.

1.3 Evidence for the Role of Insulin in Breast Cancer

Previous epidemiological studies have linked Type 2 Diabetes with an increased risk of developing breast cancer and an increase in mortality rates from the disease [39, 56-58]. However, this association was not limited to just Type 2 Diabetes. Other studies have shown that elevated levels of fasting plasma glucose and insulin, without overt clinical Type 2 Diabetes, are linked to the risk and poor outcomes of breast cancer in both pre and postmenopausal women [27, 44, 59, 60]. It is also important to note that there has been no associated risk of breast cancer development with Type 1 Diabetes [61], which further suggests that it is the insulin resistance and the associated hyperinsulinemia of Type 2 Diabetes that leads to this increased risk of breast cancer development and mortality.

1.3.1 Clinical studies

There are studies that have demonstrated a small but significant increase in the risk of breast cancer development in women with diabetes [39, 56-58]. Diabetes itself is still a very indirect measure of an insulin effect. Even though approximately 80% of individuals with Type 2 Diabetes are obese, once Type 2 Diabetes is diagnosed clinically, circulating levels of insulin are often already decreased due to a relative insulin deficiency. In addition, the

various types of therapy to treat Type 2 Diabetes, be it the biguanide metformin, sulfonylureas or insulin itself, will alter the levels of insulin, thus making it difficult to make an accurate assessment of how these individuals vary in the spectrum of breast cancer risk. Goodwin et al. was the first to demonstrate a clinically important independent adverse prognostic effect of high insulin levels in loco-regional breast cancer [60]. In this study, it was shown that women who had the highest levels of insulin had a doubled risk of recurrence of breast cancer and a tripled risk of death from the disease compared to women who had insulin levels in the lowest quartile. A similar trend was seen in women who exhibited symptoms of the metabolic syndrome and subsequently high insulin levels, who had a triple risk of recurrence of breast cancer [62]. Gunter et al. demonstrated a strong positive association between the risk of breast cancer and fasting insulin levels in postmenopausal women [44]. They showed that in these women, breast cancer incidence rates were 2.4-fold greater among those in the highest quartile relative to those in the lowest quartile of fasting insulin concentrations, even when free IGF-I, BMI, estradiol levels and other established risk factors for breast cancer were controlled. Since Type 2 Diabetes was a somewhat indirect means of evaluating the risk of breast cancer and subsequent outcomes, a more direct approach has been the measurement of fasting levels of insulin or C-peptide (which is released when insulin is processed from pro-insulin) in at risk, non-diabetic women. This more broadly reflects insulin resistance since it is a marker of insulin secretion, and can be correlated to the incidence of breast cancer as well as risk and prognosis. This was demonstrated by Eliassen et al., who reported high C-peptide levels to be correlated with a significantly reduced event-free survival in estrogen receptor (ER) and progesterone receptor (PgR) positive breast cancer [63]. It was also previously reported that women with early stage breast cancer have higher insulin levels versus the controls, and that these patients with the higher insulin levels, also exhibited clinical insulin resistance [64]. Elevated insulin was associated with an increased risk of breast cancer diagnosis in one key study in premenopausal women [27], and in several others in postmenopausal women [19, 25, 26]. Therefore, from the available data from human subjects, elevated levels of insulin most likely confer an independent risk for the development of breast cancer which is more apparent in postmenopausal women, but a more marked independent risk factor for disease progression and subsequent death.

1.3.2 In vivo studies

Studies done using *in vivo* animal models support the notion that obesity and insulin increase breast cancer development and/or growth. Supporting the idea that obesity itself is associated with an increased growth of breast cancer, ovariectomized obese C57BL/6 mice showed enhanced tumor growth when tumor cells from Wnt-1 transgenic mice were implanted subcutaneously [65]. It was also shown that obese female Zucker rats were more susceptible to 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary carcinomas compared to the lean control animals [66]. In addition, there was a surprising and significant finding that chronic administration of the human insulin analogue, aspartate (Asp) B10, which was being tested for use in humans, actually caused the formation of breast cancer in normal rats [67]. It is important to note that this analogue is known to have a higher affinity for the IGF-IR, and it was not clear whether one or both of IGF-IR and IR were responsible for this effect.

In addition to these earlier *in vivo* studies, more recent studies have opted to examine rodent models of insulin resistance in which endogenous levels of insulin are elevated to provide data that is relevant to the studies done in humans. The A-ZIP/F-1 mouse is a fatless mouse model of Type 2 Diabetes. These mice lack white fat and exhibit reduced amounts of brown fat, and are diabetic, with elevated levels of insulin, serum glucose, triglycerides and free fatty acids, and they are also known to show elevated levels of certain inflammatory cytokines [68]. Nunez et al. used this A-ZIP/F-1 mouse and crossed it to the C3(1)/T-Ag transgenic mouse model of breast cancer [69]. These double transgenic mice showed a higher incidence of breast tumor formation, decreased latency of tumor onset, larger tumors overall, and an increase in tumor multiplicity [69]. This was an important finding because these mice lack adipose tissue-derived hormones such as leptin, adiponectin and resistin, and so the role of insulin and/or IGF-I was further supported as the possible mediator of this tumorigenic effect. In another study, a diet induced model of obesity which had transplanted tumor cell lines (specifically the mouse colon 38 adenocarcinoma and the Lewis Lung LLC1 carcinoma), demonstrated increased growth which was related to the degree of the obesity as well as insulin resistance [70]. In a study done in breast tumors that were induced in IRS-$2^{-/-}$ mice which expressed the polyoma middle T-antigen in the mammary gland, these mice had

tumors that grew as expected, but interestingly, their metastatic behaviour was significantly reduced compared to the mice that did have IRS-2 present [71]. This remained the case even when these tumors were transplanted into wild-type animals [72].

1.3.3 In vitro studies

It has been shown that normal mammary epithelial cells express the insulin receptor (IR) and that in the case of breast cancer, this receptor is up-regulated [73-76]. The presence of insulin binding sites in human breast cancer cells in culture was demonstrated many years ago [77]. Further *in vitro* studies that support the role of insulin signaling in breast cancer comes from an experiment which looked at the role of the two major signaling proteins and substrates of the insulin receptor tyrosine (Tyr) kinase, more specifically, insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2). IRS-1 has been shown to be constitutively activated in breast cancer cells, and transfection of a dominant-negative IRS-1 with all of its 18 Tyr phosphorylation sites mutated into phenylalanine demonstrated reduced cancer cell growth [78].

Taken together, the epidemiological, animal and cell culture studies all support the notion that insulin resistance and the associated hyperinsulinemia promote breast tumor development, and that more evidence exists to support the direct action of insulin in breast cancer.

1.4 Mechanisms by Which Insulin Can Stimulate Breast Cancer

1.4.1 Direct action of insulin

The response of breast cancer cells to insulin can be explained by a number of factors. It is important to note that the IR and the related IGF-IR belong to the same family of Tyr kinase receptors and exhibit homology to one another. Homology between the IR and IGF-IR ranges from 45-65% in the ligand binding domains, and 60-85% in the Tyr kinase domain [79, 80]. In addition, insulin is also known to be structurally related to IGF-I [81]. Both the IR and IGF-IR are heterotetrameric structures which have two extracellular alpha subunits, containing the ligand binding domains, and two transmembrane beta subunits which contain the tyrosine kinase domains, and these subunits are bound together by a disulphide bond [81].

Functionally, the IR and IGF-IR behave like classical allosteric enzymes in which the α-subunit inhibits the tyrosine-kinase activity which is intrinsic to the β-subunit. Both the IR and IGF-IR are activated by ligand binging which leads to a de-repression of the α-subunit on the β-subunit, which leads to autophosphorylation and recruitment of the major distal signaling molecules; IRS-1 and IRS-2, and Shc [41, 82-84]. These docking proteins which contain phosphotyrosine binding domains (PTB) then bind certain src homology (SH2) domain containing signaling molecules which then results in the activation of two major signal transduction pathways: 1) the phosphatidylinositol 3-kinase (PI3K) pathway and 2) the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway [85]. The MAPK/ERK pathway is known to be responsible for stimulating gene expression and cell cycle progression, and the PI3K pathway is known to exert anti-apoptotic and pro-survival signaling mechanisms, both of which can play a role into the progression and outcomes seen in breast cancer [84, 86, 87].

PI3K is a lipid kinase that is comprised of a regulatory 85 kDa (p85) subunit and a catalytic 110 kDa (p110) subunit. Once activated, PI3K phosphorylates phosphoinositides, generating the biologically active lipids, phosphatidylinositol-3,4-bisphosphate ($PI(3,4)P_2$) and phosphatidylinositol-3,4,5-triphosphate ($PI(3,4,5)P_3$). Generation of PIP_3 results in the recruitment of pleckstrin homology (PH) domain-containing proteins such as the serine-threonine kinase Akt (also known as protein kinase B (PKB)) [88, 89], thereby leading to its translocation to the plasma membrane [90]. Akt/PKB is then activated by sequential phosphorylation at Thr308 and Ser473 residues. The Thr308 phosphorylation is mediated by another PH domain containing enzyme, phosphoinositide-dependent kinase-1 (PDK-1), but full activation of Akt/PKB requires phosphorylation on the Ser473 site. It has been recently demonstrated that PDK-2, the elusive enzyme catalyzing this phosphorylation is an mTOR-rictor complex [91]. The precise mechanism of activation of this PDK-2 is not yet clear. The positive actions of PI3K can be negatively regulated at the level of PIP_3 by phospholipid phosphatases such as the tumor suppressor phosphatase and tensin homologue (PTEN), which dephosphorylates and inactivates PIP_3. Akt/PKB activation is critical for the regulation of both glucose metabolism as well as apoptosis. Glycogen synthase kinase-3 (GSK3) was the first identified target of Akt/PKB [92]. Phosphorylation of GSK3 decreases its activity towards glycogen synthase, which then leads to an increase in glycogen synthesis [93]. It has

also been shown that Akt/PKB can phosphorylate Bcl-2 associated death promoter (BAD), a pro-apoptotic member of the Bcl-2 family of proteins, and when BAD is phosphorylated, it becomes unable to exert its pro-apoptotic function [84, 86, 87]. Although Akt/PKB has a number of different substrates within the cell, of particular importance is the increase in activity of the mammalian target of rapamycin (mTOR) pathway, which has a central role in the regulation of cellular growth and proliferation [91]. In the classical signaling cascade, Tuberosclerosis Complex 1 (also known as hamartin) and Tuberosclerosis Complex 2 (also known as tuberin) interact to form a heterodimeric complex (TSC1/2) which acts as a GTPase Activating Protein (GAP) for the small GTPase Ras Homology Enriched in Brain (Rheb), which directly binds and inactivates the mTOR complex. It should be noted that this mTOR complex is formed of mTOR and raptor (as opposed to rictor as mentioned above) and is referred to as mTORC1. Akt/PKB phosphorylates and inactivates TSC2, therefore reducing its GAP activity, which can then cause an increase in the relative amount of endogenous GTP-bound Rheb. This then relieves the inhibitory effect of TSC1/2 on mTOR. There are two main pathways that link the mTOR complex to the downstream control of messenger ribosomal nucleic acid (mRNA) translation, they are the protein 70 S6 kinase ($p70^{S6K}$) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) pathways [94-96]. Unphosphorylated 4E-BP1 binds the eukaryotic initiation factor 4E (eIF4E) thus inhibiting CAP-dependent mRNA translation. However, mTOR regulates the hierarchical phosphorylation of 4E-BP1 causing the release of eIF4E and leading to an increase in 5`-CAP-dependent mRNA translation [94]. The serine-threonine kinase, $p70^{S6K}$ plays an important role in cell growth by phosphorylating and stimulating a number of factors involved in protein synthesis, including ribosomal S6 protein as well as translational regulators. Breast cancer specimens have often demonstrated an increase in phosphorylation of these downstream targets of mTOR which have been shown to correlate with malignant progression and an associated adverse prognosis [97, 98] **(Figure 2)**.

The MAPK/ERK pathway is a signal transduction pathway that couples intracellular responses to the binding of growth factors to cell surface receptors [99, 100], and is the second most characterized pathway of the IR and IGF-IR. The pathway is complex and numerous protein components are involved. Upon activation of a Tyr kinase receptor, in our case, the insulin receptor, there is binding of the adapter molecule Grb2 and the guanyl

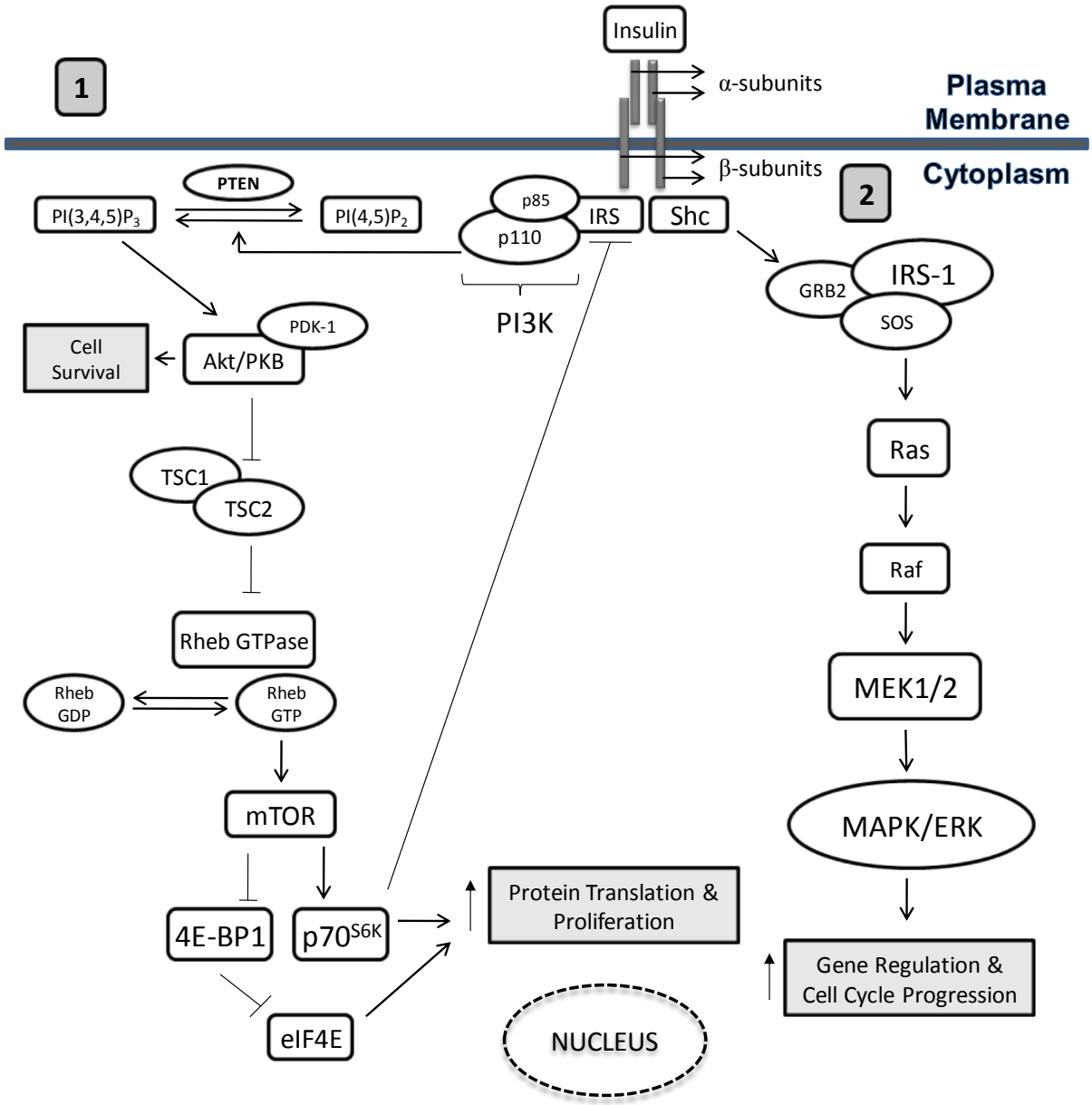


Figure 2: Insulin Signal Transduction Pathways

Binding of insulin to its receptor stimulates receptor tyrosine kinase activation by the autophosphorylation of the intracellular kinase domain, which then recruits and phosphorylates intracellular adapter proteins such as the IRS family to activate the 1) PI3K-Akt/PKB-mTOR pathway, and Shc, GRB2, SOS and IRS-1 to activate the 2) Ras-Raf-MAPK/ERK pathway. Activation of the PI3K-Akt/PKB signaling pathway subsequently stimulates mTOR which can lead to the phosphorylation of its down-stream targets; 4E-BP1 and $p70^{S6K}$, which can lead to an increase in mRNA translation and protein synthesis.

nucleotide exchange factor SOS to the phosphotyrosines on the IRS and Shc proteins. This binding then triggers the activation of the small GTPase Ras [101]. Activated Ras-GTP binds to the N-terminal regulatory domain of the serine-threonine kinase Raf thereby activating it [102]. The hydrolysis of Ras-GTP to Ras-GDP releases the active Raf. Raf kinase phosphorylates and activates MEK, a threonine-tyrosine kinase which can then phosphorylate and activate MAPK/ERK, yet another serine-threonine kinase [101, 102]. The dimeric form of MAPK can subsequently translocate to the nucleus where it can activate a number of different proteins including nuclear transcription factors which mediate cellular growth, survival and cellular differentiation **(Figure 2)**. Both the IR and IGF-IR have these signaling pathways in common. However, during evolution, mammalian IR and IGF-IR began to develop different functions. The IR became predominantly expressed in the muscle, liver and adipose tissue and became more heavily involved in glucose homeostasis and metabolism [81, 103]. On the other hand, IGF-IR was ubiquitously expressed in all tissues and its primary role became prevalent in postnatal linear growth as well as cell survival and proliferation [104].

There have been numerous studies done to establish the presence of both the IR and IGF-IR in normal breast epithelial cells [74, 82]. More interesting is the fact that both the IR and IGF-IR have been shown to be over-expressed in breast cancer cells as well as tissue specimens [75, 105, 106]. Papa et al. showed that approximately 80% of breast cancers have an increased IR expression relative to the amounts seen in normal mammary tissue [73]. When further immunohistochemistry was performed, it was shown that the IR was located predominantly in the cancer cells themselves and not the surrounding stroma [73]. While it is not specific to just breast cancer, the presence of the IR in breast cancer specimens could be used as an important prognostic variable. Mathieu et al. looked at approximately 580 node negative breast tumor specimens and examined the expression of the IR with immunohistochemistry [107]. From this they found that patients with tumors that had no detectable IR content had a decreased 5-year disease free survival compared to patients with tumors that expressed the IR [107]. However, in the same study, a small subgroup of patients (n=62) with tumors that demonstrated a high IR content had a much shorter disease free survival rate when compared to patients with a moderate IR content within the tumors [107]. In addition, Mulligan et al. evaluated the expression of the IR and its prognostic significance

in women with early stage breast cancer [108]. They found that IR was highly expressed in the majority of early stage breast cancer, but this expression was not clearly down-regulated in the presence of high insulin levels. In addition, they demonstrated that high IR expression is independently and significantly associated with favourable clinical outcomes [108].

With respect to the clinical significance of the IGF-IR in breast cancer specimens, there remains some controversy over its relevance as a prognostic variable. In a study examining approximately 200 primary breast tumors with immunohistochemistry to detect the presence of IGF-IR, it was found that even though 43.8% of these specimens had an over-expression of IGF-IR, it did not correlate with nodal status, tumor size, hormone receptor status, histological grade or prognosis [109]. However, in another study by Railo et al., approximately 120 primary breast tumors were examined and a positive correlation was seen between tumors that had IGF-IR expression as well as expression of the estrogen receptor (ER) [110]. It was also shown in this study that patients who had tumors that were IGF-IR positive and ER negative had a worse prognosis compared to tumors that were both IGF-IR and ER negative.

Another important finding that further supports this mechanism by which insulin can act directly to play a role in breast cancer is that the IR exists as two isoforms; a fetal isoform or IR-isoform A (IR-A) which is known to exhibit mitogenic activity, and an adult isoform or IR-isoform B (IR-B) which is known to mediate the metabolic effects of insulin [111, 112]. IR-A is the isoform that is missing 12 amino acid residues due to exclusion of exon 11 [111]. On the other hand, IR-B is the isoform that contains this 12 amino acid residue chain at the carboxy terminus of the alpha subunit [111, 113]. Fetal tissues express relatively more IR-A than IR-B, and this fetal pattern of IR-A over-expression is recapitulated in breast cancer [114]. It has been shown in previous studies that the IR-A has the ability to form a hybrid receptor with IGF-IR, which elicits differential ligand binding [115, 116]. In addition, compared to IR-B, IR-A has a 2-fold increase in the affinity of insulin binding [117, 118], and due to this exon deletion, the binding affinity for IGF-I is greatly augmented [114]. Insulin also has a relatively high affinity for this hybrid IGF-I/IR-A receptor [116]. Insulin-like growth factor II (IGF-II) has been shown to have a high affinity for the IR-A, but not IR-B [73]. IGF-II is a known fetal growth factor which is synthesized by cells to simulate both

growth and proliferation in both an autocrine and paracrine manner. Sciacca et al. showed that in the MDA-MB-157 human breast cancer cell line, which expresses more IR than IGF-IR, autocrine secretion of IGF-II stimulates cellular proliferation via the IR, and this effect is mediated in part by the IR-A [119].

1.4.2 Indirect action of insulin

Although the hypothesis that the mechanism of insulin to influence survival outcomes is via a direct action on breast cancer cells by affecting their growth and metastasis, it is important to note that insulin can also have indirect effects on breast cancer progression. The following potential indirect mechanisms are described below, and although important to discuss, they do not negate the proposed direct effects of insulin.

IGF Binding Proteins (IGFBPs):

The bioavailability of IGF-I in the circulation is dependent on six IGF binding proteins (IGFBPs). The majority of IGF-I is bound to IGFBP-3 which is a very large protein that is produced by the liver [120]. In general, these binding proteins serve to inhibit the action of IGF-I, however, there are some studies that suggest that certain binding proteins may in fact increase the effectiveness of IGF-I and/or exert direct growth stimulatory or growth inhibitory effects independent of IGF [121]. IGFBP-1 is a small binding protein which has been shown to play a role in the regulation of bioavailable IGF-I in relation to nutrient stores and supplies, and so it is a recognized inhibitor of IGF-I [122]. Insulin and IGFBP-1 are known to have an inverse relationship to one another, that is, insulin can rapidly suppress the synthesis of IGFBP-1, which can lead to the increase in free bioavailable IGF-I. Therefore, in states of hyperinsulinemia, there is repression of hepatic production of IGFBP-1 [123, 124], and so insulin, in essence, can indirectly increase breast cancer growth via augmented IGF-I signaling.

Protein Lipidation:

One of the signaling mechanisms involved in the mitogenic pathway is the activation of small GTP-binding proteins such as Ras and Rho. G-proteins must be localized to the membranes correctly, and this is usually accomplished by the covalent addition of a 15-20

carbon isoprenoid lipid chain at the carboxy terminus. There are two major enzymes which catalyze this reaction: 1) farnesyl transferase (FTase) and 2) geranyl geranyl transferase (GGTase) [125]. There are a number of studies that indicate that insulin can stimulate both FTase [126] and GGTase [127], which can subsequently lead to the activation of Ras and Rho-A. One particular study using MCF-7 cells, a human breast cancer cell line, showed that insulin and not IGF-I potentiated the mitogenic effect in fibroblasts of epidermal growth factor (EGF) via this pathway [126].

Estrogen:

Estrogen is the primary female sex hormone and belongs to the family of steroid hormones. In premenopausal women, estrogen is synthesized by the follicular cells of the ovary under the control of gonadotropins [128], however, in postmenopausal women, circulating estrogen is derived mainly by aromatization of androgenic steroid precursors, and can also be produced in small amounts by other tissues such as the liver, adrenal glands and breast tissue [128, 129]. The conversion of the hormone testosterone to estradiol and of androstenedione to estrone are catalyzed by the enzyme aromatase. Aromatase is localized to various tissue types such as the brain, gonads, skin, blood vessels, bone, but more importantly, adipose tissue, liver and muscle [129]. Therefore, in obese postmenopausal women who are hyperinsulinemic, it has been shown that there is an increase in adipose tissue aromatase, and subsequently higher circulating levels of estrogen in these women [130]. In addition, insulin has the ability to stimulate aromatase activity directly, thus causing an increase in the levels of bioavailable estradiol [130]. Estrogens are also bound to sex hormone binding globulin (SHBG), and hyperinsulinemia has been shown to decrease the levels of these SHBG, leading to an excess of free bioavailable estrogen [131].

Estrogen Receptor (ER):

The stimulation of breast cancer growth by the hormone estrogen is mediated by the estrogen receptor (ER). The ER is a nuclear receptor which undergoes homodimerization, sequence specific DNA binding to an estrogen response element (ERE), and transcriptional activation upon ligand binding [132]. There are a number of studies that suggest the possibility of various mechanisms for ER signaling. Non-genomic or non-nuclear actions are

thought to be mediated by ER that are localized to the plasma membrane which can rapidly stimulate the MAPK pathway, which has been observed in breast cancer cells [133]. Ligand independent activation of the ER to stimulate its actions on gene expression is one potential mechanism by which growth factors such as insulin and IGF-I can activate ER positive breast cancer cells in the absence of estrogen [11, 132]. Upon stimulation by insulin and/or IGF-I, the ER can be phosphorylated on tyrosine or serine (Ser) residues by certain kinases [134]. Campbell et al. suggested that phosphorylation of the ER alpha by Akt/PKB at the Ser167 site can also augment the transcriptional activity of the N-terminal AF1 domain of the ER, which can activate gene expression [135]. However, for optimum mitogenesis, breast cancer cells that are ER positive require a combination of estrogen and growth factor signaling [136-138].

Leptin:

Leptin is a multifunctional adipocytokine with numerous biological activities including appetite regulation, bone formation, reproductive function as well as angiogenesis [139]. Because leptin is produced by adipose tissue, in cases of obesity where there is an elevation in the amount of adipose tissue present, the levels of leptin are elevated [140]. In recent years, many studies have shown that leptin increased proliferation of breast cancer, along with many other cancer cells via multiple signaling pathways [141]. Ishikawa et al. demonstrated that the leptin receptor was not detectable in normal mammary epithelial cells by immunohistochemistry, whereas breast cancer cells showed positive staining for leptin receptors in approximately 83% of cases [142]. Yet another study that demonstrated a positive correlation between blood leptin levels and breast cancer risk found that the degree of leptin messenger ribosomal nucleic acid (mRNA) expression in adipose tissue was significantly higher in women with breast cancer compared to the controls [143]. Collectively, these studies suggest the importance of leptin signaling in breast cancer, but the exact role that leptin plays remains uncertain. A further study by Goodwin et al. examined the role of leptin as a mediator of the adverse prognostic effects of obesity in breast cancer, but did not show that leptin was independently associated with prognosis in early stage breast cancer [144]. Although leptin can be associated with both body mass index and insulin, they

could not show prognostic effects similar to those of insulin even before adjustments for insulin or body mass index [144].

1.5 Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR) is the cell-surface receptor for members of the epidermal growth factor family of protein ligands [145]. The EGFR is a member of the ErbB family of receptors, which is a sub-family of four structurally related receptor tyrosine kinases. This family includes EGFR (ErbB-1), HER2 (ErbB-2), HER3 (ErbB-3) and HER4 (ErbB4). This family of receptors can be activated by numerous growth factor ligands, with some ligands possessing the ability to activate one or more of these four receptors [146]. Upon activation by its growth factor ligands, the EGFR goes from a state of an inactive monomer into an active homodimer [147]. In addition to forming a homodimer, the EGFR is also capable of forming a heterodimer by binding to another member of the ErbB family of receptors [147]. The EGFR has an intrinsic autophosphorylation capability, and upon dimerization, there is stimulation of this intrinsic intracellular tyrosine kinase activity [148]. The activation of the receptor consequently leads to the activation of a number of signal transduction mechanisms including the MAPK/ERK [149] and PI3K/Akt [149] pathways, which can lead to DNA synthesis and cellular proliferation. Signaling through EGFR, HER2 and HER3 has been shown to promote tumor cell proliferation and survival in a variety of epithelial malignancies [150, 151]. Because of its association with many growth factors and the downstream signaling mechanisms, the ErbB family of receptors have been implicated in a number of cancers, and in particular, breast cancer.

1.5.1 HER2/Neu as a model of breast cancer

Studies have shown that mutations which lead to the over-expression of the ErbB family of receptors are linked to a number of different cancers, including breast cancer [152]. Since this family of receptors is dependent on growth factors for activation and is involved in cellular growth and proliferation, over-activity of these receptors can cause a predisposition for the development of cancer because of the potential for uncontrolled cell growth. The Human Epidermal growth factor Receptor 2 or HER2/Neu (ErbB-2) is a 185 kDa transmembrane receptor which has been shown to give an increased aggressiveness in breast

cancer [153, 154], and so named because it was originally identified as a transforming oncogene in chemically induced rat neuroglioblastomas. HER2/Neu is believed to be an orphan receptor, meaning that none of the EGF-family of ligands are able to activate it directly. It is believed that the receptor is constitutively activated, or it becomes activated upon heterodimerization with other members of the ErbB receptors [155]. The HER2/Neu gene itself is regarded as a proto-oncogene which is localized to the long arm of chromosome 17 in humans.

Approximately 20-30% of early stage breast cancers show an amplification of the HER2/Neu gene, or an over-expression of its gene product [156]. Patients with breast cancer that over-express HER2/Neu typically present with a more aggressive cancer type, an unfavorable prognosis, shorter relapse time, and lower survival rates [156, 157]. It is for these reasons that the HER2/Neu profile in many breast tumors are examined as a means of assessing prognosis [158]. Because of its prognostic role and the aggressiveness in which it presents itself in breast cancer, the HER2/Neu receptor has become a clinically important therapeutic target. Trastuzumab (marketed under the trade name Herceptin) is a humanized monoclonal antibody which is directly targeted to the HER2/Neu receptor [159]. Trastuzumab is only effective in breast cancers that exhibit a HER2/Neu over-expression. Because of the important clinical relevance that the HER2/Neu receptor plays in breast cancer, there is more investment in targeting the receptor directly as a means of treating patients with this particular form of breast cancer.

1.5.2 Signaling mechanisms

The activation of HER2/Neu can occur via multiple activating ligands, and various dimeric receptor protein combinations are possible, and this leads to signaling through this receptor being very diverse. As mentioned previously, HER2/Neu activation can lead to the initiation of the following signaling pathways: 1) PI3K/Akt, 2) MAPK/ERK and 3) the Janus kinase (JAK) and signal transducer and activators of transcription (STAT) or the JAK/STAT pathway **(Figure 3)**.

PI3K is a substrate for the oncogenic rat *Neu* protein, and activation of *Neu* has been shown to increase PI3K lipid kinase activity [160]. PI3K is composed of two subunits; the

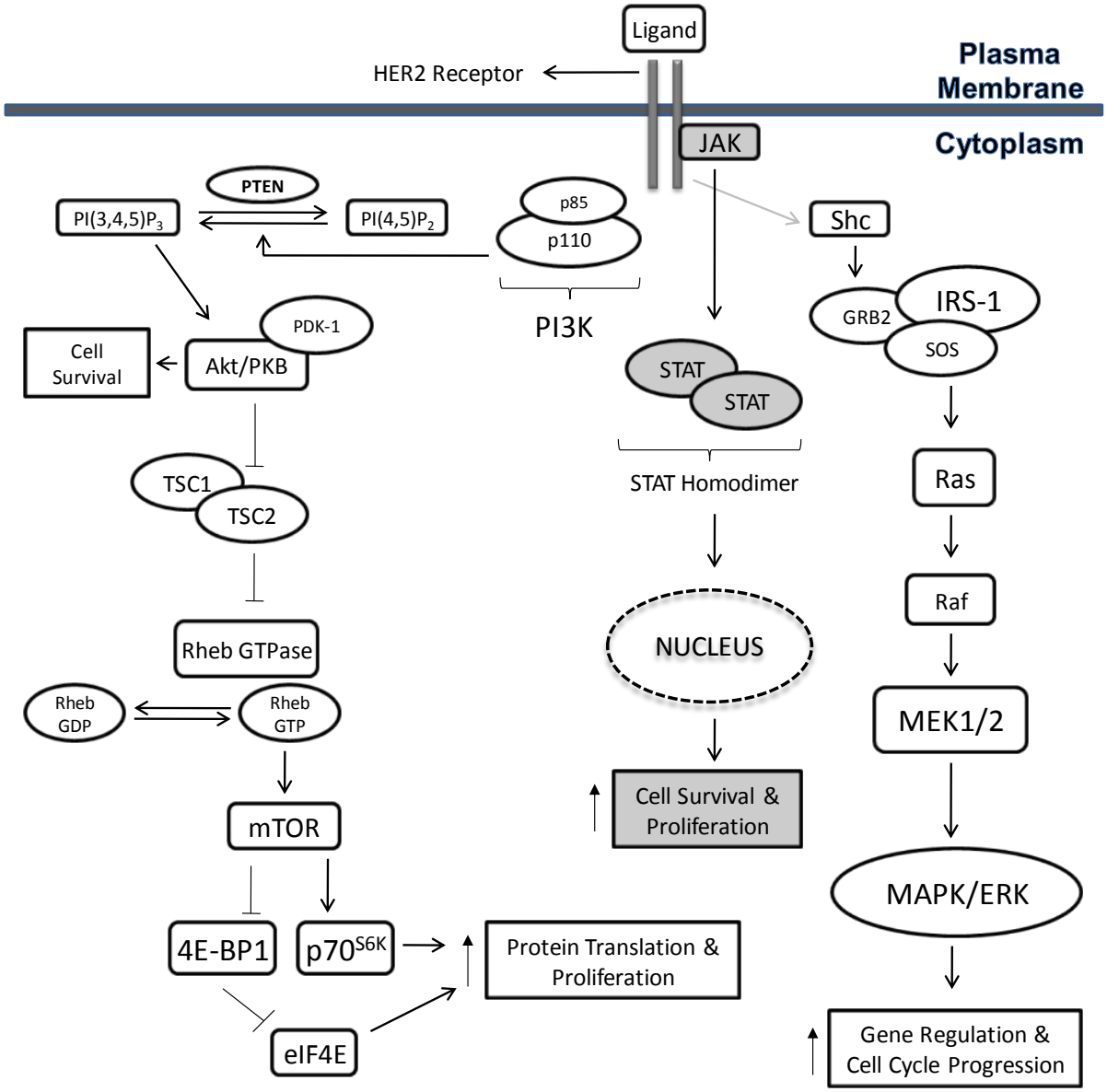


Figure 3: HER2 Signal Transduction Pathways

Various growth factors and ligand binding to the HER2 receptor can lead to the activation of three primary signal transduction pathways: 1) PI3K-Akt/PKB-mTOR, 2) Ras-Raf-MAPK/ERK, and 3) the JAK/STAT pathway. All three of these pathways can modulate many of the factors involved in tumorigenesis, and these signaling pathways can play a key role in the mechanisms of maintaining a malignant phenotype including cellular proliferation, differentiation, and cell survival.

catalytic p110 and the regulatory p85 subunit which contains both an SH2 and SH3 domain, and it is the p85 subunit which interacts with and becomes phosphorylated by activated growth factor receptors [161, 162]. It is this same p85 subunit of PI3K which has been shown in human breast cancer cell lines to become phosphorylated by HER2/Neu after ligand stimulation [163]. Some studies suggest that activation of PI3K by HER2/Neu is mediated by HER3 since the HER3 has a number of different binding domains that can interact with p85 [164, 165]. Because of the role that the PI3K/Akt pathway plays in the story of various cancers, and in our case, breast cancer, HER2/Neu over-expression is an important model of cancer to study.

There are a number of different studies which suggest the importance of the MAPK/ERK signaling pathway as an important factor in HER2/Neu signal transduction. In T47D human breast cancer cells, which express all four members of the ErbB family of receptors, MAPK activation was observed when there was treatment of heregulin (HRG)/*Neu* differentiation factor; which was identified as a candidate ligand for HER3 and subsequently HER2/Neu on the basis of its ability to induce Tyr phosphorylation on the molecule in breast cancer cells [166]. In addition, the degree of MAPK activation is reduced upon administration of an antibody which causes intracellular retention of the HER2/Neu protein to the endoplasmic reticulum [167].

Yet another signal transduction mechanism that has been shown to couple to growth factor receptor tyrosine kinases is the JAK/STAT pathway [168]. This signaling pathway takes part in the regulation of cellular responses to various cytokines and growth factors [169]. Following receptor activation by ligand engagement, the receptor associated JAKs are induced to autophosphorylate and transphosphorylate Tyr residues within the receptors cytoplasmic domain [169, 170]. These newly phosphorylated receptor sites now become docking sites for the recruitment of STAT monomers that are inactive and localized to the plasma membrane, through the interaction with the SH2 domain [169, 170]. Activation of HER2/Neu has been shown to activate STATs through JAK phosphorylation, and this subsequently leads to translocation of STATs to the nucleus where they activate the genes involved in regulating cellular proliferation and cell survival [171] **(Figure 3)**. Of particular interest is STAT3 and STAT5 which have been shown to contribute to malignant progression

by stimulating cellular proliferation [171], regulating the expression of genes involved in cell cycle progression [171], and cellular migration [172].

1.5.3 Crosstalk between the ErbB/HER family and the IGF-IR signaling pathway

There is growing evidence that both the ErbB/HER 1-4 family of receptor tyrosine kinases and the IR and IGF-IR can form homodimers or heterodimers with one another and between families for their full cellular function or for mediating resistance to certain anti-cancer therapies [152, 173-176]. One example of this is with IGF-I which was shown to stimulate a novel EGFR-dependant signal transduction pathway through the physical interaction of IGF-IR and EGFR thus activating the ERK pathway and consequently inducing proliferation in normal human mammary epithelial cells [177]. The IGF-IR has also been shown to exhibit a physical interaction with HER2 and has been implicated in mediating the resistance to the trastuzumab [173, 178, 179]. In addition, IGF-IR that has been activated has also been shown to physically interact with HER3 and HER4 in breast cancer that is resistant to gefitinib (marketed under the trade name Iressa), which is a selective inhibitor of the EGFR tyrosine kinase domain [180]. In addition to the physical interaction between the IGF-IR and EGFR and HER2, there is a crosstalk between the downstream targets of these receptors, and transactivation of similar signal transduction mechanisms that all play an important role in cellular proliferation, apoptosis, cellular migration, invasion and angiogenesis.

1.6 Metformin

1.6.1 Role in the treatment of Type 2 Diabetes Mellitus

Metformin is an orally administered anti-diabetic drug from the biguanide class of chemicals **(Figure 4)**. It was originally identified in the French lilac (*Galega officinalis*) which is a plant that has been well known for centuries to reduce the symptoms associated with Type 2 Diabetes [181]. Metformin was first described in the scientific literature in 1957 [182]. Currently, metformin is the first-line drug for the treatment of Type 2 Diabetes, the metabolic syndrome, as well as polycystic ovary syndrome, particularly in obese and overweight individuals, and it has become one of the most commonly prescribed drugs

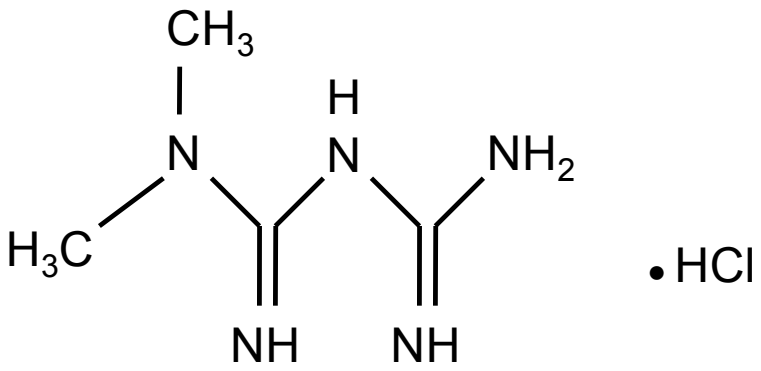


Figure 4: Chemical Structure of Metformin

Metformin (1,2-dimethyl biguanide hydrochloride) is the orally administered anti-diabetic agent used in the treatment of Type 2 Diabetes. It is in the family of biguanide compounds.

overall in North America. Unlike many other orally administered anti-diabetic drugs such as the sulfonylureas, metformin has not been shown to cause hypoglycemia [183]. It has not been shown to cause weight gain, and in some cases may in fact cause minor weight loss [184]. In the case of the metabolic syndrome, metformin has been shown to improve hyperglycemia without stimulation of insulin [185-187]. It also improved the condition of insulin resistance by reducing hepatic gluconeogenesis [187], reduces hyperinsulinemia associated with the syndrome, and also enhances skeletal muscle sensitivity to insulin [187, 188]. Metformin enhances peripheral glucose uptake, increases fatty acid β-oxidation and decreases absorption of glucose from the gastrointestinal tract [189]. This increased peripheral uptake of glucose may be mediated by an improvement of insulin binding to its receptor as per its function as an insulin sensitizer.

1.6.2 Mechanism of action of metformin

Many studies on metformin have proposed several mechanisms of action, however, activation of the AMP-activated protein kinase (AMPK) has been demonstrated to play a prominent role in mediating the effects of metformin [190]. AMPK is a heterotrimeric complex comprising subunits that together form a functional enzyme that is commonly found in a number of tissues including the liver, brain and skeletal muscle [191]. It is a central cellular energy sensor that is comprised of three protein subunits, the alpha (α), beta (β) and gamma (γ), in which each takes on a specific role for both the stability and activity of AMPK [191]. Within these subunits of AMPK lies the ability to detect fluctuations in the AMP:ATP ratio. The catalytic domain of the enzyme is located in the α-subunit, and it is here that AMPK becomes activated upon phosphorylation at the threonine (Thr172) site by an upstream AMPK kinase (AMPKK) [192] **(Figure 5)**. As the metabolic master switch in the cells, AMPK is a key regulator of a number of different intracellular processes which include, increased fatty acid β-oxidation and glycolysis, inhibition of cholesterol synthesis, stimulation of cellular glucose uptake, increased synthesis of the glucose transporter 4 (GLUT4), and the inhibition of insulin release at low glucose concentrations [193-195]. In other words, AMPK phosphorylation of its downstream targets results in an up-regulation of ATP-producing catabolic pathways and a down-regulation of ATP-consuming pathways. In addition to being activated by its up-stream kinase AMPKK, AMPK has also been shown to

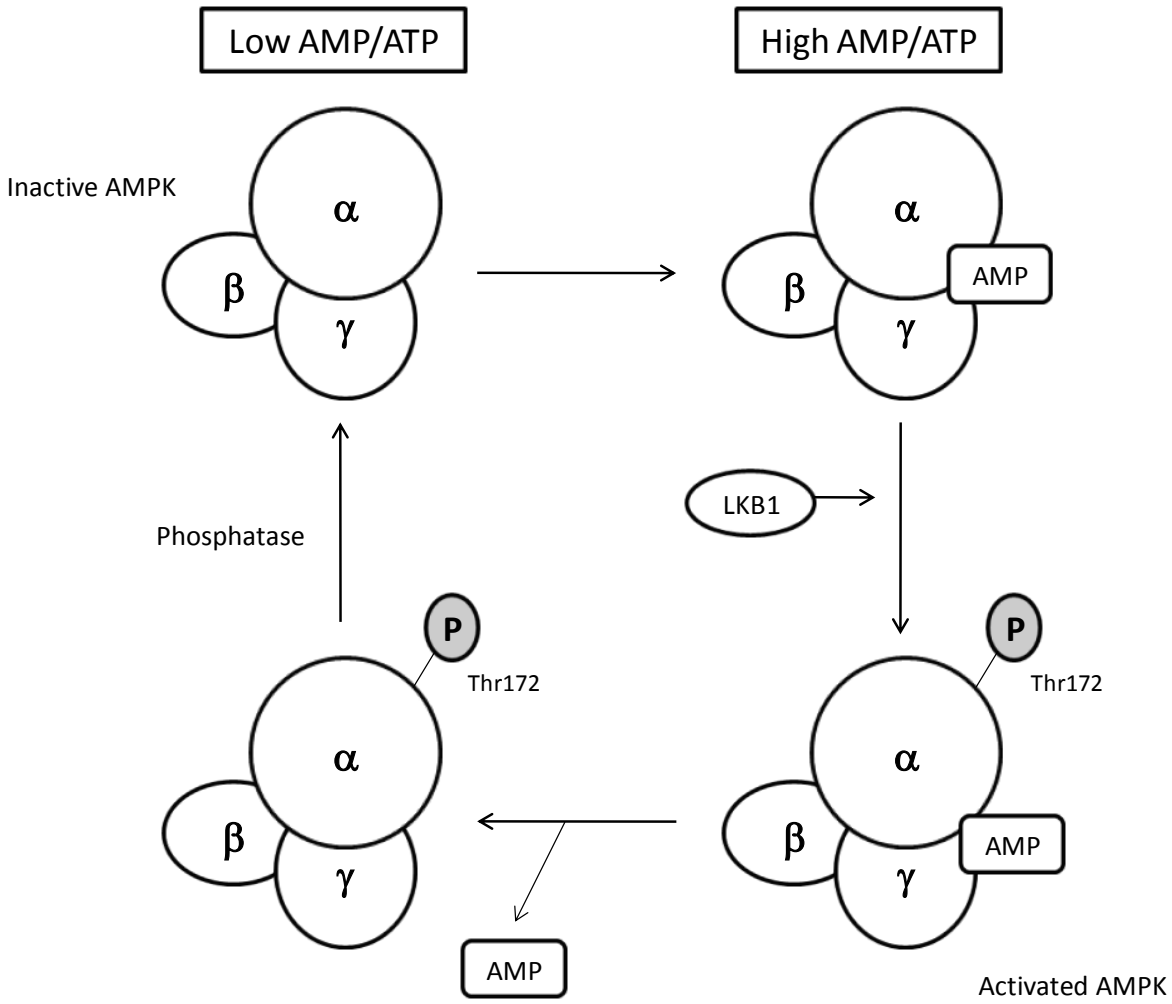


Figure 5: Regulation of the Enzymatic Activation and Inactivation of AMPK

AMPK is a heterotrimeric enzymatic complex which has the ability to detect fluctuating changes in the AMP to ATP ratio in the cell. When there is an increase in AMP/ATP, binding of AMP leads to an increased phosphorylation by LKB1 at the Thr172 site. When the cellular ratio of AMP/ATP is lowered, protein phosphatases will remove the phosphorylation on Thr172 leading to an inactivation of AMPK.

be activated by another up-stream serine/threonine kinase, LKB1 [196-199]. LKB1 is encoded by the Peutz-Jegher syndrome tumor suppressor gene and is required for activation of AMPK in response to stress [200]. At the molecular level, metformin activates AMPK through LKB1, by phosphorylating AMPK on the Thr172 site [196-199]. Metformin has been shown to directly inhibit complex I of the respiratory chain in the mitochondria which leads to a decrease in ATP synthesis, and a subsequent rise in cellular AMP:ATP ratio, which essentially mimics the conditions of cellular energy stress [201]. AMPK also plays a role as an inhibitor of acetyl-CoA carboxylase (ACC), which is the rate-limiting enzyme in fatty acid synthesis [202, 203]. ACC is the enzyme that converts acetyl-CoA to malonyl-CoA. Malonyl-CoA is an inhibitor of carnitine parmitoyltransferase-1 which helps transport fatty acids into the mitochondria for oxidation [203]. When ACC is phosphorylated and inactivated by AMPK, it leads to the increase in this fatty acid transport and oxidation [204] **(Figure 6)**.

1.6.3 Use of metformin in the treatment of breast cancer

In addition to its main function as a metabolic energy regulator, it has more recently been shown that AMPK may in fact influence aspects of other signal transduction mechanisms that are known to play a role in cellular growth and proliferation. There have been a number of different population studies which have demonstrated that treatment with metformin in Type 2 Diabetic patients was associated with a decrease in the risk of developing cancer, especially when compared to patients who were given sulfonylureas, who tended to have an increased risk of cancer-related mortality [205, 206]. In yet another study using a smaller cohort of patients, those treated with metformin demonstrated a lower incidence of cancer overall compared to individuals who were given other treatments [206]. These two independent studies have led to the further investigation of the potential use of metformin as an anti-neoplastic agent in early breast cancer patients [207, 208].

In addition, it has been shown that metformin can inhibit the growth of breast cancer cells through the activation of AMPK [209]. As well, metformin can also inhibit the initiation of protein translation, as shown by the 30% reduction of protein synthesis in MCF-7 breast cancer cells [209]. AMPK achieves this by phosphorylation and stabilization of the protein

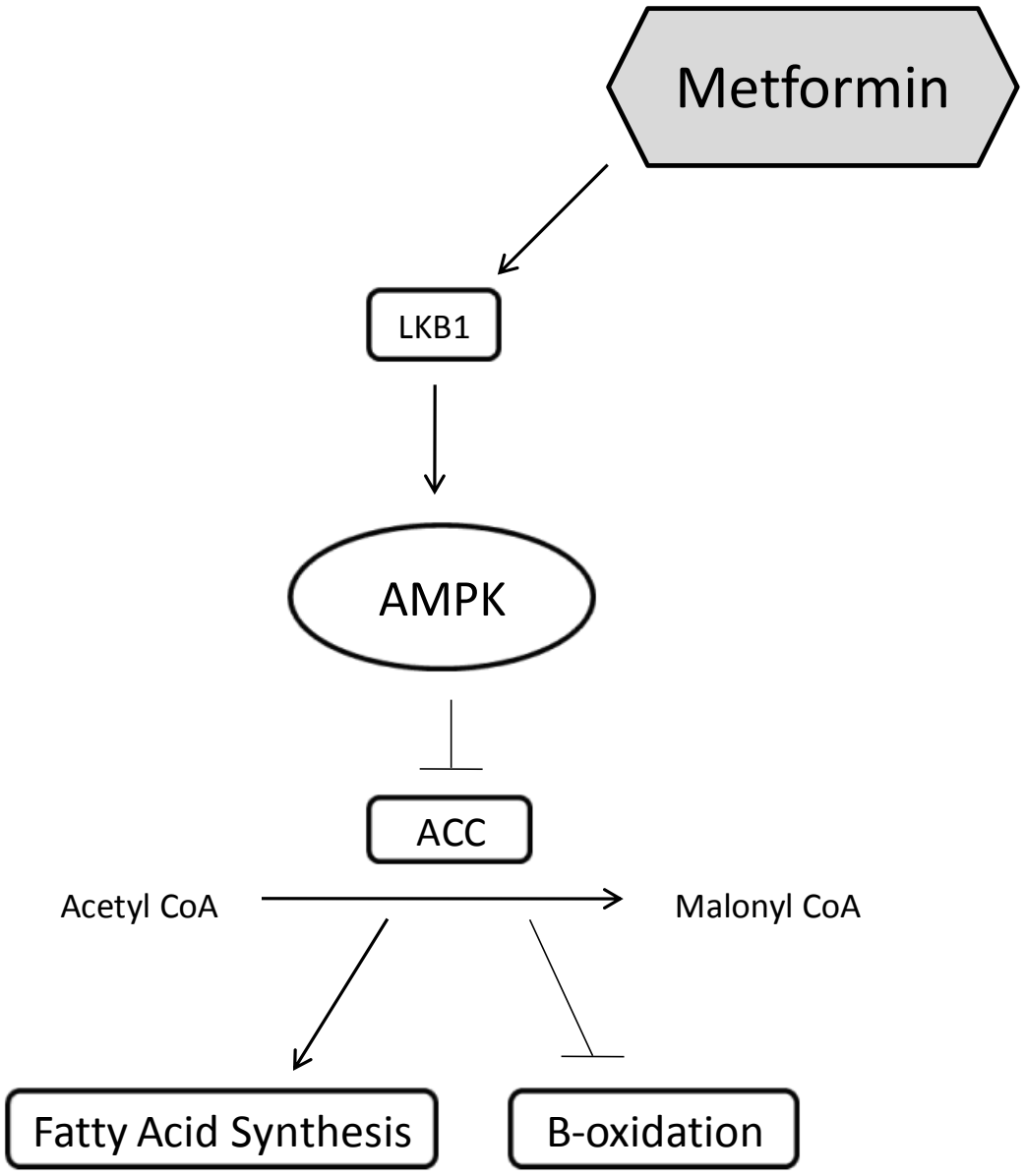


Figure 6: Molecular Targets of Metformin

Metformin has been shown to activate AMPK by acting on its up-stream kinase LKB1. Upon activation of AMPK, ACC, the rate-limiting enzyme in fatty acid synthesis, becomes phosphorylated and inactivated, and so there is a reduction in the synthesis of fatty acids, and an increase in fatty acid β-oxidation.

product of the TSC2 tumor suppressor gene [209, 210]. As mentioned previously, TSC2 negatively regulates eukaryotic protein synthesis by inhibiting mTOR. In addition to acting on TSC2, AMPK has been shown to directly inhibit mTOR, and a subsequent decrease in the phosphorylation of downstream targets $p70^{S6K}$ and 4E-BP1 was observed [209] **(Figure 7)**. As well, treatment of these cells with the AMPK inhibitor, Compound C, prevented the inhibition of protein translation, suggesting that in fact the effects of metformin on translation were mediated by AMPK [209]. When TSC2 null mouse embryonic fibroblasts ($TSC2^{-/-}$) and the human breast cancer cell line MDA-MB-231, which lacks the up-stream kinase LKB1, were treated with metformin, there was no effect on protein translation, which further supported the importance of LKB1 and TSC2 in the mechanism of action of metformin [209].

This brings us to the notion that in addition to the possible beneficial effects of lowering circulating levels of insulin by metformin in women that exhibit hyperinsulinemia along with breast cancer, there is also the potential beneficial effect of AMPK activation to counter mRNA translation, ribosome biogenesis, autophagy, as well as cellular growth and proliferation in breast cancer cells by inhibiting the PI3K/Akt/mTOR signaling pathway [211, 212]. It is this ability of metformin to activate AMPK that has also been suggested to contribute to a direct, insulin-independent mechanism that can inhibit cancer cells by inhibiting the down-stream effector mTOR [209, 210].

1.7 General Hypothesis

There is strong evidence to support the notion that high levels of insulin are associated with an increased incidence and with poor outcomes in breast cancer. Although normal mammary epithelial cells and the majority (80%) of malignant mammary epithelial cells express insulin receptors, it remains unknown whether in women with insulin resistance and hyperinsulinemia, these cells remain sensitive to insulin in contrast to skeletal muscle, adipose tissue and liver. In this state one could postulate that persistent abnormal stimulation by insulin of these cells would be present. The general objective of these studies was to examine the effect of insulin resistance and hyperinsulinemia on breast cancer development and progression using a rodent model of breast cancer. In addition, it was hypothesized that

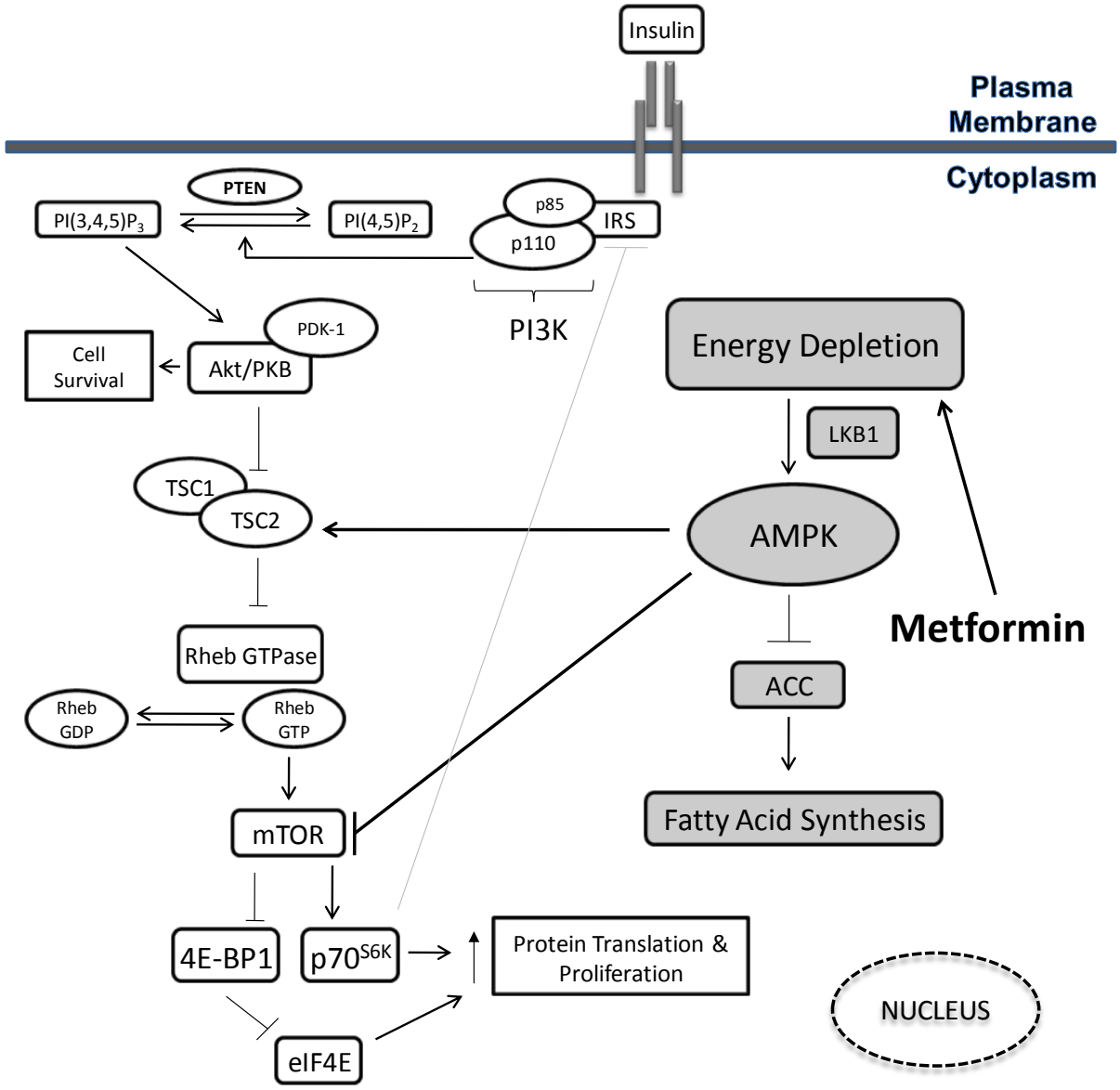


Figure 7: Molecular Targets of Metformin and the Interaction with Key Factors in Tumorigenesis

Metformin can act on the up-stream kinase of AMPK, LKB1 which can lead to the activation of AMPK. Activation of AMPK has been shown to directly activate TSC2, which is a negative regulator of mTOR. In addition, AMPK has been shown to directly inhibit the activity of mTOR, which would lead to a reduction in the activation of its down-stream targets, and a subsequent reduction in protein synthesis and cellular proliferation. So not only does metformin act to lower circulating insulin levels, but it can also act directly on these key players involved in tumorigenesis.

treatment of the insulin resistant state with the anti-diabetic and insulin lowering agent metformin will improve insulin sensitivity and lower circulating levels of insulin, thus potentially decreasing the incidence and/or improving the outcome of breast cancer.

CHAPTER 2:

The Effect of a High-Fat Diet on HER2/Neu Mammary Carcinogenesis

2.1 Introduction

Obesity is a known risk factor for the development of the metabolic syndrome also known as the insulin resistance syndrome, which in general terms is defined as insulin resistance associated with glucose intolerance, dyslipidemia and/or hypertension [30, 213, 214]. The prevalence of metabolic syndrome has increased with the increasing incidences of breast cancer, diabetes, and obesity worldwide [215]. Type 2 Diabetes is characterized by hyperglycemia, hyperinsulinemia and insulin resistance. Epidemiological studies of the association between Type 2 Diabetes and risk of breast cancer were reviewed and a modest association between these two diseases is evident. This appears to be more consistent among postmenopausal than among premenopausal women [216]. An evaluation of this link indicates that the risk is present prior to the onset of Type 2 Diabetes, in other words, at the stage of insulin resistance associated with compensatory hyperinsulinemia with normal glucose concentrations [217].

The above data support the findings relating increased levels of insulin to an increased risk of the development of and mortality due to breast cancer [50, 60]. Two other studies carefully adjusted for other prognostic variables, demonstrated that higher insulin levels increased the incidence of distant recurrence of breast cancer, i.e. the appearance of the tumor in a region of the body that is different than where it initially originated [60, 218]. Similarly, Lawlor et al. observed a modest but linear increase of breast cancer risk between the lowest and the highest quartiles of insulin level, among postmenopausal, non-diabetic women [219]. Therefore, from available data in human studies, elevated insulin likely confers a small independent risk for the development of breast cancer, but a more marked independent risk for disease progression and death.

Recently, it was reported that consumption of a moderately high fat diet had little effect on oncogene-induced, ER negative mammary tumor development in the MMTV-HER2/Neu mouse [220]. In contrast, the consumption of the same diet in another murine model of breast cancer, the MMTV-TGF-α, shortened mammary tumor latency [221]. These

authors suggested that the lack of ER in the HER2/Neu tumors as the cause of the absence of an effect of high-fat diet (HFD)/obesity on tumor development. Although there is much supporting evidence for the role that insulin can play in the development and progression of breast cancer, the exact mechanisms by which this is occurring has yet to be elucidated.

2.1.1 Hypothesis

In this component of the study, the effect of high-fat diet induced obesity, which is the most common cause of insulin resistance that is seen in human subjects, was examined *in vivo* with respect to its effects on breast cancer development and progression. In order to do this, a well characterized murine model of breast cancer, the mouse mammary tumor virus (MMTV)-HER2/Neu which develops mammary tumors that mimic a human subtype of breast cancer [222] by over-expressing HER2, was given a high-fat diet (HFD). The relationship between diet-induced obesity, insulin-mediated signaling and the effect on breast tumor growth and development was examined. We hypothesize that if there is insulin resistance associated with an increased level of circulating insulin in these mice fed a high-fat diet, then this could lead to an increase in growth of breast tumors and a decreased latency of tumor onset, as well as a maintenance of insulin signaling in the tumors compared to the metabolic target tissues.

2.2 Materials and Methods

2.2.1 Reagents

Antibodies for phospho-p70 S6 kinase (Thr389), phospho-Akt/PKB (Ser473), Akt/PKB, phospho-4E-BP1 (Thr37/47), 4E-BP1 were obtained from Cell Signaling Technology Inc. (Beverly, MA). The p70 antibody, Agarose Immunoprecipitation (IP) reagent, anti-phosphotyrosine (pY99) and anti-rabbit and anti-mouse IgG secondary antibodies all came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Insulin enzyme-linked immunosorbent assay (ELISA) kits were from Linco Research Inc. (St. Charles, MO). Anti-IRS-1 and anti-IRS-2 antibodies were obtained from Upstate/Millipore (Billerica, MA). The nitrocellulose membrane was from Invitrogen Canada Inc. (Burlington, ON). The enhanced-chemiluminescence (ECL) substrate kit was from KPL Inc. (Gaithersburg, MD).

The biotinylated rabbit anti-rat IgG and the Elite ABC-horse radish peroxidase came from Vector Laboratories (Burlingame, CA). The Ki67 antibody was obtained from Dako (Glostrup, Denmark).

2.2.2 Animals

The transgenic model of breast cancer that was used for this study was the well established mouse mammary tumor virus (MMTV) long-terminal repeat WT HER2/Neu transgenic mouse (FVB/N-Tg [MMTV-HER2/Neu] 202Mut/J Stock No. 002376) which is commercially available from Jackson Laboratories (Bar Harbor, ME). Focal mammary tumors are expected to appear at approximately 5 months of age, with a median incidence around 205 days, and these usually metastasize to the lungs [223, 224]. Mice were maintained on a 12-h light/dark cycle. All procedures were conducted according to the protocols and guidelines approved by the University Health Network Animal Care Committee.

2.2.3 Diet formulation and treatment

Forty female FVB/N HER2/Neu mice at the age of 4 weeks were randomly placed on a low-fat diet (LFD) which consisted of 10% kcal fat (n=16), and a high-fat diet (HFD) which consisted of 45% kcal fat (n=24), and both diets were from Harlan Teklad (Madison, WI) **(Table 1)**. Diets were stable for six months and stored at 4^0C. Mice were allowed *ad libitum* access to food and water throughout the treatment period.

HFD (45% kcal Fat)	% kcal From	Detailed Composition	g/kg	% of Total Fat	
Protein	20	Casein L-Cystine	245 3.5	N/A	
Carbohydrate	35	Corn Starch Maltodextrin Sucrose	85 115 200	N/A	
Fat	45	Lard Soybean Oil	195 30	Saturated Monounsaturated Polyunsaturated	36 47 17
Total	100				100

Table 1: Composition and Detailed Breakdown of HFD

2.2.4 Body weights and Magnetic Resonance Imaging (MRI)

Body weights were monitored and recorded weekly. For the assessment of fat versus lean mass, a mouse whole-body magnetic resonance analyzer was used (Echo Medical Systems, Houston, TX), at the Max Bell Research Facility at the Toronto General Hospital, and this was performed once a month.

2.2.5 Intra-Peritoneal Glucose Tolerance Test and plasma insulin concentration

Glucose tolerance tests (GTTs) were performed on mice fasted overnight using a glucose load of 1.5 g of glucose/kg body mass per injection, and was injected intra-peritoneally (i.p.). Blood glucose measurements were made at 0, 10, 20, 30, 60, 90, and 120 minutes after the injection using a Sure Step, One Touch Glucometer from Lifescan Inc. (Milpitas, CA). Tail vein blood samples were collected at 10 min after glucose administration in heparin-coated tubes. Plasma was separated from the whole blood by centrifugation at 3000 × g. Plasma insulin concentrations were assayed in duplicate by an ELISA kit (Linco Research Inc., St. Charles, MO), according to the manufacturer's instructions.

2.2.6 Tumor detection and measurements

For tumor measurements the mice were palpated twice weekly beginning at 4 months of age. Tumor size was assessed by measuring the perpendicular tumor diameters with a digital caliper. Tumor volume was calculated by the formula as follows: Tumor Volume = [((smallest diameter)^2 × (largest diameter)^2)/2)] [225] and values obtained from this calculation were then used to assess growth rates.

2.2.7 Animal sacrifice and tissue collection

The mice were euthanized by CO_2 gas when they reached one year of age or when the largest mammary tumor reached a diameter of 1.5 cm, which was in accordance with the Canadian Council on Animal Care and Cancer Endpoint Guidelines. Before sacrificing, animals were injected either with saline (vehicle) or with i.p. insulin at a dose of 2 U insulin/kg body mass (1 U = 6 nmol) 5 or 10 minutes prior to sacrifice. The skeletal muscle and the mammary tumor tissue were rapidly excised and snap frozen in liquid nitrogen, and

then stored at -80^{0}C until subsequent homogenization for immunoblot analysis. In addition, part of the tumor tissue was fixed in 10% buffered formalin for immunohistochemistry.

2.2.8 Immunoblotting and Immunoprecipitation

For immunoblot analysis, mammary tumor samples were homogenized with a hand-held glass homogenizer in ice cold lysis buffer (50 mM Tris pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Ethylene Glycol Tetraacetic Acid (EGTA), 1 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_4$, 1 μM okadaic acid, 1 mM Phenylmethylsulphonyl Fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin). Cellular debris was removed by centrifugation at 10 000 × g for 20 min at 4^{0}C. Protein amounts were determined by Bradford protein assay kit (Bio-Rad, Mississauga, ON). 30 μg of protein was re-suspended in 2× Laemmli sample buffer (4% sodium dodecyl sulfate (SDS), 20% glycerol, 125 mM Tris pH 6.8, 0.2% bromophenol blue, 10% beta-mercaptoethanol), and then boiled for 5 min and separated by 10% SDS-polyacrylamide gel and then electrophoretically transferred onto nitrocellulose membranes. The membranes were incubated for 1-h in Tris-Tween Normal Saline (TTNS) blocking buffer containing 5% bovine serum albumin (BSA), followed by sequential incubation with primary (all at a dilution of 1:1000 for p-p70^{S6K}, p-70^{S6K}, p-Akt/PKB, Akt/PKB; and 1:5000 dilution for β-actin) and secondary (at a dilution of 1:5000) antibodies. Antibody-protein complexes were visualized using enhanced-chemiluminescence (ECL) according to the manufacturer's instructions, and band intensities were quantified by scanning laser densitometry.

For immunoprecipitation (IP) 500 μg of the tumor lysates were incubated with agarose-conjugated anti-IRS-1 antibody or with anti-IRS-2 antibody followed by incubation with Protein A/G PLUS-Agarose immunoprecipitation reagent overnight at 4^{0}C with rotation. Immune complexes were collected by centrifugation for 5 min and washed three times with 1.5 mL of ice cold phosphate buffered saline (PBS). The samples were then re-suspended in 1× Laemmli sample buffer, boiled for 5 min and separated by 8% SDS-PAGE under reducing conditions and transferred onto nitrocellulose membranes. This was followed by immunoblot with anti-phosphotyrosine (pY99) (at a dilution of 1:1000) and IRS-1 or IRS-

2 antibodies (at a dilution of 1:1000) and visualization with ECL. Band intensities were quantified by scanning laser densitometry.

2.2.9 Immunostaining

Formalin-fixed paraffin-embedded tissue sections of breast tumors were cut at 4 μm, de-waxed in 3 changes of xylene and rehydrated through graded alcohols. After antigen retrieval in Tris-HCl buffer, pH 9.0 at 115^0C for 10 min and Avidin blocking, sections were incubated with Ki67 antibody at a 1:25 dilution for 1-h at room temperature and subsequently washed with TTNS. After incubation with biotinylated rabbit anti-rat IgG at a 1:200 dilution for 30 min, sections were washed and incubated with Elite ABC-horseradish peroxidase. Antibody binding was visualized with diaminobenzidine, counterstained with hematoxylin. Finally, sections were dehydrated through graded alcohol, cleared in xylene and cover-slipped. All of the slides were scanned at × 20 by ScanScope XT (Aperio Technologies, Vista, CA) that provided high resolution images (0.5 microns/pixel). Images of the whole slides were analyzed using Positive Pixel Count algorithm available in ImageScope Software, and each slide contained approximately 150,000-180,000 cells. Brown nuclear staining was considered to be positive for Ki67. Labeling index (Percentage of positive Ki67 staining) was calculated as the percentage of cells with positive nuclear staining. Immunostaining was performed randomly, and scored on tumor tissue sections from each mouse (n=7) from both the LFD and HFD groups.

2.2.10 Statistical Analysis

All results are expressed as mean ± SEM where applicable. Statistical significance was assessed by two-tailed Student's t test or ANOVA. In addition, Kaplan-Meier survival estimates were calculated for the time to the development of the first tumor, as well as second tumor in the various treatment groups (i.e. LFD vs. HFD). Analysis was done in terms of median survival times and Log-rank tests were performed to compare the survival curves of different experimental groups, and determine if there was a significant difference between them. Clinically, the median survival is the number of days at which half the n-value survived without developing the event of interest, so at this point, half the n-value had a longer and half have a shorter survival time. Mice which died from non-study related causes

(i.e. due to unknown circumstances before the experimental endpoint) had the data censored at the time of death. These statistical calculations were performed using Statistica software v. 7.0 (Statistical Analysis System, Cary, NC). A $p<0.05$ was considered statistically significant in all data analysis.

2.3 Results

2.3.1 Body weights and body fat content

To investigate the influence of diet on body weight, the HER2/Neu mice were fed with high-fat diet (HFD) or low-fat diet (LFD) (45% vs. 10% kcal from fat respectively). The diets were instituted at 4 weeks of age and were well tolerated. There were no statistically significant differences in grams of food consumed per day between the treatment groups. HER2/Neu mice showed a modest, but significant increase in body weight after 30 weeks on diet (LFD 24.4 ± 0.6 g vs. HFD 27.1 ± 1.3 g, $p<0.05$) **(Figure 8a)**. To determine whether the presence of HFD altered the body fat content of the mice, MRI analysis was performed after 8, 13, 16, 21 and 25 weeks on diet. There was a significant increase in percent of body fat in HFD compared to LFD mice at the 13 week (LFD 15.8 ± 0.7 % vs. HFD 22.9 ± 0.5 %, $p<0.01$), 21 week (LFD 15.6 ± 0.7 % vs. HFD 22.6 ± 0.7 %, $p<0.01$) and 25 week (LFD 12.9 ± 0.4 % vs. HFD 21.5 ± 0.8 %, $p<0.01$) time points **(Figure 8b)**.

2.3.2 Glucose tolerance and plasma insulin concentrations

To investigate the metabolic consequences of the HFD, intra-peritoneal glucose tolerance tests (i.p. GTTs) were performed after 28 weeks on diet. The HFD fed HER2/Neu mice showed no change in fasting blood glucose levels but a mild impairment of glucose tolerance with significantly increased values after 20, 60, 90 and 120 min of glucose loading compared to the LFD treated mice ($p<0.05$) **(Figure 9a)**. To explore dietary effects on insulin levels, plasma insulin levels were assayed using an ELISA kit. The mean insulin level in plasma samples of mice on HFD was not significantly higher than that of mice on LFD 10 min after glucose administration (LFD 5.19 ± 0.51 nmol/L vs. HFD 5.33 ± 0.38 nmol/L) **(Figure 9b)**.

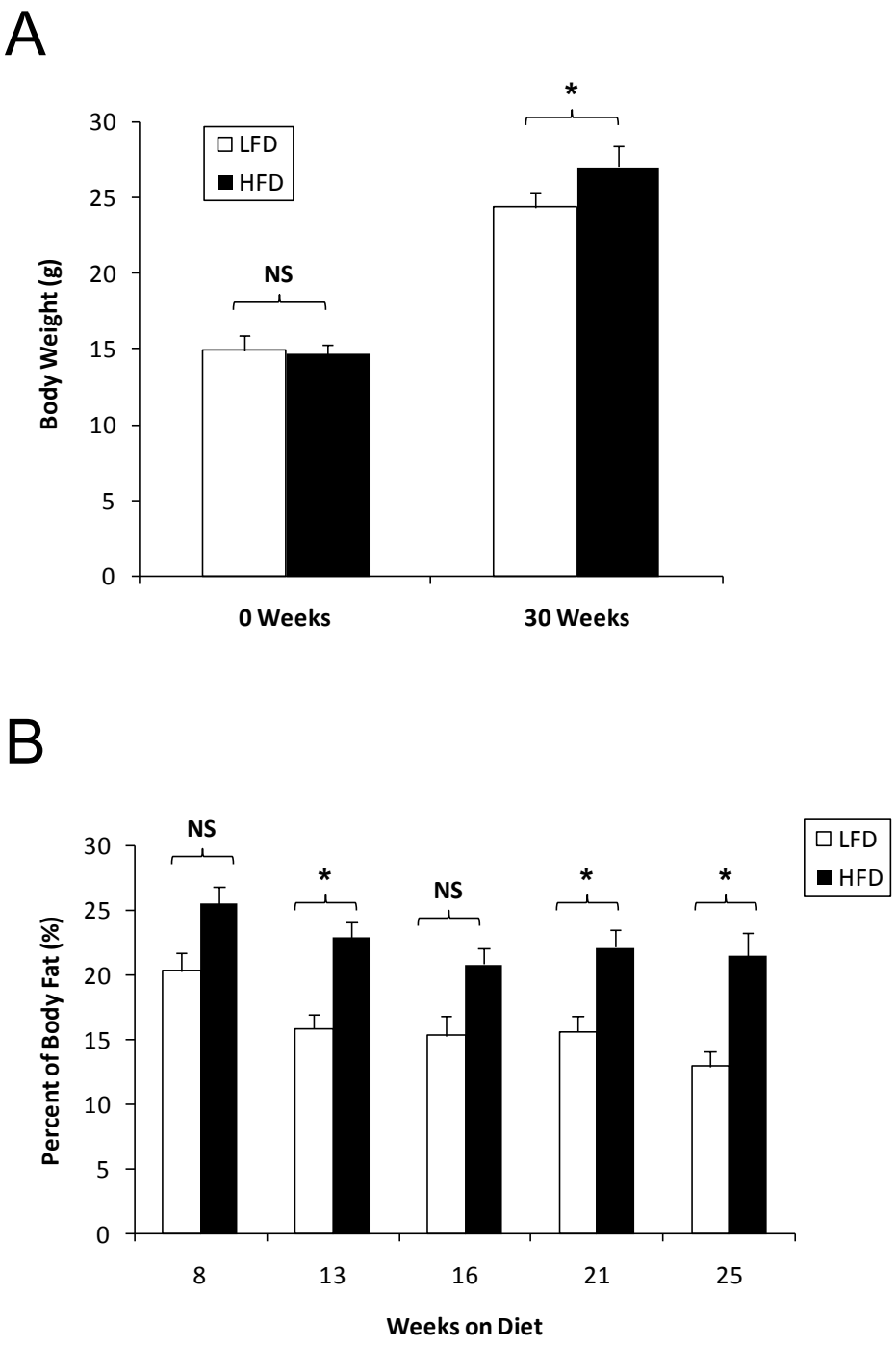


Figure 8: Body Weight and MRI Analysis of HER2/Neu Mice on a High-Fat Diet

(A) Body weights of mice maintained on a low-fat diet (LFD n=16), and on a high-fat diet (HFD n=24) for 30 weeks. *p<0.05 between LFD and HFD. **(B)** Body fat content as assessed by MRI in mice maintained on (LFD n=16) or (HFD n=24) over a 25 week period. *p<0.01 between LFD and HFD.

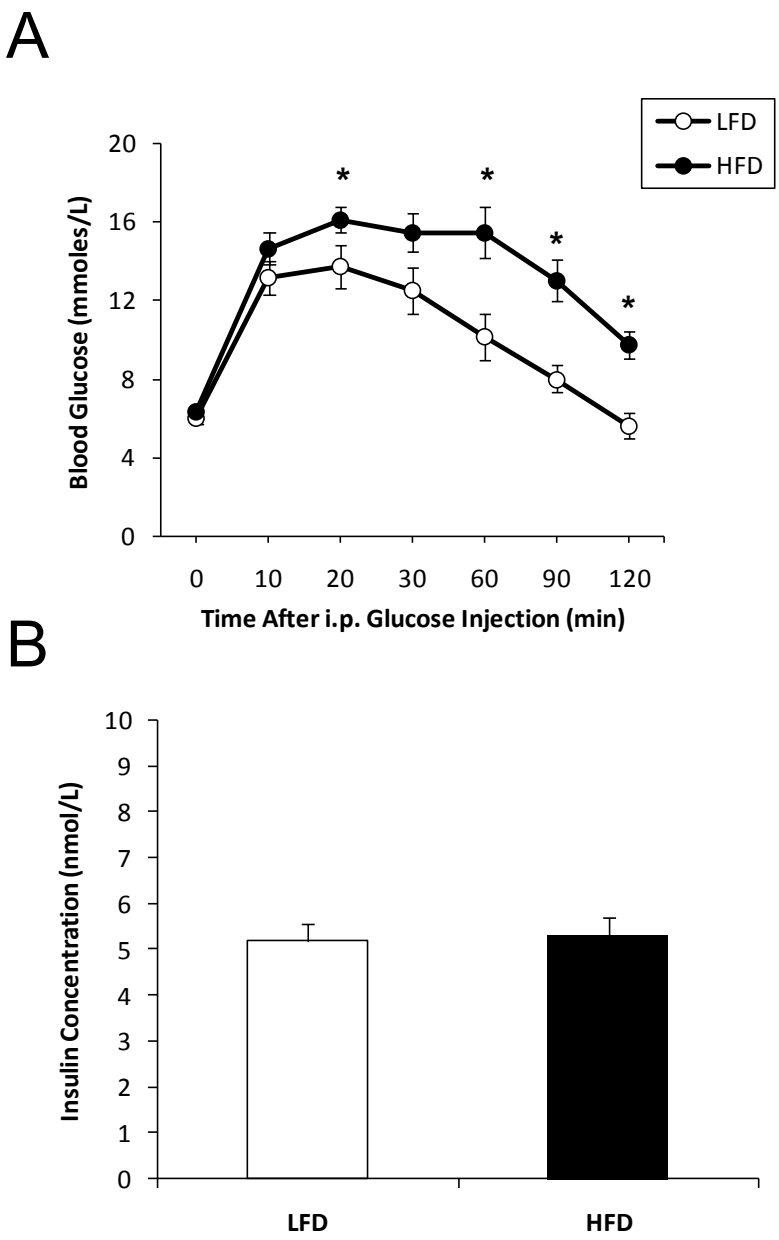


Figure 9: Intra-peritoneal Glucose Tolerance Test and Insulin Concentrations of HER2/Neu Mice on a High-Fat Diet

(A) Intra-peritoneal glucose tolerance test (i.p. GTT) after 28 weeks on diet. Following an overnight fast, glucose (1.5g/kg body weight) was administered by i.p. injection, and tail vein blood was withdrawn at 0, 10, 20, 30, 60, 90 and 120 minutes post-glucose load, and blood glucose was measured, (LFD n=16) and (HFD n=24). *p<0.05 between LFD and HFD. **(B)** Insulin concentrations measured 10 minutes post-glucose load after 28 weeks on diet, using a mouse insulin ELISA kit, (LFD n=16) and (HFD n=24).

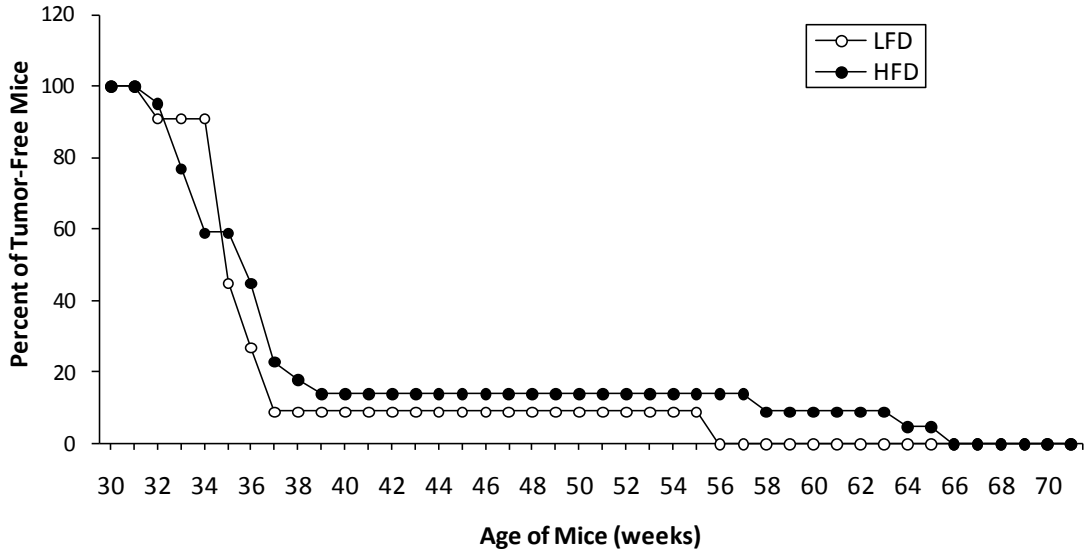
2.3.3 Onset of the first tumor and second tumor

To determine whether the HFD affected mammary gland tumor development and progression, the mice were palpated twice weekly and tumors were measured. The age of detection of the first mammary tumor in each mouse in both groups was plotted as a Kaplan-Meier "survival analysis". The median time of tumor-free survival was similar between LFD (35 weeks of age) and HFD mice (36 weeks of age) **(Figure 10a)**. Next, we examined the onset of the second tumor formation since the HER2/Neu mice often develop multiple tumors. The age of detection of the second mammary tumor of the mice in both diet groups was plotted as a Kaplan-Meier "survival analysis" similar to what was done for the onset of the first tumor. The high-fat diet decreased the latency period for the appearance of a second tumor, however this did not achieve statistical significance (survival analysis Log-rank test p=0.187) with the median time for two tumor-free survival seen earlier in these mice (37 weeks of age) than in LFD mice (41 weeks of age) **(Figure 10b)**.

2.3.4 Growth rates and average number of tumors

To assess any differences in growth rates of tumors between the treatment groups, tumor growth rates were calculated. First, we looked at the rate of tumor progression. This is the number of days between first tumor detection and the last tumor measurement (determined by the sacrifice cut-off point of 1.5 cm), and then this is averaged between each treatment group. It represents the rate at which the tumor would be progressing. There were no significant differences between the rates of tumor progression between the LFD and HFD fed mice (LFD 35 ± 7.38 days vs. HFD 38 ± 3.91 days) **(Figure 11a)**. Tumor growth rates, in absolute growth (LFD 59.2 ± 13.3 mm^3/day vs. HFD 48.2 ± 6.3 mm^3/day) and percent daily relative growth (LFD 204.8 ± 133.7 %/day vs. HFD 108.2 ± 67.5%/day) were also not significantly different between the two groups **(Figure 11b & 11c)**. However, there was 2-fold increase in the average number of tumors detected in the HFD fed mice relative to the LFD mice (LFD 1.5 ± 0.25 vs. HFD 2.7 ± 0.23, $p<0.01$) **(Figure 12)**.

A



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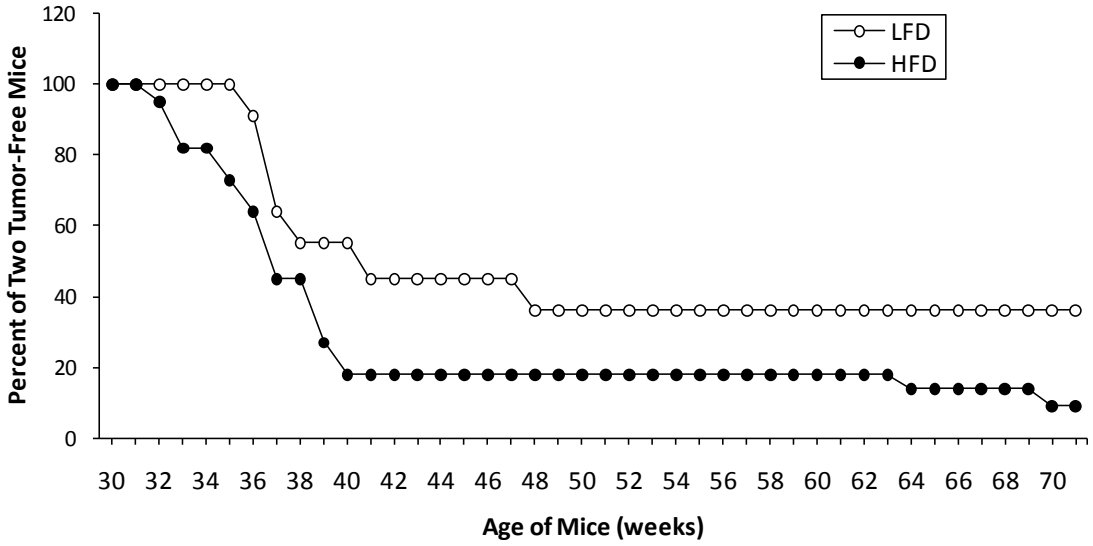


Figure 10: Survival Analysis of First Tumor Onset and Second Tumor Onset in HER2/Neu Mice on a High-Fat Diet

(A) Kaplan-Meier survival analysis of the percent of mice which are tumor-free (i.e. have no tumors at all) against the age of the mice in weeks, (LFD n=11) and (HFD n=18). **(B)** Kaplan-Meier survival analysis of the percent of mice with no tumors, or only one tumor (i.e. reflects the number of mice with two tumors or more) against the age of mice in weeks, (LFD n=11) and (HFD n=18).

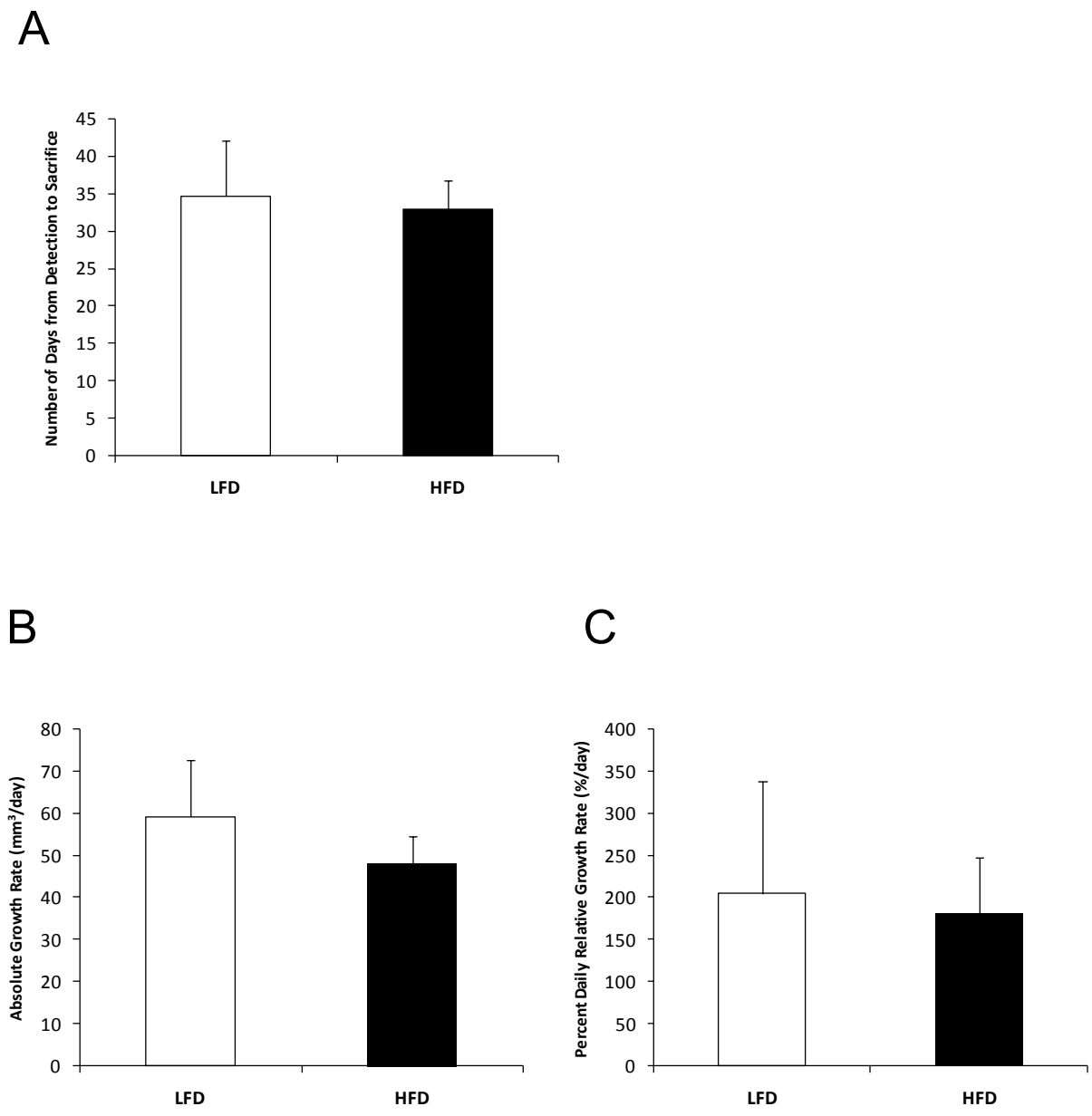


Figure 11: Growth Rates of Mammary Tumors from HER2/Neu Mice on a High-Fat Diet

(A) The number of days between the first tumor detection and last tumor measurement (i.e. at the sacrifice end-point of 1.5 cm) was averaged and plotted for each treatment group, (LFD n=10) and (HFD n=18) **(B)** Absolute growth rate of tumors (mm^3/number of days from tumor onset until sacrifice point). Volumes are calculated from caliper measurements taken twice a week, (LFD n=10) and (HFD n=18). **(C)** Percent daily relative growth rate (% change in volume/day). Volumes are calculated in the same manner as was done for absolute growth rate, (LFD n=10) and (HFD n=18).

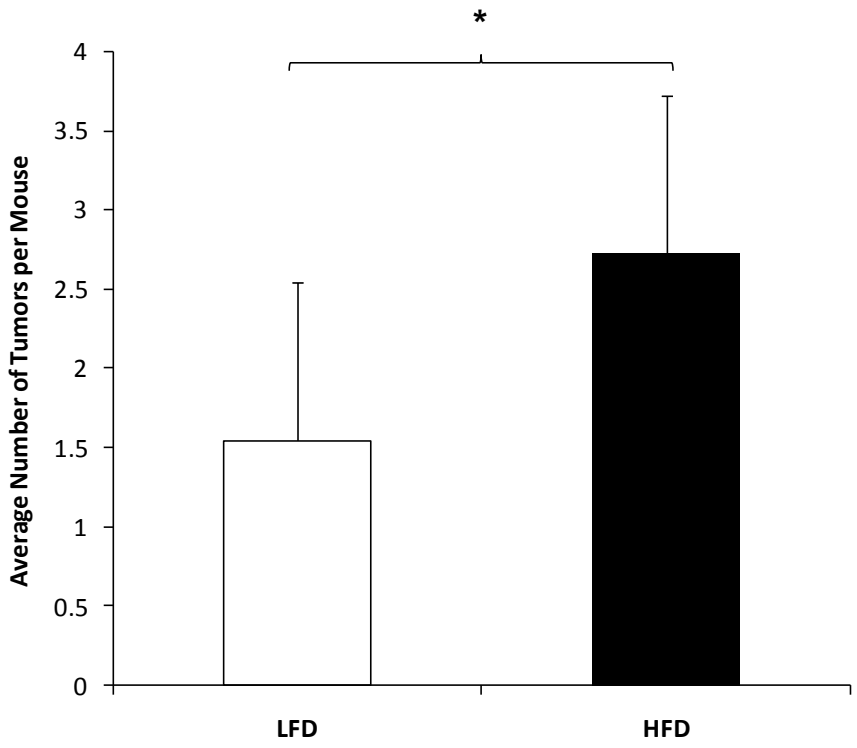


Figure 12: Average Number of Tumors that Developed per Mouse in HER2/Neu Mice on a High-Fat Diet

Average number of tumors per mouse, (LFD n=16) and (HFD n=24). *p<0.01 between LFD and HFD.

2.3.5 Immunoblots of Akt/PKB in skeletal muscle

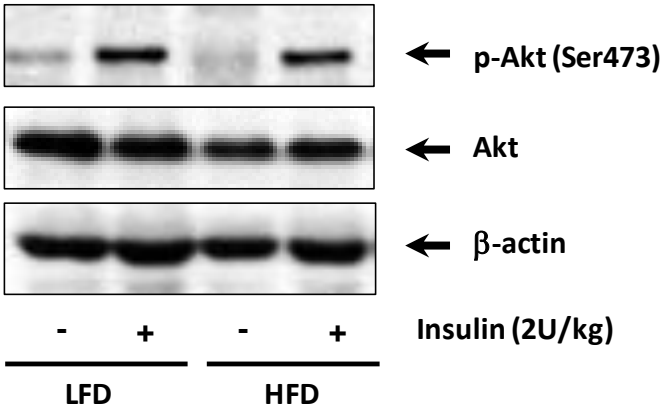
It is well documented that changes in diet can influence insulin signaling in metabolic target tissues such as liver, muscle and adipose cells. We investigated if the diet influenced signaling pathways in the skeletal muscle from these mice in order to assess the extent of insulin resistance. Skeletal muscle lysates were prepared and there was no significant difference in the phosphorylation of Akt/PKB in the muscle between the LFD and HFD at either the basal state or 5 min after acute insulin stimulation consistent with the absence of hyperinsulinemia in this model **(Figure 13b).**

2.3.6 Immunoblots of Akt/PKB, p70^{S6K}, 4E-BP1 in tumor tissue

The effect of diet changes, if any, on mammary gland tumors has not been established. In the mammary tumor lysates, there were very low levels of phosphorylation of Akt/PKB, p70^{S6K} and 4E-BP1 **(Figure 14)** in the basal state and no differences between the LFD and HFD groups. Surprisingly, at 5 min post-insulin stimulation, there was a more robust response observed in the HFD group. Thus, while phosphorylation of Akt/PKB and p70^{S6K} were weakly stimulated at 5 min in LFD mammary tumors, phosphorylation of these proteins was significantly increased in the mammary tumors from HFD mice (p-p70/p70: LFD 0.94 ± 0.10 vs. HFD + 1.82 ± 0.18, $p<0.05$) and (p-Akt/Akt: LFD 1.14 ± 0.14 vs. HFD 3.22 ± 0.15, $p<0.05$) **(Figure 15a and 15b)**.

Since it has been reported that in the early phases of weight gain, insulin signaling may actually be augmented and/or occur more rapidly [226], an additional set of experiments was performed using a 10 min duration of insulin stimulation. At 10 min post-insulin stimulation, a similar extent of stimulation of phosphorylation was observed in these tumors from the LFD group was seen (p-p70/p70: LFD (10 min insulin) 1.48 ± 0.08 vs. HFD (5 min insulin) 1.82 ± 0.18) and (p-Akt/Akt: LFD (10 min insulin) 2.36 ± 0.04 vs. HFD (5 min insulin) 3.22 ± 0.15, $p<0.05$) **(Figure 15a and 15b)**. In addition, there was a more marked phosphorylation of the other downstream targets of mTOR; 4E-BP1 in the tumor tissue in the HFD fed mice upon insulin stimulation. We found a significant increase in the 4E-BP1 phosphorylation in the HFD fed mice with a 5 minute acute insulin stimulation relative to the LFD mice that were given an acute insulin stimulation for 5 minutes, as well as 10 minutes

A



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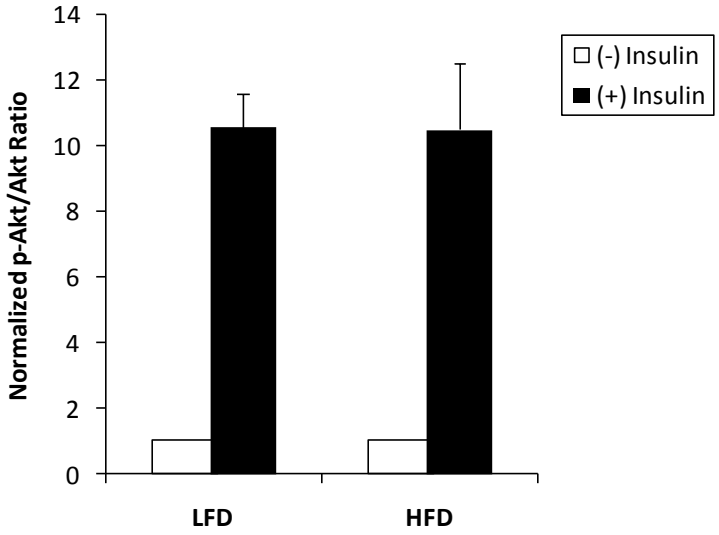


Figure 13: Immunoblot of Skeletal Muscle Lysates from HER2/Neu Mice on a High-Fat Diet

Comparison of insulin stimulated phosphorylation in skeletal muscle lysates, of the insulin signaling protein Akt/PKB 5 minutes after acute insulin injection in LFD mice versus HFD fed mice as shown by **(A)** the representative blot and **(B)** the densitometric graphs were generated from three independent experiments.

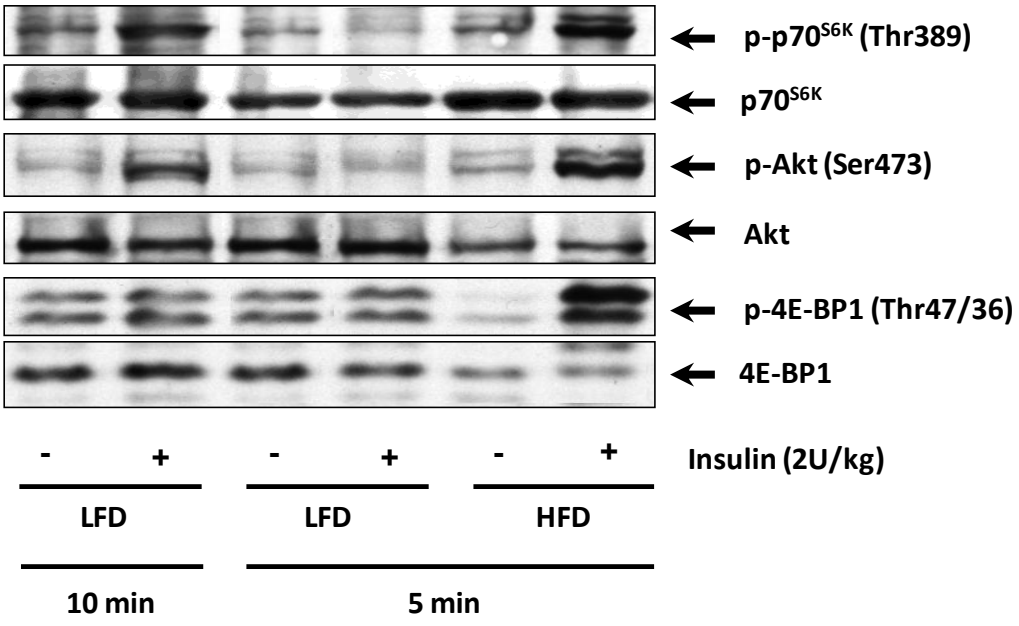


Figure 14: Immunoblot of Mammary Tumor Lysates from HER2/Neu Mice on a High-Fat Diet

Comparison of insulin stimulated phosphorylation in mammary tumor lysates, of the insulin signaling proteins $p70^{S6K}$, Akt/PKB, and 4E-BP1 in homogenized tumor lysates at 5 and 10 minutes after acute insulin injection in LFD mice versus only a 5 minute injection in HFD fed mice as shown by the representative blots.

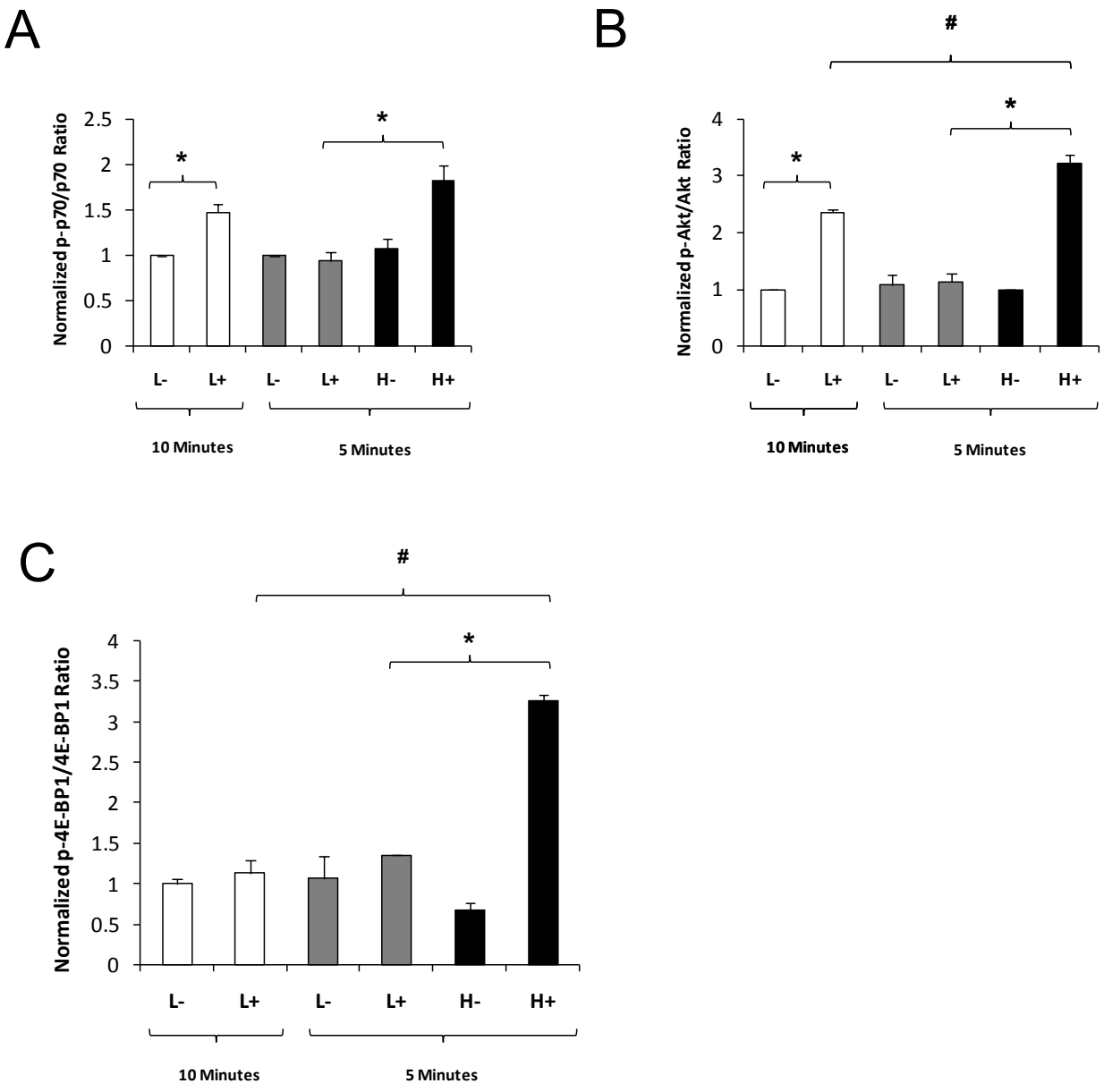


Figure 15: Densitometry of Immunoblots of Mammary Tumor Lysates from HER2/Neu Mice on a High-Fat Diet

(A) Normalized p-p70/p70 ratio in LFD fed mice at 5 and 10 minutes after acute insulin injection, and HFD fed mice 5 minutes after an acute insulin injection. This was generated from three independent experiments. *p<0.05 between LFD and HFD after a 5 minute acute insulin injection. **(B)** Normalized p-Akt/Akt ratio in LFD fed mice at 5 and 10 minutes after acute insulin injection, and HFD fed mice 5 minutes after an acute insulin injection. This was generated from three independent experiments. *p<0.05 between LFD and HFD after a 5 minute acute insulin injection. #p<0.05 between the LFD with a 10 minute insulin stimulation and the HFD after a 5 minute insulin stimulation. **(C)** Normalized p-4E-BP1/4E-BP1 ratio in LFD fed mice at 5 and 10 minutes after acute insulin injection, and HFD fed mice 5 minutes after an acute insulin injection. This was generated from three independent experiments. *p<0.05 between LFD and HFD after a 5 minute acute insulin injection. #p<0.05 between the LFD with a 10 minute insulin stimulation and the HFD after a 5 minute insulin stimulation. (L=LFD and H=HFD).

(LFD (5 min insulin) 1.35 ± 0.01 vs. HFD (5 min insulin) 3.26 ± 0.09, $p<0.05$) and (LFD (10 min insulin) 1.13 ± 0.17 vs. HFD (5 min insulin) 3.26 ± 0.09, $p<0.05$) **(Figure 15c)**.

2.3.7 *Immunoblots of IRS-1 and IRS-2 in tumor tissue*

The fact that IRS proteins are hyperphosphorylated in breast cancer [78] and their high levels signify a poor prognosis [227], prompted us to examine the tyrosine phosphorylation of IRS-1 and IRS-2 in tumors from HFD and LFD treated mice. Immunoprecipitation with IRS-1 and IRS-2 antibodies followed by anti-pY immunoblotting, showed similarly low levels of basal Tyr phosphorylation of IRS-1 and IRS-2 from mammary tumors of both LFD and HFD mice, and after 5 min of acute insulin stimulation, a pattern similar to Akt/PKB and $p70^{S6K}$ was observed **(Figure 16a)**. There was a significant increase in Tyr phosphorylation in both diet groups which was again, slightly greater in the HFD group for IRS-1 (IRS-1: LFD (5 min insulin) 1.91 ± 0.1 vs. HFD (5 min insulin 2.82 ± 0.2) **(Figure 16b)**, but identical in the two groups for IRS-2 (IRS-2: LFD (5 min insulin) 5.55 ± 0.3 vs. HFD (5 min insulin) 5.77 ± 0.2) **(Figure 16c)**.

2.3.8 Immunostaining of the proliferative marker Ki67

The lack of differences in basal and maximal insulin stimulation of growth signalling proteins, circulating insulin concentrations and tumor growth rates in the two diet groups all suggest that the high-fat diet did not affect tumor cell proliferation. To better understand this, we studied Ki67; a marker of cellular proliferation. Positively stained cells were quantified using Aperio Image Scope software and expressed as the Ki67 Labeling Index. There were no statistical differences in the percentage of positively stained nuclei in tumors from the LFD treated group and the HFD treated group of mice (LFD 5.55 ± 1.31 %vs. HFD 5.77 ± 0.95 %) **(Figure 17a & 17b).**

2.4 Discussion

In this study, a mild obesity phenotype was generated in the HER2/Neu mouse model of breast cancer without evidence of significant hyperinsulinemia or insulin resistance. It is important to note that these HER2/Neu mice are bred on a FVB background. Although some studies have documented that insulin resistance develops on a 45% kcal fat diet, other

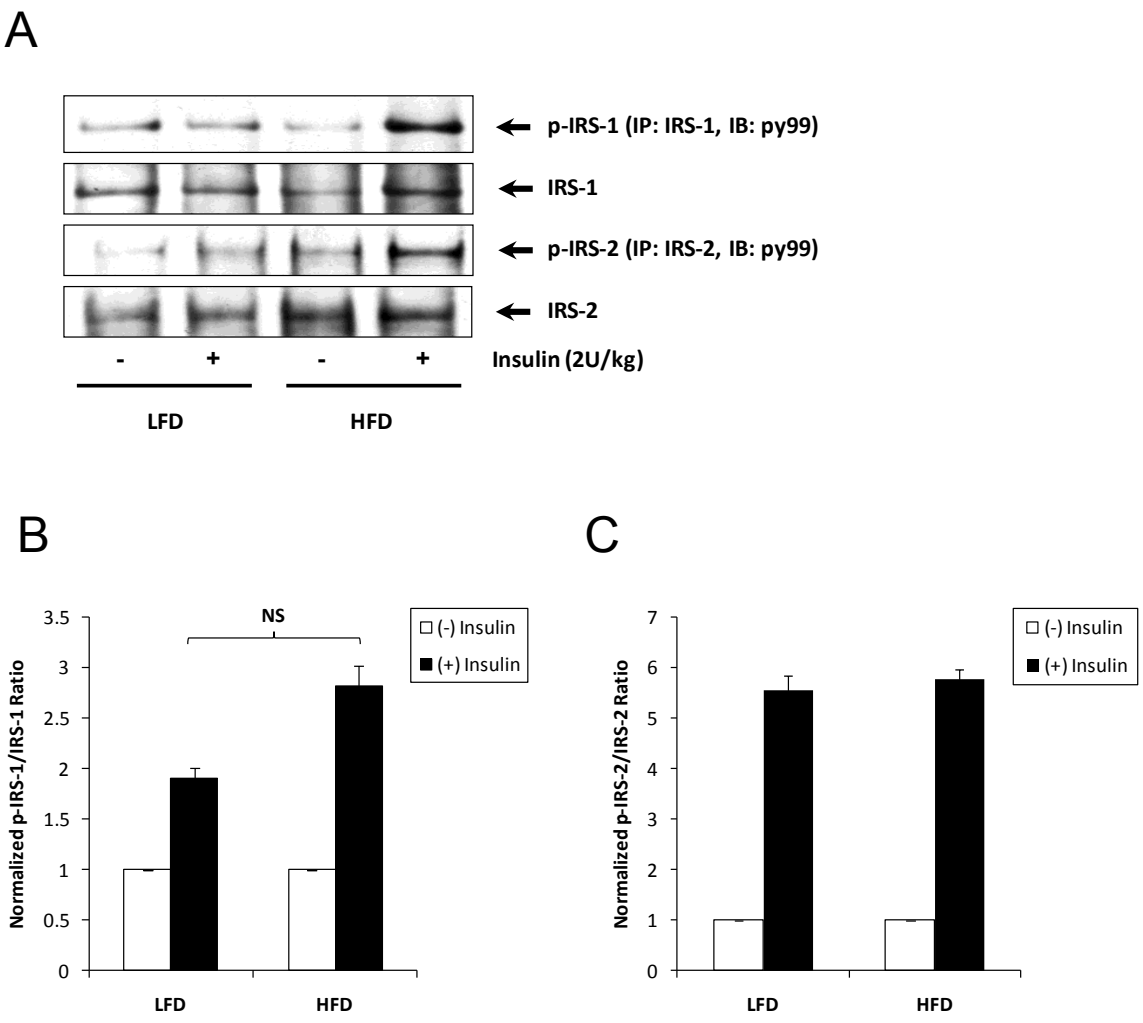


Figure 16: Immunoprecipitation of Mammary Tumor Lysates from HER2/Neu Mice on a High-Fat Diet

5 minute insulin stimulated IRS-1 and IRS-2 tyrosine phosphorylation in homogenized mammary tumor lysates from both LFD and HFD fed mice. Samples were immunoprecipitated with IRS-1 or IRS-2 antibodies coupled to protein A/G, and prepared for immunoblot analysis using antibodies against phosphorylated tyrosine (PY-99), total IRS-1 and IRS-2. **(A)** A representative blot from each experiment is shown. **(B) (C)** The densitometric graphs were generated from three independent experiments from each group.

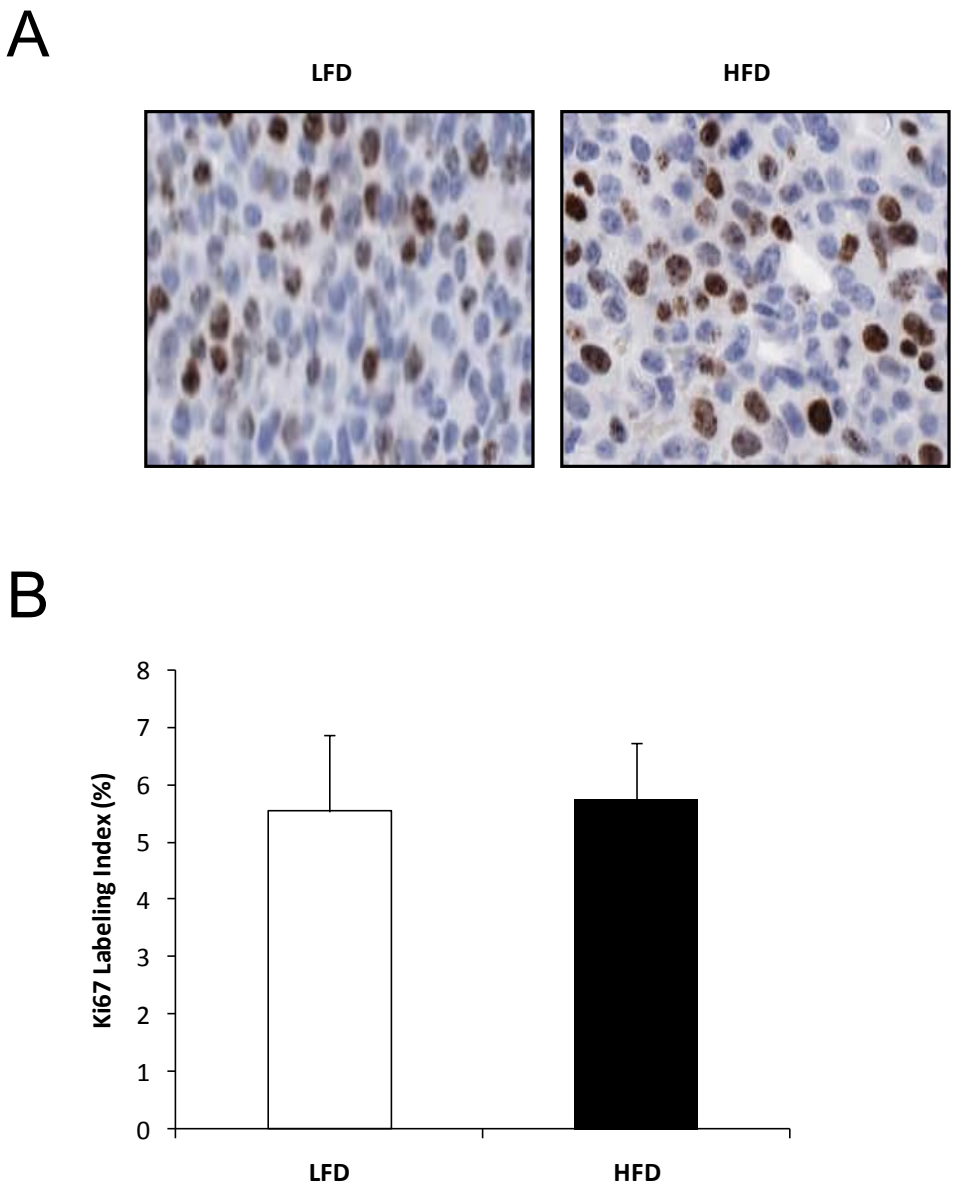


Figure 17: Immunohistochemistry Staining for the Proliferative Marker Ki67 in Mammary Tumors from HER2/Neu Mice on a High-Fat Diet

(A) Formalin fixed tumor sections were stained with an antibody for the proliferative marker Ki67 (20X magnification) between LFD and HFD. **(B)** Representation of the percentage of Ki67 expression in tumor sections from LFD and HFD fed mice (LFD n=7) and (HFD n=7).

investigators have found that a higher fat content (65% kcal) is required for the insulin resistance to develop in the FVB/N strain [Kahn CR, Personal Communication]. Previous obesity studies using this FVB/N strain demonstrated that it is somewhat obesity-resistant when fed a calorie dense diet as shown by the lack of an increase in body weight or energy expenditure when on this diet [228].

Thus, when fed the high-fat diet, we found that the HER2/Neu mice had an increased body weight and body fat content overall, and an impairment in glucose tolerance, but there was no elevation in plasma insulin concentrations. We determined insulin concentrations at 10 min post-glucose administration in an attempt to detect small differences, since early and mild insulin resistance may be accompanied by glucose stimulated hyperinsulinemia, which is not apparent in the basal state. Since there may be variability in the timing of peak insulin levels after glucose administration in the mice, we cannot completely exclude the possibility that a small increase could have occurred at 30 or 60 minutes post-glucose. At the same time, it is interesting to note the mild impairment of glucose tolerance we observed. Combined with the lack of hyperinsulinemia and the absence of insulin resistance in the skeletal muscle, the data suggest a potential impairment of insulin secretion induced by the HFD.

With respect to the development and growth of mammary gland tumors, the latency period of first tumor formation was not altered by the HFD. However, the latency for the appearance of a second tumor was decreased by the HFD. The growth rates of the mammary tumors were not altered by the HFD and since we had three different measures, namely the absolute growth rate/day and relative growth rate (% of initial size/day) and the time of detection to a pre-determined endpoint of 1.5 cm, the accelerated occurrence of a second tumor did not appear to be due to a more rapid growth of small undetectable tumors. This was confirmed by a lack of evidence for an increase in cellular proliferation in tumors between the LFD and HFD fed mice, as seen by Ki67 staining. The decreased latency observed in the occurrence of a second tumor was accompanied by a 2-fold increase in the average number of tumors per mouse in the HFD fed mice relative to the controls.

These data suggest that it is the initiation of tumor formation that is altered by the high-fat diet, rather than an alteration in tumor growth and proliferation, in other words, a

"tumor promoting effect". Tumor promotion is defined as the phenomenon whereby the development of a cancer can be promoted by factors that do not directly alter the cellular DNA [229]. So the tumor promoter which is not mutagenic can increase the frequency of tumor formation in tissues previously exposed to a mutagen or tumor initiator. For example, phorbol esters, which are diacylglycerol mimetics which can activate protein kinase C (PKC) and activate part of the phosphoinositide signaling pathway eventually leading to a stimulation of cellular proliferation, are well established tumor promoters. Another important mechanism to consider for carcinoma progression is the loss of epithelial features and the transition into mesenchymal cells which have characteristics and invasive properties of tumor cells. The first step in the epithelial mesenchymal transition (EMT) usually involves a loss of cell-cell contact by a functional inactivation of the cell adhesion molecule E-cadherin. It has been previously shown that Akt/PKB activation can regulate various cellular mechanisms including EMT. Julien et al. demonstrated that the activation of the transcription factor Snail lead to the repression of E-cadherin and this was dependant on an Akt/PKB-mediated nuclear factor-kappa B (NF-kappaB) activation, and that NF-kappaB induces Snail expression [230]. It is not clear why the effect of our high fat diet was more apparent in the case of second tumor formation and not observed for the first tumor formation. One possibility is that this effect of high-fat diet is time-dependent. The diets were initiated at 4 weeks of age in these mice, which was 32 weeks before half the HFD fed mice developed the first tumor, and 37 weeks before half of the LFD fed mice developed a second tumor. However, half of the HFD fed mice developed a second tumor only one week after the first tumor. This suggests that there may be a critical window of time when the HFD exerts its effect. Other possibilities which could account for these data is that the genetically predisposed breast cancer model we used causes the HFD effect to be relatively weak in comparison to the HER2/Neu over-expression, and that the tumor promoting mechanisms may require several weeks or even months to be established. Further experiments are required to sort out these factors.

Women in westernized countries who consume a high-fat, calorie rich diet and are relatively sedentary, have an earlier onset of puberty [231]. In addition, onset of menarche before the age of 13 is associated with an increased risk of breast cancer [231, 232]. A number of studies have also demonstrated that the earlier the onset of menarche, the earlier the onset of breast development as a result of the increase in serum estradiol levels which can

then persist into early adulthood [233, 234]. We instituted the HFD at 4 weeks of age in these female mice, which is right when they can become fertile (which is typically 4-6 weeks of age). Our HFD was given at a time that was considered childhood for these mice, and the resulting trend we observed correlates to the observations seen in human subjects [235]. However, since HER2/Neu associated breast cancer appear to be estrogen unresponsive, and this mechanism is unlikely to have contributed to these findings.

It is widely agreed that the western diet with its high saturated fatty acid content and high n-6/n-3 ratio of polyunsaturated fatty acids favours the manifestation of hyperinsulinemia in individuals who are genetically predisposed [236]. Obesity is a risk factor for postmenopausal breast cancer and is associated with shortened latency and/or increased mammary tumor incidence in animals. To study the effects of HFD and obesity, Cleary et al. used two mouse models of breast cancer. In MMTV-TGF-α mice with diet-induced obesity the latency of mammary tumors was significantly shortened [221]. In contrast, when the same protocol was tested in the MMTV-HER2/Neu breast cancer model, mammary tumor latency, incidence, metastasis and burden were similar for all groups [220]. They concluded that obesity is not a risk factor for development of ER negative breast cancer i.e. in the HER2/Neu model. This was based on the concept that hyperinsulinemia in the obese and high-fat diet fed state stimulates mammary carcinogenesis indirectly, by elevating circulating levels of estrogen via insulin binding and inhibiting SHBG [237-239] However, in human subjects and other rodent models, the elevated risk associated with hyperinsulinemia was observed for both ER+ and ER- tumors.

In addition, there are some important points to note in the protocol and interpretation of the data of Cleary et al. [220]. In the MMTV-HER2/Neu study, the HFD fed mice were divided into three groups at an early (19 weeks) age based on body weight. These were classified as obesity-resistant, overweight and obesity-prone. While age at tumor detection, i.e. latency was not significantly decreased, a trend was observed and it is not clear whether this sub-grouping obscured a significant difference. Thus mean tumor detection age of chow fed mice was 67.7 ± 4.9 weeks and those of the three HFD fed mice were 63.8 ± 6.7 weeks, 61.7 ± 3.5 weeks and 64.8 ± 5.9 weeks, all earlier. Similarly, the age at which mice were euthanized when tumors reached 20 mm in size was 80.1 ± 2.6 weeks for chow fed and 75.7

± 4.1 weeks, 72.7 ± 3.2 weeks and 69.4 ± 4.9 weeks for HFD fed groups, again all earlier. The incidence of mammary tumors showed a similar trend, chow fed 45% and 59%, 56% and 67% in the three HFD groups [220]. The number of tumors/mouse were not different, but as opposed to the calculation in the present study in which this is expressed for the entire population of mice, Cleary et al. used a denominator restricted to mice with at least one tumor, eliminating those in which tumors were not detected. This explains the discrepancy in tumor incidence reported which was greater in the HFD group. While these trends were not as marked as those observed in the MMTV-TGF-α model, it is also noteworthy that the latter model was bred on the C57BL/6 background [221], while the MMTV-HER2/Neu was on the FVB background, similar to ours [220]. In addition, the mice used by Cleary et al. were heterozygous for the transgene while the mice we used were homozygotes. Finally, the HFD was 32.5% fat while that used in our study was 45%. Taken together, our interpretation is that HFD does have an effect on latency and tumor development, but the magnitude depends on various factors, such as, the model used, the background strain and the proportion of fat in the diet.

One other possible influence is the fatty acid composition of the HFD. At least in cell culture models, the saturated fatty acid (FA), palmitic acid, induces apoptosis, while the monounsaturated FA, oleic acid, can stimulate proliferation [240]. However, studies in women have not supported a significant effect [241]. This may be because the difference in dietary fatty acid composition are not large enough in human subjects to observe small effects. The fatty acid composition of the diet used by Cleary et al. was largely palmitic acid (milk fat; approximately 20% of the 32.5%, in other words, 61% of the fat), while our HFD was 86.7% lard oil. It is not known whether these FA would have variable effects on tumor promotion.

In this study, assessment of insulin signaling in the skeletal muscle confirmed the absence of an overt insulin resistant state in these mice fed a HFD. However, interestingly, we observed an accelerated insulin response in tumors from HFD fed mice. Thus, 5 min after insulin injection, IRS-1 Tyr phosphorylation as well as Akt/PKB, p70S6K, and 4E-BP1 phosphorylation were all consistently increased. However, after 10 min the responses were similar. Although this may seem counterintuitive, in the early stages of obesity associated

with increased nutrient intake, prior to the development of insulin resistance, an accelerated response to insulin has been previously described [226]. The exact mechanism of this "priming" has not been identified, nor is it known whether this phenomenon contributes in any way to tumorigenesis.

Insulin resistance and hyperinsulinemia have been postulated to contribute to tumor formation and growth via increased levels of estrogen and/or IGF-1. However, in the absence of hyperinsulinemia, it is unlikely that these factors would have been affected and contributed to our observations. Indeed, Cleary et al. found that even in the obesity-resistant MMTV-TGF-α HFD fed mice, the HFD decreased tumor latency without altering body weight [221]. The body fat content however, was modestly increased. Furthermore the same diet on the MMTV-HER2/Neu model did not cause any change in IGF-1 level and these tumors do not express the ER. Thus, our data indicating the absence of significant insulin resistance in skeletal muscle and the absence of hyperinsulinemia combined with the results reported by Cleary et al. support the concept that HFD manifests a tumor promoting effect independent of significant obesity and insulin resistance.

Increases in adipose tissue mass, even in the presence of similar total body weight may be associated with alterations in the synthesis and secretion of various "adipokines". Two circulating factors which have been suggested to influence breast cancer are the adipokines leptin and adiponectin. While leptin stimulates breast cancer cell growth *in vitro* [242], the results of associations with increased risk in human subjects are mixed and lose significance when corrected for other factors [144].

The other adipokine is adiponectin, which is decreased in obesity and insulin resistance [243]. Recent clinical studies have shown that low serum levels of adiponectin are associated with increased risk of breast cancer in postmenopausal women [244]. Wang et al. demonstrated that the inhibitory role of adiponectin on MDA-MB-231 cell growth might be attributed to its suppressive effects on the GSK-3β/β-catenin signaling pathway [245]. Their *in vivo* studies showed that both supplementation of recombinant adiponectin and adenovirus-mediated over-expression of this adipokine substantially reduced the mammary tumorigenesis of MDA-MB-231 cells in female nude mice. These data support a potential

role of adiponectin as a negative regulator of breast cancer growth [246]. Due to inadequate volume of blood, we did not measure circulating adiponectin and so cannot rule out a potential contribution to these results.

Finally, tumor cells are known to exhibit an altered metabolism characterized by increased glucose uptake, elevated glycolysis and increased de novo fatty acid synthesis [247-250]. The enzyme fatty acid synthase (FASN) is up-regulated [251] and inhibition or knockdown by siRNA has been demonstrated to inhibit BC growth [251]. Of relevance, addition of palmitic acid was shown to circumvent the down-regulation of FASN and overcome the defect in FA synthesis. These findings raise the hypothesis that in some circumstances the dietary supply of FA may influence breast cancer growth and/or development. This could explain a degree of independence in some effects from obesity and insulin resistance.

In summary, these data show that feeding a HFD to breast cancer-prone mice promote tumor development in an apparent time-dependent manner independent of overt hyperinsulinemia and significant insulin resistance. However, tumor growth in this mild obesity phenotype is not affected. The precise mechanism of shortened tumor latency may involve an effect of increased adipose tissue mass, e.g. altered cytokines, or an effect of increased dietary FA content. Further experiments are required to define the contribution of these factors.

CHAPTER 3:

Effect of High-Fat Diet with a Fructose Supplementation and Treatment with Metformin on HER2/Neu Mammary Carcinogenesis

3.1 Introduction

In the first component of our study, we wanted to test the hypothesis that higher circulating levels of insulin caused by obesity-induced insulin resistance have an adverse effect on breast cancer growth and development. Although we observed a tumor-promoting effect of a high-fat diet (45% kcal) in the HER2/Neu rodent model we used (Chapter 2), we could not conclude that it was a result of an insulin effect since we did not observe changes in plasma insulin concentrations between the LFD and HFD fed mice. The FVB background strain of this transgenic mouse tends to elicit a mild obesity phenotype when fed a high-fat diet that consists of 45% kcal from fat alone. Because we wanted to study the insulin effect as part of our original hypothesis, we decided to follow the same study design as before, but changed the high-fat diet to one that consisted of 45% kcal from fat, that was supplemented with 15% fructose [Woo M, Personal Communication]**.** In this component of the study, by ensuring that the circulating levels of insulin would be increased with the administration of this particular diet, we wanted to also test the effect that metformin would have in lowering the insulin levels to a point comparable to the controls, and then observing any outcomes this might have on the development and progression of breast cancer.

The epidemiological association of metformin use with a lower risk of cancer mortality in people with Type 2 diabetes compared with sulphonylureas and insulin treatment suggests the potential that metformin use may lower the risk of cancer development and/or growth. The exact mechanism by which metformin treatment may cause these remains uncertain. Metformin is classically considered to function as an insulin sensitizer that promotes a reduction of circulating levels of insulin and glucose in patients that exhibit hyperglycemia and hyperinsulinemia [252, 253]. As mentioned previously, the ability of metformin to activate AMPK through the activation of its up-stream kinase LKB1 has been suggested as a possible insulin-independent mechanism by which the cancer cells can be targeted. AMPK has been shown to directly inhibit mTOR [209, 210]. In fact, several studies

have shown that AMPK can suppress mTOR signaling that has been induced by amino acids and growth factors either by directly or indirectly acting on TSC2 [254-256].

Beyond the reported effects of metformin on the classic insulin-responsive tissues, not much is known about its effects on other cell types, including cancer cells. It has been hypothesized that the inhibition of tumorigenesis by metformin and the cellular responses to AMPK activation may in fact depend upon the cellular status of various tumor suppressor genes such as p53 and LKB1. AMPK has been shown to directly phosphorylate p53 at the Ser15 site which results in its stabilization [257]. In addition, conditions of nutrient deficiency causes stabilization of p53, which can in turn induce autophagy in the cell [258]. Recent *in vivo* work by Buzzai et al. to evaluate the potential anti-neoplastic capabilities of metformin has demonstrated that metformin selectively inhibited p53-null colorectal tumor growth, but the effect was not seen in the wild-type p53 tumor cells [259]. The explanation for this observation is that when metformin inhibits ATP synthesis and activates AMPK, it stimulates a change in metabolic pathways that requires active p53, and it has been suggested that in the presence of this tumor suppressor, p53, metformin may promote malignant cell survival, and is therefore more effective in the treatment of cancers that are p53-deficient. However, other studies into the importance of p53 with regards to the effects of metformin on tumorigenesis suggest that metformin acts independently of p53 status. Zhuang et al. recently showed that metformin inhibited proliferation of most breast cancer cell lines and it did so independent of the estrogen receptor, HER2 or p53 status [260]. Another recent study looked at the effect of metformin on the growth of human cancer cells, specifically the Lewis Lung LLC1 carcinoma transplanted in C57BL/6 mice that were given a control diet or a high-energy diet which caused an increase in body weight and hyperinsulinemia, demonstrated that metformin significantly reduced the effect of the high-energy diet on tumor growth [261] . The mice that were given the control diet did not show any change in the tumor growth rate with metformin treatment. This study suggested that metformin reduced the increased IR activation by this high-energy diet, and there was indeed activation of AMPK.

It is important to note, taking these findings into consideration, that the presence or absence of certain molecular markers may be causing this differential response to metformin

as an anti-cancer therapy. To further support this point are the findings by the hallmark study of Anisimov et al. who first reported that systemic treatment with metformin increased the lifespan of the same rodent model of breast cancer we used in our study, the MMTV-HER2/Neu [262, 263]. There was an apparent delay in the development of spontaneous breast tumor formation, and the tumors that appeared in the metformin treated group were smaller in size relative to the controls. In this study, both groups were fed a regular chow diet. The authors concluded that metformin did not influence the incidence of the mammary tumors in these animals, however, there was a decrease in tumor multiplicity and an increase in tumor latency with metformin treatment. This suggests the possibility that metformin may play a role in early tumor initiation events. Anisimov et al. went on to study the effect of metformin on lifespan in a different model, the outbred female SHR rodent model [264]. Metformin treatment in these particular mice significantly increased the mean life span as well as the maximum life span. However, it did not decrease the incidence of spontaneous tumor formation, suggesting that the HER2 protein itself may in fact be a key target of metformin during its action on breast cancer.

In order to elucidate this point further, a recent *in vitro* study using human MCF-7 and SKBR3 breast cancer cells were used to examine the role the HER2 oncoprotein plays in metformin action [265]. The authors found that ectopic over-expression of HER2 significantly enhanced metformin-induced breast cancer cell growth inhibition, and metformin treatment also down-regulated HER2 protein levels, by up to 85% in both a dose and time-dependent manner. This study suggests that the metformin-induced suppression of HER2 over-expression occurs through an AMPK-independent inhibition of $p70^{S6K}$, and that the presence or absence of these hallmarks, such as HER2 and $p70^{S6K}$ hyperactivation, may be a reason why metformin-based treatment of early breast cancers can elicit alternative responses. These results are in agreement with recent findings that metformin can exert an AMPK-independent anti-tumoral effect in prostate cancer cells [266]. In this particular study, the authors found that metformin caused a strong dose-dependent inhibition of cellular proliferation in all prostate cancer cell lines, blocking the cell cycle by means of a significant decrease in cyclin D1 protein levels [266]. In addition, this was confirmed *in vivo* when metformin was administered i.p. into nude mice, or orally through the drinking water. What was shown was that there was a tumor growth inhibition of 35% with the i.p. animals and

55% in mice given metformin in the drinking water [266]. The data are consistent with and confirm the results of Zakikhani et al. in which AMPK activation by metformin was observed in epithelial cells and a reduction in cellular proliferation and protein synthesis was also seen [210]. In the MCF-7 cells, metformin was shown to be a growth inhibitor rather than an insulin sensitizer [210].

Another important mode of action by which metformin may exert its anti-cancer effect is by the regulation of fatty acid synthesis. Many cancer cells, including breast cancer demonstrate a marked increase in de novo fatty acid synthesis as a result of an increased expression of FASN [267]. It has been shown previously that high levels of FASN are associated with the malignant phenotype seen in various cancers, including ovarian cancer, as well as breast cancer [268], and reduction of FASN can cause a reduction in cellular proliferation and induce death through apoptosis [268]. One particular *in vitro* study using a number of different human breast cancer cell lines demonstrated a correlation between FASN expression and HER2 over-expression, and that at the transcriptional level, when FASN was inhibited, there was a repression of HER2 [269]. As mentioned previously, when metformin leads to activation of AMPK, it leads to a decrease in the gene expression of FASN, and an inactivation of ACC [190]. In prostate cancer cells, activation of AMPK and a consequent reduction of FASN and ACC resulted in a decrease in cellular proliferation [270].

There exists much supporting evidence for the potential beneficial role that metformin may have as an anti-cancer therapy, however, the exact mechanisms by which this is occurring still remains to be elucidated. The evidence that insulin influences breast carcinogenesis is extremely important because it raises the possibility of adopting insulin reduction for therapeutic and chemopreventative purposes in breast cancer. Therefore, there exists the need to explore this potential anti-cancer activity as well as determining the exact therapeutic avenue by which it can be used.

3.1.1 Hypothesis

We decided to test the effect of a new high-fat diet formulation in the same transgenic HER2/Neu over-expressing mouse to see if the insulin resistant state can be induced. If so, we hypothesize that the increased levels of insulin will accelerate tumor formation and/or

growth of breast cancer in this model. In addition, we will treat a subset of these mice on their respective diets with the anti-diabetic drug metformin, and we expect that metformin will improve insulin sensitivity in the high-fat diet treated mice, and decrease the breast cancer growth to that which is observed in the mice fed a low-fat diet.

3.2 Materials and Methods

3.2.1 Reagents

Antibodies for phospho-AMPK (Thr172), AMPK, phospho-ACC (Ser79), and ACC were obtained from Cell Signaling Technology Inc. (Beverly, MA). The anti-rabbit and anti-mouse IgG secondary antibodies all came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Metformin (1,1-dimethylbiguanide hydrochloride) was from MP Biomedicals (Solon, OH). Insulin enzyme-linked immunosorbent assay (ELISA) kits were from Linco Research Inc. (St. Charles, MO). The biotinylated rabbit anti-rat IgG and the Elite ABC-horse radish peroxidase came from Vector Laboratories (Burlingame, CA). The Ki67 antibody and the Dako Cytomation Fluorescent Mounting Medium was obtained from Dako (Glostrup, Denmark). The anti-phospho-FOXO1A antibody was obtained from Abcam Inc. (Cambridge, MA), and the FOXO1A antibody was from ABR-Affinity Bioreagents (Golden, CO). The cy-3 congugated goat anti-rabbit IgG was from Zymed Laboratories Inc. (San Francisco, CA). All remaining reagents and protocols that were used were similar to what was described in Chapter 2.

3.2.2 New high-fat diet formulation

Female FVB/N HER2/Neu mice at the age of 8 weeks were randomly placed on a low-fat diet (LFD) which consisted of 5% kcal fat, and a high-fat diet which consisted of 45% kcal fat that was supplemented with 15% fructose (which will now be referred to as HFF), and both came from Harlan Teklad (Madison, WI) **(Table 2)**. Diets were stable for six months and stored at 4^0C. Mice were allowed *ad libitum* access to food and water throughout the treatment period.

HFD (45% kcal Fat + 15% Fructose)	% kcal From	Detailed Composition	g/kg	% of Total Fat	
Protein	20	Casein DL-Methionine	240 3.6	N/A	
Carbohydrate	35	Fructose Sucrose	303 101	N/A	
Fat	45	Soybean Oil Coconut Oil, hydrogenated	142.5 80	Saturated	45
				Monounsaturated	15
				Polyunsaturated	40
Total	100				100

Table 2: Composition and Detailed Breakdown of HFF

3.2.3 Metformin treatment

Beginning at 10 weeks of age, that is, two weeks after the respective diet treatments were instituted, a subset of these female HER2/Neu mice were treated with metformin through the drinking water at a dose of 300 mg/kg body mass/day. There was no statistically significant difference in the mL of water consumed between the metformin treated groups versus the non-metformin treated groups. A stock solution of the metformin treated water was stored covered at 4^0C, and was prepared fresh every 14 days. The water given to the mice from this stock solution was changed every 3 days.

3.2.4 Study design

We performed this component of the study in two separate parts, with similar experimental designs. The first set of animals was obtained in the year 2006 and initially consisted of LFD (n=24) and HFF (n=32), and within these groups, we treated a subset of mice with metformin as follows: LFD + metformin treatment (n=8) and HFF + metformin treatment (n=12). This group of animals became our first cohort. In the year 2007, another set of animals was obtained and the experimental design was followed to what was done for the first cohort. This new set became our second cohort of animals. This time, we had n-values as follows: LFD (n=16) and HFF (n=24), and within these groups, we treated a subset of mice with metformin as follows: LFD + metformin treatment (n=8) and HFF + metformin treatment (n=12). With respect to the statistical analysis and survival curves, the data were

analyzed as separate cohorts, and then analyzed as pooled data. When both cohorts were combined, the *n*-values were as follows: LFD (*n*=24), HFF (*n*=32), LFD + metformin (*n*=16), and HFF + metformin (*n*=24).

3.2.5 Intra-Peritoneal Insulin Tolerance Test

Insulin tolerance tests (ITTs) were performed on mice fasted for 6-h using an insulin load of 2 U insulin/kg body mass per injection, and were injected intra-peritoneally (i.p.). Blood glucose measurements were made at 0, 10, 20, 30, 60, 90, and 120 minutes after the injection using a Sure Step, One Touch Glucometer from Lifescan Inc. (Milpitas, CA).

3.2.6 Immunofluorescence and confocal microscopy

Formalin-fixed paraffin-embedded tissue sections of breast tumors were cut at 4 μm, de-waxed in 3 changes of xylene and rehydrated through graded alcohols. After blocking in 10% goat serum, tissue sections were incubated with anti-phospho-FOXO1A (Ser256) antibody or with FOXO1A antibody at a 1:100 dilution at 4^0C overnight and subsequently washed with (PBS). After incubation with cy-3-conjugated goat anti-rabbit IgG for 1-h, sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and mounted in Dako Cytomation Fluorescent Mounting Medium. Fluorescent images were visualized by Confocal laser scanning microscopy, and image analysis was performed using a Zeiss LSM-510 imaging system, Apochromat × 63 objective lens (Carl Zeiss, Oberkochen, Germany). The percentage of p-FOXO1A and FOXO1A expression was calculated with Image-Pro Analyzer software on sections with approximately 1000 cells per section. For each experimental condition and treatment group, three animals were used with three sections per mouse.

3.2.7 Statistical Analysis

All results are expressed as mean ± SEM where applicable. Statistical significance was assessed by two-tailed Student's *t* test or ANOVA. In addition, Kaplan-Meier survival estimates were calculated for the time to the development of the first tumor, as well as second tumor in the various treatment groups (i.e. LFD, HFF, LFD + metformin and HFF + metformin). Analysis was done in terms of median survival times and Log-rank tests were

performed to compare the survival curves of different experimental groups and determine if there is a significant difference between them. Clinically, the median survival is the number of days at which half the mice develop the event of interest, so at this point, half the *n*-value has a longer and half a shorter survival time. Mice which died from non-study related causes (i.e. due to unknown circumstances before the experimental endpoint) had the data censored at the time of death. These statistical calculations were performed using Statistica software v. 7.0 (Statistical Analysis System, Cary, NC). A $p<0.05$ was considered statistically significant in all data analysis.

3.3 Results

3.3.1 Body weights and body fat content

To investigate the influence of diet on body weight, the HER2/Neu mice were fed with high fat diet plus fructose (HFF: 45% kcal from fat + 15% fructose supplement) or low fat diet (LFD: 5% kcal from fat). The diets were instituted at 8 weeks of age and were well tolerated. There were no statistically significant differences in grams of food consumed per day between the treatment groups. HER2/Neu mice showed a significant increase in body weight after 30 weeks on diet (LFD 25.1 ± 0.3 g vs. HFF 33.8 ± 1.0 g, $p<0.01$) **(Figure 18a)**. In addition, the subset of mice that were treated with metformin also showed a significant increase in body weight after 30 weeks on diet (LFD + metformin 25.4 ± 0.9 g vs. HFF + metformin 30.2 ± 1.0 g, $p<0.01$) **(Figure 18a)**. In addition, there was a slight decrease in body weight of the HFF + metformin treated mice compared to the HFF mice alone, however, this difference was not statistically significant **(Figure 18a)**. To determine whether the presence of HFF altered the body fat content of the mice, MRI analysis was performed after 37 weeks on diet. There was a marked increase in percent of body fat in HFF mice compare to LFD mice at this time point (LFD 11.0 ± 0.016 % vs. HFF 32.9 ± 0.019 %, $p<0.01$) and (LFD + metformin 8.6 ± 0.013 % vs. HFF + metformin 25.5 ± 0.014 %, $p<0.01$) **(Figure 18b)**. Although there was a trend to lower fat content in the percent body fat between the LFD mice and the LFD + metformin treated mice, however, there was a significant reduction in the percent of body fat present in the HFF + metformin treated mice

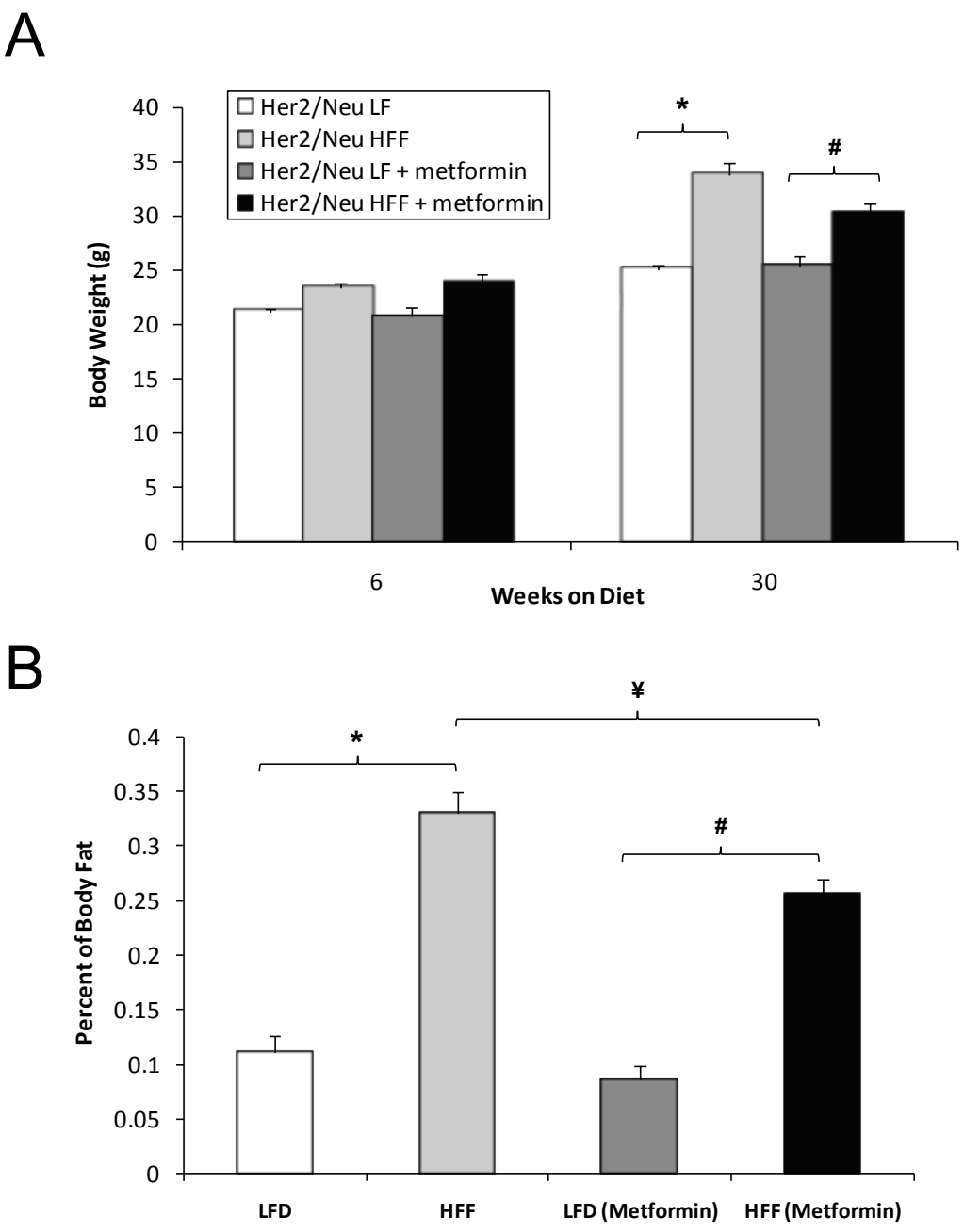


Figure 18: Body Weight and MRI Analysis of HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

(A) Body weights of mice maintained on a low-fat diet (LFD n=16), and on a high-fat fructose diet (HFF n=24), and on a LFD with metformin treatment (LFD + metformin n=8), and on a HFF with metformin treatment (HFF + metformin n=12) for 30 weeks. *p<0.01 between LFD and HFF. #p<0.01 between LFD + metformin and HFF + metformin. **(B)** Body fat content as assessed by MRI in mice maintained on (LFD n=16), (HFF n=24), (LFD + metformin n=8) and (HFF + metformin n=12) after 37 weeks on the respective diets. *p<0.01 between LFD and HFF. #p<0.01 between LFD + metformin and HFF + metformin. ¥p<0.05 between HFF and HFF + metformin.

relative to the untreated HFF mice (HFF 32.9 ± 0.019 % vs. HFF + metformin 25.5 ± 0.014 %, $p<0.05$) **(Figure 18b)**.

3.3.2 Glucose tolerance and plasma insulin concentrations

To investigate the metabolic consequences of the high fat fructose diet, intra-peritoneal glucose tolerance tests (i.p. GTTs) were performed after 30 weeks on diet. There was no change in fasting blood glucose levels of the HFF fed HER2/Neu mice both with and without metformin treatment relative to the LFD mice. However, there was an impairment of glucose tolerance in the HFF mice compared to the LFD with significantly increased blood glucose values after 20, 30, 60, 90 and 120 min of glucose loading ($p<0.05$) **(Figure 19a)**. In addition, there was a significant improvement in glucose tolerance in the HFF + metformin treated mice relative to the HFF mice alone at the 20, 30, 60, 90 and 120 min post-glucose administration ($p<0.05$) **(Figure 19a)**. As expected, there was no effect of metformin on the glucose tolerance in the LFD treated subset relative to the LFD group alone since it exhibited a normal i.p. GTT profile **(Figure 19a)**. To explore dietary effects on insulin levels, plasma insulin levels from each of the four treatment groups, at 30 weeks on diet, at both the basal state and 10 min post-glucose administration were assayed using an ELISA kit. There was a significant increase in plasma insulin concentration at both the basal (LFD 9.25 ± 0.44 nmol/L vs. HFF 11.57 ± 0.98 nmol/L, $p<0.05$) and 10 min post-glucose administration (LFD 9.37 ± 0.67 nmol/L vs. HFF 12.37 ± 0.66 nmol/L, $p<0.05$) in the HER2/Neu mice that were not treated with metformin **(Figure 19b)**. In the subset that was treated with metformin, there was a significant increase in insulin concentration between the LFD and HFF at the basal state (LFD + metformin 6.25 ± 0.89 nmol/L vs. HFF + metformin 7.98 ± 0.32 nmol/L, $p<0.05$), however, there was no difference between plasma insulin concentrations in the LFD and HFF mice which were treated with metformin at the 10 min post-glucose administration (LFD + metformin 7.71 ± 0.99 nmol/L vs. HFF + metformin 8.10 ± 0.76 nmol/L) **(Figure 19b)**. More importantly, at the basal state in both the LFD and HFF mice, there was a significant reduction of plasma insulin concentration upon treatment with metformin (LFD 9.25 ± 0.44 nmol/L vs. LFD + metformin 6.25 ± 0.89 nmol/L, $p<0.05$) and (HFF 11.57 ± 0.98 nmol/L vs. HFF + metformin 7.98 ± 0.32 nmol/L, $p<0.05$), and a significant reduction of plasma insulin concentrations at 10 min post-glucose administration in the HFF mice with

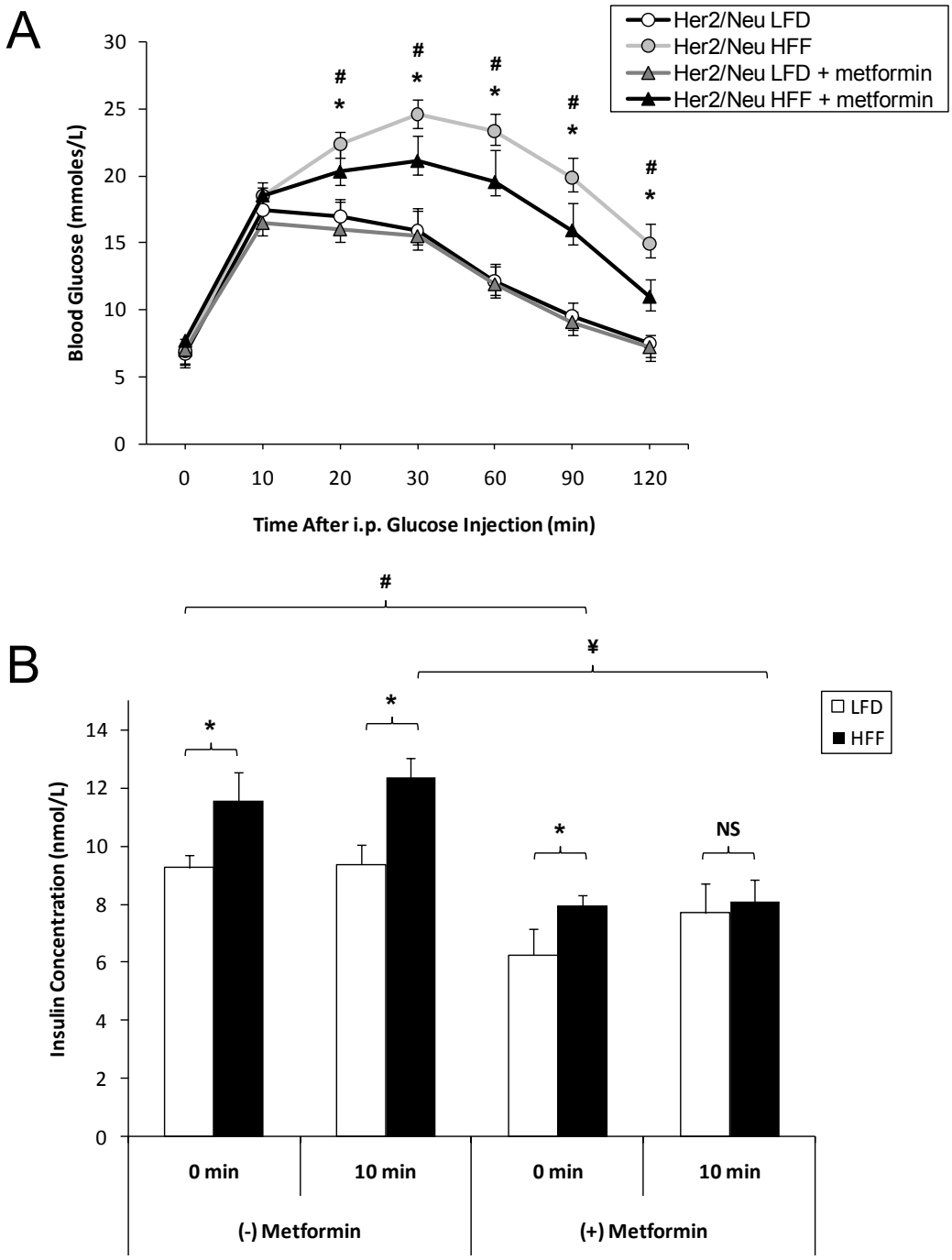


Figure 19: Intra-peritoneal Glucose Tolerance Test and Insulin Concentrations of HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

(A) Intra-peritoneal glucose tolerance test (i.p. GTT) after 30 weeks on diet. Following an overnight fast, glucose (1.5g/kg body weight) was administered by i.p. injection, and tail vein blood was withdrawn at 0, 10, 20, 30, 60, 90 and 120 minutes post-glucose load, and blood glucose was measured, (LFD n=16), (HFF n=24)., (LFD + metformin n=8) and (HFF + metformin n=12). *p<0.05 between LFD and HFF. #p<0.05 between HFF and HFF + metformin. **(B)** Insulin concentrations measured at 0 and 10 minutes post-glucose load after 30 weeks on diet, using a mouse insulin ELISA kit, (LFD n=16), (HFF n=24), (LFD + metformin n=8) and (HFF + metformin n=12). *p<0.05 between LFD and HFF. #p<0.05 between LFD and LFD + metformin and between HFF and HFF + metformin at the basal state. ¥ p<0.05 between HFF and HFF + metformin at 10 minutes post-glucose load.

metformin relative to the HFF mice alone (HFF 12.37 ± 0.66 nmol/L vs. HFF + metformin 8.10 ± 0.76 nmol/L, $p<0.05$) **(Figure 19b)**.

3.3.3 Insulin sensitivity

To further confirm the presence of insulin resistance suggested by the elevated levels of circulating plasma insulin, intra-peritoneal insulin tolerance tests (i.p. ITTs) were performed after 40 weeks on diet. There was a marked impairment of insulin sensitivity of the HFF treated mice relative to the LFD treated mice at the 20, 30, 60, 90 and 120 min after insulin administration ($p<0.05$) **(Figure 20)**. In addition, there was no significant difference seen between the LFD mice and the LFD + metformin treated subset, nor were there any significant differences seen in the insulin sensitivity between the LFD + metformin treated mice and the HFF + metformin treated mice **(Figure 20)**. However, there was a significant improvement of insulin sensitivity in the HFF mice upon treatment with metformin relative to the HFF untreated mice indicated by lower glucose levels which were noted, at the 20, 60 and 90 min time points after insulin administration ($p<0.05$) **(Figure 20)**.

3.3.4 Growth rate and average number of tumors

To assess any differences in growth rates of tumors in all four treatment groups, tumor growth rates, in absolute growth (LFD 25.8 ± 5.4 mm^3/day, HFF 38.5 ± 8.3 mm^3/day, LFD + metformin 34.5 ± 8.2 mm^3/day, and HFF + metformin 28.7 ± 6.6 mm^3/day) and percent daily relative growth (LFD 56.6 ± 14.5 %/day, HFF 66.8 ± 15.1 %/day, LFD + metformin 47.9 ± 7.6 %/day, and HFF + metformin 37.7 ± 5.6 %/day) were calculated, and there were no statistically significant differences in either the absolute or percent relative growth rates among these tumors **(Figure 21a & 21b)**. The rate of tumor progression was also calculated. This is the number of days between the first tumor measurement and the last tumor measurement (which is determined by the sacrifice cut-off point of 1.5 cm), and then this is averaged for each treatment group. Although there was a trend towards slower tumor progression induced by metformin in both groups, there were no significant differences among any of the four treatment groups with respect to this parameter of tumor progression (LFD 54 ± 6.61 days, HFF 49 ± 5.47 days, LFD + metformin 58 ± 5.28 days, and HFF + metformin 55 ± 5.01 days) **(Figure 22a)**. In addition, the average number of tumors that

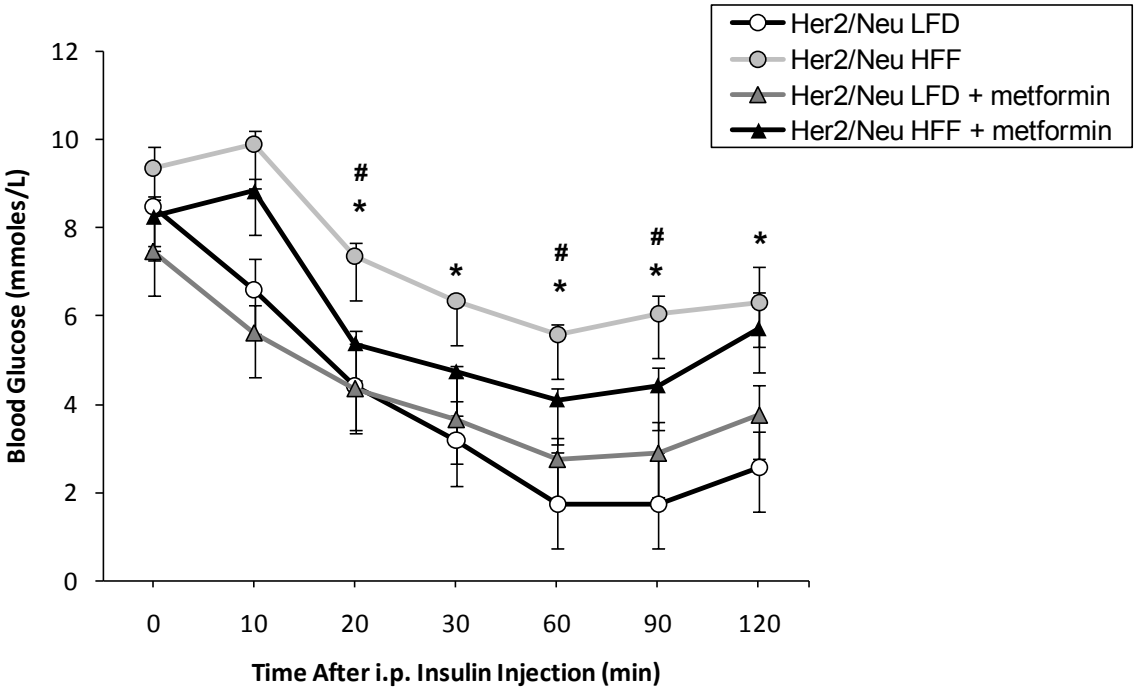
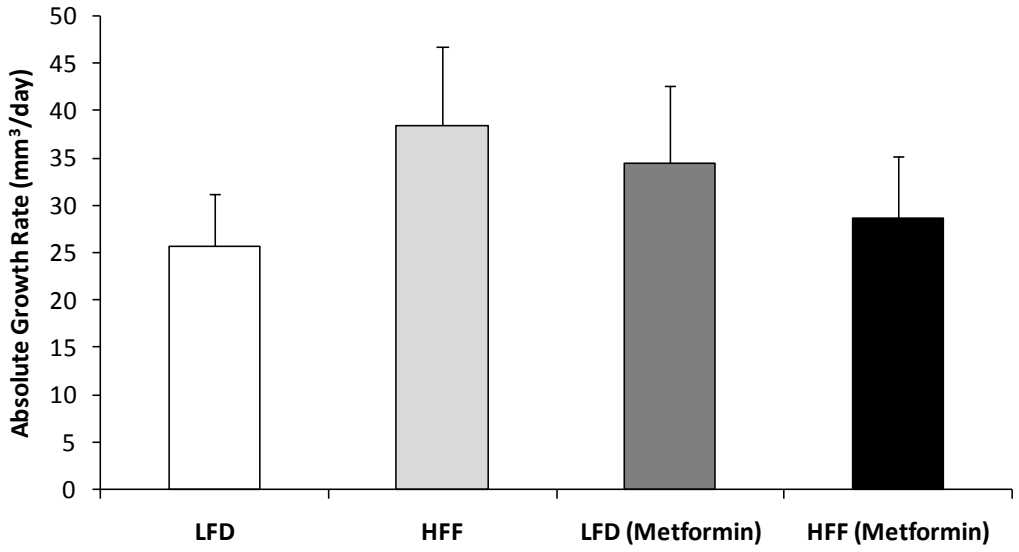


Figure 20: Intra-peritoneal Insulin Tolerance Test in HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

Intra-peritoneal insulin tolerance test (i.p. ITT) after 40 weeks on diet. Following a 6-h fast, insulin (2U insulin/kg body weight) was administered by i.p. injection, and tail vein blood was withdrawn at 0, 10, 20, 30, 60, 90 and 120 minutes post-insulin, and blood glucose was measured, (LFD n=14), (HFF n=20), (LFD + metformin n=7) and (HFF + metformin n=11). *p<0.05 between LFD and HFF. #p<0.05 between HFF and HFF + metformin.

A



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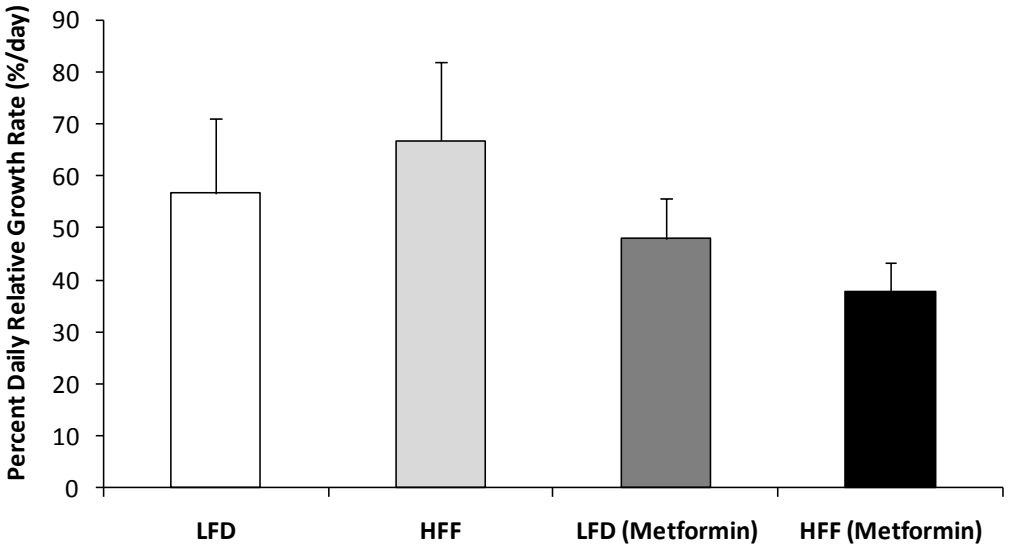
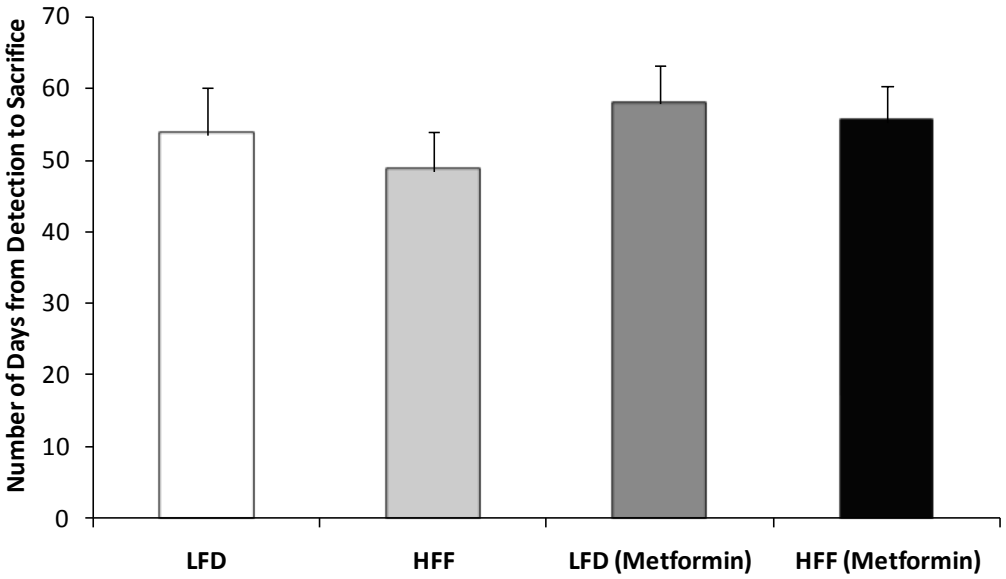


Figure 21: Absolute Growth Rate and Percent Daily Relative Growth Rate of Tumors in HER2/Neu Mice on a High-Fat Fructose Diet with Metformin

(A) Absolute growth rate of tumors (mm^3/day from tumor onset until sacrifice point). Volumes are calculated from caliper measurements taken twice a week, (LFD n=20), (HFF n=27), (LFD + metformin n=16) and (HFF + metformin n=20). **(B)** Percent daily relative growth rate (% change in volume/day). Volumes are calculated in the same manner as was done for absolute growth rate, (LFD n=20), (HFF n=27), (LFD + metformin n=16) and (HFF + metformin n=20).

A



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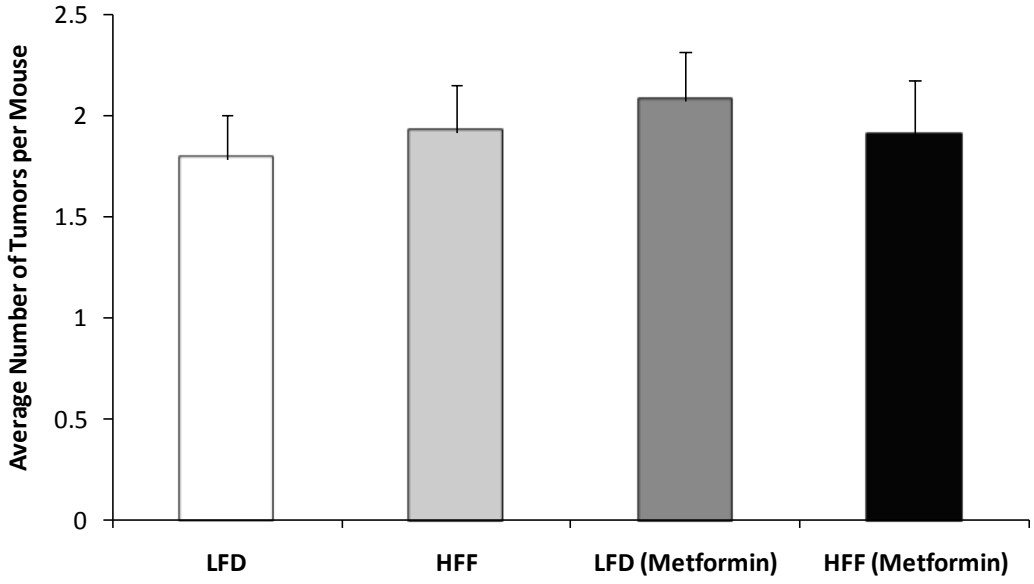


Figure 22: Rate of Tumor Progression and the Average Number of Tumors that Developed per Mouse in HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

(A) The number of days between first tumor detection and last tumor measurement (i.e. at the sacrifice end-point of 1.5 cm) was averaged and plotted for each treatment group, (LFD n=20), (HFF n=27), (LFD + metformin n=16) and (HFF + metformin n=20). **(B)** Average number of tumors per mouse, (LFD n=20), (HFF n=27), (LFD + metformin n=16) and (HFF + metformin n=20).

develop in these mice in the various treatment groups was assessed, and there was also no significant difference seen in the multiplicity of tumors among any of the four treatment groups (LFD 1.8 ± 0.21, HFF 1.9 ± 0.23, LFD + metformin 2.1 ± 0.24, and HFF + metformin 1.9 ± 0.27) **(Figure 22b)**.

3.3.5 Onset of first tumor in the first cohort

To determine whether the HFF and the treatment with metformin affected mammary gland tumor development, the mice were palpated twice weekly and tumors were measured. The age of detection of the first mammary tumor in each mouse in all four treatment groups was plotted as a Kaplan-Meier "survival analysis". In the first cohort of animals, there was no significant difference in the median time of tumor-free survival (LFD 253 days, HFF 217 days, LFD + metformin 232 days, and HFF + metformin 210 days) as assessed by Log-rank tests **(Figure 23)**.

3.3.6 Onset of first tumor in the second cohort

The same statistical analysis was performed in the second cohort of animals, and once again, the age of detection of the first mammary tumor in each mouse in all four treatment groups was plotted as a Kaplan-Meier "survival analysis". In the second cohort of animals, there was a significant difference in the median time of tumor-free survival between the LFD fed mice relative to the LFD mice that were treated with metformin. It appears that the metformin treatment in this control diet seemed to delay the onset of the first tumor (LFD 178 days vs. LFD + metformin 225 days, $p<0.05$). There were no other significant differences observed between the HFF treated animals relative to the LFD or compared to the HFF + metformin subset of mice (HFF 188 days, HFF + metformin 213 days) **(Figure 24)**.

3.3.7 Onset of first tumor in combined cohort

When all tumor onset data from each cohort was pooled together as one, the same statistical analysis was performed as before to see if there were any significant changes as the *n*-value increased. With respect to the combined cohort, there were no statistically significant differences in the median time of tumor-free survival (LFD 194 days, HFF 216 days, LFD +

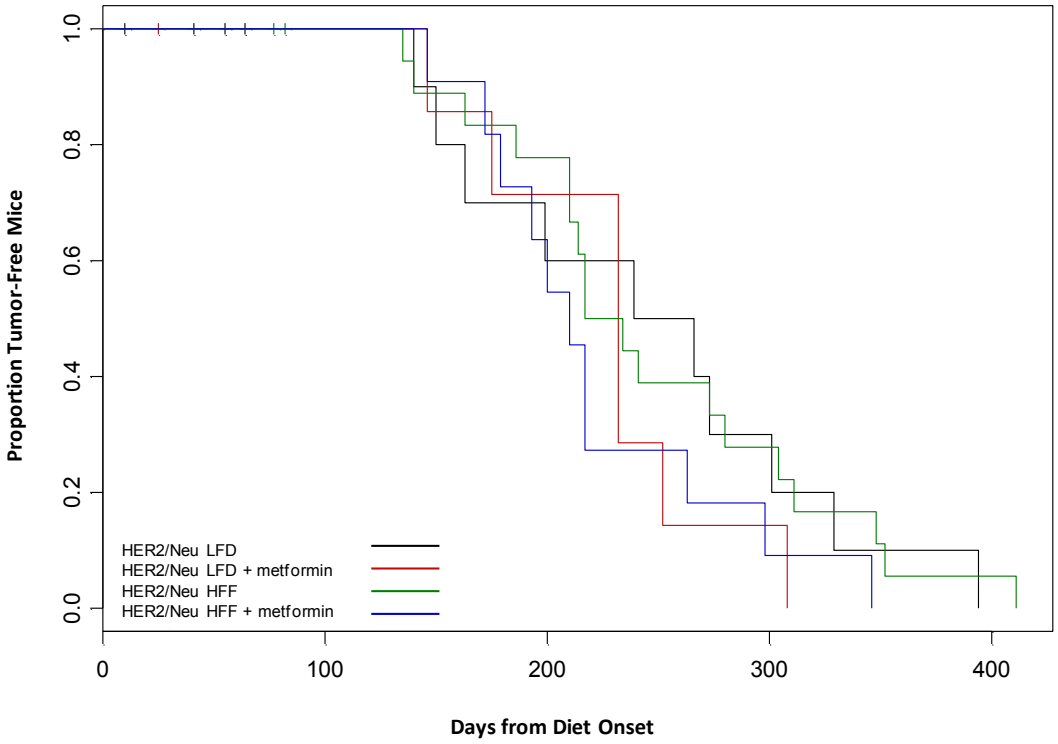


Figure 23: Survival Analysis of First Tumor Onset of HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment, in the First Cohort

Kaplan-Meier survival analysis of the percent of mice which are tumor-free (i.e. have no tumors at all) versus the number of days that the mice were on their respective diets, (LFD n=15), (HFF n=20), (LFD + metformin n=8) and (HFF + metformin n=11).

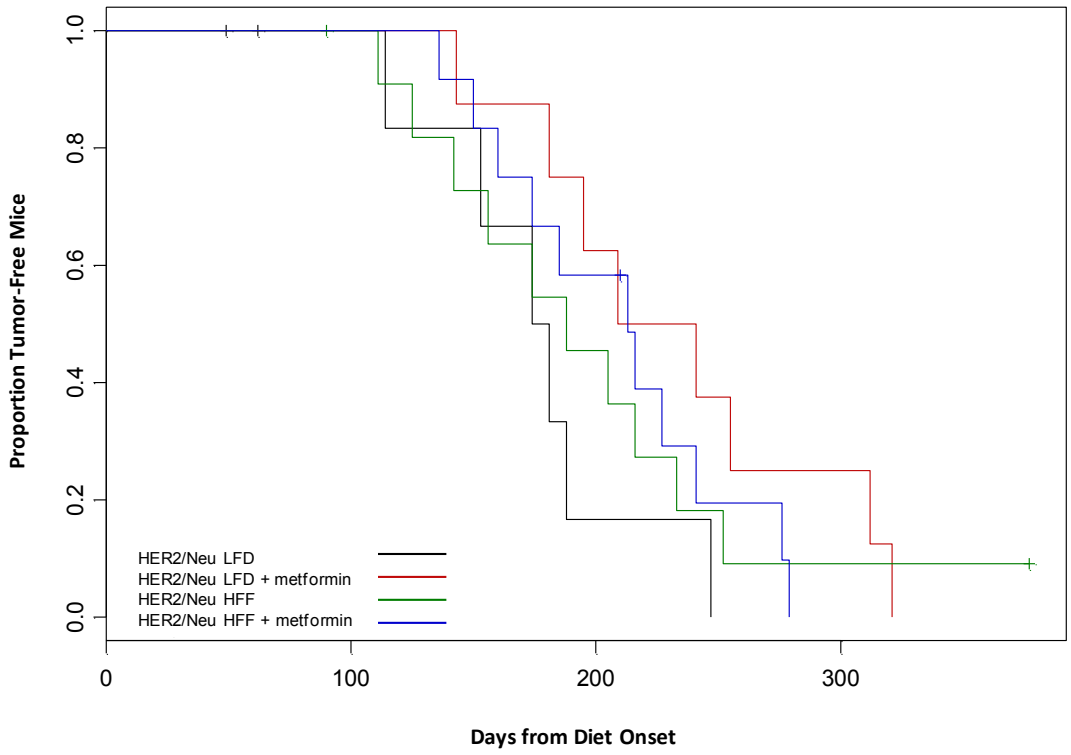


Figure 24: Survival Analysis of First Tumor Onset of HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment, in the Second Cohort

Kaplan-Meier survival analysis of the percent of mice which are tumor-free (i.e. have no tumors at all) versus the number of days that the mice were on their respective diets, (LFD n=8), (HFF n=12), (LFD + metformin n=8) and (HFF + metformin n=12). *$p<0.05$ for the median survival time between LFD and LFD + metformin.

metformin 232 days, and HFF + metformin 213 days) as assessed by Log-rank tests **(Figure 25)**.

3.3.8 Onset of second tumor in first, second and combined cohort

In addition to investigating any differences in tumor latency for the first tumor, we also examined the onset of the second tumor formation. The latency period for the appearance of a second tumor showed no significant differences between any of the four treatment groups in either the first or second cohort, or when all data was pooled into one combined cohort. The median time for two tumor-free survival is as follows for each cohort; first cohort: (LFD 301 days, HFF 303 days, LFD + metformin 300 days, and HFF + metformin 270 days); second cohort (LFD 266 days, HFF 261 days, LFD + metformin 250 days, and HFF + metformin 279 days); and combined cohort: (LFD 266 days, HFF 301 days, LFD + metformin 265 days, and HFF + metformin 270 days).

3.3.9 Immunoblots of Akt/PKB in skeletal muscle and tumor tissue

We investigated if the HFF diet as well as the treatment with metformin influenced signaling pathways in both the mammary tumors as well as a classic metabolic target tissue, namely skeletal muscle. First, we looked at the phosphorylation of Akt/PKB in the skeletal muscle in both the LFD and HFF mice without and with metformin treatment, in the basal state and after 10 min acute insulin stimulation. In the HFF mice relative to the LFD, the basal state showed no differences in the phosphorylation of Akt/PKB. However, 10 min post insulin stimulation, there was a reduced phosphorylation of Akt/PKB in the muscle from the HFF mice compared to the LFD mice (LFD 8.24 ± 0.05 vs. HFF 6.02 ± 0.71, $p<0.05$) **(Figure 26b)**. The same experimental conditions were applied to the skeletal muscle from the subset of mice that were treated with metformin. The basal state of the phosphorylation of Akt/PKB from the skeletal muscle of mice that were treated with metformin showed no significant difference between the LFD and HFF **(Figure 26b)**. However, there was an increase in the phosphorylation of Akt/PKB in the LFD treated mice 10 min after acute insulin stimulation relative to what was observed in the non-metformin treated subset (LFD 8.24 ± 0.05 vs. LFD + metformin 10.43 ± 0.46, $p<0.05$) **(Figure 26b)**. Furthermore, there was a substantial increase in the phosphorylation of Akt/PKB in the HFF mice treated with

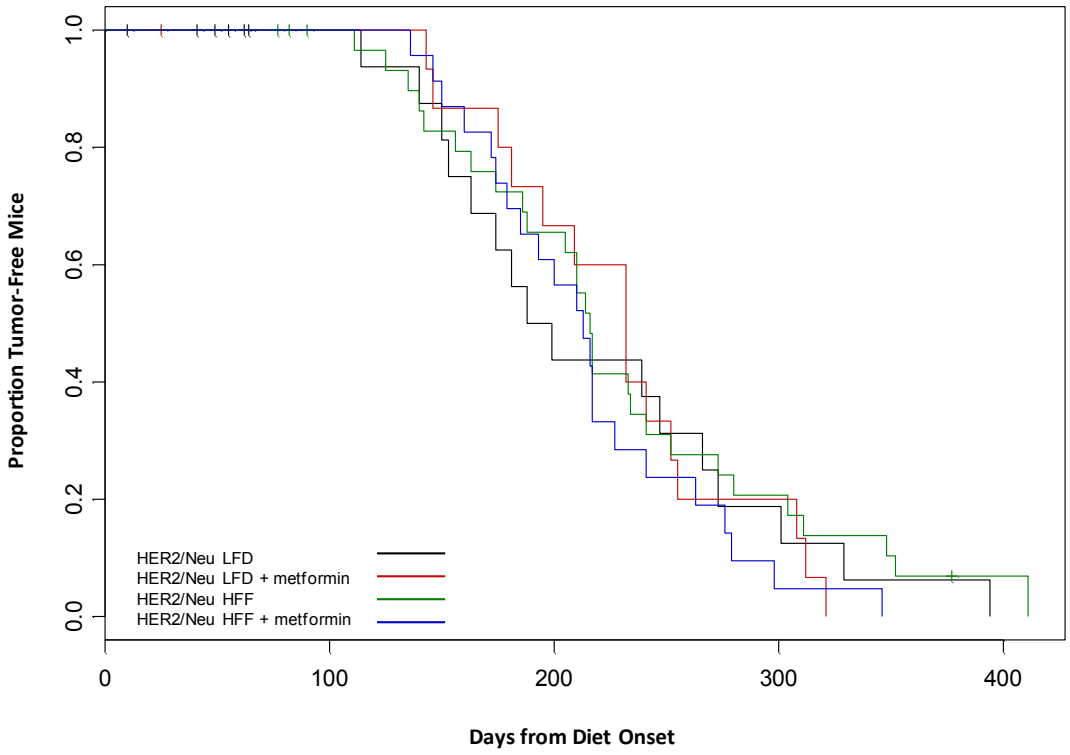


Figure 25: Survival Analysis of First Tumor Onset of HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment, in the Combined Cohort

Kaplan-Meier survival analysis of the percent of mice which are tumor-free (i.e. have no tumors at all) versus the number of days that the mice were on their respective diets, (LFD n=23), (HFF n=32), (LFD + metformin n=16) and (HFF + metformin n=23).

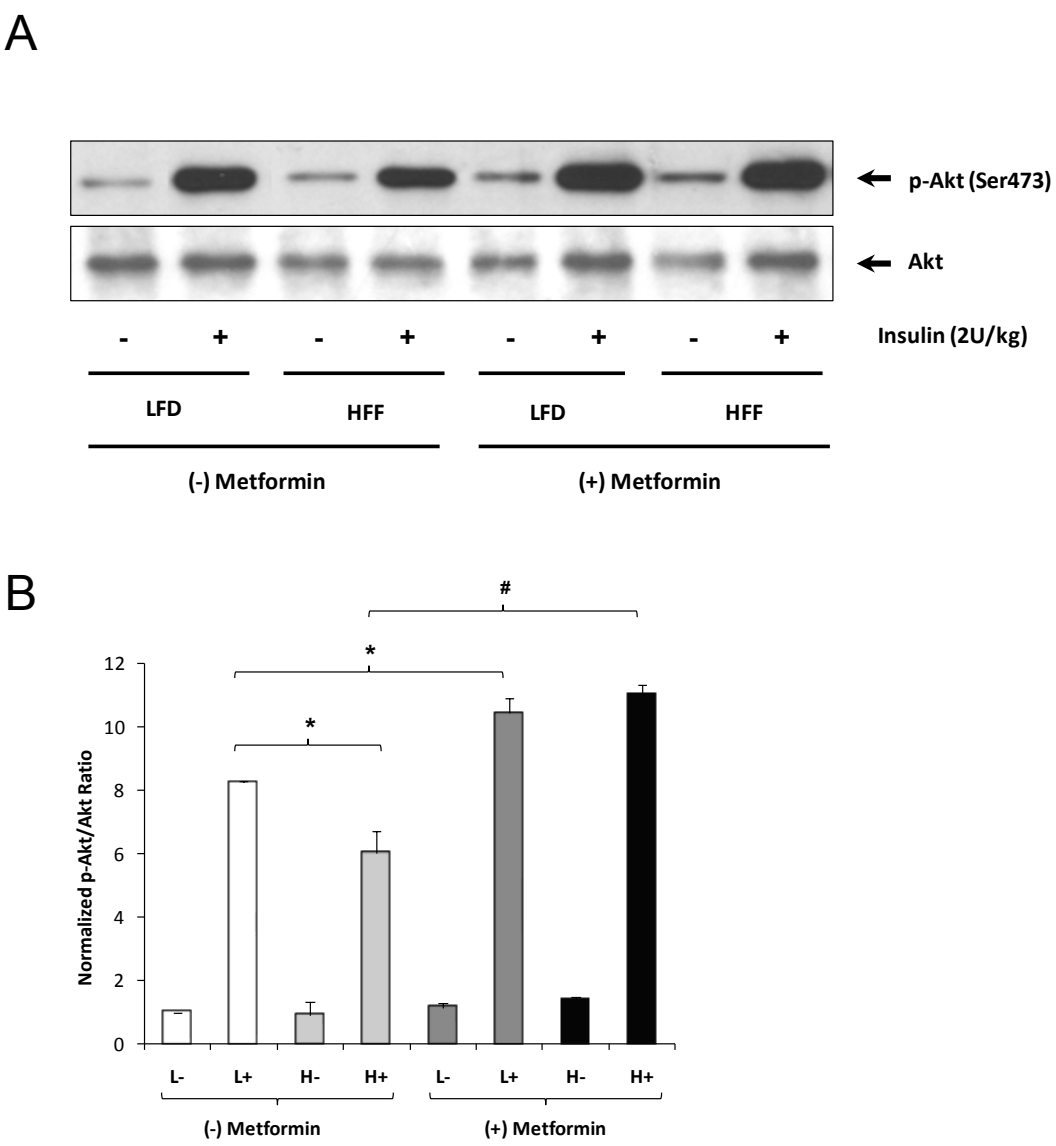


Figure 26: Immunoblot of Skeletal Muscle Lysates from HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

Comparison of insulin stimulated phosphorylation in skeletal muscle lysates, of the insulin signaling protein Akt/PKB 10 minutes after acute insulin injection in LFD and HFF fed mice, as well as in LFD and HFF fed mice that were treated with metformin as shown by **(A)** the representative blot and **(B)** the densitometric graphs were generated from three independent experiments. *$p<0.05$ between LFD and HFF 10 minutes after insulin stimulation and without metformin treatment, as well as between LFD and LFD + metformin 10 minutes after insulin stimulation. #$p<0.05$ between HFF and HFF + metformin 10 minutes after insulin stimulation. (L=LFD and H=HFF).

metformin treatment relative to the HFF fed mice alone 10 min after an acute insulin stimulation (HFF 6.02 ± 0.71 vs. HFF + metformin 11.0 ± 0.32, $p<0.05$) **(Figure 26b)**.

Next, we looked at the phosphorylation of Akt/PKB in the mammary tumors. There were no significant differences observed in the phosphorylation of Akt/PKB in the basal state between the LFD and HFF without metformin treatment and with metformin treatment. However, there was an approximate 2-fold increase in the phosphorylation of Akt/PKB in the tumor tissue in mice fed a HFF diet without metformin treatment relative to the control diet after 10 min of acute insulin stimulation (LFD 2.75 ± 0.25 vs. HFF 6.60 ± 0.09) **(Figure 27b)**. In the insulin stimulated state with metformin treatment, the mammary tumors from both the LFD and HFF mice demonstrated increased phosphorylation of Akt/PKB relative to the basal state, but showed no significant differences between each other (LFD + metformin 7.44 ± 0.25 vs. HFF + metformin 5.74 ± 0.1). As well, there was a substantial increase in the phosphorylation of Akt/PKB in the LFD treated with metformin relative to the LFD alone in the 10 min after the acute insulin stimulation (LFD 2.75 ± 0.25 vs. 7.44 ± 0.20) **(Figure 27b)**.

3.3.10 Immunoblots of AMPK in skeletal muscle and tumor tissue

It is well documented that metformin treatment can promote the phosphorylation of its target AMPK in the skeletal muscle [271]. We wanted to examine the effect that metformin had in both the skeletal muscle to demonstrate that it was in fact working, as well as in the mammary tumor to document if there were the expected changes in AMPK phosphorylation levels in this tissue type. First, we took the skeletal muscle lysates from the four treatment groups in the basal state i.e. without insulin stimulation. There were no significant differences in the phosphorylation of AMPK between the LFD and HFF without metformin treatment, as well as between the LFD + metformin and HFF + metformin subsets **(Figure 28b)**. However upon treatment with metformin, there was a 4-fold increase in the phosphorylation of AMPK in the metformin treated subsets relative to the non-metformin treated groups (LFD 1.0 ± 0 vs. LFD + metformin 4.1 ± 0.07, $p<0.05$) and (HFF 1.7 ± 0.06 vs. HFF + metformin 4.8 ± 0.35, $p<0.05$) **(Figure 28b)**.

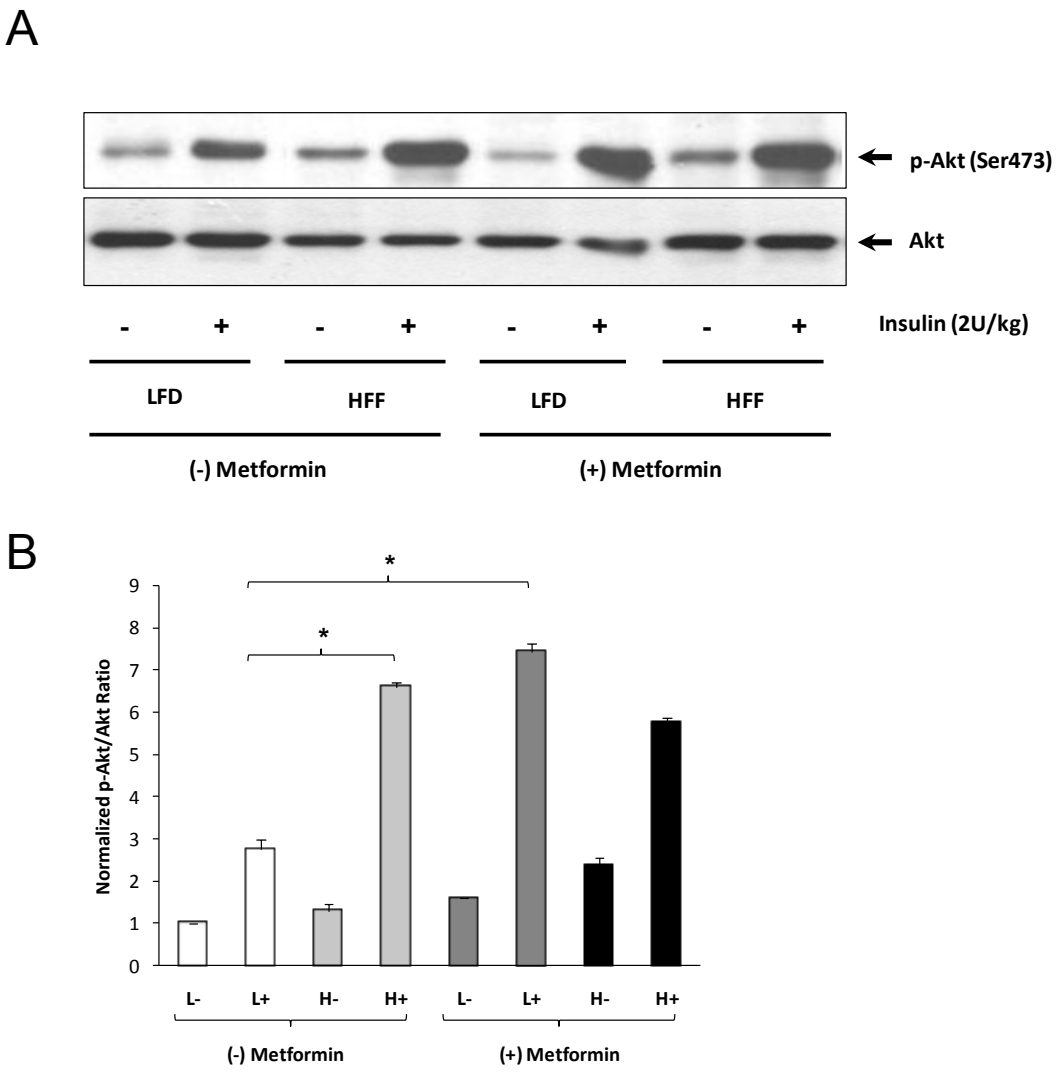


Figure 27: Immunoblot of Mammary Tumor Lysates from HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

Comparison of insulin stimulated phosphorylation in mammary tumor lysates, of the insulin signaling protein Akt/PKB 10 minutes after acute insulin injection in LFD mice versus HFF fed mice, as well as in LFD mice and HFF fed mice that were treated with metformin as shown by **(A)** the representative blot and **(B)** the densitometric graphs were generated from three independent experiments. *p<0.05 between LFD and HFF 10 minutes after insulin stimulation and without metformin treatment, as well as between LFD and LFD + metformin 10 minutes after insulin stimulation. (L=LFD and H=HFF).

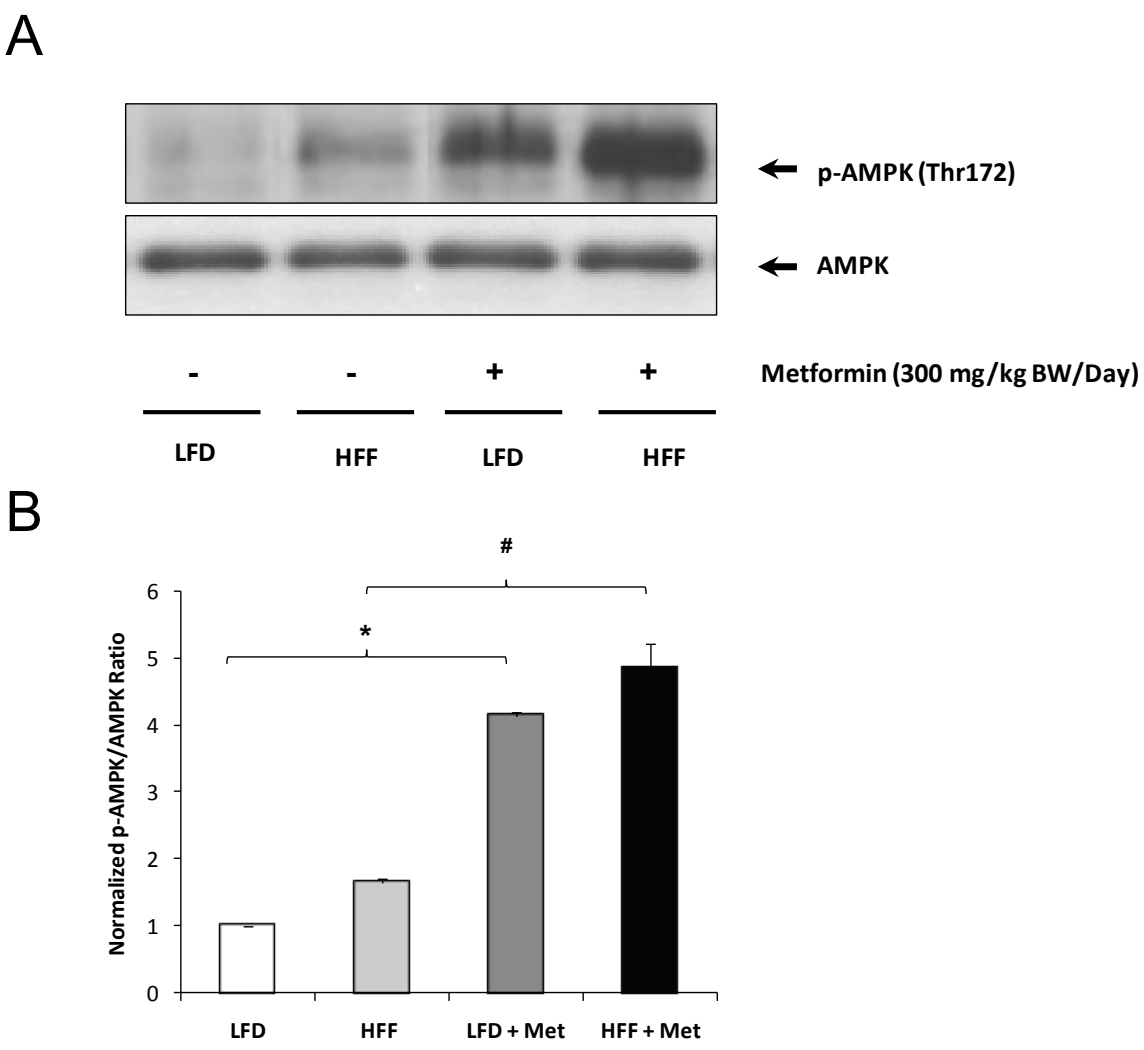


Figure 28: Immunoblot of Skeletal Muscle Lysates from HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

Comparison of AMPK phosphorylation in skeletal muscle lysates in LFD mice versus HFF fed mice, as well as in LFD mice and HFF fed mice that were treated with metformin as shown by **(A)** the representative blot and **(B)** the densitometric graphs were generated from three independent experiments. *$p<0.05$ between LFD and LFD + metformin. #$p<0.05$ between HFF and HFF + metformin.

Next, we looked at the phosphorylation of AMPK in the mammary tumors. In the four treatment groups in the basal state, we saw a similar increase in phosphorylation of AMPK in the metformin treated subset of mice, similar to that observed in skeletal muscle, and although there were no significant differences between the LFD and HFF treated group without metformin (LFD 1.0 ± 0 vs. HFF 1.2 ± 0.14) or between the LFD and HFF fed mice that were treated with metformin (LFD + metformin 2.2 ± 0.10 vs. HFF + metformin 2.4 ± 0.18) **(Figure 29b)**, there was a 2.5-fold increase in the phosphorylation of AMPK in the metformin treated subset relative to the non-metformin treated group (LFD 1.0 ± 0 vs. LFD + metformin 2.2 ± 0.10, $p<0.05$) and (HFF 1.2 ± 0.14 vs. HFF + metformin 2.4 ± 0.18, $p<0.05$) **(Figure 29b)**.

3.3.11 Immunoblots of ACC in tumor tissue

To further investigate the molecular effects of metformin in these HER2/Neu mice, we blotted for Acetyl-CoA Carboxylase (ACC) which is the rate limiting enzyme in fatty acid synthesis as mentioned previously. With AMPK activation induced by the action of metformin, ACC should become phosphorylated and thus inactivated [190]. In the mammary tumor lysates, when LFD and HFF fed mice were compared with and without metformin, there was a 4-5 fold increase in the phosphorylation of ACC in both the LFD and HFF fed mice that were treated with metformin relative to the LFD and HFF mice which were not treated (LFD 1.0 ± 0 vs. LFD + metformin 4.1 ± 0.13, $p<0.05$) and (HFF 1.5 ± 0.11 vs. HFF + metformin 4.8 ± 0.23, $p<0.05$) **(Figure 30b)**.

3.3.12 Immunofluorescence of FOXO1A in tumor tissue

In addition to investigating the phosphorylation of Akt/PKB in the mammary tumor lysates, mammary tumor sections were stained with FOXO1A as a means of assessing the signaling mechanism in more detail. Forkhead Box O1A (FOXO1A) is a transcription factor that contains a distinct forkhead domain, and is a target phosphorylated by Akt/PKB, on Ser256 [272]. Therefore, upon insulin stimulation, and subsequent activation of Akt/PKB, FOXO1A will become phosphorylated and will then translocate from the nucleus to the cytoplasm where its transcriptional activity is inhibited [272]. Thus, this is a method of further assessing the Akt/PKB activity in the mammary tumor tissue from these mice.

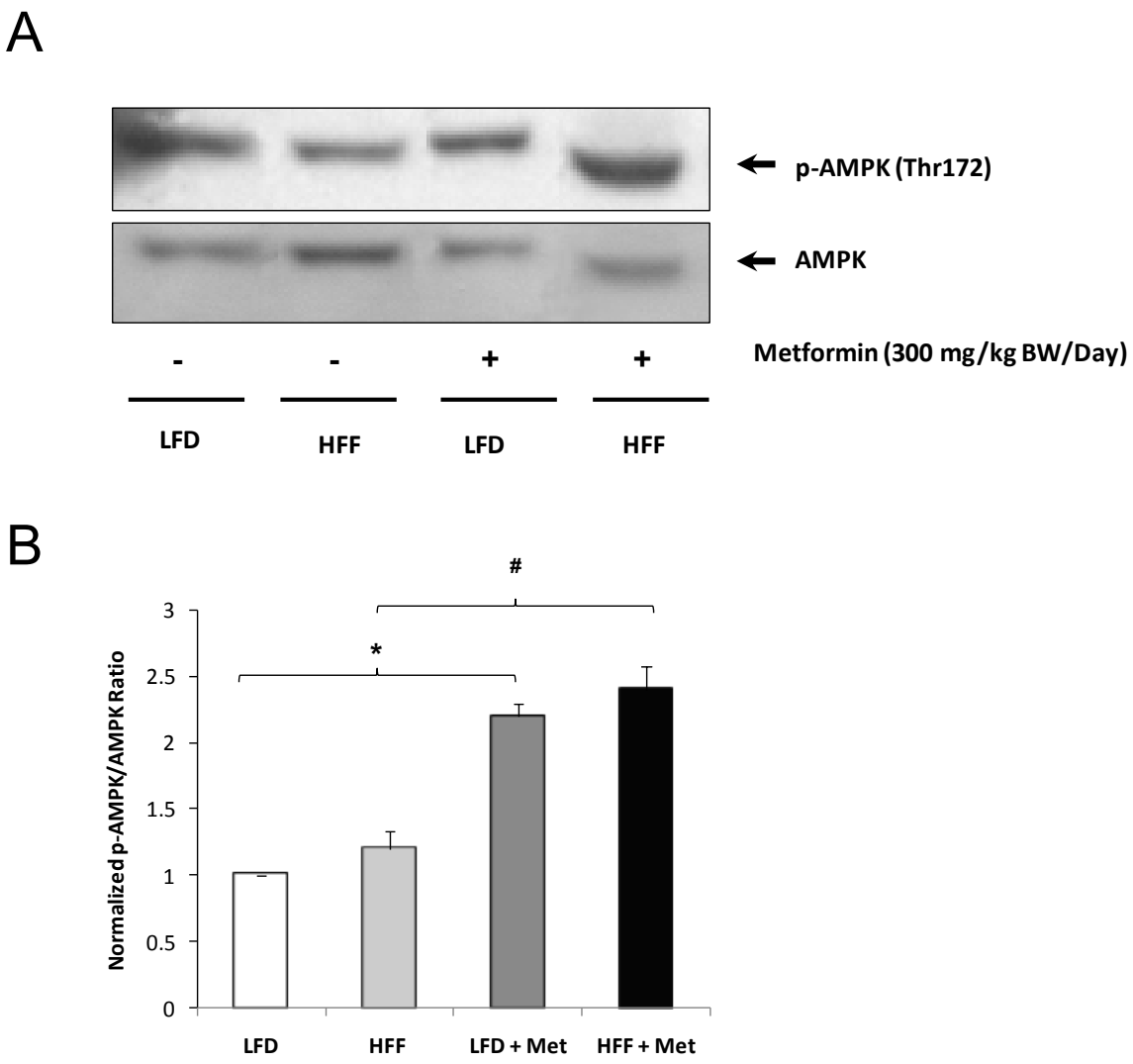


Figure 29: Immunoblot of Mammary Tumor Lysates from HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

Comparison of AMPK phosphorylation in mammary tumor lysates in LFD mice versus HFF fed mice, as well as in LFD mice and HFF fed mice that were treated with metformin as shown by **(A)** the representative blot and **(B)** the densitometric graphs were generated from three independent experiments. *$p<0.05$ between LFD and LFD + metformin. #$p<0.05$ between HFF and HFF + metformin.

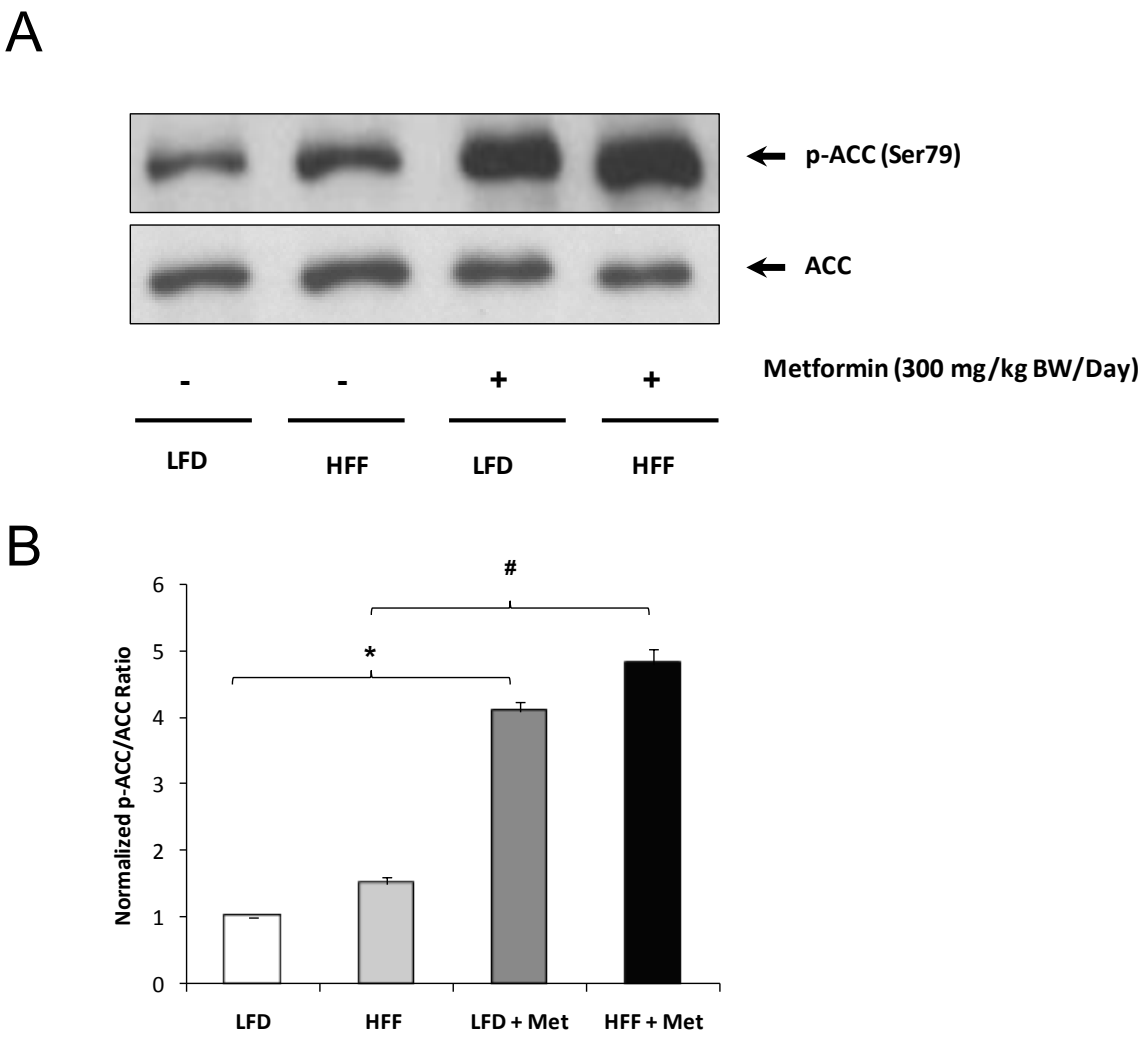


Figure 30: Immunoblot of Mammary Tumor Lysates from HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

Comparison of ACC phosphorylation in mammary tumor lysates in LFD mice versus HFF fed mice, as well as in LFD mice and HFF fed mice that were treated with metformin as shown by **(A)** the representative blot and **(B)** the densitometric graphs were generated from three independent experiments. *p<0.05 between LFD and LFD + metformin. #p<0.05 between HFF and HFF + metformin.

Confocal microscopic images were taken of the sections of mammary tumors that were stained with phospho-FOXO1A in each of the four treatment groups under basal conditions, and 10 minutes after insulin stimulation **(Figure 31)**. The percentage of total phospho-FOXO1A protein localized to the nucleus or cytoplasm was corrected for the total area of nuclei in each group and plotted as a percentage **(Figure 32)**. The nuclear fraction of p-FOXO1A was significantly higher in the basal state in all groups compared to the insulin stimulated condition, except for the HFF + metformin treated group, where the nuclear fraction of the insulin-stimulated condition was slightly higher than the basal state. With respect to the cytoplasmic fraction of p-FOXO1A, it was significantly higher in each of the insulin-stimulated conditions relative to the basal state in each of the four treatment groups **(Figure 32)**. However, there were no significant differences in the cytoplasmic fraction of p-FOXO1A between the LFD and HFF (LFD 34 ± 3.1 % vs. HFF 37 ± 4.1 %) as well as between the LFD + metformin and the HFF + metformin (LFD + metformin 28 ± 3.8 % vs. HFF + metformin 27 ± 1.8 %), but there was a slight decrease in the cytoplasmic fraction in the metformin treated subgroups compared to the non-metformin treated groups which did not attain statistical significance **(Figure 32)**.

3.3.13 Immunostaining of the proliferative marker Ki67

Positively stained cells were quantified using Aperio Image Scope software and expressed as the Ki67 Labeling Index. There were no statistical differences in the percentage of positively stained nuclei in tumors between any of the four treatment groups (LFD 8.45 ± 0.7 %, HFD 7.97 ± 1.6 %, LFD + metformin 8.29 ± 2.7 %, and HFF + metformin 8.02 ± 1.0 %). **(Figure 33a & 33b).**

3.4 Discussion

In this study, we present results demonstrating that consumption of a high-fat plus fructose diet had no marked effect on oncogene-induced, ER negative mammary tumor development in mice over-expressing HER2/Neu. Despite a significant impact of this high-fat plus fructose diet on body weight, body fat content, glucose tolerance, as well as on the circulating levels of plasma insulin, there were no significant differences in the latency of mammary tumor development or in tumor incidence compared to the low-fat diet fed

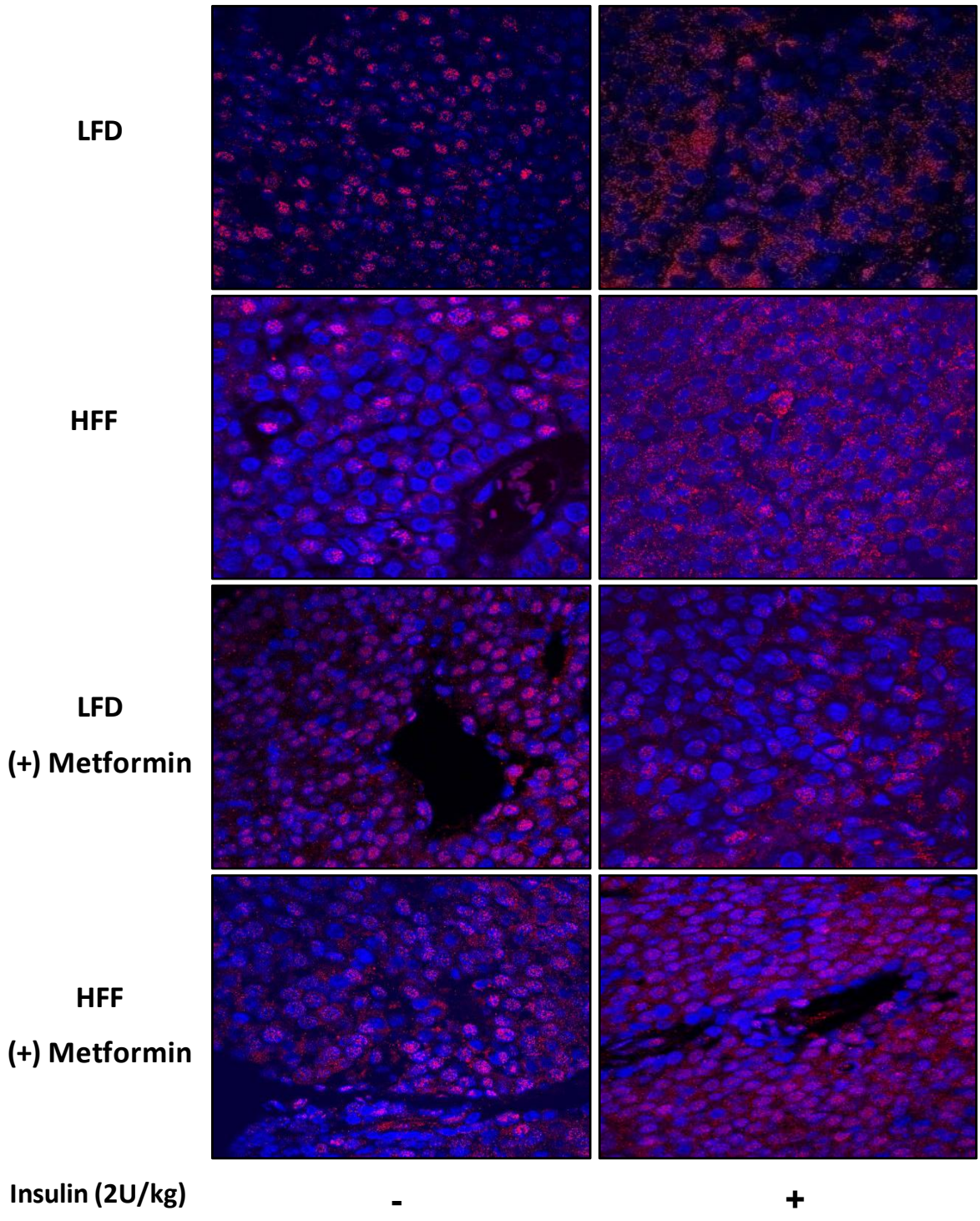


Figure 31: Immunofluorescence for FOXO1A in the Mammary Tumors from HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

Confocal microscopy images of the sections of mammary tumors that were stained with phospho-FOXO1A in each of the four treatment groups under basal conditions, as well as 10 minutes after an acute insulin stimulation. The nuclear DAPI staining is represented by blue.

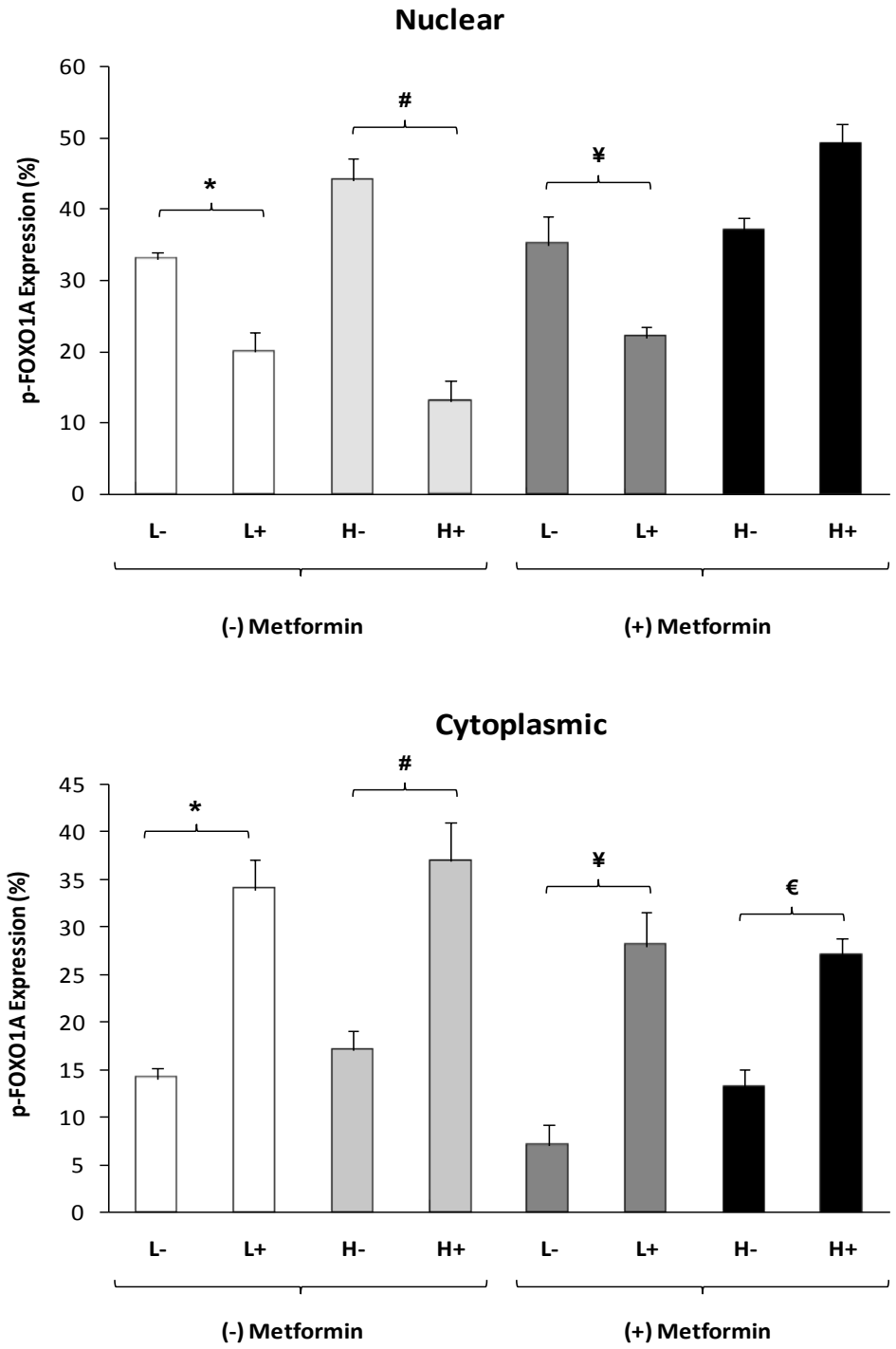
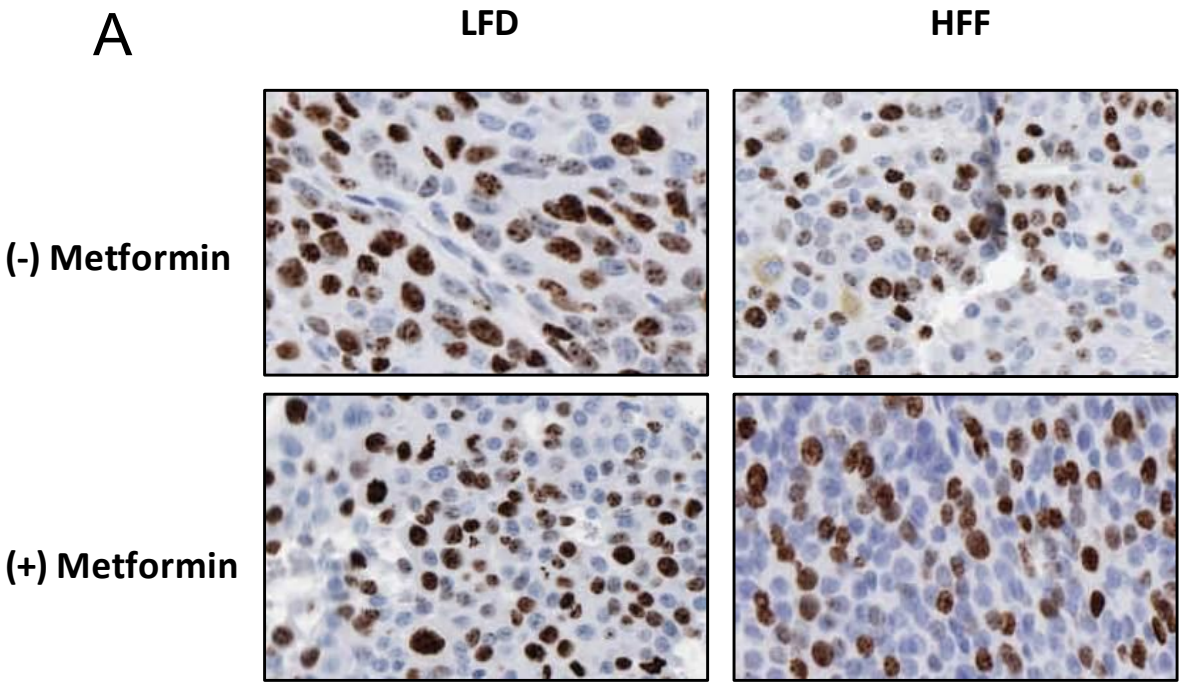


Figure 32: Immunofluorescence for FOXO1A in the Mammary Tumors from HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

The percentage of total phospho-FOXO1A protein localized to the nucleus or cytoplasm was corrected for the total area of nuclei in each treatment group and plotted as a percentage. *p<0.05 between LFD and LFD 10 minutes after insulin stimulation and without metformin treatment. #p<0.05 between HFF and HFF 10 minutes after insulin stimulation and without metformin treatment. ¥p<0.05 between LFD and LFD 10 minutes after insulin stimulation with metformin treatment. €p<0.05 between HFF and HFF 10 minutes after insulin stimulation with metformin treatment. (L=LFD and H=HFF).



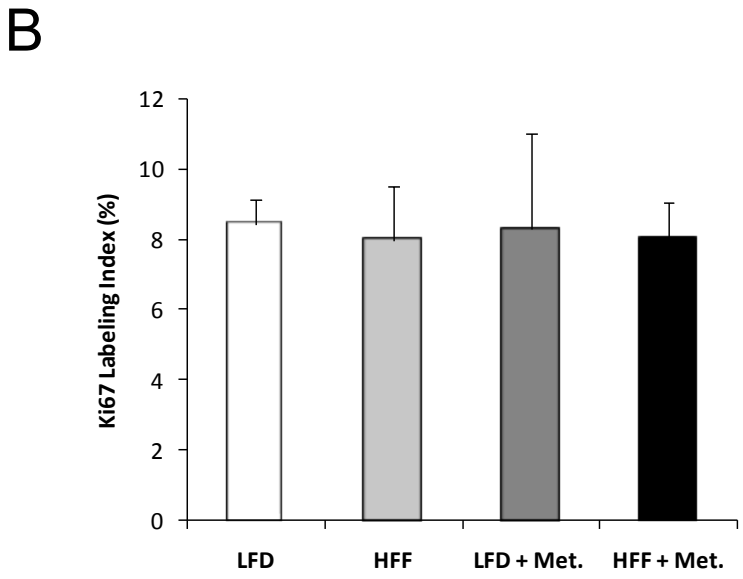


Figure 33: Immunohistochemistry Staining for the Proliferative Marker Ki67 in Mammary Tumors from HER2/Neu Mice on a High-Fat Fructose Diet and with Metformin Treatment

(A) Formalin fixed tumor sections were stained with an antibody for the proliferative marker Ki67 (20X magnification) between LFD and HFF both with and without metformin. **(B)** Representation of the percentage of Ki67 expression in tumor sections from each of the four treatment groups (LFD n=7), (HFF n=7), (LFD + metformin n=7) and (HFF + metformin n=7).

animals. In addition, there were no differences observed in the growth rate measurements of tumors between the LFD and HFF treated mice despite the presence of hyperinsulinemia and insulin resistance confirmed by the decreased phosphorylation of Akt/PKB in the skeletal muscle from mice fed the HFF. This finding is in contrast to that of our previous study in which we observed that a high-fat diet (45% kcal from fat alone) which did not induce insulin resistance or hyperinsulinemia, had an apparent modest tumor promoting effect.

The experimental protocols instituted in each study were similar except for the age of the mice in which the diet treatment was given. In our first study, the HFD was administered at 4 weeks of age, whereas in our second study, the HFF was given to the mice at 8 weeks of age. In addition, one other major difference was in the composition of the high-fat diet. Apart from the addition of fructose, the primary source of fat in the previous HFD came from lard and soybean oil, however, the HFF composition was primarily soybean oil with hydrogenated coconut oil, so there was a much higher content of polyunsaturated and saturated fats in this diet compared to the monounsaturated and saturated fats that made up the HFD in the first study. Since we did not observe an induction of insulin resistance and subsequent hyperinsulinemia in the first study in this particular FVB background strain of HER2/Neu mice, we supplemented the high-fat diet with 15% fructose in order to generate elevated circulating plasma insulin concentration. This was also necessary in order to test the hypothesis that metformin has therapeutic benefit on the breast cancer by lowering the elevated levels of insulin.

The observation that the HFF, i.e. adding fructose to high-fat, did not affect tumor latency in contrast to the first HFD remains unexplained. There would however, clearly be differences in the metabolic effects mediated by adding fructose to the fat. It has been well documented that a diet higher in fruits and vegetables is associated with lower rates of cancer development [273], but this notion has been assumed to be due to the antioxidant or growth inhibitory compounds present in these foods. The potential inhibitory effects of fructose requires further study in this particular model, as well as other models of breast cancer.

AMPK activation is a possible therapeutic target for cancers with an activated Akt/PKB signaling pathway. As mentioned previously, AMPK has been shown to inhibit

mTOR signaling downstream of Akt/PKB, and inhibition of the mTOR pathway has been reported to inhibit tumor growth *in vitro* and metastasis in animal models [274]. Our results with the AMPK activator metformin are interesting in the sense that treatment and improvement of the insulin resistant state had no effect on the latency or incidence of mammary tumor development. In all aspects of the physiological data, such as body weight, body fat content, glucose tolerance and insulin sensitivity, treatment of metformin in mice that were given the HFF diet improved the insulin resistant state as expected. However, there was no effect seen in the latency of tumor onset between mice on the LFD and HFF that were treated with metformin. In addition, there were no observed differences in the growth rates of the mammary tumors, as well as multiplicity of tumors upon metformin treatment. In addition, there was no change in the cellular proliferation as shown by the Ki67 staining.

Interestingly however, we observed in the second cohort of animals, that metformin did indeed elicit some protective effects in the LFD treated group as shown by the onset of tumor latency becoming delayed compared to the group of animals given the LFD alone **(Figure 24)**. This finding is consistent with the results reported by Anisimov et al. who studied the effects of metformin in the same HER2/Neu mouse model on a regular chow diet [262]. Using a similar experimental design, Anisimov et al. also investigated the effect of metformin on the development of tumors in the SHR mouse model [264]. In this model, metformin did not suppress the development of spontaneous tumors. Proposed explanations for this observation is that because the SHR mice develop cancer much later in life compared to the HER2/Neu mice, the metformin may have extended the life span to the point where the cancer was allowed to develop in this particular strain [275]. It was also suggested that metformin may have pleiotropic effects in early life, therefore causing the SHR mice to develop cancer later in life [275]. However, a recent study looked into the possible role of the HER2 oncoprotein itself, and it demonstrated that it represents an important cellular target involved in the anti-neoplastic actions of metformin [265]. In this study, the authors found that the ectopic over-expression of the HER2 oncogene was sufficient to enhance metformin-induced cellular growth inhibition in human breast cancer cells, and that supplementation with metformin lead to the down-regulation of the HER2 oncoprotein expression in both a dose and time dependant manner [265]. It is also possible that there is a threshold effect for insulin and that even with metformin treatment, the insulin levels in the HFF insulin resistant

mice remain above this threshold level to induce mammary tumors. Another potential explanation is that this particular high-fat diet provides a large amount of free fatty acids (FFAs) which are necessary for tumor growth, and thus, even with the inactivation of ACC by metformin, the tumor growth is maintained by exogenous lipids.

We also demonstrated for the first time that the mammary tumor cells do not develop insulin resistance along with the classical metabolic target tissues. This was evidenced by the immunoblot analysis of Akt/PKB in the tumor tissue, which showed a sustained phosphorylation of Akt/PKB in the HFF mice. Upon metformin treatment, the phosphorylation of Akt/PKB in the HFF fed mice was reduced slightly in the insulin stimulated state, however, tumors from the LFD fed mice that were treated with metformin under conditions of insulin stimulation demonstrated an increase in the phosphorylation of Akt/PKB. In addition, the mammary tumors from all four treatment conditions in the basal state were similar. The FOXO1A immunofluorescence staining also reiterated the finding that the mammary tumors seem to remain sensitive to insulin in the insulin resistant state, as shown by the elevated cytoplasmic fraction of FOXO1A upon insulin stimulation in the HFF mice. This observation is consistent with the lack of IR down-regulation in tumors from human subjects which we reported previously [108]. This maintenance of insulin signaling may explain the observation in human subjects that hyperinsulinemia influences the prognosis of breast cancer. Other immunoblot data of the skeletal muscle demonstrated that there was indeed insulin resistance, and that in both skeletal muscle and mammary tumors metformin was in fact active as shown by the phosphorylation of AMPK as well as ACC.

Recent evidence also indicates the importance of cyclin-dependant kinase (CDK) inhibitors in addition to the activation of AMPK and down-regulation of cyclin D1, as a means by which metformin can cause cell cycle arrest in cancer cells [260]. Zhuang et al. went on to further provide a potential molecular mechanism to explain the ability of metformin having a beneficial effect on cancer prevention and treatment. They proposed that metformin arrests cell proliferation by activating AMPK which leads to a loss of cyclin D1. This decrease in cyclin D1 leads to the release of sequestered CDK inhibitors such as p27 and p21 which then go on to bind the cyclin E/CDK2 complex. The cyclin E/CDK2 complex is responsible for allowing the cell to go from the G1 into the S phase of the cell cycle. In cell

lines which were resistant to metformin mediated cell cycle arrest, there was an absence or very low levels of the CDK inhibitor p27, and it has been shown previously that loss of p27 expression is present in some cancers, and is correlated with a poor prognosis [276]. This is also the case when there is a down-regulation of the p53-p21 axis.

Another recent study by Phoenix et al. looked into the effects of metformin in various human breast cancer cell lines paying close attention to the hormonal characteristics of each [277]. In this study, there was confirmation of the reduction of cellular proliferation upon AMPK stimulation by metformin, and they also found that this result was different depending on the ER status of the cell line. The findings suggested that metformin treatment reduced cell growth in both ER positive and ER negative breast cancer cells, but that the ER negative cells were less sensitive to this metformin effect than the ER positive cells [277]. Since the HER2/Neu mouse model is ER negative, perhaps the lack of an effect by metformin on proliferation, as shown by the Ki67 staining, is due to this fact.

The mechanism of action of metformin as an anti-neoplastic agent may still be related to a direct action of the drug on cancer cells via inhibition of the mTOR pathway, or by the indirect effect of lowering insulin levels. It remains plausible that these two biological pathways both contribute to its potential anticancer activity. Furthermore, the activation of AMPK may also have other targets in addition to mTOR/$p70^{S6K}$, such as ACC which contribute to growth inhibition. Finally, the link between AMPK activation and down-regulation of cyclin D1 remains to be identified. Further work is necessary in order to determine which pathways are the key to the potential therapeutic effect of metformin.

CHAPTER 4:

General Discussion

4.1 Summary of Findings

- Our first high-fat diet (HFD) which consisted of 45% kcal of fat caused an increase in body weight and fat content, and an impairment of glucose tolerance, but it did not cause a significant increase in plasma insulin concentration.
- There was no difference in the onset of the first mammary tumor with the HFD, nor was there any change in the growth rates of the tumors.
- There was a tendency towards an earlier onset of a second tumor in mice that were given the HFD, and there was a 2-fold increase in the overall number of tumors that formed in these mice.
- There was no insulin resistance in the skeletal muscle in mice fed the HFD, however, there was an apparent acceleration in the phosphorylation of Akt/PKB, p70S6K and 4E-BP1 in the mammary tumors from HFD fed mice upon insulin stimulation, at 5 min post-insulin stimulation, while maximum stimulation appeared to be similar by 10 minutes.
- There was a trend to an increase in the insulin-stimulated phosphorylation of IRS-1 in the mammary tumors from the HFD mice.
- There was no difference in the cellular proliferation in the mammary tumors from mice fed the HFD as seen by the Ki67 staining.

- Our second high-fat diet formulation which consisted of 45% kcal of fat and 15% fructose (HFF) caused a more overt obese phenotype with significant increase in body weight, fat content, an impairment of glucose tolerance, elevated levels of plasma insulin concentrations along with an impairment of insulin sensitivity.
- Treatment of mice fed a HFF diet with metformin caused a slight decrease in body weight, a significant decrease in body fat content, along with an improvement of glucose tolerance and a decrease in plasma insulin concentrations, along with an improvement of insulin sensitivity.

- There was no difference seen between any of the four treatment groups when the growth rates of the mammary tumors were assessed, and there was no difference in the average number of tumors that developed per mouse.
- There was no difference in the onset of the first mammary tumor in the first cohort and the combined cohort, however, there was a significant delay in the onset of the first mammary tumor in the low-fat diet (LFD) fed mice that were treated with metformin relative to the LFD fed mice alone, in the second cohort. In contrast, in the same cohort, metformin did not affect tumor latency in the HFF group.
- There was insulin resistance present in the skeletal muscle from mice given the HFF, and treatment with metformin corrected this insulin resistant state.
- In contrast, the mammary tumors appeared to remain sensitive to insulin in an insulin resistant state.
- There was a significant increase in the phosphorylation of AMPK in the skeletal muscle and mammary tumors from mice that were treated with metformin relative to their controls.
- There was a significant increase in the phosphorylation of ACC in the mammary tumors from mice that were treated with metformin relative to their controls.
- The immunofluorescence staining of FOXO1A correlated with the immunoblot data of Akt/PKB activity seen in the mammary tumors.
- There was no difference in the cellular proliferation in the mammary tumors from the mice in any of the four treatment groups demonstrated by the Ki67 staining.

4.2 Discussion

Epidemiological studies demonstrate that breast cancer is the most common type of cancer diagnosed in women and is a significant cause of morbidity and mortality. While there are numerous risk factors known to be associated with increased breast cancer risk, we chose to focus specifically on obesity induced insulin resistance and hyperinsulinemia and the role that this can play as a potential adverse factor with respect to the development and progression of breast cancer. Previous work in the area has demonstrated effects on promoting breast cancer development and progression, and adverse outcomes have been shown in clinical studies. Our general objective of this study was to examine in greater detail

the molecular mechanisms involved in this effect using a rodent model of breast cancer, and we also wanted to test the hypothesis that if improvements to the insulin resistant state are made with the use of the anti-diabetic agent metformin, there would be beneficial effects on the outcomes of breast cancer.

In the first component of the study, we observed a mild obese phenotype in the HER2/Neu mice that were given the HFD (45% kcal fat), but there was no difference in the plasma insulin concentrations compared to the control animals. In addition, tumor growth in this mild obesity phenotype was not affected. However, we did observe a tumor-promoting effect of this HFD as seen by the shortened tumor latency of the second mammary tumor. The precise mechanism of shortened tumor latency may involve an effect of increased adipose tissue mass, e.g. altered cytokines, or an effect of increased dietary FA content. In the second component of the study we observed a more overt obese phenotype in the same HER2/Neu model when they were given the HFF diet (45% kcal fat + 15% fructose). With this diet treatment, we observed an increase in plasma insulin concentrations and a well defined state of insulin resistance. There appeared to be, however, no effect of the HFF diet on mammary tumor latency in these mice. One possible explanation for this difference in findings may be due to the exact breakdown and proportions of various fatty acids which make up the total fat content in these respective diets. In the HFD, the fat source was primarily derived from lard, which is mainly oleic acid and stearic acid, monounsaturated and saturated fatty acids respectively. However, in the HFF diet, the main fatty acid that is found in the total fat source comes from soybean oil and hydrogenated coconut oil, which cause it to be high in both saturated and polyunsaturated fatty acids. Hardy et al. demonstrated a proliferative effect of oleic acid in the MDA-MB-231 breast cancer cell line [240], and because the HFD appears to have a more substantial oleic acid content compared to the HFF diet, this could potentially be a cause of our observations. In addition, the differences we observed in tumor latency between the diets could also be due to the fact that diets which are rich in medium chain triglycerides such as lauric acid, which is the primary fatty acid in coconut oil, can elicit a protective effect from the development of cancer [278]. However, diets which are rich in n-6 fatty acids such as linoleic acid can cause tumor promotion [279-281]. Perhaps in the HFF diet, the high lauric acid content from the coconut oil was sufficient to counteract any potentially harmful effects that the linoleic acid could

have caused on tumor latency and incidence [281]. Because the HFD did not have lauric acid, but did have a significant content of linoleic acid due to the soybean oil, the coconut oil may in fact have fatty acids that are beneficial with respect to the protection from cancer development.

It is also important to note the direct effect that FFAs can have on pancreatic β-cells. FFAs are important to the normal function of the β-cell, its capacity to compensate for insulin resistance and its failure in Type 2 Diabetes [282-284]. If the fatty acid supply in the islet becomes compromised, there is a loss of glucose-stimulated insulin secretion [285], however, this process is rapidly reversed upon replacement by exogenous FFAs [285]. If there is a chronic excess of FFAs, the saturated FFAs can cause a reduction in insulin biosynthesis and secretion, as well as β-cell apoptosis [283, 285, 286]. If this is the case, then the difference in the fatty acid profile of our two diets may explain the difference in the plasma insulin concentrations between the two components of our study. As mentioned previously, since there may be variability in the timing of peak insulin levels after glucose administration in the mice, we cannot completely exclude the possibility that a small increase in insulin could have occurred at 30 or 60 minutes post-glucose in the first component of the study (Chapter 2). At the same time, it is interesting to note the mild impairment of glucose tolerance we observed. Combined with the lack of hyperinsulinemia and the absence of insulin resistance in the skeletal muscle, the data suggest a potential impairment of insulin secretion induced by the HFD. However, in the high-fat fructose diet (HFF), the variation in the fatty acid composition may not have caused an impairment in insulin secretion, since we did observe an increase in plasma insulin concentration in the HFF fed mice compared to the LFD mice.

Perhaps due to the nature of our particular study design and model of breast cancer, we did not observe a definite correlation with previous clinical studies and rodent *in vivo* studies. However, we still cannot rule out the importance of insulin and its signaling cascades on the outcomes of breast cancer. Given the evidence that both obese and Type 2 Diabetic patients have an increased risk for the development of breast cancer, and have a subsequently poorer prognosis, efforts must be taken to ensure proper education of at risk individuals about the importance of weight control and metabolic risk factor reduction with regards to the primary prevention of breast cancer.

The Human Epidermal growth factor Receptor 2 or HER2/Neu (ErbB-2) is a 185 kDa transmembrane receptor which confers an increased aggressiveness of breast cancer [153, 154], and is named because it was originally identified as a transforming oncogene in chemically induced rat neuroglioblastomas. Approximately 20-30% of early stage breast cancers show an amplification of the HER2/Neu gene, or an over-expression of its gene product [156]. Patients with breast cancer that over-express HER2/Neu typically present with a more aggressive cancer type, an unfavourable prognosis, shorter relapse time, and lower survival rates [156, 157]. One of the most extensively studied *in vivo* models of breast cancer is the MMTV-WT HER2/Neu mouse. Direct evidence in support of a role of *Neu* in mammary tumorigenesis has come from observations made in this specific transgenic model. These mice over-express the HER2/Neu specifically in the mammary gland, and they develop focal mammary tumors at about 5 months of age [223, 224]. Constitutively activating mutations of *Neu* are also frequently observed in these tumors [224]. One major caveat of this model is that the onset of tumorigenesis is driven by Tyr kinase signaling. Thus the effects of hyperinsulinemia, as in the second component of the study where we observed insulin resistance, may be less evident in this model since insulin also exerts its action via Tyr kinase activity. It is important to note that regardless of the diet or metformin treatment in these mice, they all developed mammary tumors, and perhaps the lack of differences in the growth rate measurements is due to the fact that this model of cancer is very aggressive and it tends to overshadow any small differences we may have otherwise found in another breast cancer model.

Current evidence in the literature has advanced our understanding of AMPK and specifically targeted mTOR therapy, and has led to a better understanding of the potential relevance between the lower incidence of cancer and metformin use in diabetic patients [205]. The high prevalence of the metabolic syndrome in North American women, and the correlations of high circulating insulin levels with an adverse prognosis in breast cancer, prompted this study of the effects of treatment with metformin on lowering circulating insulin levels and the outcomes on breast cancer growth and development. To briefly summarize, metformin has been in use for over 40 years as an anti-diabetic agent which improves glycemic control through enhancing insulin sensitivity in the muscle as well as in liver. As an insulin sensitizer, causing a reduction in insulin resistance, there is an

improvement in insulin signalling in metabolic tissues and a subsequent lowering of circulating levels of insulin [188]. There is also evidence that metformin can inhibit hepatic gluconeogenesis [287] and this is another way in which insulin levels are decreased. The decreased insulin concentrations may contribute to the reduction of the mitogenic and cellular growth promoting effects of insulin on various cell types [138].

Metformin acts via activation of AMPK via its upstream kinase LKB1 [190, 287] at the molecular level, and it has been shown that this activation regulates pathways that are associated with the control of cellular proliferation [288]. A number of different studies have shown that the activation of AMPK leads to a suppression of mTOR signaling induced by growth factors and amino acids [255, 256, 289] either directly or indirectly. This is another reason why we have chosen metformin as a potential therapeutic target for breast cancer. There are other potential mechanisms in various *in vitro* studies using metformin which demonstrated that it leads to the reduction of protein synthesis [210, 290] as well as fatty acid synthesis [190], and cyclin D1, important in cell cycle progression [260, 266].

The use of metformin as a means to correct the insulin resistant state and thereby lower circulating insulin levels was effective, however the outcomes on the latency of the mammary tumor development was interesting. In HER2/Neu mice that were fed the HFF diet, and then treated with metformin, there was no difference in tumor latency, multiplicity, growth rate or cellular proliferation when compared to the untreated HFF animals. However, consistent with previously reported studies using the same mouse model on a regular chow diet with metformin treatment, we found that metformin delayed the onset of the first mammary tumor in the LFD group in the second cohort. Metformin may be acting via AMPK activation which would inhibit mTOR and ACC. It is plausible that nutritional excess as in the HFF diet can somehow mitigate these actions of metformin despite the fact that AMPK phosphorylation was stimulated by metformin in both diet groups. For example, palmitic acid can overcome inhibition of cell growth seen in the context of inhibition of de novo fatty acid synthesis by down-regulation of FASN or ACC [251, 268, 269, 291]. In addition, examining the phosphorylation of mTOR and its downstream targets 4E-BP1 and $p70^{S6K}$ will help to answer some crucial questions regarding the exact molecular mechanism involved in the metformin effect in this particular breast cancer model. The lack of effect of

metformin in the HFF diet group as opposed to the LFD group may be related to the extent of lowering of insulin levels. Thus, metformin lowered insulin in both diet groups, but these remained lower in the LFD group, indeed below the "normal" values. There may be a critical level to achieve in order to induce a potential effect on tumor formation.

Importantly, we have demonstrated for the first time that mammary tumor cells remain sensitive to insulin under conditions of metabolic insulin resistance as shown by the Akt/PKB immunoblots and confirmed by the FOXO1A staining in the mammary tumors from the HER2/Neu mice that were fed a high-fat fructose diet. Although further investigation is necessary, it is a particularly significant finding with respect to our hypothesis of the role of insulin in breast cancer, and supports the clinical and epidemiological data that have been published on this subject.

Given all the evidence, as well as our own preliminary findings in a rodent model using metformin as a potential neo-adjuvant therapy in breast cancer, there is still a great deal of work that needs to be done to further document efficacy as well as to elucidate the exact biochemical changes which may be occurring. Such studies will provide the information required to imitate clinical trials using agents which enhance insulin sensitivity.

4.3 Future Directions

Metformin is known to activate AMPK and we have demonstrated *in vivo* that AMPK is activated by metformin in breast cancer. As mentioned previously, the activation of AMPK and the subsequent inhibition of mTOR has been implicated as the mechanism by which metformin can directly inhibit tumor growth [259]. In addition to metformin being an activator of AMPK, conditions of cellular stress and hypoxia, which both deplete the cellular supply of ATP, have been shown to also activate AMPK [292]. It has been shown that many solid tumors tend to suffer a hypoxic environment, but still manage to survive by utilizing a compensatory mechanism which includes increased anaerobic metabolism, i.e. glycolysis [247-249]. Therefore, it is possible that a tumor which is hypoxic and/or which has adapted to a condition of hypoxia, will become resistant to the effects of metformin.

There is accumulating evidence demonstrating that the hypoxia inducible factor-1 (HIF-1) functions as a key regulator of the adaptive response to hypoxia in cancer tissues, and a number of different studies have shown the up-regulation of hypoxia inducible genes in various different tumor types [293]. As mentioned earlier, the role of the HER2 receptor in human breast cancer is of great importance. A study by Laughner et al. demonstrated that HER2 signaling induced HIF-1 protein synthesis [294], which could potentially explain the mild effect of metformin in the HER2/Neu mice that we observed. This raises the question of whether the inhibitory activity of metformin is abolished in conditions of hypoxia. By culturing various human breast cancer cell lines, which are differentiated by ErbB2 expression as well as AMPK and LKB1 expression, under conditions of hypoxia and then treating them with and without metformin at various time points will allow the investigation into whether there is in fact a proliferative or anti-proliferative effect of differing concentrations of metformin.

Oxygen deprivation leads to the activation of HIF-1 which in turn can induce vascular endothelial growth factor- A (VEGF-A) [295]. Examining whether VEGF expression levels change under hypoxic conditions with and without metformin treatment in various breast cancer cell lines may be one way to examine this relationship. It may be possible that elevated amounts of VEGF can keep the cells alive and/or induce angiogenesis under metformin treatment. To test this hypothesis, treating the breast cancer with VEGF receptor specific inhibitors both with and without metformin treatment under hypoxic conditions could shed light on this possibility.

It will be important to further examine whether metformin-related inhibition of tumor growth associated with AMPK activation is due to either or both the inhibition of mTOR, or the ability of AMPK to phosphorylate and inactivate ACC, which leads to a decrease in fatty acid synthesis and lipogenesis in breast cancer cells. It has been previously demonstrated that the addition of palmitic acid can compensate for any defects in ACC activity by supplying essential fatty acids [291]. Therefore, it would be interesting to examine the effects of adding palmitic acid to various human breast cancer cell lines and once again look at the effects that adding metformin will have with regards to proliferation and apoptosis. As well, transfecting human breast cancer cells with a constitutively active AMPK in order to mimic the effects of

metformin treatment and examining if this has an anti-proliferative or apoptotic effect, and whether this effect depends on inhibition of fatty acid synthesis will be imperative. It will also be of importance to examine the profile of cyclin D1 in the mammary tumor tissue from these mice in the various treatment conditions, as well as the CDK inhibitors p27 and p21 to observe any correlation to the work by Zhuang et al. [260].

Lastly, due to the potential role for the IR in cancer, examining the contribution of insulin action in the mammary gland *in vivo* will be important. Generating a mammary gland specific conditional insulin receptor knockout mouse would allow further investigation into the hypothesis that mice lacking the IR in the mammary gland would possibly exhibit a lower incidence of tumor development and a slower growth of tumors when crossed with a rodent model of breast cancer. These studies would shed further light on the role that insulin plays in breast cancer outcomes, as well as further elucidating the therapeutic role that metformin can have, not only as an insulin sensitizer, but as a direct inhibitor of targets crucial to tumorigenesis.

CHAPTER 5:

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