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Chapter

Metformin Activity against Breast Cancer: Mechanistic Differences by Molecular Subtype and Metabolic Conditions

Reema S. Wahdan-Alaswad and Ann D. Thor

Abstract

Obesity and type 2 diabetes increase the risk of and reduce survival in breast cancer (BC) patients. Metformin is the only anti-diabetic drug that alters this risk, with a reduction in BC incidence and improved outcomes. Metformin has AMP-kinase (AMPK) dependent and independent mechanisms of action, most notably affecting the liver and skeletal muscle. We and others have shown that metformin also downregulates protein and lipid synthesis; deactivates various receptor tyrosine kinases; alters cell cycle transcription/translation; modulates mitochondrial respiration and miRNA activation; targets key metabolic molecules; induces stem cell death and may induce apoptosis or autophagy in BC cells. Many of these anti-cancer effects are molecular subtype-specific. Metformin is most potent against triple negative (basal), followed by luminal BCs. The efficacy of metformin, as well as dose needed for the activity, is also modulated by the extracellular glucose concentration, cellular expression of the glucose transporter protein 1 (GLUT1), and the organic cation transporter protein 1 (OCT1, which transports metformin into cells). This chapter summarizes the diverse clinical and preclinical data related to the anti-cancer effects of metformin, focused against breast cancer.

Keywords: metformin, breast cancer, TGF-β, STAT3, and PI3K/AKT/mTOR, FASN, MiR-193b, cancer stem cells, EGFR, cholesterol, glucose

1. Introduction

Metabolic dysregulation of carbohydrate and lipid metabolism is frequent in cancer cells, facilitating growth and survival through adaptive mechanisms. Otto Warburg was the first to recognize that cancer cells favor glycolysis as compared to oxidative phosphorylation for the generation of energy (ATP) [1]. While the former is less efficient in terms of energy production per molecule of glucose, it also generates precursor molecules (amino acids, fatty acids, etc.) for replication and facilitates survival under oxidative stress [2]. This is in contrast to normal cells, which typically use oxidative metabolism to derive more energy (ATP) per molecule of glucose [3, 4]. Nearly a century later, we now recognize that cancer cells may utilize either aerobic or anaerobic respiration. The majority of cancer cells also have alterations of mitochondrial respiration, further providing a selective advantage to
Metformin facilitate cancer growth and survival [5]. More specifically, it may increase intracellular reactive oxygen species by disruption of the mitochondrial electron transport chain to reduce the mitochondrial membrane potential in BC or act directly to inhibit the mitochondrial respiratory-chain complex 1 (MRCC1) [6–8].

Chronic energy excess and physical inactivity lead to systemic alterations of carbohydrate and fatty acid metabolism characterized by systemic hyperglycemia, hyperinsulinemia with insulin resistance followed by hypoinsulinemia, an increase in inflammatory cytokines and adipokines, alterations of steroid and growth hormones, and downregulation of immune surveillance and tissue oxygenation [3, 9, 10]. These changes are frequent but variable in patients with obesity and type 2 diabetes and can be modified by drugs, exercise, body weight, socioeconomic factors, access to healthcare, genetic risk, and other factors. Patients with these disorders are at an increased risk of cardiovascular disease, cancer, and other diseases associated with significant morbidity and mortality. In the U.S., there are ~13.8 million type 2 diabetics, 5 million undiagnosed diabetics, and 41 million persons with prediabetes/metabolic syndrome [11–13]. Obesity is a frequent comorbidity, often proceeding diabetes by years or decades.

Energy-sensing systems are integral to maintaining homeostasis in normal and transformed cells. Energy deprivation is frequent in cancer cells due to an inadequate vascular supply to meet the needs of increased cell replication. In energy-stressed cells, AMPK is allosterically modified by binding to AMP and ADP, rendering them targetable by AMPK kinases. AMPK activation induces signaling, upregulates energy production, and inhibits energy programming for cell growth and motility. In cancerous cells, this shift often fails to occur even with stress. As a result, cancer cells typically prioritize replication and motility to favor cancer growth and metastasis. Drugs that activate AMPK, most notably metformin, reengage the AMPK failsafe to inhibit proliferation and motility. Thus, metformin provides a unique and generally less-toxic approach to combat the emergence or growth of cancers through inhibition of cell replication. This is particularly important for patients with obesity and type 2 diabetes, who lack homeostasis and experience wide swings in systemic glucose, insulin, and other energy precursors and growth factors that contribute to systemic energy stress.

2. Metabolic dysregulation, breast cancer, and metformin

Abundant epidemiologic and clinical data have shown that obesity and type 2 diabetes increase the risk and severity of cardiovascular disease and human cancer. Each of these chronic metabolic disorders as a single variable significantly increases the risk of breast cancer (BC) [10, 14]. In combination, the risk is increased by 20–50%, depending on the severity of disease and other variables. It is highest in women with abdominal (central) obesity in the postmenopausal setting, in women of all ethnic backgrounds [15–17]. Obesity also promotes BC in premenopausal women of color, especially African Americans and Latinos [18–23]. In patients with obesity and diabetes, BC also presents at a higher disease stage and is more resistant to treatment, resulting in a shorter disease-free interval and a significantly higher mortality rate [24, 25].

Steroid receptor-positive BC (luminal A) and basal (triple negative) BC cells are the most responsive to extracellular glucose at or above 7 mM of glucose to promote cell replication, tumor growth, and motility. In contrast, steroid receptor-positive BC cells that also express high HER2 (luminal B) and steroid receptor-negative, HER2 positive (the HER2 subtype) are less responsive to hyperglycemia, even at levels associated with untreated type 2 diabetes (10 mM glucose or higher) [26].
Glucose directly promotes signaling in epithelial cells or can act indirectly by interacting with molecular signaling proteins, such as the insulin-like growth factor (IGF-1), sex hormones, and adipokines [3, 27, 28]. Insulin and insulin-like growth factors are frequently increased in newly diagnosed BC patients [28–31]. These potent growth factors promote BC growth and are associated with a worse prognosis, both in overweight and ‘normal’ weight women [24, 29, 32–34]. Epidemiological and clinical data show that obesity and type 2 diabetes are particularly associated with luminal A (estrogen and progesterone responsive) as well as triple negative BCs [19, 21, 27, 35, 36].

Metformin (N’, N’-dimethylbiguanide) is the most frequently used drug to treat patients with metabolic syndrome (prediabetes) and type 2 diabetes worldwide. It has been used successfully for over six decades and has a very favorable benefit-risk profile [37]. Metformin is stable at room temperature with a long shelf life, is inexpensive and orally administered, and has low rates of significant toxicity or drug-drug interaction. Metformin is best known for its effects on liver and skeletal muscle cells, where it downregulates insulin resistance, lowers serum insulin, stimulates insulin receptor tyrosine kinase activity, inhibits hepatic glucose output (thus lowering A1C), increases glucose uptake by skeletal muscle cells, and can alter fatty acid metabolism.

Epidemiologic data show a significant lowering of cancer risk in patients with metabolic dysregulation (obesity, diabetes, or metabolic syndrome) who take metformin [29, 34, 38, 39]. Metformin use by BC patients has also been associated with improved treatment response and survival. In one meta-analytic study of BC patients with diabetes, metformin use was associated with a 65% improvement in BC-specific survival as compared to nonusers [40]. The anticancer properties of metformin are in contrast to other antidiabetic agents, including sulfonylureas and insulin, which promote cancer growth [9].

It is also taken for its ‘antiaging’ properties in individuals without obesity or metabolic dysregulation, particularly outside of the US [10, 41, 42]. Numerous clinical trials are currently underway in BC patients to evaluate the benefit of metformin combined with or following the administration of other therapeutic agents [29, 32, 33, 43–46]. Studies designed to test the benefit of metformin in patients in only specific molecular subtypes of BC have not been performed, although some have looked at molecular cohort interactions as a secondary goal [30, 47–51]. There are limited data on the use of metformin in metabolically ‘normal’ BC patients. However, our preclinical data suggest that metformin is most active in all molecular subtypes with physiological levels of extracellular glucose [26]. This evidence provides a rationale for testing metformin in otherwise healthy BC patients.

3. AMPK-dependent mechanisms of metformin action in BC

Cellular uptake of metformin requires expression and functionality of the organic cation transporter 1 (OCT1) protein, which in some individuals or BCs may be altered (more or less effective in transporting metformin into the cell) by polymorphism or genetic error [52]. Polymorphisms have also been associated with a decrease in metformin efficacy in diabetic patients [53–55]. In BC cells, we have demonstrated that OCT1 expression is associated with the anticancer activity in vivo [44]. Once inside the cell, metformin may directly interact with the metabolic sensor AMPK to induce activation, restoring homeostasis and blocking cellular replication and motility under low energy (stress) conditions. The AMPK ‘switch’ is also influenced by the intracellular AMP:ATP ratio, which in turn is influenced by fatty acid oxidation and...
Metformin

glucose metabolism. Thus, metformin can indirectly affect AMPK, through reduction of gluconeogenesis and thus changing of the AMP:ATP ratio. These mechanisms are represented in Figure 1. These processes are modulated by P53 status. It is mutated in many BCs, particularly tumors that are high grade, late-stage or nonluminal in subtype. In BCs that are P53 competent, AMPK activation (from metformin or other triggers) upregulates P53 tumor suppressor activity as a downstream target. This induces activation of cell cycle checkpoint proteins, to inhibit cell proliferation [56]. In P53 incompetent cells, AMPK activation from metformin may be less effective through P53 mechanisms. Given the numerous other actions of metformin, as well as the molecular subtype specificity of the drug, we postulated that P53 status alone would not have a major impact on anticancer effects of metformin. We have demonstrated that this is the case in preclinical studies of numerous BC cell lines [57]. In other cells, metformin may induce cell cycle arrest and death through activation of apoptotic pathways and downregulation of p53 [58, 59] or PARP cleavage, especially in triple negative BC [60, 61].

Activation of mTOR-dependent protein synthesis and cell growth (downstream of the PI3K/Akt signaling axis), along with AMPK, provides a robust signaling platform for BC cell growth, proliferation, and chemotherapy resistance. In addition to activating AMPK, metformin inhibits mTOR and downstream signaling components of this critical pathway. Mutation of the PI3K catalytic subunit (PIK3CA) occurs in 20-35% of BCs [62, 63]. Mutation or loss of the tumor suppressor gene PTEN has also been demonstrated in 40% of BC [64, 65]. Metformin can also inhibit gluconeogenesis and mTOR signaling independent of AMPK and the tuberous sclerosis 2 (TSC2) gene in some experimental systems (in hepatic cells that lack AMPK or its kinase, LKB1). In this model system, metformin induces

Figure 1. Metformin AMPK-dependent mechanism of action on breast cancer. Metformin activates AMPK directly through insulin-like growth factor (IGF-I) or insulin receptor, which in turn can activate PI3K/Akt/mTOR or RAS/Raf/MEK/ERK to increase cell growth, survival, angiogenesis, migration, and invasion. Metformin indirectly activates AMPK, which activates mTORC2, CREB, and gluconeogenesis. Lastly, glucose can enter BC cell through GLUT-1, and metformin can directly downregulate GLUT-1 receptor.
downregulation of hepatic gluconeogenesis through non-AMPK–associated mechanisms [66, 67].

Signaling systems and thus metformin sensitivity by dose or mechanism vary by the molecular subtype of BC as well as unique genomic changes in each patient’s BC. For example, we have shown that metformin-induced partial S phase arrest increased P-AMPK and reduced P-EGFR, P-MAPK, P-Src, cyclin D1, and cyclin E, with the induction of PARP cleavage and apoptosis only in triple negative BCs [44]. In this tumor subtype, metformin specifically targets Stat3 and is not dependent on mTOR signaling [44]. In non-triple negative BCs (luminal and HER2), metformin induces partial cell cycle arrest at the G1 checkpoint, reduces cyclin D1 and E2F1 expression, and inhibits AMPK, MAPK, Akt, and mTOR activity [44, 57, 68]. Metformin-associated AMPK activation may also inactivate the insulin receptor substrate 1 (IRS1), which in turn regulates IGF-IR and PI3K/Akt signaling pathways to block the progrowth effects of hyperinsulinemia and insulin-like growth factors typically associated with type 2 diabetes [66, 67, 69].

Metformin is unique in the breadth and complexity of AMPK-dependent direct and indirect targets that inhibit cancer. Several new mechanisms fall into the rapidly expanding field of immuno-oncology. Metformin-induced activation of AMPK activates the programmed death ligand-1 (PD-L1) at S195, reducing stability and membrane localization and thus increasing PD-L1 degradation [70]. Metformin also promotes cytotoxic T cell lymphocyte activity in tumor tissue and enhances tumor-associated immune surveillance [6, 70, 71]. Additionally, metformin upregulates pro-inflammatory cytokines (tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), IL-1β, the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB), the hypoxia-inducible factor 1-alpha (HIF-1α), and the vascular endothelial growth factor (VEGF), reviewed in [72, 73]).

AMPK-dependent mechanisms of action have been validated using clinical trial–derived BC samples as well as preclinical model systems, reviewed in detail elsewhere [43]. Some of these were especially important to spur the expansion of metformin use in BC patients. The timing, dose, and duration of metformin treatment in BC patients with or without other chemotherapy are actively under investigation. Neoadjuvant metformin, in particular, has shown benefit with a higher rate of complete pathological response, as compared to similar BC patients [74].

3.1 Metformin targets cell cycle proteins in AMPK-dependent manner in breast cancer

AMPK plays an integral role in the regulation of cell cycle and cell division. The ability of metformin to activate AMPK thus has a significant inhibitory effect on cell-cycle associated proteins. This mechanism is represented in Figure 2.

Expression profiling of BC derived from metformin-treated patients as compared to controls has shown consistent downregulation of many gene encoding proteins involved in mitosis, including kinesins, tubulins, histones, Aurora, as well as Polo-like kinases and ribosomal proteins (critical for protein and macromolecular biosynthesis, respectively) [75]. Given the targeted effects of metformin, it is not surprising that its actions are synergistic with drugs like paclitaxel that induce defects in mitotic spindle assembly, chromosome segregation, and cell division. In combination, metformin and paclitaxel dramatically increase the number of cells arrested in G2-M and apoptosis, as compared to either agent alone [76]. Metformin may also induce GO/G1 arrest due to activation of AMPK, downregulation of cyclin D1, and enhanced binding of CDK2 by p27Kip1 and p21Cip1 [60, 61], especially in non-triple negative cells. Some have shown that metformin sensitivity to GO/G1
Metformin arrest is linked to overexpression of p27\textsuperscript{kip1} and p21\textsuperscript{cip1} [60, 61]. We have demonstrated that metformin induces cycle arrest at the G1 checkpoint in luminal A, B and HER2 BC [75] associated with a reduction of cyclin D1 and E2F1 expression, with no changes in p27\textsuperscript{kip1} or p21\textsuperscript{waf1}. While these authors describe how metformin can increase CDK chemical inhibitors to control BC growth [57, 61], others have utilized cell cycle-dependent kinases (CDK) inhibitors with metformin and report that this combination should be used with caution [77].

In addition to downregulating cell replication under stress, metformin upregulates the cellular DNA-damage response, resulting in a decline in the mutational burden for those cancer cells that survive. Mechanisms underlying this effect include selective activation of the ataxia telangiectasia mutated (ATM) gene as well as ATM targets, such as protein kinase CHK2 gene and attenuation of reactive oxygen species ROS that result in DNA damage [78]. Algire et al. have postulated that downregulation of ROS production and thus somatic mutation are likely contributing mechanisms for the reduction in cancer risk associated with metformin use [8].

In summary, AMPK plays a central regulatory role in human cells, including BC where it regulates energy metabolism, cell growth and motility, response to insulin and growth factors, and estrogen production. Metformin induces AMPK activation in a robust manner, to affect numerous target pathways and intermediate molecules. The activity of AMPK and thus metformin can be modified by interacting factors including hormones, growth factors, and energy sensors. Selective targeting of AMPK-dependent pathways has shown less efficacy than metformin alone against BC [79], consistent with the findings that not all mechanisms of metformin action are AMPK dependent.
4. AMP-independent mechanisms of action on metformin

4.1 Metformin action on glucose and metabolism

Upregulation of bioavailable glucose, insulin, and other growth factors increase the risk and promote BC aggression [16, 23, 27, 80, 81]. In addition to shifts in host metabolism, glycolytic reprogramming occurs in breast epithelial cells during malignant transformation. This process is accentuated by systemic dysregulation of carbohydrate and lipid metabolism, as bioavailable sugars and fat typically increase in these patients. Glycolytic reprogramming includes dependence on aerobic respiration, providing less-efficient energy (ATP) production per molecule of glucose from and incomplete oxidative phosphorylation. Cancer cell reprogramming includes activation of numerous signaling intermediaries, including phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), mammalian target of rapamycin (mTOR), phosphatase and tensin homolog (PTEN), and AMPK [82–84]. Changes in other factors including c-MYC, hypoxia-inducible factor 1-alpha (HIF1α), epidermal growth factor receptor (EGFR), tumor protein 53 (P53), and the Met receptor may also facilitate cancer cell dependence on aerobic glycolysis [16, 85–87].

We have focused on the effects of extracellular glucose and other carbohydrates, combined with or without metformin using BC cell lines and animal models of obesity, metabolic syndrome, and mammary tumorigenesis, summarized in Figure 3 and detailed elsewhere [26, 47, 49, 52, 57, 68, 88–93]. Importantly, most in vitro studies of metformin use commercially purchased media containing ~17 mM glucose (incompatible with human life, above concentrations achieved in diabetes). This is significantly higher than serum derived from normal persons (~5 mM), metabolic syndrome patients (~7 mM), or uncontrolled diabetes (~10 mM) [26]. We have shown that all molecular subtypes of BC cells grown with high glucose media require significantly more metformin to achieve the same anticancer efficacy (i.e., much higher EC50 of metformin) [26]. Normalization of glucose concentration in the culture media significantly reduced the EC50 of metformin for all BC cell types to induce BC growth inhibition or death. This hyperglycemic override of metformin action by dose makes biologic sense, given the ability of glucose to enter cells and promote many of the same pathways we have shown that are critical to metformin action. Similar issues may arise in animal models, particularly if the animals are overfed or obese. In both mouse and rat model systems, we have achieved plasma metformin concentrations equivalent to the normal range in humans, by providing it in the drinking water. We have also shown that metformin accumulates in the cytoplasm, markedly higher than serum levels in mammary tumor cells with functional and sufficient OCT1 protein [26].

Luminal A and some subsets of triple negative BC cell lines show the greatest increase in proliferation when cultured in media with supraphysiologic glucose or insulin. In contrast, luminal B and HER2 BC cells were significantly less responsive to glucose or insulin, even at the highest concentrations examined. This responsiveness pattern was similar to the cellular response to metformin by molecular BC subtype, with triple negative being the most responsive. From a molecular standpoint, triple negative BC cell responsivity to high glucose and metformin by dose was unique (efficacy at lower EC50s). Triple negative BC cells are especially dependent on glucose/glucosamine (metabolized through glycolysis) and lipids for energy and building block production, cell division, phenotypic aggression, and motility [94]. When grown with media containing supraphysiologic glucose, they upregulate specific genes, including EGFR, P-EGFR, IGF1R, P-IGF1R, IRS2, cyclin D1, and cyclin E expression, and inhibit AMPK/P-AMPK and p38 in a dose-dependent manner [26]. With the addition of metformin, there is a downregulation of these
Metformin

Figure 3. Metformin action on glucose and metabolism breast cancer. Metformin enters the BC cell through OCT1 transporter to attenuate inner membrane fluidity/permeability, the Krebs cycle (TCA), and complex I of the mitochondria. Metformin can also block downstream signaling intermediates involved in the PI3K/Akt/mTOR or RAS/RAF/MEK/ERK signaling pathways, which can control BC cell growth. Lastly, metformin blocks GLUT1 transporter and key enzymes that are involved in carbohydrate synthesis.

genes and the upregulation of genes associated with cell killing and growth control [49, 94]. Our report showed that glucose promotes phenotypic aggression and reduces metformin efficacy by targeting key enzymes that are required for glucose metabolism in TNBC. Such enzymes include G6PD, Fructose-2-6-BP, PGK, PGM, ENO, PKM2, and LDH-A (shown in Figure 3 and reviewed in [49]). Further, we reported that metformin attenuated the expression of over 20 critical genes involved in glucose metabolism, glucose transporters, gluconeogenesis, and tricarboxylic acid cycle [49]. Metformin-associated gene expression changes also reduced phenotypic aggressiveness and stem-like progenitor cell pool [26, 49, 90, 92]. Metformin treatment also restricted cell proliferation with S phase arrest, motility (through downregulation of intermediate filament proteins), and increased apoptosis (through activation of both the intrinsic and extrinsic pathways) [26, 47, 57, 88, 89, 92]. Metformin significantly inhibits carbohydrate induced pro-oncogenic metabolic and biologic characteristics of triple negative BC cells [26]. Altogether, metformin's ability to target key glucose transporters, such a GLUT1, along with key genes involved in glucose and carbohydrate metabolism, highlights the role that this agent may play to control highly aggressive malignant BC cells via downregulation of the cellular metabolic machinery.

We have also shown that inhibition of lipid biosynthesis was requisite to the anticancer effects of metformin in triple negative BC cells. It downregulates both
fatty acid synthase (FASN) and the cholesterol biosynthesis pathway, as detailed below. Other studies have focused on interactions between obesity, weight gain, hormonal status, and BC, and more specifically if metformin could be used to disrupt this process. Using a rat model of mammary tumor development after exposure to a carcinogen, animals were overfed and then segregated into lean and obese. Both subsets were subjected to ovary removal, half were given metformin, and they were followed for the development and progression of mammary tumors [52, 93, 95]. Obese rats experienced marked changes in metabolism, akin to metabolic syndrome. Mammary tumors from these obese rats showed enhanced tumor growth and tumor-associated glucose uptake, 50% higher than nonobese rats in association with upregulation of the progesterone receptor. In contrast, the lean rats preferentially deposited excess nutrients in mammary (nontumor) and peripheral tissues. Metformin abrogated systemic metabolic dysregulation, reduced tumorigenesis, tumor progression, and tumor-associated PR expression in obese rats. Similar changes in body weight and obesity are frequent after female menopause has been observed in BC of postmenopausal females with obesity, providing additional clues for the use and timing of metformin associated with BC risk and treatment for future study.

4.2 Metformin action on cholesterol, EGFR signaling, and lipid rafts

The mevalonate pathway, also known as the β-hydroxy β-methylglutaryl-CoA (HMG-CoA) reductase pathway, is critical for cancer cell survival. Inhibition of the pathway by statins or other agents has been shown to have anticancer effects [96, 97]. In contrast, elevated cholesterol has been strongly associated with BC risk, a worse BC-associated outcome and chemotherapeutic resistance. This reflects the pivotal role of lipids including cholesterol in cancer survival and growth, including upregulation of signaling through membrane-bound receptors, facilitation of intracellular signaling pathways, and serving as an anchor for intracytoplasmic filaments to promote motility and invasion and as a precursor for cellular metabolism to generate energy and facilitate replication [98, 99]. We have shown that triple negative BC cells are especially dependent on the upregulation of lipid and cholesterol biosynthesis [100].

Statins are widely prescribed for patients with high cholesterol or lipid abnormalities, most often to reduce the risk of cardiovascular disease. Statins also benefit women to reduce the risk and disease progression of BC. Two population-based studies from Northern Europe are particularly compelling. A Finnish study involving over 30,000 women showed that statin use, pre- or post-BC diagnosis, reduced BC-specific mortality by about 50% [101]. A large Danish study showed a benefit for BC patients as well, with significantly lower recurrence rates in statin users as compared to nonusers. They also reported that lipophilic statins (rather than hydrophilic satins) had the most anti-BC activity [102]. A recent study from MD Anderson Cancer Center suggests that statin use is particularly beneficial for BC patients with triple negative tumors, especially in patients with higher stage disease [95]. Their data are consistent with our preclinical data, showing significant upregulation of lipid metabolism-associated gene triple negative BC as compared to other molecular subtypes. See for further discussion elsewhere [103]. A major issue with statin use is toxicity, which reportedly occurs in up to half of patients. Some statin drugs are also expensive and thus may be unaffordable by many patients.

Metformin, in contrast, is relatively nontoxic and inexpensive. We have demonstrated that metformin has potent effects in lipid and cholesterol biosynthesis in BC cells. More specifically, it inhibits transcriptional activation of HMGCo-A
Metformin (the enzyme targeted by statins), as well as over 20 other genes in the cholesterol biosynthesis pathway. We have also shown that it induces translational activation of downstream signaling, including the genes ACA2, HMGCS1, HMGCR, MVK, MVD, LSS, and DHCR24 (Figure 4). Through broad inhibition of cholesterol biosynthesis in triple negative BC, metformin induces a significant reduction of membrane-associated and intracellular cholesterol and reduces GM1 lipid rafts through decreased synthesis and destabilization (disassociation). GM1 lipid raft stability has a profound effect on some receptors that rely on GM1 lipid rafts (like EGFR) for stability, ligand binding, and thus activation, resulting in downstream signaling. We have shown that metformin inhibits cholesterol biosynthesis and raft production, reducing membranous EGFR and its activation associated with downstream signaling in TNBC [91]. We have also shown that in combination, metformin and the statin-mimetic MβCD were synergistic in attenuating cholesterol biosynthesis and cell proliferation [91]. Others have validated our observation that metformin downregulates genes involved in cholesterol biosynthesis, reporting downregulation of HMGCR, LDLR, and SREBP1 [104]. A particularly exciting corollary of these findings is the potential of metformin to synergize with receptor tyrosine kinase inhibitors (RTKIs) against BC. This is an underexplored area of breast oncology research with tremendous translational potential, given the growing use of RTKIs against BC.

Figure 4.
Metformin action on cholesterol synthesis and lipid rafts. Metformin blocks epidermal growth factor receptor (EGFR), human epidermal growth factor receptors 2/3 (HER2/HER3), which in turn can block key enzymes involved in cholesterol synthesis pathway. Metformin and statins both can inhibit rate limiting step HMG-CoA Reductase, HMGCR. Metformin can also decrease cellular membrane rigidity, increase fluidity, and decrease cholesterol content to allow for the internalization of EGFR, HER2, or HER3 receptors. Internalization of these receptors is through GM1 lipid rafts, which are degraded and allow for BC cell death.
4.3 Metformin action on miRNA and FASN signaling

MiRNAs are endogenous, short (21-25) nucleotide sequences that control gene expression during post-transcriptional translation. It has previously been reported that more than half of human genes are regulated by miRNAs [105]. A growing body of evidence has highlighted the role of miRNAs as master regulators of metabolic processes, such as lipid and cholesterol synthesis [92, 105, 106]. Perturbations of these processes are important for tumor development. Modulation of these regulators using synthetic antagonirs to block the activity of specific miRNAs is an important new area of breast research. Metformin exerts some of its anticancer activity through modulation of miRNAs that target genes in metabolic and other pathways (Figure 5) [92, 107, 108]. miRNAs have been reported to be potential biomarkers for BC (i.e., miR-9, miR-10b, and miR-17-5p), whereas others reportedly have prognostic (i.e., miR-148a and miR-335) or predictive relevance (i.e., miR-26a, miR-30c, miR-187, and miR-339-5p) [109].

We have shown that metformin increases several members of the miR-193 family. It upregulates miR-193b, which in turn targets and downregulates the FASN 3’UTR. FASN is an important component of de novo fatty acid synthesis. Using an miR-193b mimic, we induced a drastic reduction in fatty acid synthase (FASN) protein expression as well as increased growth inhibition and apoptosis of TNBC [92]. A separate expression profiling study of metformin-treated TNBC cells has shown similar results [106]. These data show that inhibition of FASN and fatty acid biosynthesis contributes to the potency of metformin against BC cells.

Figure 5. Metformin action on lipid synthesis and miRNAs metformin blocks EGFR, HER2, and HER3, which in turn can block key enzymes involved in cholesterol synthesis pathway as described in Figure 4. Metformin can also block acetyl-CoA carboxylase (ACC), which in turn can decrease fatty acid synthase (FASN). Metformin can also increase a myriad of miRNAs (shown in green). One of these miRNAs (miR-193b) can target FASN, which can decrease fatty acid synthesis in BC cells. Additionally metformin can block FASN and increase BC cell death.
4.4 Metformin action on PI3K/Akt/mTOR signaling in breast cancer

The PI3K/Akt/mTOR pathway plays a central role in regulating protein synthesis, cell proliferation, tumorigenesis, angiogenesis, tumor growth, and metastasis [63]. While AMPK-dependent phosphorylation is frequently described in metformin-mediated inhibition of the PI3K/Akt/mTOR signaling pathway, AMPK activation is not mandatory for these effects; see schematic in Figure 6 [57]. We have shown that metformin inhibits Akt and mTOR and inhibits cellular proliferation and colony formation and causes a partial G1 cell cycle arrest in all ER-positive, HER2 normal or abnormal BC cell lines examined [57]. Metformin-mediated inhibition of the PI3K/Akt/mTOR signaling pathway has also been shown to induce inhibition of cell replication, S phase arrest, and apoptosis, with a reduction in E2F1 and cyclin D1 expression in triple negative BC cell lines [57].

4.5 Metformin action in STAT3 signaling

TNBC shows high activation of the signal transducer and activator of transcription 3 (STAT3) signaling pathway, which in turn promotes cell growth, invasion, migration, metastasis, angiogenesis, immune evasion, and drug resistance and inhibits apoptosis [88]. We have shown that metformin specifically targets STAT3 signaling to reduce P-STAT3 at both Ser727 and Tyr705 phosphorylation sites but not STAT3 expression in TNBC, schematically represented in Figure 6. In combination with a Stat3 inhibitor, metformin significantly downregulated STAT3

Figure 6. Metformin action in breast cancer. Metformin can block receptor tyrosine kinase (RTK), such as EGFR, HER2, and HER3. Metformin further blocks downstream signaling intermediates involved in PI3K/Akt/mTOR or RAS/Raf/MEK/ERK signaling pathway, such as AKT, mTOR, MEK, or ERK, which can decrease cell growth, angiogenesis, and migration/invasion. Further, metformin can further block cytokine and growth factor receptors such as the TGF-RII. Metformin can block IL-6/STAT3 pathway and TGF-signaling pathway, which in turn can decrease cell growth, angiogenesis, migration/invasion, inflammation, and EMT.
expression and was synergistic in reducing cell growth and the induction of apoptosis in TNBC [88]. Given that TNBC also shows an upregulation/activation of the PI3K/Akt/mTOR signaling pathways, we then combined metformin with an mTOR inhibitor rapamycin, to determine if it would reduce metformin efficacy. Significant interactions with metformin were not observed; thus, mechanisms underlying its effects are not dependent on mTOR.

The JAK/STAT pathway is upregulated by obesity-associated mechanisms that promote BC growth. Others have demonstrated that metformin attenuates Janus kinase (JAK)/STAT3 signaling at Ser515 and Ser518 within the Src homology 2 domain of JAK1 [110]. Metformin has also been shown to preferentially inhibit nuclear translocation of NK-xB and phosphorylation of STAT3 in cancer stem cells (CSCs) as compared to non-CSCs [111]. Given the procarcinogenic and prometastatic role that JAK/STAT pathways play in TNBC, the development of therapeutic strategies to attenuate these pathways using metformin may provide benefit with limited toxicity.

4.6 Metformin and TGF-β signaling in TNBC

A subset of TNBC subclassified as mesenchymal-stem like/claudin-low (MSL/CL) characteristically shows high expression and activation of TGF-β signaling, phenotypic aggression, and a worse outcome. In addition to TGF-β receptor 2 expression, BC in this group shows upregulation of Smad2, Smad3, ID1, and ID3 [90]. They are especially responsive to TGF-β ligand 1 (TGF-β1), resulting in cell proliferation, migration, and invasion. MSL/CL cell lines also demonstrate downregulation of several growth factor receptors in response to metformin, including fibroblast growth factor receptors (FGFR2 and FGFR3), hormone receptors (AR, ESR1, and PGR), and claudin integral membrane proteins of tight junctions (CLDN3, CLDN4, and CLDN7) in the MSL/CL BC subtypes [90]. Metformin directly attenuated TGF-β signaling pathway by downregulating activation of Smad2/Smad3, ID1, and ID3 (Figure 6). In combination with TGF-β inhibitors (TβRI-KIs; LY2197299 or SB431542), metformin synergistically enhanced cell death in MSL/CL BC cells [90]. Overall, these data suggest that targeting TGF-β signaling using metformin with or without a TGF-β inhibitor may provide benefit for patients with MSL/CL BCs.

The process of epithelial-mesenchymal transition (EMT) is also common in TNBC and has been associated with biologic aggression and stem-like properties. Metformin reportedly inhibits EMT in a metastatic canine model of mammary cancer [112]. Others have shown that metformin reduces EMT through blockade of transcription factors like ZEB1, TWIST1, and SNAIL (Slug) [113–115]. Given that TGF-β pathway activation and EMT promote breast cancer stem cells (BCSC), therapeutic resistance, dormancy, and a poor outcome [113], and that metformin has been shown to block these in TNBC, inhibitors against TGF-β-induced EMT combined with metformin may provide benefit in some TNBC patients.

4.7 Metformin action on breast cancer and angiogenesis, and the microenvironment

Clinical studies have demonstrated that diabetic patients treated with metformin are less likely to develop cardiovascular disease, independent of glycemic control. It is unclear whether this outcome reflects downregulation of hyperglycemia and systemic inflammatory triggers or vascular damage, or whether metformin has a direct effect on endothelial cells, vascular resistance, elasticity, and damage
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[12, 80, 116]. In the context of breast cancer, it has long been demonstrated that high-stage and grade cancers with a worse prognosis have the capacity to upregulate peri- and intratumoral neo-angiogenesis [117]. The induction of new vessels provides metabolic and oxygen delivery advantages to the cancer cells, facilitating survival and growth. Neo-angiogenesis is also associated with an increased capacity of the BC to metastasize, particularly to distant sites including the visceral organs and brain. We have demonstrated a reduction in vascular density and growth, in association with metformin treatment in preclinical models. Others have shown that metformin is associated with reduced tumor angiogenesis in many different cancer cell types. Metformin and alternate biguanides, such as phenformin, down-regulate VEGF-dependent activation of ERK1, inhibiting neo-angiogenesis and reducing microvessel density (MVD) [118]. Wang et al. have shown that metformin also downregulates the expression of two other genes, platelet-derived growth factor B (PDGF-B) and fibroblast growth factor (FGF-2), to reduce angiogenesis [119]. Downregulation of PDGF-B also restricts BC cell proliferation, survival, and migration, [117]. Metformin’s effect on the microenvironment and angiogenesis has also been shown to enhance chemo-sensitivity, via a reduction in MVD leakage and cancer cell hypoxia in vivo [117]. Thus, metformin’s effects go beyond the cancer cell itself and include the peri- and intratumoral microenvironment and neovasculature.

Figure 7. Metformin action on breast cancer stem cells. Metformin can block a myriad of signaling pathways involved in BCSCs, including WNT, transforming growth factor (TGF), NOTCH, hypoxia inducible factor (HIF), and STAT3 signaling pathways. These pathways are thought to enrich for BCSC through the enrichment of CD44 positive receptor and aldehyde dehydrogenase (ALDH+) and decrease in CD24 expression. Metformin can be given as a monotherapy or combinatorial therapy with alternate chemotherapeutic agents, which in turn can induce BCSC death with an increase in apoptosis, cell cycle arrest, and DNA damage. Overall, reduction in BCSCs can result in reduction of tumor growth and prevention in therapy-mediated relapse.
4.8 Metformin action on breast cancer stem cells

Cancer stem cells (CSCs), also known as tumor-initiating cells (TICs), are the progenitor cells that give rise to BC as well as heterogeneity within transformed populations. CSCs are maintained as a subpopulation within the neoplasm that perpetuates clonal expansion and may facilitate dormancy, metastasis, chemoresistance, and relapse. Among the molecular BC subtypes, TNBC shows the highest enrichment of CSCs, identified by expression patterns with flow cytometry as CD44+, CD24−/low CSC [120]. BC CSCs are particularly sensitive to metformin, which induces rapid cell death facilitated through a number of pathways involved in cell differentiation, renewal, metastasis, and metabolism (Figure 7). It directly targets key CSC gene signatures such as Notch 1, NFκB, Sox2, KLF-4, Oct4, Lin28, MMP-9, and MMP-2 [121]. Metformin attenuates CSCs in resistant BC, through repression of let-7 miRNA [121]. Its ability to attenuate key metabolic genes, such as FASN via upregulation of miR-193b, also contributes to its anti-CSC activity as stem cells are heavily dependent on aerobic glycolysis [92].

The capacity of metformin to induce CSC cell death has significant clinical relevance, given their role in therapeutic resistance, dormancy, and disease progression. Metformin reduces cancer recurrence through the preferential killing of differentiated rather than undifferentiated CSCs [122]. In combination with chemotherapy, metformin is especially active against BC CSCs [111]. In studies of trastuzumab-resistant BC cells as well as xenograft models, the combination of trastuzumab and metformin significantly reduced CD44+, CD24−/low CSC subpopulations and reduced tumor volume [111, 123, 124]. In combination with doxorubicin, paclitaxel, or carboplatin, metformin can also eradicate CSCs and reduce the effective dosage required of the highly toxic chemotherapeutic agents, minimizing patient risk [111, 123].

5. Clinical evidence with metformin in breast cancer prevention and treatment

The pleiotropic oncostatic effects of metformin have been explored as an adjuvant therapeutic option for the management of BC [43, 125, 126]. Epidemiological studies have demonstrated associations between metformin use in patients with type 2 diabetes and decreased cancer incidence and cancer-related mortality [10]. Several observational and randomized trials have evaluated a number of biomarker changes after metformin administration, increasing the footage of metformin as an off-label agent for BC. Over 11 ongoing and 13 completed clinical trials have tested the efficacy of metformin as a monotherapy or in combination with chemotherapy and/or radiotherapy for the management of BC (reviewed in [43, 127]). Goodwin et al. have shown that after six months of metformin treatment, a reduction in insulin by 22% had improved metabolic indices, such as insulin sensitivity, body weight, and cholesterol levels in nondiabetic patients with early-stage BC [29]. This information suggests that metformin is effective in the nondiabetic population. These data and other clinical trials further provide support in using metformin as an adjuvant agent as it is the only agent that does not promote BC but actually retards tumor growth. In addition, these clinical trials further support the need to screen for metabolic dysfunction and evaluate whether or not metformin should be integrated into the treatment for BC therapy. Further, BC patients receiving 1500 mg/day of metformin showed a significant reduction in insulin levels and insulin resistance [44, 128]. The effect of metformin in response to neoadjuvant chemotherapy has been examined in diabetic BC patients. This study included 2529
women with BC and confirmed that metformin could achieve higher pathological complete response with neoadjuvant therapy relative to non-metformin users [129]. Dowling et al. have further examined neoadjuvant metformin in a prospective window of opportunity study [32]. Clinical and biological effects of metformin on nondiabetic BC patients were evaluated. These patients were treated with 500 mg of metformin three times daily for 2 weeks. Significant attenuated expression of the insulin receptor was observed in treated breast tumors and had high expression of OCT1 (organic cation transporter 1) [32]. The effect of metformin in nondiabetic BC patients was previously reviewed [43]. Systemic reviews and meta-analyses, highlighting a summary of studies involving metformin therapy in nondiabetic patients and diabetic patients, were reviewed in [43].

5.1 Metformin dose recommended for breast cancer patients

Pharmacokinetic profiling of mouse tumors provided preclinical analysis of appropriate human doses to provide efficient inhibition of tumor growth [130]. Based on this evidence, metformin-mediated activation of AMPK and antitumor function was dependent on cellular uptake of the drug, which is primarily controlled by membrane transporters OCT1, OCT2, and OCT3 [131]. Based on the high expression of OCT transporters, 850 mg/day of metformin is required to inhibit tumor growth efficiently. If a tumor expresses low levels of OCT transporter, then 2250 mg/day is recommended [132]. Additionally, a dose of metformin of 500–850 mg/day is typically recommended with standard chemotherapy (including anthracyclines, platinum, taxanes, and capecitabine) for first- or second-line therapy (please see https://www.drugbank.ca/drugs/DB00331). The combination of metformin with a chemotherapeutic agent is recommended for a number of cycles until progression is unacceptable or toxicity develops.

5.2 Indications and contraindications for metformin use for breast cancer

Metformin is not approved for clinical use by the FDA and is still considered investigational for the treatment for BC. While metformin is well established as an inexpensive, well-tolerated, and effective for the treatment of diabetes, adjuvant use of metformin for BC remains to be defined. Current clinical trials have not outlined indications and contraindications for metformin use as adjuvant therapy for BC. Generally, metformin hydrochloride tablets are contraindicated in patients with (1) severe renal impairment (eGFR below 30 mL/min/1.73 m2), (2) hypersensitivity to metformin, and (3) acute or chronic metabolic acidosis including diabetic ketoacidosis. Additionally, current clinical trials with metformin have been listed (https://clinicaltrials.gov/ct2/show/NCT01310231 and https://clinicaltrials.gov/ct2/show/NCT01101438). The NCIC CTG MA.32 Phase III randomized clinical trial has completed enrollment of 3649 nondiabetic women receiving standard surgical, chemotherapeutic, hormonal, biologic, and radiation treatment for T1-3, N0-3, M0 breast cancer. This trial has provided preliminary findings [33] and has not defined clear indications and/or contraindications for metformin use as adjuvant therapy for breast cancer.

6. Conclusions

A preponderance of clinical, epidemiological, and scientific evidence indicates that metabolic dysregulation of carbohydrate and lipid metabolism promote BC pathogenesis and a worse outcome, for women who have the disease [9, 10, 30, 40, 45, 129, 133].
One of the therapeutic agents commonly used in patients with metabolic syndrome or type 2 diabetes, metformin, has demonstrated significant anti-BC activity. Metformin inhibits gluconeogenesis, reduces circulating levels of glucose, increases insulin sensitivity, and reduces hyperinsulinemia associated with insulin [134]. These factors have been associated with BC prognosis. Several mechanisms of metformin action involve AMPK-dependent and AMPK-independent signaling pathways, and these effects are remarkably broad and potent. Its ability to target metabolic dysregulation of carbohydrate and lipid metabolism as well as cancer stem cells appear to be equally important in its anticancer activity against BC [129, 133–137]. Furthermore, the effects of metformin are unique among molecular subsets of BC. A better understanding of these mechanisms will facilitate targeted applications in patients with specific subtypes, fostering the goal of more personalized cancer care.

A number of clinical trials are underway to evaluate metformin in BC patients [30, 44–46, 50, 136]. Most have been designed to evaluate its efficacy, in combination with various chemo- or radiotherapy agents; see (https://clinicaltrials.gov/ct2/results?term=cancer+AND+metformin). Most ongoing or completed clinical trials have evaluated metformin's effect on cellular proliferation or death, pathological response rate, progression-free or overall survival. Some have also sought to compare its efficacy in patients with or without metabolic dysregulation, as a secondary aim. None have specifically been designed to evaluate interactions with CSCs, or in selected molecular subtypes, although correlative studies have provided some data in this regard. The ALTTO trial has shown that metformin improves outcomes for patients with diabetes and either HER2+ or hormone receptor positive BC [30]. The NCIC Clinical Trials Group (NCIC CTG) MA.32 has shown benefit from metformin, as compared to placebo on outcomes in early stage BC [33]. It demonstrated efficacy with improvements in body weight, insulin, glucose, and leptin levels in BC patients examined, regardless of baseline BMI or fasting insulin levels [33].

In conclusion, metformin is a unique drug with a long track record of human use, which has demonstrated robust efficacy against type 2 diabetes and metabolic dysregulation. Epidemiologic data show independent and significant benefit in preventing cardiovascular disease and cancer in these patients. Metformin is an inexpensive oral agent that is currently available worldwide. It is generally well tolerated and has a low risk-benefit ratio. Epidemiological and clinical data have shown that metformin reduces BC incidence and mortality in women with metabolic dysregulation, obesity, and type 2 diabetes. This subpopulation of women is at significantly higher risk for BC, particularly in the postmenopausal setting. Preclinical and clinical evidence shows that metformin inhibits BC cell replication and tumor growth, decreases tumor aggression, reduces the stem cell pool, and slows motility/metastasis and can promote cell death through apoptosis, autophagy, or upregulation of immunity. Metformin has unique effects on molecular subsets of BC, with the aggressive triple negative BC showing the most sensitivity and lowest EC50 data. TNBC is particularly sensitive to metformin's downregulation of fatty acid and cholesterol biosynthesis, glucose transport, and carbohydrate metabolism. This cancer subtype is typically the most aggressive and is less responsive to traditional chemotherapy; thus, metformin's potency may provide significant benefit especially in these patients.

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Conflict of interest

The authors have declared that no conflict of interest exists.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ACAA2 | Acetyl-coenzyme A acetyltransferase 2 |
| ADP | Adenosine di-phosphate |
| AKT | Protein kinase B |
| ALTTO | Adjuvant lapatinib and/or trastuzumab treatment optimization |
| AMP | Adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| AMPKK | AMP-activated protein kinase kinase |
| ATP | Adenosine triphosphate |
| AR | Androgen receptor |
| BC | Breast cancer |
| BCSCs | Breast cancer stem cells |
| CDK | Cyclin-dependent kinase |
| CL | Claudin-low |
| CLDN | Claudin integral membrane proteins of tight junctions |
| CSC | Cancer stem cells |
| DHCR24 | 24-dehydrocholesterol reductase |
| ESR1 | Estrogen receptor |
| HIF-1α | Hypoxia-inducible factor 1-alpha |
| EC | Effective concentration/Inhibitory concentration |
| EGFR | Epidermal growth factor receptor |
| EMT | Epithelial mesenchymal transition |
| FASN | Fatty acid synthase |
| FDA | Food and Drug Administration |
| FGFR2 | Fibroblast growth factor receptor 2 |
| FGFR3 | Fibroblast growth factor receptor 3 |
| GLUT1 | Glucose transporter 1 |
| GM1 | GM1 gangliosidosis marker |
| HER2 | Human epidermal growth factor receptor 2 |
| HER3 | Human epidermal growth factor receptor 3 |
| HMGCo-A | β-Hydroxy β-methylglutaryl-CoA |
| HMGCS1 | Hydroxymethylglutaryl-CoA synthase |
| HMGCR | 3-Hydroxy-3-Methylglutaryl-CoA Reductase |
| ID1 | Inhibitor of differentiation-1 |
| IGFIR | Insulin-like growth factor receptor |
| IGF1 | Insulin-like growth factor-1 |
| IRS1 | Insulin receptor substrate 1 |
| IL-1β | Interleukin 1 beta |
| IL-6 | Interleukin-like 6 |
| JAK | Janus kinase |
| LDLR | Low-density lipoprotein receptor |
| LKB1 | Liver kinase B1 |
| LSS | Lanosterol synthase |
| MAPK | Mitogen-activated protein kinases |
| MβCD | Methyl-β-cyclodextrin |
| MTOR | Mammalian target of rapamycin |
| MRCC1 | Mitochondrial respiratory-chain complex 1 |
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Author details

Reema S. Wahdan-Alaswad and Ann D. Thor*
Department of Pathology, University of Colorado Anschutz Medical Campus, Aurora, Colorado, United States of America

*Address all correspondence to: ann.thor@cuanschutz.edu

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