THESIS

INHERENT AEROBIC CAPACITY AND SUSCEPTIBILITY TO BREAST CANCER DEVELOPMENT

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ABSTRACT

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Physical inactivity is one of the risk factors for developing breast cancer. Aerobic capacity is an objective measure of an individual's activity behavior as physical exercise improves their ability to consume, transfer, and utilize oxygen. Variability in responses to the same physical exercise program led scientists to determine that there are two components of aerobic capacity—inducible and inherent. The latter became possible to study when two models with high (HIAC) and low (LIAC) inherent aerobic capacity were created.

A number of studies conducted on these models showed that not only do these strains differ in their exercise performance but also in their susceptibility to disease. LIAC animals gain more weight and exhibit reduced fatty acid oxidation compared with their HIAC counterparts, especially on a high-fat diet. Based on these observations, my working hypothesis is that inherent aerobic capacity underlies an individual's metabolic flexibility. Metabolically inflexible cells exhibit increased glucose utilization, anabolic metabolism, as well as cell proliferation and survival. Interestingly, similar factors are also associated with carcinogenesis. HIAC animals appear to be metabolically more flexible on a systemic and cellular level than their LIAC counterparts. Additionally, our

laboratory previously showed that LIAC animals exhibited greater incidence, multiplicity, and lower latency of breast tumors than HIAC upon carcinogen administration.

To reveal the underlying mechanisms of their different carcinogenic responses, we analyze protein expression patterns in the mammary gland and tumors of HIAC/LIAC models. We demonstrate that LIAC animals upregulate pathways associated with glucose utilization, protein and fatty acid synthesis, as well as other carcinogenic signatures, whereas HIACs are associated with energy sensing, fatty acid oxidation, and cell cycle arrest. Consequently, we propose that higher inherent aerobic capacity renders cells metabolically more flexible and reduces their susceptibility to breast cancer development.

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CHAPTER 1:

Overview

1.1. Introduction

Cancer is considered to be one of the oldest, multifaceted, and lethal diseases around the world. Cancer strikes at various locations, but in women it most commonly occurs in the breast. There are a plethora of factors underlying carcinogenesis: some of them we can control, whereas other factors—we cannot. The factors we can control put the ball in our court when it comes to protecting ourselves from cancer. In modern times, obesity, presumably resulting from excess caloric intake and physical inactivity is a risk factor for many diseases—and cancer is most certainly one of them. However, simply increasing physical exercise—when used as a preventative measure or a treatment—does not equally benefit all individuals. Therefore, a goal of my thesis project was to investigate the crosstalk among obesity, exercise trainability, and the development of breast cancer, a process referred to as mammary carcinogenesis, in the rodent model.

1.2. Carcinogenesis as a disease

Cancer is the second most common cause of death, yielding only to cardiovascular diseases (1). And yet, in figurative language, whenever someone refers to a problem that has detrimental properties and spreads very fast, they use “cancer” as an example—“Corruption is cancer of the society,” they say (2). It is true that cancer is

an illness that does not have a universal cure nor even 100% reliable tools for its early detection (3). It originates from a single cell or from a disorder that affects many cells as a result of environmental and/or intrinsic factors. It also can exhaust the organism (cancer cachexia) as it grows and spreads, and can affect essentially any part of the body, including but not limited to stomach, brain, skin, blood (4). Tumors differ not only by the type of tissue or location from which they originate but also by what caused the oncogenic transformation that gave rise to them (5). Given such observations, carcinogenesis, rather than cancer or tumor, is the disease process.

Carcinogenic transformation confers a growth advantage rendering the clone of cells that arise autonomous in terms of their survival (6). The cells acquire the ability to grow and continuously divide regardless of availability of various growth factors (7). A cancer cell can become more responsive to mitogenic signals by increasing the number or sensitivity of receptor kinases, and consequently, enter the cell cycle even upon trace amounts of growth factors outside the cell. Apart from endocrine stimuli, cancer cells often utilize paracrine and even autocrine signals by producing the growth factors themselves or inducing surrounding cells to secrete growth factors for them. Most commonly, a tumor cell can upregulate the elements of the mitogenic pathway(s) that enable it to go through all stages of cell division, gaining increasing replicative potential. Cancer cells often have elevated levels of cyclins as well as their dependent kinases. However, in order to uphold the proliferating phenotype, a cell needs to not only unceasingly drive the process forward by upregulating oncogenes but also to avoid the negative feedback mechanisms of regulation by downregulating tumor suppressors and the cell death pathways with which they interact. A cancer cell often

hinders the factors that impede progression of the cell cycle, such as retinoblastoma protein or cyclin-dependent kinases inhibitor proteins (CKIs), by decreasing their amount, expression of defective forms, or preventing their activation via post-translational modifications. Similarly, cancer cells can evade apoptotic cell death by upregulating anti-apoptotic proteins, such as inhibitors of apoptosis and B cell lymphoma/leukaemia-2 protein (BCL-2), and simultaneously downregulating the pro-apoptotic proteins (BCL-2-associated X protein (BAX), tumor suppressor p53) signaling pathways. Another form of negative control is the Hayflick limit—the finite number of cell divisions that a single cell can undergo based on the length of its telomeres (8). A tumor cell is capable of upregulating telomerase activity and thus increasing telomere length, which renders a cell able to go through an increased number of cell divisions. Therefore, a single cell multiplies and a tumor expands in size with successive rounds of cell replication. To ensure their survival, cells require resources from the external environment, constant availability of which they attempt to ensure via a number of pathways including promoting angiogenesis. With an enlarging network of blood vessels and increased supply of nutrients, oxygen, and other resources, tumors can grow rapidly and acquire a more aggressive phenotype. Plasticity in reorganization of their cytoskeleton in addition to pro-survival phenotypic change enables cancer cells to achieve metastatic potential: the cells become able to abandon the site of their origin and travel via the bloodstream or lymphatic system to distant sites, which they invade (7).

The mechanisms of carcinogenesis encompass genetic mutations, alterations in signaling networks, and metabolic rewiring (7). Mistakes in replication of DNA often take

place in cells with high proliferative potential, for example stem cells, and with or without environmental factors, for instance genotoxic compounds or UV exposure (6). The introduction of mutations in this manner can be passed on to daughter cells. Such mutations can be indifferent or exert a significant effect on physiology of the cell: they can promote formation of other mutations (by affecting DNA repair systems) (9); they can distort functional elements of signaling pathways resulting in deactivation or overactivation. For example, mutations in Ras gene often render its protein product constitutively active and so, the downstream mitogen-activated protein kinase/extracellular signal–regulated kinases 1 and 2 (ERK1/2) pathway also becomes overactivated (10). Another example is that cells unbalance metabolism via overexpression of hexokinase drives glycolysis) (11). Alterations in energy homeostasis and intracellular signaling may also occur due to environmental stimuli as well as circulating hormones, cytokines, and nutrients (12).

Nevertheless, a single cell becomes cancerous owing to not one but a combination of influential mutations and/or other factors. Epigenetic changes also can render favorable physiological conditions for cancer progression which are heritable across generations of cells (13). Such favorable circumstances for execution of aforementioned hallmarks of cancer arise from a crosstalk between dysregulated signaling pathways and cellular energy metabolism. Interestingly, by shifting the normal physiology of the cell towards a cancerous one, mutagenesis is also promoted (14). Overall, oncogenic transformation of the cell happens by the means of defective intracellular signaling and imbalanced energy metabolism orchestrated by intrinsic and environmental factors.

1.3. Risk factors for cancer that we can control

People have control over exposure to environmental influences that increase the risk for cancer development. Apart from ionizing radiation and infectious diseases, up to 50% of cancer risk factors are associated with lifestyle choices. Risk factors include use of tobacco products, overconsumption of alcohol, drug abuse, unbalanced diet, overeating, and low levels of physical activity. (15). For example, cigarettes and processed food contain many carcinogens—compounds that render the organism susceptible to cancer—such as nitrosamines, polycyclic aromatic hydrocarbons (PAHs), aflatoxins, dioxins, and acrylamides. Apart from affecting DNA structure, they are also able to deregulate intracellular signaling pathways as well as metabolism (16). The deregulation of some pathways has been implicated in the development of obesity, type 2 diabetes, cardiovascular, and neurodegenerative diseases. Thus, an intricate crosstalk among such diseases, including cancer, exists (17). Women diagnosed with obesity have poorer prognosis and higher mortality from breast cancer (18-24). Mammary adenocarcinomas arise in a tissue, in which they are often surrounded by a large number of adipocytes and associated stroma. In obese individuals, adipocytes may exert effects that favor cancer progression, such as increased inflammation, mechanical tension, paracrine signaling, and increased nutrient supply (24). There is an active discussion about who was first—the chicken or the egg—in terms of obesity and cancer formation. However, there is increasing interest in including weight loss via caloric restriction, increased physical activity, and/or administration of pharmacological mimetics of these interventions as adjuvants during and after the treatment of breast cancer (19, 25, 26).

1.4. Aerobic capacity as a measure of physical activity levels

In the case of increasing physical activity for extended periods of time, monitoring and maintaining this new behavior is challenging for humans. Moreover, it is clear that one exercise program is not appropriate for everyone. For some, a specific intensity and type of physical exercise may be inadequate or even detrimental. Needs are also certain to change during disease progression as well as during and following treatment. Much of the work on physical activity in cancer patients is based on self-assessments which are notoriously inaccurate (27).

Therefore, an increasing number of exercise scientists are looking for objective measures to evaluate the effects of physical activity on the physiology of an individual. One such assessment metric is cardiorespiratory fitness, or aerobic capacity. This parameter determines the ability of the body to consume, transfer, and utilize oxygen, which corresponds to an individual's capacity to perform and respond to physical exercise. It is measured as the maximal volume of oxygen that a body can consume during exercise with gradually increasing intensity that leads to final exhaustion (28). People and animals with higher aerobic capacity perform better exercise-wise than those with lower maximal aerobic capacity. At the same time, regular exercise programs increase the individual's aerobic capacity. Intensity of each type of exercise can be measured also by the percentage of maximal oxygen uptake required to perform that task successfully (29). Therefore, people who differ in their aerobic capacity and will be subjected to one particular exercise program will display variability in their performance. Depending on intensity of the exercise, individuals with higher aerobic capacity might

benefit from such a program, whereas those with lower aerobic capacity might even experience adverse effects from the same program (30). Thus, aerobic capacity becomes an important yet still quite elusive factor of an organism's responsiveness to physical exercise, especially in terms of its medical applications.

Physical activity is generally associated with positive effects on human health and well-being. Physical exercise is a common preventative measure for various disorders, both physiological and psychological (31). Currently obesity and physical inactivity are thought to predispose to chronic diseases that account for 60% of annual mortality (32). Consequently, some individuals tend to subject themselves to inconvenient and often exhausting training programs in order to improve their health via increasing their cardiorespiratory fitness which is associated with lower morbidity and mortality. However, trainability, or the scope of improvement, of aerobic capacity in response to the same exercise program varies among people. In fact, family studies in the 1960s as well as genomic analyses demonstrated that up to 50% of an individual's trainability has a heritable component (33). Since individuals with higher aerobic capacity are able to benefit more from physical exercise in all aspects, then what if people with a higher level of its inherent component could also benefit as much without prior challenging exercise programs?

1.5. Model organisms for studying aerobic capacity

Many questions exist about how aerobic capacity, both inherent and induced, affect an individual's health and response to disease. In order to facilitate the deconstruction of these questions, Drs. Steven Britton and Lauren Koch created two rat

lines by divergent artificial selection based their performance in a treadmill running-to-exhaustion test. Two extremes of animals who ran the longest distances as well as those that ran the shortest were bred with animals of the likewise endurance. Over 30 generations, two outbred populations of rats were created that differed over 300% in their running ability and maximal oxygen uptake. Such drastic difference in their exercise performance can be detected in animals without any prior training. Hence, animals that do better exercise-wise have high inherent aerobic capacity (HIAC) and those that do worse have low inherent aerobic capacity (LIAC). Apart from their disparity in exercise capacity, these animals also differ morphologically: HIAC animals are leaner and smaller than LIAC animals. Over 15 years of investigating these models, other differences have been noted. LIAC animals are prone to various chronic disease risks. LIAC rats have early predisposition to metabolic syndrome, cardiovascular diseases, neurodegenerative and even behavioral disorders. In contrast, HIAC rats are protected from such developments. The pathophysiology of LIAC animals seems to be correlated with metabolic inflexibility, i.e., these animals appear to have intrinsic deficiencies in metabolic processes that require oxygen. Interestingly, these populations not only differ in their inherent aerobic capacity (IAC) levels, but also are models of health (HIAC) and disease (LIAC) (34).

Recently, our laboratory demonstrated that LIAC animals are susceptible to the development of breast cancer, whereas the HIAC strain appears to be protected without being subjected to any form of physical exercise or training during the development of cancer. LIAC animals unlike HIAC exhibited higher incidence (47.3 vs. 14.0%; $p < 0.001$) and multiplicity (0.85 vs. 0.18 cancers per rat; $p < 0.0001$) of breast cancer as

well as a shorter latency to tumor occurrence (Mantel hazard ratio = 4.01; 2.02–7.93, 95% confidence interval (95% CI); $P = 4 \times 10^{-4}$). Mammary glands of HIAC animals had upregulated intracellular pathways that are associated with energy sensing (AMP-activated kinase (AMPK), silent mating type information regulation 2 homolog 1 (SIRT1)) and anti-cancer effects (cyclin-dependent kinase inhibitor 1 (p21), cyclin-dependent kinase inhibitor 1B (p27)). LIAC animals had elevated expression of pathways in their mammary glands that often are implicated in cancer development, for instance 3-phosphoinositide-dependent protein kinase-1/protein kinase B (PI3K/AKT) signaling that exerts mitogenic and pro-anabolic effects (35). Therefore, inherent aerobic capacity connects energy metabolism regulation and susceptibility to mammary carcinogenesis.

However, to date, nothing has been reported about the molecular basis of differences between tumors from animals with high versus low inherent aerobic capacity. This issue is the basis of my thesis project. Understanding the molecular mechanisms underlying effects of inherent aerobic capacity on risk of cancer formation will not only illuminate how cardiorespiratory fitness contributes to the carcinogenic process, but also may reveal improved approaches to the treatment of people who suffer from breast cancer and metabolic disorders, such as obesity. It is said, “An ounce of prevention is worth a pound of cure” (Benjamin Franklin). Accounting for inherent and induced aerobic capacity may not only inform cancer prevention, but also cancer diagnostics, early detection and treatment, and in so doing, save lives.

CHAPTER 2:

Critical Analysis of Literature on

Breast Cancer, Inherent Aerobic Capacity, and Metabolic Flexibility

2.1. Introduction

Breast cancer is the most commonly diagnosed type of cancer and second leading cause of cancer death among women in the US. The probability of developing breast cancer in women is 12.4%, that is 1 in 8 women will be diagnosed with breast cancer during their lifetime. It is estimated that in 2018 there will be 40,920 deaths from this type of cancer out of 266,120 diagnosed cases (1). There are several factors in breast cancer prognosis that have considerable impact on long-term morbidity and mortality that are associated with lifestyle choices. These include dietary pattern and amount of food consumed relative to energy expenditure. Chronic positive energy balance resulting in obesity is associated with poor prognosis in women with breast cancer, whereas a physically active lifestyle and participation in exercise training (which increases aerobic capacity) improves prognosis (20, 21, 23).

Targeting obesity with pharmacological agents as well as dietary energy restriction to induce intentional weight loss have been reported to have both positive and indifferent impacts on breast cancer prognosis (19, 25). Similarly, increasing physical activity levels in obese breast cancer patients has been reported to improve prognosis in some patients but have no effect in others (20). Studying mechanisms that underlie such contrasting effects of these intervention strategies is complicated since

mammary carcinogenesis is a complex disease involving genetic and environmental factors in its progression. Inherent and acquired factors are also the basis of susceptibility to obesity, which affects breast cancer development and prognosis. What is relatively unappreciated is that genetic and non-genetic mechanisms are operative in determining physical activity behaviors and how an individual responds to a program of exercise designed to improve fitness (aerobic capacity).

Aerobic capacity is a lens through which to study the effects of physical activity on cancer. Aerobic capacity is defined as the ability to transport oxygen from atmosphere to a cell's mitochondria in order to perform physical work as well as other related body functions. Increased aerobic capacity is a strong predictor of reduced risk to various diseases, including breast cancer (36), and of all-cause mortality (37). Because of the complexity of these processes, well-chosen animal models can play a pivotal role in deconstructing and identifying the mechanisms that account for improvement in prognosis. However, many animal models for studying complex diseases are inappropriate. Deficiencies include: disease simulation which mostly resembles a response to injury; specific gene approaches that contrast with the polygenic nature of the disease; introducing mutagenic development of the disease that has a different natural history than observed clinically; and isolating from external environmental factors of influence, both positive and negative (38). In this review, we will focus on two related animal models that show great potential in studying the mechanisms that underlie effects of aerobic capacity on mammary carcinogenesis. These models were developed for high and low aerobic capacity levels by divergent selection of outbred rats based on treadmill running ability (39). These models differ by

polygenic patterns of inheritance and show great potential in studying how exercise-induced improvement in aerobic capacity impacts the metabolic rewiring associated with breast cancer (35, 40, 41).

2.2. Pathophysiology of breast cancer

2.2.1. General information about breast cancer

Breast cancer is a complex group of diseases and is classified based on tumor size, lymph node involvement, histological grade, and menopausal status. Invasive ductal carcinoma comprises 80% of breast tumors, followed by lobular, tubular, medullary, and other special types of the disease. Gene expression profiles evaluated using hierarchical cluster analysis identified three molecular subtypes of breast cancer:

- Luminal A and B, which have higher expression of estrogen and progesterone receptors ([ER+ | PR+] HER2-/+); the carcinomas have a better prognosis, and respond positively to hormone therapy;
- HER2 positive, which overexpress epidermal growth factor receptor 2 ([ER- PR-] HER2+++) and upregulate its signaling, have high histological grade and a poor prognosis, and are mostly treated with chemotherapy;
- Basal-like, or triple negative, as they do not express any of aforementioned key receptors ([ER- PR-] HER2-), are considered to be the most aggressive with a poor prognosis, diagnosed at an early age and as large tumors with high histological grade and lymph node involvement commonly found (42-44).

With improvements in genetic and biochemical techniques, data on gene copy number alterations and gene expression profiles have been integrated with molecular signatures and pathological surrogates, so that breast cancer can be further divided into ten molecular subtypes. This provides an opportunity to improve treatment of breast cancer via tailored approaches according to molecular subtype. This is referred to as precision medicine. However, with all the therapeutic advances made in this field, breast cancer still remains the second leading cause of death from cancer and the most commonly diagnosed type of cancer in women (42-44).

2.2.2. Metabolic rewiring at the center of breast carcinogenesis

In addition to the classic hallmarks of cancer (sustained proliferative signaling, replicative immortality, invasion and metastasis, resistance to growth suppressors, evasion from cell death program and immune destruction, genomic instability, induction of angiogenesis and inflammation), tumor cells are also characterized by reprogrammed intermediate metabolism, i.e. metabolic rewiring (7). This was initially recognized in the 1920s and is referred to as the Warburg effect. Otto Warburg showed that cancer cells upregulate glycolysis despite availability of ambient oxygen. Increased glycolytic flux provides metabolic intermediates that are diverted to anabolic pathways, such as pentose phosphate pathways, synthesis of glycogen and nonessential amino acids. These anabolic pathways are also induced by the intermediates of the tricarboxylic acid (TCA) cycle, which are driven by the formation of oxaloacetate from carboxylation of pyruvate via glycolysis and of α-ketoglutarate via catabolism of glutamine. Such anabolic metabolism is also enabled by cellular signaling: cancer cells upregulate PI3K-

AKT-mTOR signaling that leads to not only protein synthesis, but also activation of hypoxia-inducible factor-1α (HIF-1α) and sterol regulatory element-binding protein 1 (SREBP-1) and a corresponding increase in glycolytic flux and fatty acid synthesis (45-47).

Even though breast cancer cells utilize other energy substrates, like amino acids or fatty acids, their main metabolism is shifted towards glucose utilization as upregulating glycolysis allows cells to increase their anabolic metabolism (45, 47, 48). This shift in metabolism is associated with amplification and/or overexpression of the oncogene transcription factor MYC which increases glucose metabolism by upregulating GLUT-1, lactate dehydrogenase, and other genes involved in glycolysis (47). Other changes in metabolism that shift breast cancer cell towards glycolysis include higher expression of: the β-subunit of the mitochondrial H^+-ATP synthase (β-F1-ATPase), heat shock protein 60 (Hsp60), the glycolytic glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and lactate dehydrogenase (45, 49).

2.2.3. Manipulation of metabolic reprogramming alters breast cancer development

A number of investigators have sought to determine whether metabolic reprogramming is essential to the carcinogenic process. Breast cancer cells overexpress hexokinase 2 and its knockout or depletion suppresses the malignant phenotype (50). Variable expression patterns of glucose transporter types have also been detected in breast tumor tissues and cancer cell lines and are associated with HIF-1, estrogen, and growth factor signaling. Increased GLUT-1-mediated glucose

uptake has been observed in 55 breast carcinomas, whereas typical for adipose and muscle tissues, GLUT-4 was overexpressed in various human malignant breast tissues, but not in breast cancer cell lines (46). Inhibition of pyruvate dehydrogenase kinase (PDK) activity not only reversed the glycolytic phenotype in metastatic breast cancer cells, but also reduced their growth and proliferation (48). Additionally, liver-metastatic breast cancer cells exhibit higher rates of conversion of glucose-derived pyruvate into lactate by increased expression of PDK and activity of its upstream factor—HIF-1α (51). Disruption of the HIF-1α pathway reversed the glycolytic phenotype (51) as well as decreased glucose uptake and viability of breast cancer cells (46). Interestingly, higher levels of HIF-1α as well as higher lactate production correlated with invasiveness of breast cancer cell lines (52). Breast cancer cells also overexpress and activate fatty acid synthase (FASN), involved in *de novo* fatty acid synthesis, and inhibitors of this synthase reduce viability and induce cell death as well as increasing susceptibility to anti-tumor drugs (46). Another study on 24 breast carcinomas revealed that FASN was significantly increased in tumor samples, whereas the levels of carnitine palmitoyltransferase I (CPT-1), a rate limiting enzyme that facilitates transport of long-chain fatty acids into mitochondria for oxidation, were decreased in the cytoplasm as well as nuclei (53, 54). In addition to FASN, increased levels and activity of acetyl-CoA-carboxylase (ACC) were also reported and point to importance of higher lipogenic profile for breast cancer cells survival (55, 56). Collectively, these findings are consistent with an obligatory role of metabolic reprogramming in the development and progression of breast cancer.

2.3. Comparing cancer metabolic reprogramming with the concept of metabolic flexibility

Metabolic homeostasis is characterized by diurnal oscillation in the amount of carbohydrate relative to fat that is oxidized as a fuel source and is reported as the respiratory quotient (RQ). This parameter represents cellular fuel partitioning between glucose (high RQ) and fatty acid oxidation (low RQ). Kelley and colleagues discovered that individuals that suffer from obesity or type 2 diabetes fail to change their RQ from low to high during the transition from the fasted to fed state. That is, obese people retain a glycolytic phenotype. The authors pointed out that inflexibility in regulating oxidation of glucose and fatty acids was related to insulin resistance and coined the term “metabolic inflexibility” to describe this phenomenon (57).

Reduced oxidative enzyme capacity and lower activity of CPT-1 as well as accumulation of triglycerides in muscle underlie metabolic inflexibility. In particular, skeletal muscles in obese individuals favored glucose utilization and lipogenesis instead of fat oxidation during fasting, unlike their lean counterparts (57). This predominance of glucose utilization as well as an anabolic profile were similar to that observed in breast cancer cells, especially based on downregulated CPT-1 (53, 54) and general pro-lipogenic profile (46, 55, 56). Therefore, we interrogated the idea that metabolic rewiring of cancer cells has features that overlapped with metabolic inflexibility. This could have translational importance in that it might provide an avenue by which to break the link between obesity and breast cancer.

2.3.1. Metabolic flexibility on a whole-body level

In metabolically flexible individuals, transition from fasting to feeding is associated with a switch from predominantly oxidative fatty acid metabolism to more glucose oxidation as well as glycolytic energy production in skeletal muscles upon high energy demand (Fig. 1) (57, 58). This switch is aimed to maintain metabolic homeostasis and shift from catabolic to anabolic metabolism, so that energy from dietary macronutrients can be efficiently stored in adipose tissue as well as in skeletal muscle and liver (58).

After a meal, there is a rise in the insulin/glucagon ratio owing to the stimulation of pancreatic β-cells by circulating glucose. In turn, insulin acts to decrease plasma glucose levels by promoting its uptake (via stimulating expression and translocation of glucose transporters, such as GLUT-4, to the plasma membrane of the cells) and utilization as well as preventing its *de novo* production. Peripheral tissues, such as skeletal muscles and liver absorb free glucose and store it in the form of glycogen. Adipose tissue, at this point, switches from lipolysis to lipid synthesis as insulin inhibits hormone-sensitive lipase. In adipocytes and hepatocytes, dietary fatty acids are re-esterified into triglycerides and release of non-esterified fatty acids is reduced. Altogether, it leads to clearance and lowers circulating free fatty acid and triglyceride levels. Therefore, the post-meal levels of glucose and lipids are maintained (59).

In contrast, when energy substrates are scarce or demand for them increases, the cells switch to fatty acid oxidation in response to a drop in the insulin/glucagon ratio. Glucagon promotes glycogenolysis and ketogenesis in the liver at the expense of

lipogenesis. Adipose tissue conducts lipolysis of its stored lipids and release of fatty acids into the circulation (60). Heart and skeletal muscles also switch to triglyceride breakdown and utilization of fatty acids as their predominant fuel but adipose tissue remains the main source of free fatty acids (60, 61).

Altogether, uptake, esterification, and release of free fatty acids underlies the role of adipose tissue as the main buffer that stores fatty acids during the fed state and supplies the peripheral organs with fatty acid during a fast, and thus, is an important determinant of metabolic flexibility (58, 60).

2.3.2. Cellular level of metabolic flexibility

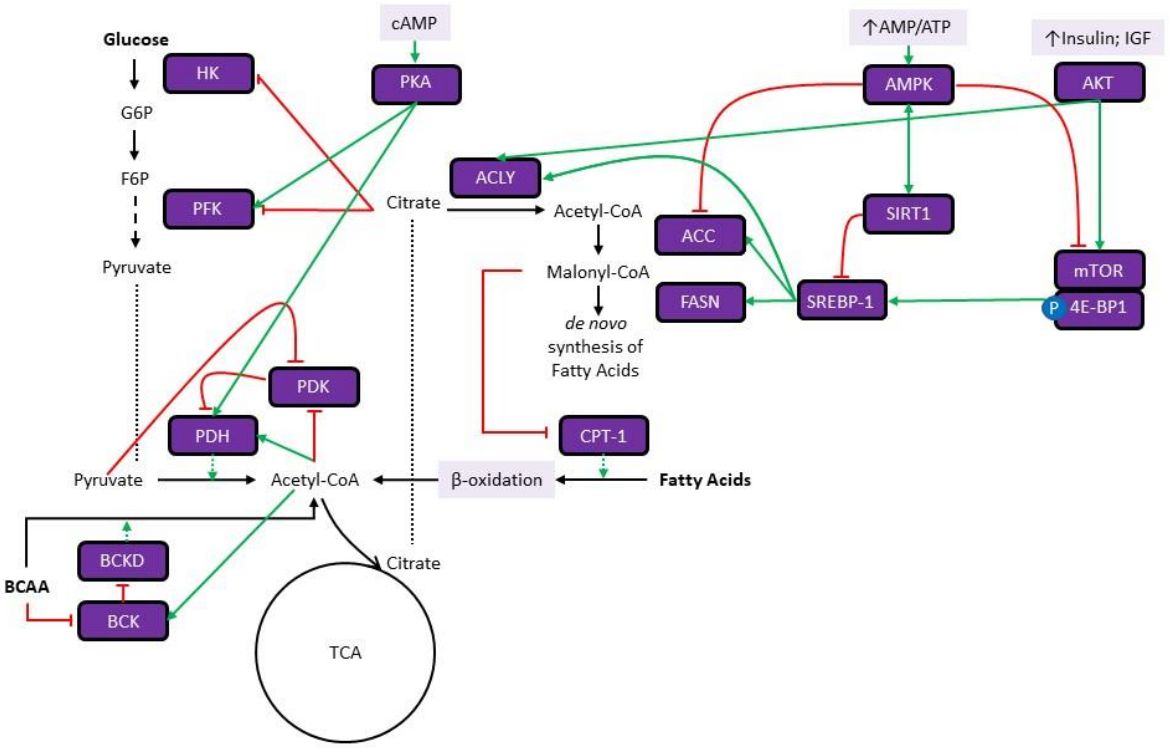


Figure 1. Metabolic flexibility in energy substrate utilization during transition from fasted to fed state.

Utilization of the main energy substrates, such as glucose or fatty acids, is reciprocally regulated upon their availability or energy demand. During fasting, fatty acids are oxidized while glucose utilization and fatty acid synthesis are blunted: high rates of β-oxidation provide acetyl-CoA which inhibits PDH and activates PDK, and thus, downregulates oxidation of glucose. Elevated AMP/ATP ratio activates AMPK which, via mTOR inhibition and SIRT1 activation, inactivates SREBP1 and its target, FASN-regulated de novo synthesis of fatty acids. AMPK also inactivates ACC and lowers level of malonyl-CoA. Uninhibited by malonyl-CoA, CPT-1 promotes further β-oxidation of fatty acids. Conversely, during feeding, glucose is oxidized in TCA promoting elevation in cytoplasmic citrate levels. In turn, citrate blunts β-oxidation by elevating levels of acetyl-CoA (via ACLY) and of malonyl-CoA (via ACC) which inhibits CPT-1. Utilization of BCAA is regulated by BCKD, which can be inhibited by its kinase, BCK. Increased cellular concentrations of energy substrates promote their own oxidation by inhibiting rate-limiting factors, such as PDK, BCK, or CPT-1. Signaling pathways, such as AMPK-SIRT1, are regulating energy deficit response and promote catabolic metabolism, whereas PKA and AKT-mTOR—anabolic response and glucose metabolism.

ACC, acetyl-CoA carboxylase; AMPK, 5' AMP-activated kinase; BCAA, branched-chain amino acids; BCKD, branched-chain ketoacid dehydrogenase; BCK, BCKD kinase; ACLY, ATP citrate lyase; CPT-1, carnitine palmitoyltransferase 1; G6P, glucose 6 phosphate; HK, hexokinase; PFK, phosphofructokinase; PDH, pyruvate dehydrogenase; PDK, PDH kinase; FASN, Fatty acid synthase; SREBP-1, sterol regulatory element-binding protein 1; SIRT1, silent mating type information regulation 2 homolog 1; mTOR, mechanistic target of rapamycin; 4E-BP1, 4E-binding protein; AKT, protein kinase B; PKA, cAMP-dependent protein kinase; β_2-AR, β_2-adrenergic receptor; IGF, insulin-growth factor.

At the cellular level, metabolic flexibility can be described through the lens of Randle Cycle as well as work of McGarry and colleagues (61-64). Feeding promotes mitochondrial glucose oxidation and lipogenesis at the expense of β-oxidation of fatty acids. Higher rates of glycolysis lead to production of pyruvate that activates pyruvate dehydrogenase (PDH) by deactivating pyruvate dehydrogenase kinase (PDK). Thus, the TCA cycle is constantly refilled with acetyl-coenzyme A (CoA) and produces increasing amounts of citrate, which in turn, is transformed back to acetyl-CoA by ATP-citrate lyase (ACLY) in the cytoplasm. Acetyl-CoA carboxylases (ACCs) are activated by and convert acetyl-CoA into malonyl-CoA. The resulting rise in the content

of malonyl-CoA not only is utilized for lipogenesis, but also inhibits CPT-1—the rate limiting enzyme that normally allows mitochondria to take up fatty acids for oxidation, thus downregulating β-oxidation (61-64).

A fasting-induced decrease in insulin and insulin-like growth factor 1 (IGF-1) levels activates forkhead box protein O (FOXO) transcription factors activity due to downregulated AKT leading to autophagy and/or stress resistance. Low levels of nutrients, especially proteins, downregulate mTOR signaling which is also achieved via the activation of the AMPK pathway. AMPK is activated by increased ratio of AMP/ATP in response to limited nutrients availability which also leads to increased ratio of oxidized to reduced nicotinamide adenine dinucleotides NAD^+/NADH activating Sirtuins, such as SIRT1, that deacetylate proteins and improve mitochondrial function (60). During fasting, energy-stress-induced AMPK can relieve inhibition of fatty acid oxidation by blocking ACC activity that lowers malonyl-CoA levels. Higher rates of β-oxidation result in the cellular accumulation of acetyl-CoA, ATP, and NADH. These molecules hinder PDH by allosteric inhibition and/or by activation of its kinase (PDK), and thus, uncouple glycolysis from glucose oxidation. In addition, the concomitant rise in cytoplasmic citrate that inhibits phosphofructokinase-1 and indirectly—hexokinase 2, by accumulation of glucose-6-phosphate, impedes glycolysis.

Branched-chain amino acids (BCAA) (leucine, isoleucine, valine), even though they are quantitatively less important, are considered to be an alternative fuel source during long-term caloric restriction as well as during exercise (60, 65, 66). BCAAs catabolism leads to a rise in acyl-CoA esters as well as NADH levels which reduce

BCAA breakdown to α-keto acids by inhibiting branched-chain ketoacid dehydrogenase (BCKD) complex. It can also be inhibited by its kinase (BCK), but α-keto acids downregulate it when BCAAs are abundant. In this way, BCAAs catabolism takes place only upon surplus of dietary protein or during prolonged starvation, but not during short-term caloric restriction (61). However, during caloric excess, the latter exert a feedback regulation and inhibit BCAA catabolism, too (60). Also, on a high-fat diet, insulin resistance is associated with disruptive BCAA metabolism and buildup of its metabolites. Altogether, they lead to aberrant β-oxidation and incomplete breakdown of fatty acids, all of which switch to glucose metabolism and reactive oxygen species (ROS) production (67).

Overall, the summary of metabolic flexibility in a cell is depicted in Fig. 1. A transition between fed and fasting states is ensured by metabolic flexibility in the switching among fuels and replenishing of substrate stores, thus maintaining the energy balance (61-64).

2.3.3. Metabolic flexibility during rest-to-activity transition

Physical exercise also requires metabolic flexibility to switch between glucose and fatty acid catabolism (58). Acute exercise is a potent activator of AMPK as it increases the AMP/ATP ratio, and favors fatty acid as a predominant fuel for oxidation. AMPK activation also increases translation of peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1-alpha (PGC-1α) which promotes mitochondrial biogenesis and expression of the enzymes of the electron transport chain (ETC). In addition, PGC-1α regulates oxidative phosphorylation via coactivation

of nuclear receptors, lipid uptake and oxidation in response to SIRT1-mediated deacetylation, and glucose uptake via GLUT-1/4 as well as gluconeogenic genes expression (68). Intensity and duration of exercise relies on the metabolic flexibility to switch from fatty acid to glucose oxidation (58).

Fatty acid oxidation contributes significantly to bouts of endurance exercise, while quantitatively and proportionally less during high intensity exercise (58). High-intensity exercise switches the muscle from utilization of fatty acids to glucose oxidation because supply of oxygen is limited. During even higher intensity exercise, anaerobic glycolysis becomes the predominant way to produce energy. Since insulin levels are generally low during physical exercise, such transition occurs in an insulin-independent manner (58). In anaerobic glycolysis, increasing levels of pyruvate are diverted from mitochondrial oxidation and converted into lactate and NAD^+ production. The latter maintains redox potential and substrate flux that stimulates glycolysis (69). Exercise also shifts cells, cardiomyocytes in particular, to glucose metabolism by stimulating sympathetic nervous system: β-adrenergic receptor stimulation leads to production of cyclic AMP and subsequent activation of cAMP-dependent protein kinase A (PKA). This enzyme stimulates phosphofructokinase and PDH forcing a cell to utilize glucose as a fuel regardless of availability of fatty acids (70). Atypical lipid metabolism causes triglyceride buildup, endoplasmic reticulum stress, mitochondrial dysfunction, and elevated ROS (71). Likewise, insulin may lead to the same outcomes via AKT signaling (70). Therefore, fatty acids' input into exercise performance is significant, but as exercise intensity and duration increase, the contribution of fatty acids declines (58, 60).

2.3.4. Metabolic flexibility in transition from energy supply to energy demand

Whole-body metabolic flexibility has been well described from the perspective of skeletal muscle, liver, and adipose tissue metabolism in accordance with fluctuations in energy requirements and energy substrate availability, for example during the transition between the fed and fasting states. When caloric availability is sufficient, animals preferentially utilize glucose, replete glycogen stores, and deposit lipids. However, acute scarcity of energy substrates, for example during sleeping or between meals, promotes lipolysis and fatty acid catabolism, which are intensified during the resting to exercise transition. Glucose utilization may increase with the intensity of the exercise and therefore, energy demands increase, up to the point of anaerobic glycolysis-derived energy acquisition (58). So, living beings seem to shift their physiology based on availability of energy sources. This flexibility in fuel selection underlies adaptability of the organism to maintain its normal physiological function and well-being.

2.3.5. Pathophysiology of metabolic inflexibility

Ad libitum feeding in humans is frequently associated with overconsumption of calorically dense processed foods that leads to a surplus of energy beyond the amount necessary for optimal cellular function and metabolic homeostasis. Evidence exists that smaller, more frequent meals are more conducive to the maintenance of energy homeostasis than larger and less frequent meals (72, 73). Such excess of dietary energy can overwhelm mitochondria diminishing their capacity for metabolic flexibility in fuel selection for oxidation and has been linked to development of metabolic diseases (61). Inability of cells to adjust oxidation to availability of particular fuels has been

observed in various cell types—from skeletal muscles and hepatocytes to adipocytes and immune cells—in a range of metabolic diseases, including obesity, diabetes, heart disease, nonalcoholic steatohepatitis, poly-cysticovarian syndrome, and even during physical inactivity (61). As mentioned before, Kelley and colleagues first described metabolic inflexibility in its association with insulin resistance (57).

Using an analogy with rush hour traffic, Muoio et al. proposed that carbon overload leads to “metabolic gridlock,” and thus, metabolic inflexibility. Catabolism of glucose, fatty acids, and even BCAAs lead to production of acetyl-CoA that enters TCA, as well as NADH that feeds electron transport chain, which altogether enables ATP generation. In normal physiology, metabolic signals match production of ATP to its demand in a reciprocal and coordinated manner by PDH, CPT-1, and BCKD that play the role of traffic lights. Higher rates of fatty acid utilization impede glucose and BCAA oxidation, and vice versa. Such metabolic flexibility suggests that during resting conditions and low energy demand, mitochondria function the best when acetyl-CoA is produced from one fuel at a time. However, chronic overload causes signal failure leading to persistent influx of energy fuels and a consequent traffic jam at the critical sites where the roads converge. Thus, mitochondria develop metabolic gridlock, in which the mitochondrial membrane potential increases and leads to elevated ROS production, resulting in the impairment of metabolic network activity via inhibiting disulfide bonds in proteins. This decreases the energy sensing function and ultimately leads to the development of metabolic diseases (61).

A number of studies place lower metabolic flexibility at the core of pathophysiology of insulin resistance, obesity, and cancer (58, 60, 61). High-fat, refined-carbohydrate diet, and physical inactivity, as well as even enhanced sympathetic nervous system activity are considered to be factors that lead to development of an insulin resistant phenotype (74). During such metabolic challenge, glucose utilization is favored, and a cellular increase in malonyl-CoA level leads to lipid storage, in the form of triglycerides, at the expense of fatty acid oxidation owing to inhibited CPT-1 (74). Consequently, cells store fats as well as other lipid metabolites, such as diacylglycerol and ceramides (58). After feeding, insulin-resistant patients demonstrate higher levels of circulating glucose, whereas after fasting, they fail to switch to fatty acid oxidation compared with healthy individuals (58, 60, 61). Even caloric restriction during insulin resistance renders skeletal muscles as well as other tissues unable to switch to fatty acid oxidation (75). On a high-fat diet, skeletal muscles of people that suffer from type 2 diabetes appear to have lower mitochondrial content and rely on metabolizing glucose.

2.4. Metabolic flexibility and inherent aerobic capacity

In rodents, metabolic inflexibility can be induced by fuel overload, such as feeding a high-fat diet, as well as other manipulations that lead to the development of metabolic syndrome and associated diseases (61). In response to this dietary challenge, metabolically inflexible animals are prone to obesity and insulin resistance as they are unable to increase their fatty acid oxidation rates at the expense of glucose (76). Interestingly, such susceptibility to metabolic diseases as well as protection from

them upon feeding the high-fat diet has been reported in two artificially selected rat strains that differ in their inherent aerobic capacity.

2.4.1. Developing animal models that differ by the level of inherent aerobic capacity

The ability of the organism to respond to exercise is defined by its capacity to transfer and utilize oxygen, and this capacity has two components—intrinsic, or inherent, (77-79) and adaptational, or induced (80, 81). Considering the complexity of this trait, Drs. Lauren Koch and Steven Britton developed animal models by divergent artificial selection (82). In this way, the contrasting variation of alleles was concentrated at the extremes and fixed at loci controlling the selected genotypes throughout generations, and thus such models are able to represent the complexity of quantitative traits that show continuous variation, such as height, blood pressure, and aerobic capacity (83).

To initiate these models, 96 males and females of N:NIH stock of genetically heterogeneous and outbred rats were subjected to a treadmill running protocol to select for the levels of endurance exercise capacity (78, 82). For the first week, the animals were exposed to treadmill running exercise gradually increasing each day to reach the ability to run for 5 min at a speed of 10m/min on a 15º slope as such intensity is considered to not affect significant changes in aerobic capacity and is consistent with the goal to select for low rather than zero aerobic capacity (82, 84, 85). During the second week, the exercise intensity was increased while animals ran till exhaustion. Animals also were evaluated for their treadmill running capacity by their total distance

run. Later, the 13 best and 13 worst performers of each sex were bred, and their offspring were subjected to the same capacity test at 10 weeks of age. Within-family selection was performed to avoid inbreeding and when the cycle of rotational breeding was completed, the matings were stopped to avoid sibling mating (level of inbreeding was ~0.96%/generation)(82).

The first generation showed over 3-fold difference in the running capacity for both males and females. By generation 6, female and male high capacity runners increased their performance by 140% and 131%, respectively, while the low line decreased their running capacity by 18% and 11%, respectively. Overall divergent response to selection was 171%. Selection for HIAC appeared to be of relatively larger magnitude (82). Perhaps, it is also due to zero being a limit for the low trait as a minimal aerobic capacity threshold may be under bigger evolutionary pressure, whereas at the high-end natural selection has no constrains in genetic variation (82, 83, 86).

Interestingly, differences in inherent aerobic capacity produced differences in other characteristics, such as body weight. By generation 6, LIAC were heavier (204 ± 4 g for females and 291 ± 4 g for males, $p < 0.001$) than HIAC (170 ± 2 g for females and 251 ± 3 g for males, $p < 0.001$) (82). Other characteristics for which these animals were incidentally selected, referred to as correlated responses as they are based on genetic components of phenotypic correlation produced by pleiotropy, appeared to be associated with traits responsible for health and disease (34, 83). Therefore, these HIAC and LIAC were used also as contrasting models for disease risks. After 36 generations of selection for inherent aerobic capacity, these animals

differed more than eight-fold in their treadmill running capacity as well as risks to development of various diseases. LIAC models are susceptible to the development of metabolic syndrome, neurodegenerations, cognitive impairment, fatty liver disease, cancer, and even shortened lifespan, while HIACs are resistant to them (87).

2.4.2. Higher inherent aerobic capacity corresponds to higher metabolic flexibility

On a chow diet, animals with high inherent aerobic capacity (HIAC) have higher oxidation of fatty acids, probably due to higher mitochondrial content in muscle and liver tissue, and generally greater adaptability to control food and energy intake when given diets that varied in their carbohydrate and fat content (88, 89). Animals with low inherent aerobic capacity (LIAC) displayed preference for carbohydrate oxidation and generally reduced whole-body fatty acid oxidation in addition to increased fatty acid trafficking to adipose depots, which was connected to elevated food intake and general positive energy balance (89). The weight of evidence indicates that LIAC animals are heavier and fatter than HIAC. This greater body weight and rates of weight gain, and increased feeding efficiency in LIAC animals appear to reflect metabolic inflexibility and were associated with susceptibility to metabolic diseases (89-91). HIAC animals also consumed more oxygen than LIAC animals regardless of the diet consumed and effectively utilized lipids for their activity fuel, based on their low RQ (90, 92).

On a systemic level, LIAC animals also distinctively exhibit markers of metabolic syndrome such as hypertriglyceridemia, hyperinsulinemia, higher circulating levels of free fatty acids, increased body weight gain, and visceral obesity (93-95). Evidence that LIAC animals are prone to obesity has also been reported (94-96). Additionally,

a number of studies reported that LIAC rely more on anaerobic glycolysis, mobilizing more glycogen, producing lactate, accumulating acetylcarnitines, which are markers for carbohydrate oxidation and impaired oxidation of fats (65, 97).

On a chow diet, HIAC animals show three times higher mitochondrial fatty acid oxidation in oxidative skeletal muscle despite LIAC animals had greater mitochondrial area measured by electron microscopy. However, the cytochrome *c* protein/total mitochondrial area ratio was greater in HIAC strain regardless of the diet. Thus, LIAC animals have larger mitochondrial area but not better mitochondrial function. Excess of dietary fat increased fatty acid oxidation—both complete (CO_2 production) and incomplete (production of acid-soluble metabolites)—up to no significant differences between populations. These observations were made after high-fat diet exacerbated LIAC's insulin resistance, whereas HIAC animals were protected from such effects (98).

Systems-level computational modeling confirms that HIAC and LIAC animals demonstrate differences in fuel utilization. Throughout the course of transient aerobic exercise, LIAC animals favor glucose utilization, whereas HIAC animals utilize fatty acids. However, after sustained periods of exercise, LIAC upregulate fatty acid oxidation and partly compensate for an initial deficit. Therefore, LIAC animals need more time to acclimate to intensity and duration of exercise in order to decrease the disparity in fuel utilization with HIAC counterparts. The authors also determined that whole-body metabolic phenotype of LIAC is associated with ~50% lower mitochondrial activity and 5-fold reduced mitochondrial fatty acid transport activity (99). Elevated rates of lipid metabolism correspond to more efficient coupling of acetyl-CoA from β-oxidation

processes with TCA cycle leading to metabolic benefits (100). HIAC animals had higher rates of hepatic fatty acid uptake and β-oxidative flux on high-fat diet (94). In contrast, LIAC animals reduce mitochondrial capacity for fatty acids oxidation, 2-pyruvate oxidation, as well as long-chain acylcarnitines (65, 94).

3-week caloric restriction in 50% food reduction induces HIAC animals to lose more weight than LIAC animals which seem to be preserving their fat mass. Although caloric restriction does reduce physical activity in both groups, elevated rates of energy expenditure in HIAC animals remain even during caloric restriction and regardless of sex, age, or body size. Therefore, LIAC animals are metabolically thrifty as they conserve their energy to a greater extent than HIAC animals (97).

Enzymatic control over fuel selection during exercise differs between HIAC and LIAC owing to increased control over fatty acid transport factors (CPT-1, CD36) in HIAC animals. CPT-1 activity was upregulated in HIAC animals, whereas LIAC animals displayed higher lactate transport. LIAC animals exhibit not only a reduction in mitochondrial density in white but not red gastrocnemius muscle, but also significantly greater acetylation and phosphorylation of enzymes involved in fatty acid transport and β-oxidation during exercise. In contrast, HIAC animals enrich proteins of oxidative phosphorylation and fatty acid metabolism (99). Another study demonstrates that LIAC rats display reduced hepatic mitochondrial content (by reduced citrate synthase activity and cytochrome *c* protein), reduced capacity to oxidize fatty acids yet increased *de novo* lipogenic profile. LIAC livers display elevated levels of SREBP-1 as well as its downstream target, ACC, in contrast with HIAC. Thus, the authors suggested that

activity of CPT-1 was reduced due to elevated malonyl-CoA levels in LIAC livers (101). Finally, HIAC animals activate AMPK pathway regardless of voluntary wheel-running program, high-fat diet, or ovariectomization (102), whereas high-fat diet significantly reduces AMPK activity in LIAC strain which was associated with insulin resistance (103).

Overall, susceptibility as well as certain preference for beneficial effects of physical activity that are present in HIAC animals appear to be connected to their ability to maintain energy homeostasis and metabolic flexibility. The latter is best demonstrated during transitions from feeding to fasting or from resting to acute exercise when external factors induce the body to respond by selecting certain energetic fuels to meet its requirements. In turn, inability to shift metabolism upon high or low energy demand as well as availability of energy resources is associated with various diseases risks, including obesity and cancer (58, 60, 61). Animals that did not undergo any physical training and differ in their inherent capacity for oxygen-dependent physiological processes seem to have different flexibility in fuel utilization as well as susceptibility to the same aforementioned diseases (99).

2.4.3. Conclusions

Based on our literature analysis, a crosstalk among the metabolic rewiring associated with the cancer development, the metabolic fuel oxidation pathway associated with inherent aerobic capacity, and the metabolic paradigm underlying metabolic flexibility emerges. Animals with LIAC are associated with predominant glucose utilization (89, 94, 99), even glycolytic signature (65, 97, 104) and concomitant

de novo lipogenesis (89, 99, 101). Breast cancer cells also exhibit the same profile (46, 51-55). Glucose utilization is correlated with *de novo* synthesis of fatty acids, and switching from the synthesis of the latter to their utilization upon certain energy demand is regulated by metabolic flexibility (58, 60, 61). Lower metabolic flexibility leads to development of metabolic diseases, such as insulin resistance and obesity (58, 60, 61, 105), which positively correlate with breast cancer development (20, 21, 23). Again, LIAC animals show significant susceptibility to these diseases, unlike HIAC animals (89-91, 91, 93-95, 98). In contrast, HIAC animals metabolize predominantly fatty acids upon energy demands (90, 92, 97-99). Fatty acid transport is crucial for fuel selection in HIAC and LIAC animals. Unlike LIAC, HIAC animals can switch between fatty acid and glucose metabolism in a physiological manner (99). Interestingly, HIAC animals also correlate with factors associated with anti-tumor effects. For example, HIAC cells upregulate AMPK (102, 103, 106) which not only is associated with fatty acid catabolism (60, 68) but also with anti-tumor effects (43, 107, 108). Therefore, HIAC and LIAC animal models reveal the common ground between metabolic homeostasis, cancer, and aerobic capacity.

Cancer cells, in general, preferentially oxidize glucose and glutamine as energy substrates. However, there are other features of malignancy associated metabolism present in tumor cells depending on cancer type: utilization of intermediates from glycolysis and/or TCA cycle for biosynthesis and reduced production of NADPH; aberrant glucose and amino acids uptake; plasticity in energy substrate acquisition; changes in gene regulation driven by metabolites; increased demand for nitrogen, and metabolic communication with tumor microenvironment (7, 60). High-fat diet leads to

upregulated uptake of fatty acids via stimulated CD36 expression of the tumor cells as well as their metastasis (109). Animal-derived dietary amino acids are also associated with cancer progression (110). However, fasting reduces incidence and development of cancer by downregulating AKT signaling and thus, reducing mTOR activity and altering levels of insulin or IGF-1 (60, 110). Increased body weight and low levels of physical activity are also associated with cancer (24, 111). Obesity, insulin resistance, type 2 diabetes, and metabolic syndrome in general are common risk factors for neoplastic transformation owing to insulin/IGF-1 signaling, and inflammation, respectively (24, 111, 112). Thus, it suggests that metabolic inflexibility positively correlated with cancer (60).

Cancer cells exhibit a decrease in metabolic flexibility to the point of metabolic "addiction" for specific nutrients. Even though such rigidity renders them vulnerable to metabolic fluctuations, the cells compensate with overstimulated growth and proliferation. PI3K-AKT pathway renders cells more reliant on glucose, whereas MYC—on glutamine. Metabolic interventions that would make these substrates less available for the tumor cells have been one of the therapy targets. Cancer cells meet their energetic requirements from mainly glycolysis but also from oxidative phosphorylation, or even both. Either way, tumor cells rely more on glucose metabolism as decreasing amounts of available glucose have a detrimental effect on tumor progression (113).

Physical activity is associated with a reduction in risk for cancer development. Such advantageous effects are associated with an increase in an individual's aerobic capacity based on the fact that moderate levels of activity exert a beneficial effect. Endurance exercises at 40% of individual's V_{O_2max} are recommended to exert detectable

improvement in cancer prognosis. Increases in aerobic capacity have also been associated with improvement of quality of life and general psychological and physical benefits for patients with cancer (114). Lower levels of aerobic capacity have been associated with markers of metabolic syndrome as well as a hormonal profile favoring cancer progression. Male patients with low values of $V_{O_2}max$ have higher serum concentrations of IGF-1 and lower concentration of its binding protein (IGFBP-3) and of testosterone—factors that are associated with progression of prostate cancer (115). Exercise interventions improved physical parameters, metabolic condition, and even quality of life of breast cancer patients following an increase in their aerobic capacity. Interestingly, physical condition as well as levels of aerobic capacity in women with breast cancer have been determined to be lower than population norms (116).

Previously, our laboratory demonstrated that female HIAC animals had not only smaller fat depots but also better cancer prognosis. Tumor multiplicity and incidence were lower compared with LIAC animals. At the same time, the cancer latency of the latter was significantly lower than such of HIAC animals. Animals with higher IAC upregulated their AMPK activity and downregulated PI3K-AKT pathway, and thus, showed reduced activity of mTOR signaling in their mammary gland. The authors proposed that these pathways as well as higher IAC are associated with protection from mammary carcinogenesis (35).

CHAPTER 3:

Difference in Network Signaling in Mammary Cancers Occurring in Rats with Low or High Inherent Aerobic Capacity

3.1. Introduction

Lifestyle choices represent risk factors for breast cancer, which is the second leading cause of death in the United States among women. One in eight women will develop this malignancy in their lifetime; and it is expected that over a hundred women already diagnosed will die every day in 2018 (1). Such habits as heavy alcohol consumption, unhealthy diet, sedentary way of life, and especially obesity have been associated with breast cancer morbidity and mortality. In contrast, regular physical activity is considered a beneficial behavior that reduced not only breast cancer risk, but also the susceptibility to the development of obesity, cardiovascular diseases, and type 2 diabetes, which are common comorbidities in breast cancer patients (117, 118). The range of physical activity behaviors are quite diverse for humans (119), which makes it of interest to determine what underlies this variation. Knowing intrinsic mechanisms that render the human body not only more or less physically active but also comparatively responsive to physical activity-mediated health benefits is translationally important in cancer prevention and treatment.

Assessment of physical activity levels has an inherent bias due to human error in self-reporting the amount, type, and intensity of physical activity. However, an individual's aerobic capacity—which can be measured as maximal oxygen uptake—is a

more objective and reliable factor that predicts exercise-induced health benefits (28). Low levels of aerobic capacity have been found to be a strong predictor of all-cause morbidity and mortality. Although, higher levels of aerobic capacity are associated with better cancer prognosis, the evidence is quite mixed (114), and thus, requires more thorough investigation in order to identify the conditions of positive response to a particular program of exercise.

Regular moderate to high intensity physical activity leads to increased maximal oxygen uptake levels and corresponds to the induced component of aerobic capacity. However, improvement in aerobic fitness has a heritable component which accounts for about 50% of the variability among individuals in their responsiveness to the same defined exercise training program (33, 120). The impact of this heritable component of aerobic capacity on disease outcomes, particularly cancer, is poorly understood.

In order to provide a means for the systematic investigation of intrinsic fitness, Drs. Koch and Britton developed experimental animal models using artificial two-way selection in the outbred N:NIH strain of rat. Rats were selected for their performance in a graded treadmill running test. Britton/Koch obtained two rat strains with high and low levels of IAC—high capacity runners, or HIAC, and low capacity runners, or LIAC, respectively. Not only did these animals diverge in their inborn aerobic capacity and ability improve aerobic fitness in response to exercise training, they also exhibited different susceptibility to disease risks that span from metabolic to cardiovascular and neurodegenerative diseases (34). LIAC animals develop insulin resistance and are prone to obesity owing to altered mitochondrial function, energy utilization, and energy

expenditure. Subsequently, metabolic disorders appear to emerge from lower metabolic flexibility of low IAC rats (121).

Such disparity in maintaining metabolic homeostasis by HIAC/LIAC strains indicates their suitability for studying molecular mechanisms that underlie the nature of IAC and its connection to health and disease (34). However, the majority of work on aerobic capacity has been done on muscle tissue as it is the predominant hub linking physical activity to its effects on the cells (122-126). Nevertheless, low IAC has been reported to result in pathophysiologic changes in other tissues (87, 127, 128). Recently, our laboratory showed that low inherent aerobic capacity was associated with higher risk of mammary carcinogenesis in comparison to rats with high inherent aerobic capacity (35).

HIAC and LIAC animals respond differently to carcinogen (1-methyl-1-nitrosurea (MNU)) administration—LIAC animals are more prone to formation of breast cancer unlike their HIAC counterparts. Different rates of carcinogenic response in these two strains were observed in the absence of any prior physical training, which is consistent with an intrinsic origin of these effects (35). Our laboratory demonstrated different levels of protein expression within the mammary gland of animals with low or high IAC: HIAC animals featured more activated AMPK and downregulated AKT pathways compared with LIAC, and resulted in decreased mTOR signaling pathway activity (35). As detailed in Chapter 2, the signaling activity in the mammary gland that distinguished between LIAC and HIAC is characteristic of the differences in metabolic flexibility associated with type 2 diabetes and obesity. In this chapter, we report on characteristics of

the mammary cancers that occurred in the experiments previously reported (35) and investigate whether the pattern of signaling activity observed in mammary carcinoma in HIAC and LIAC is similar to or distinct from that observed in the mammary gland.

This chapter will be the basis of a manuscript to be submitted to Cancer Prevention Res. Fall 2018.

3.2. Materials and Methods

3.2.1. Animals

Male and female rats categorized by phenotype as with high inherent aerobic capacity (HIAC) and with low inherent aerobic capacity (LIAC) from generation 29 of selection were tested for their maximum running capacity at 10 weeks of age according to our standard selection procedure (65). Briefly, endurance trials were performed on an inclined (15 degrees) motorized treadmill (Columbus Instruments, OH) on 5 consecutive days. The treadmill velocity began at 10 m/min and was increased by 1 m/min every 2 min until exhaustion. Exhaustion was operationally defined as the third time a rat could no longer keep pace with the speed of the treadmill and remained on the shock grid for 2 s. At this point the total distance run was recorded. The single best distance of the 5 trials was considered as the performance indicator most closely associated with the heritable component of endurance running capacity. Using these data, 8 breeding pairs of LIAC and of HIAC rats were selected. Whereas the founder population had a capacity to run for 355 ± 144 m (23.1 min) until exhaustion, the generation 29 LIACs averaged 260 ± 60 (18.3 min) and the HIACs ran for 2106 ± 273 (74.6 min). The female offspring of these breeding pairs were used for the carcinogenesis experiment.

3.2.2. Carcinogen administration and tumor detection

A carcinogenesis experiment was conducted in which 21-day old female LIAC and HIAC rats were injected intraperitoneally with 1-methyl-1-nitrosurea (MNU, 70mg/kg, Ash Stevens, Detroit, MI) according to published procedure (129). A total of 56 LIAC and 56 HIAC female rats were assigned to each group serially as they became available from the breeding colony. The statistical power of this study was 80% to detect an effect size of 0.5 in incident cancer between the LIAC and HIAC groups based on an incidence of 50% in the N:NIH founder population determined in a preliminary experiment.

Rats were group-housed (3 per cage) in solid bottomed polycarbonate cages and fed a standard laboratory diet for rodents (Harlan 2918 Teklad Lab Animal Diet). All rats remained sedentary, i.e., physical activity was limited to spontaneous activity within their group housed setting, throughout the study. Animal rooms were maintained at 22 ± 1°C with 50% relative humidity and a 12-h light/12-h dark cycle. Rats were weighed weekly and were palpated for the detection of mammary tumors twice per week starting from three weeks post carcinogen. The study was terminated 33 weeks post carcinogen injection.

At necropsy, following an overnight fast, rats were euthanized over a 3-hour time interval, between 8 a.m. and 11 a.m. via inhalation of gaseous carbon dioxide. The sequence in which rats were euthanized was stratified across groups to minimize the likelihood that order effects would masquerade as treatment associated effects. After the rat lost consciousness, blood was directly obtained from the retro-orbital sinus

and gravity fed through heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) into EDTA coated tubes (Becton Dickinson, Franklin Lakes, NJ) for plasma. The bleeding procedure took approximately 1 min/rat. Thereafter, the unconscious rat was euthanized by cervical dislocation. Rats were then skinned and the skin to which mammary gland chains were attached was examined under translucent light for detectable mammary pathologies at 5x magnification. All detectable mammary gland pathologies were excised, weighed, and a cross section prepared for histological classification according to published criteria (130). The remainder of each mammary pathology weighing more than 100 mg was snap frozen in liquid nitrogen. After excision of detectable pathologies, the abdominal-inguinal mammary gland chains were excised and processed as whole mount preparations per published method (130). The left mammary gland chain was prepared on glass and immediately fixed in 10% neutral buffered formalin, whereas, the right chain was spread out on transparency film (3M Corporation) and immediately frozen in liquid nitrogen. With a team of 8 technicians, the average time from cervical dislocation to fixing or freezing tissue was 4 min.

This experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee and conducted according to the committee guidelines at Colorado State University.

3.2.3. Immunohistochemical assessment of hormone receptor status

Progesterone receptor status of mammary carcinomas was determined using our previously published procedure (131).

3.2.4. Protein expression profiling

A comprehensive list of signaling molecules and pathways linking exercise-induced aerobic capacity and the development of breast cancer in the rat mammary cancer model, was compiled based on published work (35, 132-137). This list provided the rationale for subsequent experiments on tissue.

3.2.5. Western blotting

Western blotting was used in a manner analogous to the use of protein array technology. Our method has been reported (137). The chemiluminescence signal was captured using a ChemiDoc densitometer (Bio-Rad) that was equipped with a CCD camera having a resolution of 1300 x 1030. Quantity One software (Bio-Rad) was used in the analysis. The Quantity One software has a warning algorithm that notifies the user if pixel density is approaching saturation so that all signals used for analysis are in the linear range. All Western blot signals were within a range where the signal was linearly related to the mass of protein and actin-normalized scanning density data were used for analysis.

3.2.6. Data evaluation

Differences in cancer burden were evaluated by the nonparametric Kruskal-Wallis test (138). Data from Western blot analyses were evaluated via unsupervised and supervised multivariate techniques for hypothesis generation per our previously published approach (139). Principal component analysis (PCA) was used to determine relationships between LIAC and HIAC with no prior knowledge of class

membership. Orthogonal projections to latent structures for discriminant analysis (OPLS-DA), a supervised, class-based method where class membership is assigned and used to elicit maximum data separation, was applied using the class information to partition variation into predictive and orthogonal components The contribution of each component partitioned into between-class (predictive) and within-class (orthogonal) variance was also estimated (140-143). Scatter plot of the first two score vectors for the PCA models were drawn, along with 95% confidence ellipses based on Hotelling's multivariate T^2, to identify outliers that might bias the results. For OPLS-DA, class separation was shown in several ways:

- Biplot was used to co-chart scores and loadings from the OPLS-DA model for their simultaneous display and interpretation. Thus, this plot displays similarities and dissimilarities among observations and permits interpretation of the observations in terms of the variables. Observations (proteins) situated near variables (HIAC/LIAC) are high in those variables and are low in variables situated opposite. Observations close to the plot origin have average properties. Variables close to the plot origin do not contribute to the formation of the scores in question, i.e. they are poorly described by the model components.

- S-plot was constructed to identify influential proteins in the separation of treatment groups. S-plots based on the first principal component show reliability (modeled correlation) plotted against feature magnitude (loadings or modeled covariance). If protein concentrations have variation in correlation and covariance between classes, this plot will assume an S-shape, with heavily influential

features separating from other features at the upper right and lower left tails of the feature cloud within the model space (141, 143).

- Variable importance in the projections (VIP) plot was also computed. VIP expresses the influence on Y (class assignment, i.e., LIAC versus HIAC) of every variable x_k. The VIP for each variable is expressed with its respective 95% confidence interval. VIP can be compared with one another. VIP greater than 1 are considered most relevant to accounting for class separation, whereas, VIP values less than 0.5 are considered unimportant. VIP scores between 0.5 and 1.0 are considered to be in a gray area relative to interpreting their importance to class separation. All multivariate analyses were done using SIMCA-P+ v.12.0.1 (Umetrics, Umea, Sweden).

3.3. Results

In order to extend our understanding of how inherent aerobic capacity was affecting tumor development, we focused several analyses on the tumors that were induced.

3.3.1. Characteristics of induced tumors

Tumor size and hormone receptor status are indicators of breast cancer prognosis. As shown in Fig. 2, cancer burden (tumor mass per number of rats in the group) was markedly higher in rats with low inherent aerobic capacity: 1.14 ± 0.45 g/rat in LIAC vs. 0.21 ± 0.16 g/rat in HIAC, $p < 0.001$.

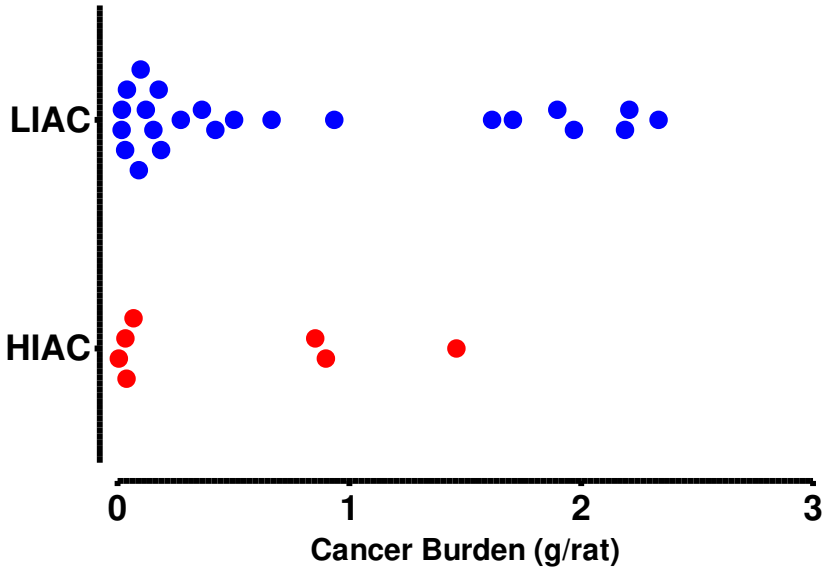


Figure 2. Effect of inherent aerobic capacity on tumor burden.

Total tumor mass per rat was computer and is displayed for rats with high (HIAC) or low (LIAC) inherent aerobic running capacity. Cancer burden was 0.21 ± 0.16 g/rat, $p < 0.001$, in HIAC, which was significantly lower than in LIAC (1.14 ± 0.45, $p < 0.001$)

Next, we stained all tumors classified as adenocarcinomas for PR as an indicator of functional steroid hormone signaling. We took the data for the percent of cells with positive staining and co-graphed it with tumor mass and the day post carcinogen on which the carcinoma was detected (Fig. 3). While the mean percent PR staining did not vary statistically by aerobic capacity status, visual inspection of the bubble chart shows wide variation in PR staining in the LIAC group indicating the presence of both steroid hormone receptor responsive and non-responsive tumors in the LIAC group, whereas the PR staining of HIAC tumors was more uniform and indicative of tumors with a more favorable prognosis. These findings suggest differences in the cells of origin within the mammary epithelium that give rise to mammary carcinomas and point to the potential

value of studying how aerobic capacity affects cell differentiation within the mammary gland.

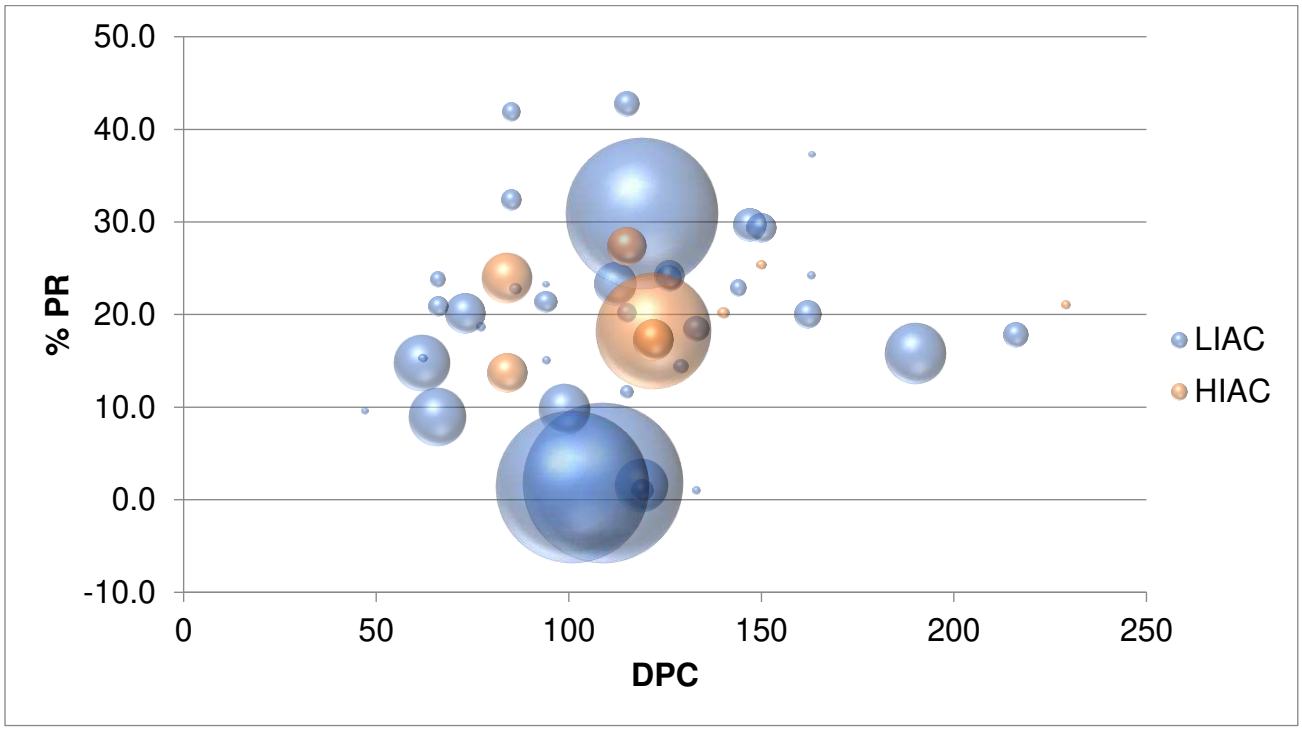


Figure 3. Effect of inherent aerobic capacity on tumor hormone receptor status.

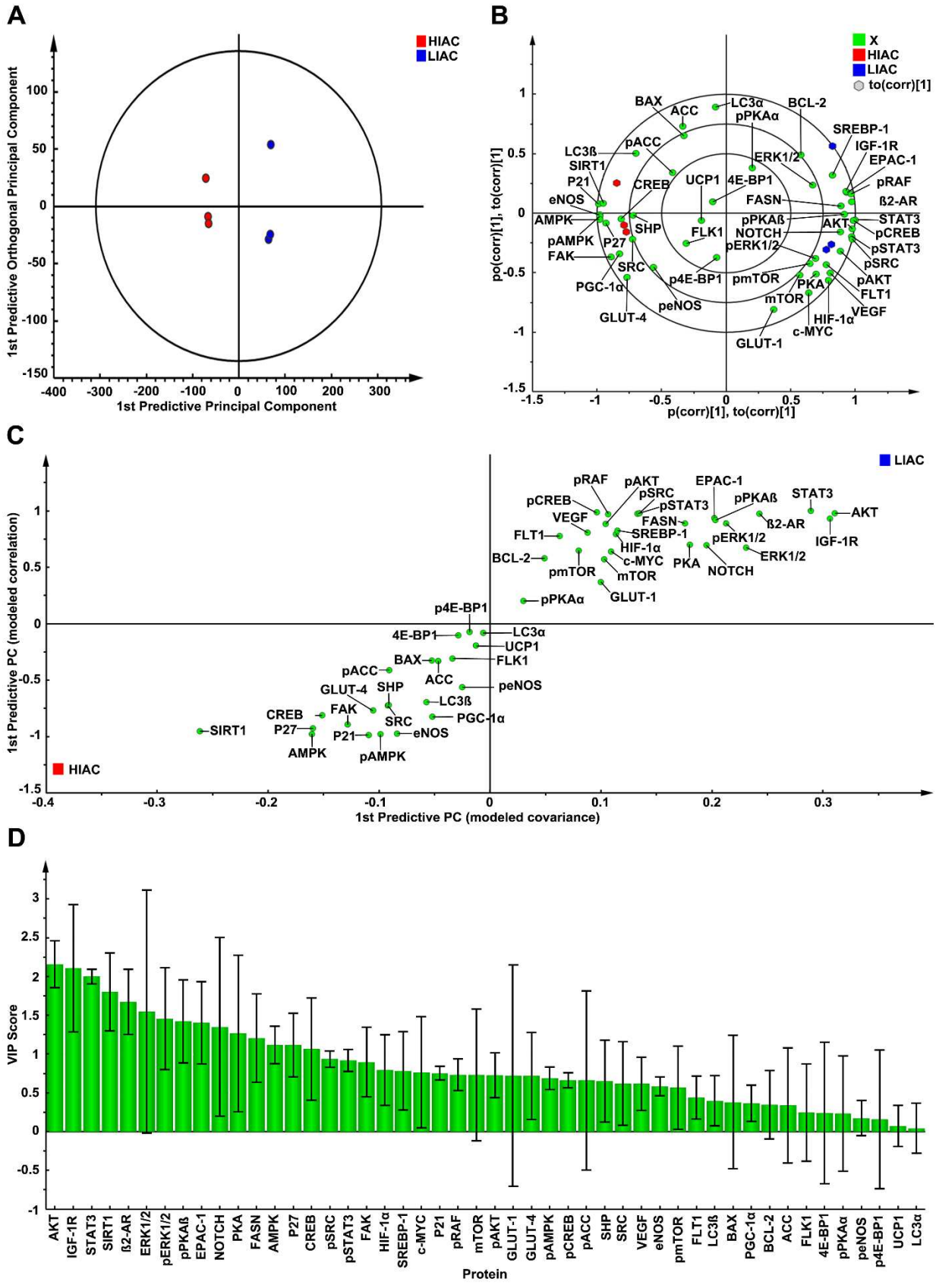
For all palpable mammary carcinomas, percent of cells staining positive for progesterone receptor (% PR) was determined. This data, plus the mass of the tumor determined gravimetrically at necropsy and the date (days post carcinogen, DPC) that the mass was initially detected by palpation were used to construct a bubble plot with the size of each bubble reflecting relative tumor mass.

3.3.2. Mammary tumor protein expression

While the number of mammary tumors suitable for analysis in the HIAC group was limited because of tumor size, three HIAC tumors were available and matched to three tumors in the LIAC group based on weight, time of detection post carcinogen

injection, and PR status. Our underlying assumption was that while protein expression patterns were dissimilar in mammary glands from LIAC versus HIAC, the occurrence of a tumor would result in tumors with similar patterns of protein expression (144).

The protein expression data were mean-centered, Pareto-scaled, and evaluated using unsupervised PCA (Fig. 4A). There was complete separation between groups, thus favoring the dissimilarity hypothesis. The model had 2 components with cross-validated predictive ability, $Q^2(Y)$ = 46.7%, and total explained variance, $R^2(X)$=81.8%. The same data set was then subjected to supervised OPLS-DA and cross-validated predictive ability of the model, $Q^2(Y)$, increased to 99.9% with 74% of total variance, $R^2(X)$, explained. The biplot for this model (Fig. 4B) shows not only the scores plot depicting the separation among tumors in LIAC versus HIAC, but also the proteins most influential in accounting for the observed separation. These effects were further quantified via S-plot analysis (Fig. 4C) and variable importance for projection analysis (VIP, Fig. 4D).


A
HIAC
LIAC
1st Predictive Orthogonal Principal Component
1st Predictive Principal Component
B
X
HIAC
LIAC
to(corr)[1]
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BAX
ACC
LC3α
pPKAα
BCL-2
SREBP-1
IGF-1R
EPAC-1
LC3ß
pACC
ERK1/2
SIRT1
P21
CREB
UCP1
4E-BP1
pRAF
eNOS
FASN
ß2-AR
AMPK
SHP
FLK1
pPKAß
AKT
STAT3
pAMPK
P27
NOTCH
pCREB
FAK
pERK1/2
pSTAT3
SRC
pSRC
PGC-1α
p4E-BP1
pmTOR
PKA
pAKT
FLT1
mTOR
peNOS
VEGF
GLUT-4
HIF-1α
c-MYC
GLUT-1
C
LIAC
HIAC
1st Predictive PC (modeled correlation)
1st Predictive PC (modeled covariance)
pRAF
pAKT
EPAC-1
pCREB
pSRC
pPKAß
STAT3
pSTAT3
AKT
VEGF
FASN
ß2-AR
FLT1
SREBP-1
pERK1/2
IGF-1R
HIF-1α
BCL-2
c-MYC
PKA
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NOTCH
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UCP1
BAX
FLK1
pACC
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peNOS
GLUT-4
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SREBP-1
c-MYC
P21
pRAF
mTOR
pAKT
GLUT-1
GLUT-4
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eNOS
pmTOR
FLT1
LC3ß
BAX
PGC-1α
BCL-2
ACC
FLK1
4E-BP1
pPKAα
peNOS
p4E-BP1
UCP1
LC3α

Figure 4. Analysis of protein expression in the mammary carcinoma using a supervised clustering algorithm

Effects of high (HIAC) or low (LIAC) inherent aerobic running capacity on patterns of protein expression in the mammary carcinoma (n=3/group) were assessed by multivariate discriminant analysis. Initially, inherent clustering patterns were determined by unsupervised analysis through PCA and complete separation of treatment groups was observed.

A. To determine contributing sources of variation, the scatter plot represents supervised analysis of the 2-class OPLS-DA model, which rotates the model plane to maximize separation due to class assignment. Complete separation of the 2 classes was observed.

B. To determine the proteins responsible for class separation multivariate analysis was used to construct a biplot that identified influential proteins responsible for the separation between classes.

C. An S-plot was constructed by plotting modeled correlation in the first predictive principal component against modeled correlation from the first predictive component. Upper right and lower left regions of S-plots contain candidate proteins with both high reliability and high magnitude discriminatory proteins.

D. To determine the statistical reliability of the effects, variable importance plots were generated in which jack-knifed confidence intervals (JKCI) were created on the magnitude of covariance in the first component for the analytes assessed. Proteins with JKCI including 0 were considered not to account for separation.

These data indicate that CKIs—p21 and p27—were significantly higher in HIAC tumors, whereas higher expression of pERK1/2, pAKT, pmTOR was associated with LIAC tumors. Relative to apoptosis, higher BAX expression was associated with HIAC tumors while higher BCL-2 expression was associated with LIAC tumors, although those associations, i.e. as indicated by the VIP plot, were weak relative to the effects exerted by p21 or p27. The focus of analysis then shifted to examining what regulatory proteins were associated with the tumor locations on the scores plot using the vector locations on the biplot. This examination revealed higher levels for activated AMPK and

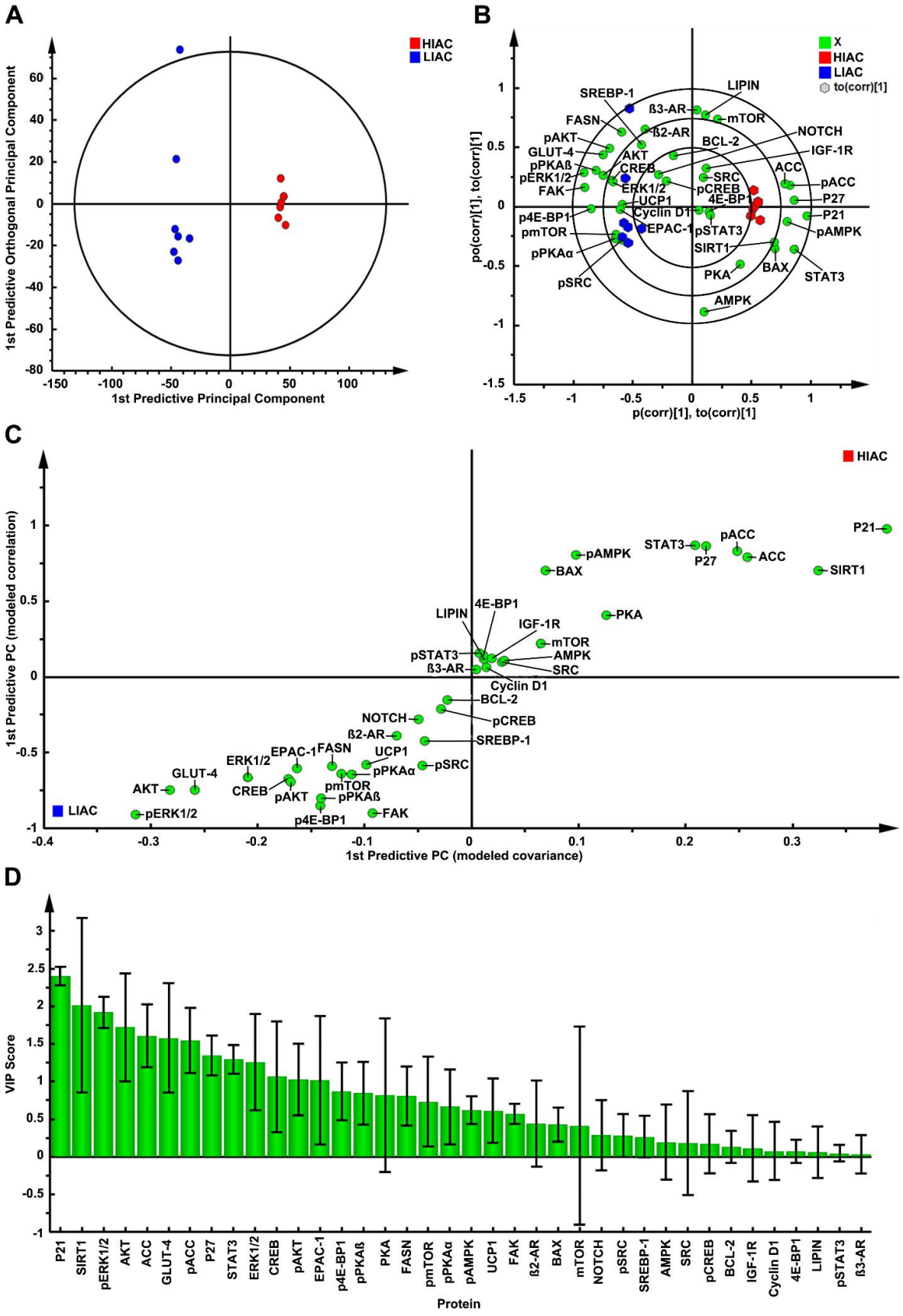
of SIRT1 in immediate proximity to the score values for the tumors and the vectors for p21 and p27, whereas patterns of protein expression characteristic of Notch and activated PKA co-localized with LIAC tumors and the factors associated with glycolytic phenotype. Higher levels of proteins associated with *de novo* lipid synthesis (FASN, SREBP1) were also associated with LIAC.

3.3.3. Mammary gland protein expression

Next, we analyzed the mammary gland for the expression of the similar array of proteins for which mammary tumors were evaluated. Our analyses were confined to pathology free mammary tissue which is comprised predominately of mammary epithelial cells, adipocytes and the stroma in which the mammary epithelium is embedded. The expression data were mean-centered and Pareto-scaled.

Our first objective in examining the protein expression data in the mammary gland was to explore the question of whether the pattern of expression was similar or dissimilar in LIAC versus HIAC mammary gland. Our expectation was that the patterns would differ given that LIAC glands were more susceptible to tumor occurrence than HIAC glands.

Data were subjected to unsupervised PCA (Fig. 5A). PCA resulted in complete separation with 100% classification accuracy supporting the dissimilarity hypothesis. The same data set was then subjected to supervised OPLS-DA and cross-validated predictive ability of the model $Q^2(Y)$ was 96.9% with 65.3% of total variance, $R^2(X)$, explained.


A
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1st Predictive Principal Component
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SREBP-1
LIPIN
ß3-AR
mTOR
FASN
ß2-AR
NOTCH
pAKT
BCL-2
IGF-1R
GLUT-4
AKT
pPKAß
CREB
ACC
pERK1/2
SRC
pACC
ERK1/2
pCREB
FAK
4E-BP1
P27
UCP1
Cyclin D1
P21
p4E-BP1
EPAC-1
pSTAT3
pAMPK
pmTOR
SIRT1
pPKAα
BAX
PKA
STAT3
pSRC
AMPK
C
HIAC
LIAC
1st Predictive PC (modeled correlation)
1st Predictive PC (modeled covariance)
P21
pACC
STAT3
pAMPK
P27
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BAX
SIRT1
4E-BP1
LIPIN
PKA
IGF-1R
mTOR
pSTAT3
AMPK
ß3-AR
SRC
Cyclin D1
BCL-2
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ß2-AR
SREBP-1
ERK1/2
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pPKAß
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UCP1
FAK
ß2-AR
BAX
mTOR
NOTCH
pSRC
SREBP-1
AMPK
SRC
pCREB
BCL-2
IGF-1R
Cyclin D1
4E-BP1
LIPIN
pSTAT3
ß3-AR

Figure 5. Analysis of protein expression in the mammary gland using a supervised clustering algorithm

Effects of high (HIAC) or low (LIAC) inherent aerobic running on patterns of protein expression in the mammary gland (n=7/group) were assessed by multivariate discriminant analysis. Initially, inherent clustering patterns were determined by unsupervised analysis through PCA and complete separation of treatment groups was observed.

A. To determine contributing sources of variation, the scatter plot represents supervised analysis of the 2-class OPLS-DA model, which rotates the model plane to maximize separation due to class assignment. Complete separation of the 2 classes was observed.

B. To determine the proteins responsible for class separation multivariate analysis was used to construct a biplot that identified influential proteins responsible for the separation between classes.

C. An S-plot was constructed by plotting modeled correlation in the first predictive principal component against modeled correlation from the first predictive component. Upper right and lower left regions of S-plots contain candidate proteins with both high reliability and high magnitude discriminatory proteins.

D. To determine the statistical reliability of the effects, variable importance plots were generated in which jack-knifed confidence intervals (JKCI) were created on the magnitude of covariance in the first component for the analytes assessed. Proteins with JKCI including 0 were considered not to account for separation.

The biplot for this model (Fig. 5B), S-plot analysis (Fig. 5C), and VIP analysis (Fig. 5D) indicated that p21, p27, BAX, activated AMPK, ACC, and SIRT1 were more highly expressed in HIAC versus LIAC and localized with HIAC mammary glands in the biplot. On the other hand, proteins associated with mTOR activation (pmTOR, pAKT, p4E-BP1) and activation of PKA (β_2-AR, pPKAα/β, pSRC) localized with LIAC mammary glands and were more highly expressed. However, some of the proteins involved in PKA-mediated signaling (pSTAT3, pCREB) localized in the center of the

biplot indicating that they had little effect in accounting for separation, which is also apparent in the S-plot and the VIP plot.

3.4. Discussion

Given that clinical evidence indicates an inverse association between breast cancer risk and intensity/volume of physical activity as well as that both exercise intensity and/or volume are positively associated with aerobic capacity, our mechanistic studies focused on the pathways that have been implicated in linking induced aerobic capacity to the development of chemically induced mammary carcinogenesis in the rat (35, 132-137). The fact that differences in protein expression in mammary gland and mammary tumors were sufficient to permit 100% discrimination between LIAC and HIAC indicates that paracrine and autocrine regulation are likely to be playing a role in accounting for differences in the carcinogenic response (35).

The strongest evidence for differential expression between LIAC and HIAC was for oncogenic signaling, i.e. AKT, mTOR, ERK1/2, signatures of G_1/S arrest, such as p21 and p27, as well as lipid metabolism (ACC, FASN, SREBP1) based on the VIP rankings (Fig. 4). Consistent with these observations, activation of energy sensors, such as AMPK and SIRT1, was detected in HIAC carcinomas, both of which have been implicated in the resistance of HIAC in other disease model systems (92, 145). On the other hand, the activation of EPAC- and PKA-related signaling was apparent in mammary carcinomas of LIAC.

Our experiments indicate the merit of:

1) determination of the dominant cellular process involved in inhibiting carcinogenesis with primacy given to examining effects of LIAC/HIAC status on the G1/S checkpoint in the cell cycle;

2) examination of the role of mTOR network activity, a signaling cascade de-regulated in the majority of human breast, with a focus on regulation of mTOR via sensors of both intracellular and extracellular energy/nutrient status;

3) investigation of the role of cAMP-EPAC-1/PKA network activity, which has received limited attention in the breast cancer field, but that plays a central role in energy homeostasis, particularly aspects that relate to fuel availability to support skeletal muscle contraction during physical activity.

3.5. Conclusions

Our analyses show that mammary glands of animals with LIAC exhibit an anabolic signature with stronger correlation to pathways that are central in oncogenic transformation, such as cAMP-PKA/EPAC-1, AKT-mTOR, ERK1/2, SREBP1, FASN. Their expression was even higher in tumors of these animals. Protein expression profiles characteristic of cell proliferation, cell survival, and accumulation of lipids and proteins were predominant in LIAC. Overall, the expression of AKT, SIRT1, β_2-AR, p21, p27, PKA, ERK1/2, EPAC-1 were the most influential factors in tumors based on classification by IAC. The magnitude of expression of these proteins was greater in tumors than mammary gland, a finding consistent the pathological differences that exist

between these two tissues (146, 147). Generally, HIAC had a phenotype associated with higher rates of energy expenditure in mammary gland and tumors, a finding consistent with improved metabolic flexibility as discussed in Chapter 2. Accordingly, we postulate that IAC is associated with the main factors of cellular level of regulation of metabolic flexibility, such as AMPK and SIRT1; AKT, mTOR, ERK1/2, PKA, Notch signaling pathways, and their targets that regulate the switch between fatty acid and glucose metabolism (60). Our results are consistent with genomic studies in skeletal muscles that identified AMPK and mTOR signaling networks as well as energy sensing, cell cycle, hypoxia, and other elements as candidate mechanisms associated with IAC (33).

CHAPTER 4:

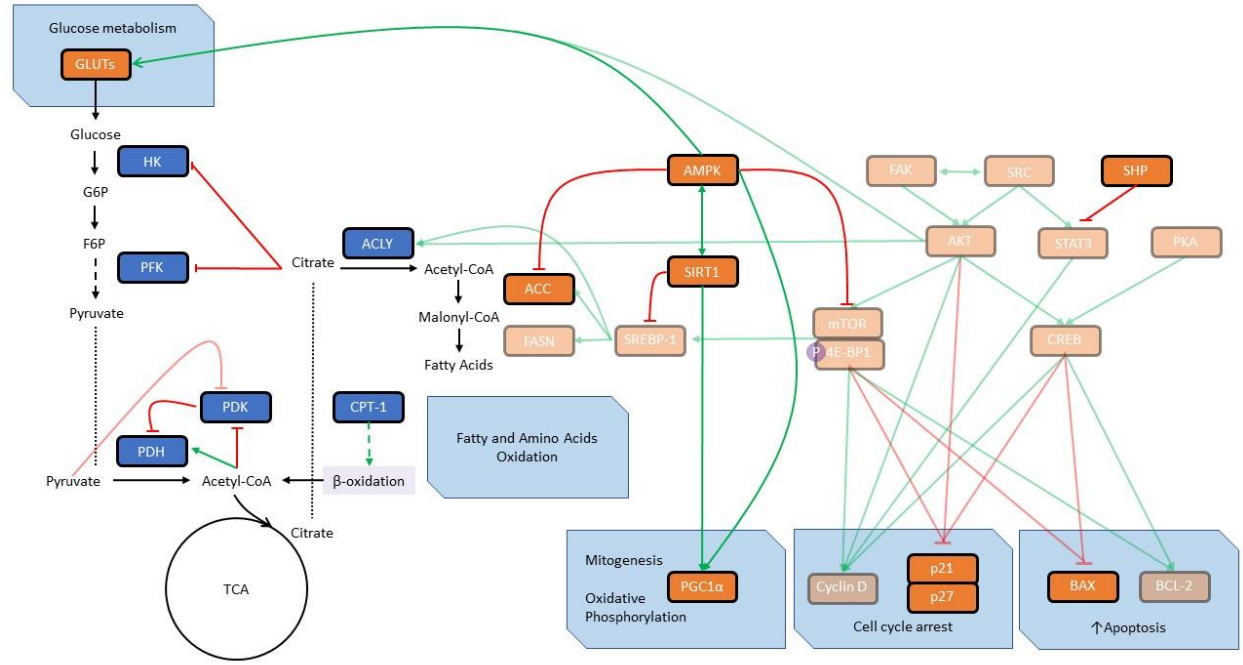
Synthesis and Conclusions

4.1. Synthesis

Animals with HIAC versus LIAC differ in their response to chemically induced mammary carcinogenesis not only in the magnitude of the response but also in the signaling networks that are activated in the induced tumors. The differences in signaling appear to be conserved between mammary gland and mammary tumor, although the magnitude of activation was generally greater in the tumor than in uninvolved mammary tissue. These differentially regulated intracellular signaling profiles have been associated not only with aerobic fitness and cancer development, but also with metabolic flexibility (Fig. 6.).

Factors such as AMPK, SIRT1, ACC, and p21/p27 that were associated with HIAC have been linked with the positive health benefits of aerobic exercise (148, 149). In contrast, LIAC animals had signaling profiles associated with anaerobiosis and muscle hypertropy: IGF1-R-AKT (150, 151), ERK1/2 signaling (151, 152), cAMP-PKA (153) and cAMP-EPAC-1 (154). Therefore, it seems that LIAC animals not only favor glucose metabolism, but also rely on breakdown of glucose via glycolysis as a prominent fuel source. Such a predilection for a single fuel source is the hallmark of metabolic inflexibility.

A.


Glucose metabolism
GLUTs
Glucose
HK
G6P
F6P
PFK
Pyruvate
Citrate
ACLY
Acetyl-CoA
ACC
Malonyl-CoA
FASN
Fatty Acids
SREBP-1
AMPK
SIRT1
FAK
SRC
SHP
AKT
STAT3
PKA
mTOR
4E-BP1
CREB
PDK
PDH
CPT-1
Pyruvate
Acetyl-CoA
β-oxidation
Fatty and Amino Acids Oxidation
Citrate
TCA
Mitogenesis
PGC1α
Oxidative Phosphorylation
Cyclin D
p21
p27
Cell cycle arrest
BAX
BCL-2
↑Apoptosis

B.

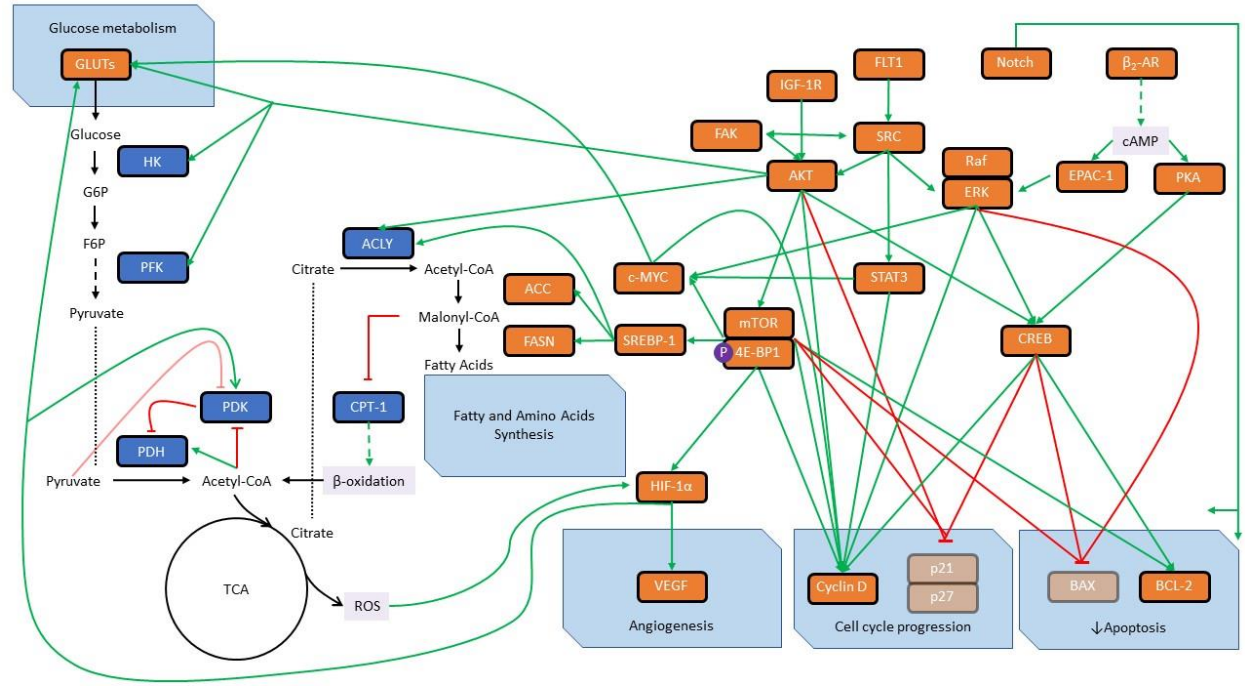

Glucose metabolism
GLUTs
Glucose
HK
G6P
F6P
PFK
Pyruvate
Citrate
ACLY
Acetyl-CoA
ACC
Malonyl-CoA
FASN
Fatty Acids
SREBP-1
c-MYC
mTOR
4E-BP1
IGF-1R
FLT1
Notch
β2-AR
FAK
SRC
cAMP
AKT
Raf
ERK
EPAC-1
PKA
STAT3
CREB
PDK
PDH
CPT-1
Pyruvate
Acetyl-CoA
β-oxidation
Fatty and Amino Acids Synthesis
Citrate
TCA
ROS
HIF-1α
VEGF
Angiogenesis
Cyclin D
p21
p27
Cell cycle progression
BAX
BCL-2
↓Apoptosis

Figure 6. Metabolic and signaling pathways crosstalk in tissues of animals with (A) high inherent aerobic capacity (HIAC) and animals with (B) low inherent aerobic capacity (LIAC).

A. Greater fatty acid oxidation is upregulated by activity of AMPK and SIRT1 at the expense of glucose metabolism. Acetyl-CoA from TCA and β-oxidation activate PDK and impedes glycolysis from glucose oxidation by inhibiting PDH, whereas post-TCA citrate inhibits HK and PFK attenuating glycolysis and undergoes ACLY-mediated conversion into acetyl-CoA. Further carboxylation of cytosolic acetyl-CoA into malonyl-CoA is impeded by AMPK-induced inhibition of ACC. AMPK also downregulates lipogenesis (FASN, ACC) by inhibiting mTOR-SREBP-1 pathway and induces glucose uptake by upregulating GLUTs. SREBP-1 is also downregulated by SIRT1 which is in crosstalk with AMPK. In addition, inactive SRC, STAT3, AKT- and PKA-CREB factors and corresponding signaling pathways are associated with HIAC tissues. Altogether, such metabolic and signaling pattern leads to cel cycle arrest (via p21 and p27), induction of apoptosis (via increase in BAX and decrease in BCL-2 levels), as well as increase in mitochondrial function (via PGC-1α).

B. Glucose metabolism is upregulated in LIAC tissues at the expense of fatty acid oxidation leading to production of ROS and anabolic metabolism: AKT signaling induces glycolysis via GLUTs, HK, PFK; GLUTs are also induced by AKT-mTOR-mediated upregulation of HIF-1α and c-MYC factors; AKT-mTOR-SREBP1-stimulated ACLY and ACC promotes conversion of malonyl-CoA which inhibits CPT-1 and subsequent β-oxidation of fatty acids. In turn, AKT-mTOR-SREBP1-stimulated FASN utilizes malonyl-CoA and increases lipogenesis. AKT is downstream target of SRC and FAK which can affect activity of one another. SRC also activates STAT3 and ERK1/2. cAMP, produced from stimulation of β_2-AR stimulation, activates EPAC-1, which also activates ERK1/2. cAMP also activates PKA. Common downstream target of AKT, ERK1/2, and PKA is CREB. All of these as well as Notch signaling pathway induce angiogenesis (via VEGF), cell cycle progression (via stimulation of cyclin D and inhibition of p21 and p27), and downregulate apoptosis (by inducing BCL-2 and lowering BAX).

Factors detected in our experimental analyses are depicted in orange, whereas those obtained from literature analyses are in blue.

GLUTs, glucose transporters; HK, hexokinase; PFK, phosphofructokinase; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; TCA, tricarboxylic acid; ROS, reactive oxygen species; ACLY, ATP-citrate lyase; CPT-1, carnitine palmitoyltransferase I; ACC, acetyl-CoA-carboxylase; FASN, fatty acid synthase; SREBP-1, sterol regulatory element-binding protein 1; AMPK, AMP-activated kinase; SIRT1, silent mating type information regulation 2 homolog 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; c-MYC, oncogenic transcription factor; FAK, focal adhesions kinase; mTOR, mechanistic target of rapamycin; 4E-BP1, 4E-binding protein; AKT, protein kinase B; HIF-1α ,

hypoxia-inducible factor-1α; VEGF, vascular endothelial growth factor; Raf, rapidly accelerated fibrosarcoma kinase; ERK1/2, mitogen-activated protein kinase, extracellular signal-regulated kinases 1 and 2; p21, cyclin-dependent kinase inhibitor 1; p27, cyclin dependent kinase inhibitor 1B; FLT1, vascular endothelial growth factor receptor 1; IGF-1R, insulin growth factor 1 receptor; SRC, non-receptor tyrosine kinase SRC; STAT3, signal and transducer of transcription 3; β_2-AR, β_2-adrenergic receptor; EPAC-1, the guanine-nucleotide exchange proteins activated by cAMP 1; PKA, cAMP-dependent protein kinase; CREB, cyclic-AMP-responsive element binding protein (CREB); BCL-2, B cell lymphoma/leukaemia-2 protein; BAX, BCL-2-associated X protein.

Collectively our findings are consistent with the thesis that the maintenace of a pro-glycolytic in which glucose is the preferred fuel source is a metabolic state favored in tumors, in multiple tissues of obese, insulin resistant organisms, and in animals and people with low aerobic capacity. As such, this provides a unappreciated framework for better understanding how obesity and low aerobic fitness, a hallmark of physical inactivity, are associated with higher cancer risk and poorer cancer prognosis. The molecullar underpinnings of this hypothesis, based on integrated analysis of the literature (Chapter 2) and our experimental findings (Chapter 3), are summarized in Fig. 6.

The lower cancer risk and better prognosis associated with being of normal weight and aerobically fit are linked to metabolic flexibility in which the oxidation of glucose and fatty acids fluctuate with the energy status of the organism. On the other hand, increased cancer risk and poorer cancer prognosis that are linked to obesity and low aerobic fitness are associated with preferential oxidation of glucose irrespective of the energy status of the organism.

4.2. Future directions

Based on our results, the further analyses of cancer development in terms of inherent aerobic capacity and corresponding metabolic flexibility can be conducted from various perspectives. We have identified influential proteins involved in energy metabolism, proliferation, and other cellular events which need deeper investigation in order to shed light on the whole signaling network involved metabolic regulation of these diverse conditions. Since HIAC and LIAC animals did not undergo any metabolic challenge apart from carcinogen administration, it would be valuable to observe how exercise (intensity, duration, and type), caloric restriction, and the feeding of a high fat diet affect the carcinogenic response and metabolic flexibility as a function of inherent aerobic capacity status. Further analysis of protein expression patterns in tumors and mammary glands of animals using untargeted approaches may also reveal new relationships for data driven hypothesis testing.

To our knowledge, the work conducted in our laboratory was the first and so far is the only research directed toward studying carcinogenesis in HIAC and LIAC models (35). Further utilization of these rat strains in cancer research will shed more light on the molecular aspects of the regulation of carcinogenesis by aerobic capacity and energy homeostasis. The obtained results have significant potential for translational impact.

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