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Metformin and Breast Cancer: Molecular Targets

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Abstract
Metformin has been the first-line drug for the treatment of type II diabetes mellitus for decades, being presently the most widely prescribed antihyperglycemic drug. Retrospective studies associate the use of metformin with a reduction in cancer incidence and cancer-related death. However, despite extensive research about the molecular effects of metformin in cancer cells, its mode of action remains controversial. The major molecular targets of metformin include complex I of the mitochondrial electron transport chain, adenosine monophosphate (AMP)-activated protein kinase (AMPK), and mechanistic target of rapamycin complex 1 (mTORC1), but AMPK-independent effects of metformin have also been described. Breast cancer is one of the leading causes of cancer-related morbidity and mortality among women worldwide. Several studies have reinforced a link between breast cancer risk and diabetes. Moreover, metformin significantly reduces breast cancer risk, compared to patients who are not using metformin and is independent of diabetes status. In this review, we summarize the current molecular evidence to elucidate metformin's mode of action against breast cancer cells.

Keywords Breast cancer · Metformin · Diabetes · mTOR · AMPK

Introduction

Metformin, a biguanide, has been for decades the first-line treatment for type II diabetes mellitus (T2DM), as noted in several international guidelines, including the ADA-EASD guidelines [1]. This drug is presently the most widely prescribed antihyperglycemic drug, due to its ability to suppress hepatic gluconeogenesis and reduce blood glucose levels [2]. Metformin is also prescribed for treating some metabolic disorders such as polycystic ovary syndrome [3] and gestational diabetes mellitus [4]. The extensive use of this drug with nearly 120 million prescriptions worldwide each year is due to its favorable benefit-risk profile [5]. Indeed, it is widely recognized that metformin improves glycemic control with a good safety profile, weight neutrality or weight loss, lack of associated hypoglycemia, lower propensity for hyperlactatemia [6, 7] when compared with other biguanides such as phenformin and buformin (that led to their withdrawal in the 1970s, due to toxicity related to lactic acidosis) [7, 8], reduced cardiovascular mortality and low cost [1].

Epidemiological studies have demonstrated a correlation between T2DM and a higher incidence of malignancies, especially cancers of the liver, pancreas and endometrium (with approximately a twofold increased risk), as well as cancer of the colon, kidneys, bladder and breast (with smaller associations; about 1.2–1.5 fold) [9, 10].

Among these, breast cancer affects more than one million women every year, and although survival rates are improving, it still represents a major public health issue [11]. It is the most common cancer and the second cause of death from cancer in women [12]. Several studies have reinforced a link between breast cancer risk and T2DM [13]. Interestingly, in 2005, a first report of a reduced risk of cancer in T2DM patients treated with metformin, in comparison with patients treated with other hypoglycemic therapies, was published [14]. Since then, numerous preclinical, epidemiological and clinical studies have clearly associated the use of metformin with a reduction in cancer incidence and cancer-related death, and in vitro studies have provided mechanisms that explained the antitumor effects of this drug [15–21].

In relation to breast cancer, numerous studies reported an in vitro negative effect of metformin on the growth of breast cancer cell lines [22]. Additionally, rodent studies have shown protective effects of metformin in estrogen receptor (ER) -

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xenograft experiments and human epidermal growth factor receptor-2 (HER-2) + transgenic mammary carcinoma models [22]. However, in vivo human studies provided, to a certain degree, contradictory results. For example, in a phase II clinical trial evaluating the association of metformin with aromatase inhibitors in pretreated postmenopausal patients with hormone receptor positive metastatic breast cancer, metformin did not enhance the efficacy of aromatase inhibitors [23]. In contrast, in a phase III clinical trial evaluating the use of metformin on the outcome of patients with HER-2 + breast cancer it was shown that metformin could improve the prognosis of these patients [24]. Furthermore, in a recent systematic review with meta-analysis evaluating the association of metformin and breast cancer incidence and mortality in patients with T2DM, no effect of metformin on the incidence of breast cancer was observed; however, metformin could improve overall survival in T2DM patients with breast cancer [25]. In contrast, a population-based study evaluating long-term metformin use and breast cancer stage at diagnosis failed to show any association between these outcomes [26]. Thus, even if these studies are promising and support the concept that metformin possesses anticancer effects, there is still a lack of conclusive results from clinical trials corroborating the original epidemiological studies and the multiple antitumor effects verified in vitro. Additionally, clinical evidence for metformin antitumor activity in non-diabetic patients is also missing. Presently, more than 200 ongoing clinical trials, in almost all type of cancers, assessing the potential of metformin as an adjuvant or neoadjuvant chemotherapy agent or as an enhancer of classic chemotherapy, are registered on www.clinicaltrials.gov. Of these, many clinical trials evaluating metformin use in breast cancer are underway. Hopefully, these studies will shed light on the anticancer properties of metformin.

Despite extensive research about the molecular effects of metformin in cancer cells, the mode of action explaining its anti-tumor and chemopreventive effects remains controversial. In this review, we summarize the current knowledge concerning the molecular mechanisms involved in the anticancer effect of metformin in breast cancer.

Metformin as an Anticancer Agent

Accumulating evidence from in vitro and in vivo studies supports the fact that anticancer effects of metformin can be divided into two non-exclusive categories: an indirect/systemic effect, by reducing blood glucose and insulin levels (i.e., an antidiabetic effect), which involves both AMPK (AMP-dependent kinase)-dependent and -independent mechanisms, and a direct effect on cancer cells, involving also AMPK-dependent and -independent mechanisms [18] (Fig. 1). AMPK thus plays a major role in the anticancer effect of metformin, but mechanisms not involving AMPK have also been described. For this reason, we will describe the mechanisms of action/molecular targets involved in the beneficial effect of metformin against breast cancer in two separate categories: AMPK-dependent and AMPK-independent.

AMPK-Dependent Mechanisms of Action of Metformin

Metformin enters the cells primarily through the Organic Cation Transporter family of transporters (OCT1, OCT2 and OCT3) [27–31]. However, it is also a substrate for human multidrug and toxin extrusion 1 (MATE1) and 2 (MATE2) expressed in the liver, kidney and skeletal muscle [32]. Metformin limits oxidative phosphorylation by directly inhibiting complex I of the mitochondrial respiratory chain [19, 27–29, 33, 34], thus reducing the amount of ATP produced. This decrease in ATP levels leads to the accumulation of AMP with a consequent increase in the AMP-to-ATP ratio, resulting in the activation of LKB1, an upstream kinase, that phosphorylates and activates AMPK [20, 21, 27–29, 35, 36]. AMPK is a key regulator of energy homeostasis that plays a major role in sensing the overall energy state of the cell and shifting the metabolism accordingly [20, 35, 37, 38]. AMPK activation leads to a shift from anabolism, which consumes ATP, to catabolism, that generates it [18, 20, 21, 35, 37–39]. This AMPK-dependent effect is involved in the antihyperglycemic effect of metformin [23, 40]. However, this same mechanism could partially explain some of its anticancer properties, either by disrupting the cancer cell bioenergetics or by interfering with cell proliferation.

Effects on Metabolism

The most well-known and famous effect of metformin is its antidiabetic effect. Although some controversy exists concerning the role of AMPK in the antidiabetic effect of metformin [41], activation of this key energy-sensing enzyme seems to play a crucial role [20].

Metformin-mediated AMPK activation leads to the modulation of targets that restore energy homeostasis by enhancing glucose uptake into the skeletal muscle [42] and by inhibiting hepatic gluconeogenesis [43], resulting in an antihyperglycemic effect. Moreover, AMPK phosphorylates and inhibits several enzymes involved in lipid and sterol biosynthesis, such as acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS) and HMG-CoA reductase (14,16,19,25). By inhibiting lipogenesis and sterol biosynthesis, metformin, through AMPK, not only limits ATP consumption, but also limits the production of new cell membranes, essential for cell cycle progression and tumor proliferation [21, 28]. Furthermore, by preventing the synthesis of phospholipids,

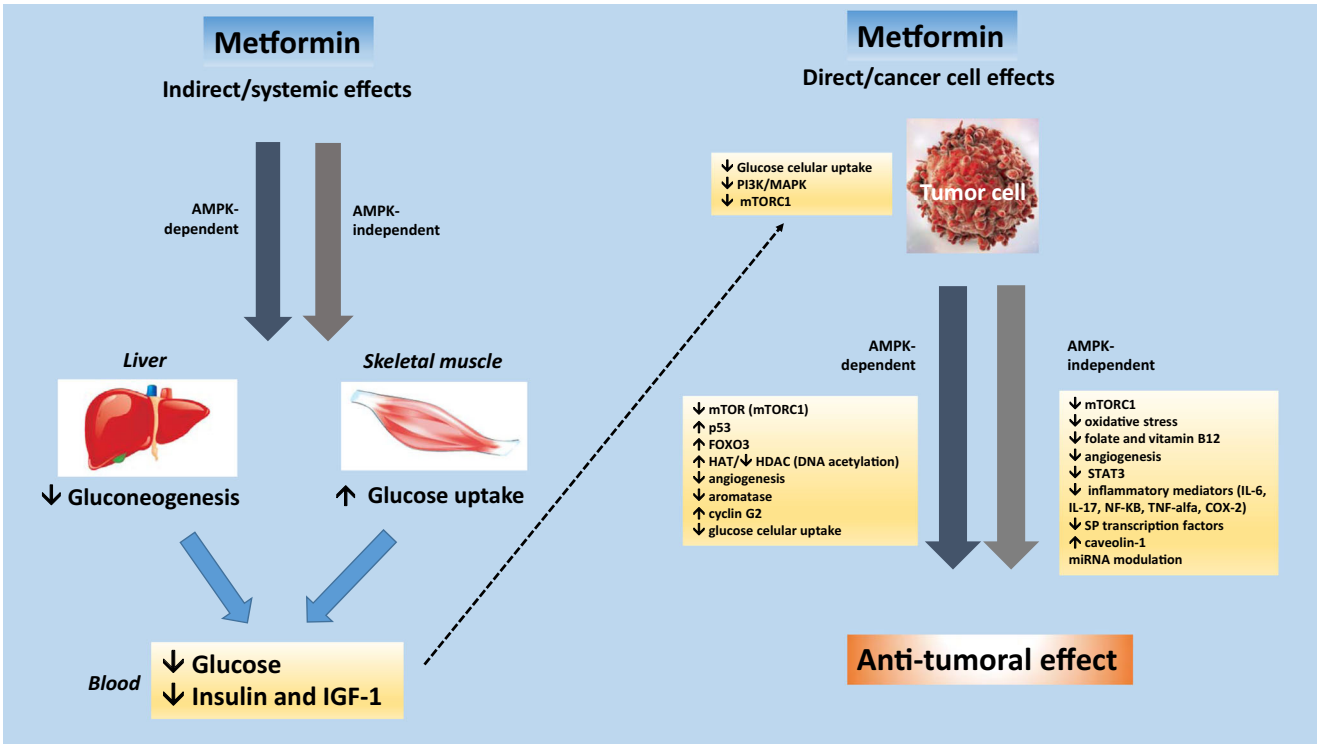

Fig. 1 Two distinct but not exclusive mechanisms contribute to the antitumoral effect of metformin: an indirect/systemic effect, mediated by a decrease in insulinemia and glycaemia, and a direct effect on cancer cells, by targeting various pathways including tumor metabolism, inflammation and angiogenesis. Both the indirect and the direct effects of metformin on cancer cells include adenosine monophosphate (AMP)-activated protein kinase (AMPK)-dependent and -independent mechanisms

metformin inhibits the formation of lipid rafts, which are membrane microdomains used, in breast cancer cells, to overexpress HER-2 receptors and that thus promote cell growth [21].

Aromatase (CYP19A1) is an enzyme capable of converting circulating androgens into estrogen in a process known as aromatization. The stromal cells of the breast tissue contain aromatase in their endoplasmic reticulum, making them capable of producing estrogen locally, which is known to stimulate cell growth and proliferation [44]. In ER-positive breast cancer cells, aromatase is overexpressed, leading to excessive estrogen production and cell proliferation [44]. AMPK phosphorylates aromatase and inhibits it, thereby inhibiting tissue-specific estrogen production and paracrine signaling [28, 44].

Nevertheless, recent advances demonstrate that metformin could also exert its glucose-lowering effect by mechanisms other than AMPK activation, namely targeting glycerophosphate dehydrogenase, reducing cAMP levels and altering the gut microbiome [45]. In relation to the first of these effects, in the liver, metformin directly inhibits mitochondrial glycerophosphate dehydrogenase, reducing the conversion of lactate and glycerol to glucose and thus reducing gluconeogenesis [46, 47]. Moreover, metformin also indirectly inhibits gluconeogenesis: the accumulation of AMP directly inhibits adenylate cyclase, reducing the amount of cAMP, which in turn greatly reduces glucagon-mediated gluconeogenesis [47].

The metformin-induced reduction in gluconeogenesis, coupled with the increased glucose consumption in the muscle and other tissues [18, 20, 40], leads to an indirect reduction in insulin production and signaling [27, 36, 40]. Since epidemiological data highlight an association between T2DM and cancer, it is not a surprise that metformin can exert an antitumor effect through its action on hyperinsulinemia [18]. Several plausible explanations link hyperinsulinemia and cancer. First, insulin may act as a growth factor promoting cancer development [48]. Indeed, insulin has mitogenic properties and excessive activity leads to an increased breast cancer risk [27, 49]. Of note, high levels of insulin can interfere with and generate resistance to transtuzumab, a HER-2 specific monoclonal antibody used in breast cancer treatment. Therefore, metformin can be combined with transtuzumab to prevent insulin resistance and create a new therapeutic alternative [21, 39]. Furthermore, hyperinsulinemia originates increased levels of insulin-like growth factors (IGFs), leading to an increase in IGF-1 levels and activation of IGF-1 receptors [50, 51]. IGF-1 plays a pivotal role in fetal development, adolescent growth, and adult tissue homeostasis; it also regulates glucose and lipid metabolism [52]. In agreement with its mitogenic action, IGF-1 is frequently overexpressed in cancer cells, including breast cancers [53].

Insulin/ IGF-1 signaling is therefore a mechanism mediating the promoting effect of T2DM on cancer. Activation of

insulin and IGF-1 receptors induces two major intracellular transduction cascades, resulting in cell growth and enhanced cell survival: the phosphoinositide3-kinase (PI3K) and the mitogen-activated protein-kinase (MAPK) signaling pathways [54]. Metformin has been found to inhibit IGF-1, with a consequent decrease in PI3K and mTOR signaling, in both in vitro and in vivo breast cancer models [55, 56].

Moreover, cancer cells typically take up high levels of glucose in order to create a fuel-rich environment for cancer progression [48]. Metformin, as a glucose-lowering agent, cuts off supplies for cancer cells, thus leading to an inhibition of tumor growth [57]. To conclude, a decrease in blood glucose, insulin and IGF-1 levels is involved in the antitumor effect of metformin in T2DM.

Effects on Cell Cycle

One of the best characterized pathways that explains the antitumor effect of metformin consists in the inhibition of mTOR through AMPK-mediated phosphorylation [20, 27, 36, 40]. mTOR is a serine/threonine kinase that regulates a plethora of cellular processes [58]. Most importantly, mTOR is activated by growth-promoting stimuli, such as an abundance of nutrients or insulin signaling, and regulates cell cycle progression and cell proliferation [58]. Activation of the mTOR pathway through ER signaling leads to cell survival and resistance to chemotherapy, tamoxifen and HER-2-targeted therapy in breast cancer cells [21].

mTOR forms two macromolecular complexes, mTORC1 and mTORC2, each with different effects [21, 58]. mTORC1 regulates protein synthesis and ribosome biogenesis, therefore modulating the temporal control of cell growth, and mTORC2 regulates the actin cytoskeleton, thus playing a role in the spatial control of cell growth [58]. AMPK leads to inhibition of mTORC1 through several distinct mechanisms. First, AMPK phosphorylates the Raptor protein, part of mTORC1, which results in its inhibition [18, 28, 36, 58]. Furthermore, AMPK also phosphorylates and activates TSC2, which is a negative modulator of mTORC1 [18, 19, 36, 58]. Moreover, because both mTORC1 and mTORC2 are stimulated by insulin [59], the indirect reduction in insulin release caused by metformin, secondary to the inhibition of gluconeogenesis by AMPK, can lead to mTORC1 and mTORC2 inactivation. Contrary to mTORC1, the effects of metformin on mTORC2 are not so clear, because mTORC2 is positively modulated by TSC2 [60]; these opposing effects can contribute to the conflicting effects of metformin. Nevertheless, the combination of these effects results in an inhibition of protein synthesis and in cell cycle arrest in G0/G1 phase [19, 36, 40]. In both ER+ and ER- breast cancer cell lines, metformin has been shown to lead to cell cycle arrest [27], which could potentially be explained through this mechanism.

Apart from mTOR inhibition, AMPK is also known to phosphorylate p53, which stabilizes its conformation and leads to its activation [18–20, 28, 39]. p53 is a tumor suppressor that plays a major role in apoptosis, cellular senescence and autophagy. p53 acts on a positive-feedback loop with AMPK in which it activates AMPK subunit β1, resulting in increased AMPK activity [20]. In addition, p53 acts in a synergic manner with AMPK to inhibit mTOR and arrest cell cycle progression [20]. In a work by Li et al, p53 knockdown/mutation in MCF-7 breast cancer cells led to resistance to the antitumor effects of both metformin and phenformin; however, MDA-MB-231 breast cancer cells treated with CP/31398, a p53 reactivator, were highly susceptible to metformin-induced growth inhibition, apoptosis and senescence. This observation suggests that p53 expression is required so that metformin can exert its antitumor effects [61]. However, the precise mechanism by which p53 responds to AMPK activation is not fully elucidated and requires further studies.

Effects on Gene Expression

FOXO3 is a transcription factor that regulates the expression of multiple genes associated with DNA repair, apoptosis, energy metabolism, stress resistance and regulation of cell cycle progression [40, 49]. After being synthesized in the cell cytoplasm, FOXO3 needs to be translocated to the nuclear compartment [38]. It has been established that FOXO3 loss of function, with cytoplasmic sequestration, leads to tumorigenesis [49]. mTORC2 phosphorylates and activates AKT, a serine/threonine kinase that plays a critical role in cell survival and glucose homeostasis [21, 38]; among the three existing AKT isoforms (AKT1, AKT2 and AKT3), AKT2 is most often involved in breast cancer cell survival and proliferation [62]. AKT phosphorylates FOXO3 at the Ser253 residue, leading to its inactivation and cytoplasmic sequestration [40]. Metformin, through an AMPK-mediated gluconeogenesis inhibition, reduces insulin release and activity, which prevents the activation of mTORC2 which, in turn, keeps FOXO3 active [38]. Furthermore, AMPK phosphorylates FOXO3 at the Ser413 residue, which leads to an increase in its activity [40, 49].

In a work by Hu et al, the administration of low-dose metformin to breast (BT-549 cell line) and ovarian cancer cells (OVCA429 cell line) resulted in increased FOXO3 nuclear localization together with p53, which led to cell cycle arrest and downregulated the expression of the stemness markers CD44, Nanog, Oct-4 and c-Myc. This observation led to the suggestion that low-dose metformin could induce a reprogramming of cancer cells into their non-cancerous phenotype [49]. However, this hypothesis warrants further exploration and confirmation.

AMPK-dependent mechanisms are also involved in the epigenetic effect of metformin. Indeed, there is evidence that AMPK modulates histone acetylation, both directly and indirectly, thus altering gene expression patterns through epigenetic regulation of various chromatin functions [35, 37]. After being translocated from the cytoplasm to the nucleus, AMPK can phosphorylate specific histone residues, which directly triggers the activation of histone acetyltransferases (HAT), in turn enhancing histone acetylation [35, 49]. However, AMPK can also phosphorylate histone deacetylases (HDAC), which promotes their export from the nucleus towards the cytoplasm, and thus HAT activity is not counteracted [35]. Finally, as mentioned previously, AMPK phosphorylates and inactivates ACC. By inhibiting the carboxylation of acetyl-CoA to malonyl-CoA, AMPK leads to an increase in the acetyl-CoA available as a substrate for HAT, which leads to increased histone acetylation and alteration of gene expression [37].

AMPK-Independent Mechanisms of Action of Metformin

miRNAs

MiRNAs, which regulate gene expression at the post-transcriptional level, are key regulators of many biological processes, such as cell proliferation, differentiation, apoptosis, stress response and angiogenesis. Accordingly, miRNAs have an important role in the regulation and development of many cancers, such as breast cancer [63]. Indeed, miRNAs behave either as oncogenes or tumor suppressor genes, thereby promoting or inhibiting cancer progression [28].

Growing evidence shows that metformin can exert anticancer effects through miRNA modulation [64–67]. Particularly in breast cancer cells, metformin has been found to interfere with the expression of several distinct miRNA that modulate the activity of distinct molecules, as shown next.

A recent work concluded that metformin inhibits the growth and invasiveness of breast cancer cells by upregulating miR-200c expression, which targets AKT2 [62]; as stated above, AKT2 promotes breast cancer cell survival and proliferation. Also, metformin was found to cause a reduction in miR-21-5p expression in breast cancer cell lines, mouse breast cancer xenografts and breast cancer patients, which enhanced the expression of critical upstream activators of the AMPK, calcium-binding protein 39-like and Sestrin-1, leading to AMPK activation and inhibition of mTOR signaling [68]. In addition, downregulation of miR-27a (which targets AMPKα2) appears to play a vital role in metformin-induced inhibition of MCF-7 cell growth [69]. In another work, metformin-reduced breast cancer cell viability and its effect on PTEN and EZH2 expression were concluded to be related to increased miR-26a expression [70]. Additionally, metformin induced an increase in the expression of miR-193 family members in triple negative breast cancer cells, and miR-193b directly targets and inhibits FAS [64], thus acting synergistically with the AMPK-dependent effects.

In breast cancer cells, upregulation of the oncogene c-Myc promotes the expression of lactate dehydrogenase A, a detoxifying enzyme whose expression is linked to cancer chemoresistance. Metformin was found to modulate the levels of c-Myc and IRS-2, and this correlated with changes of miRNA-33a levels [71].

Dicer, an enzyme belonging to the RNase III family, cleaves pre-miRNA into miRNA. DICER modulation, mir33a upregulation and c-MYC targeting were concluded to have an important role in the anticancer effects of metformin in human breast cancer cell lines [72]. The transcriptional axis including DICER and miRNAs could thus be one of molecular mechanisms underlying the anticancer effects exerted by metformin.

Oxidative Cellular Status

Reactive oxygen species (ROS), which are chemically reactive species that cause damage to cellular structures such as DNA, lipids and proteins, are produced intracellularly through multiple mechanisms, one of their main cellular sources being the mitochondrial respiratory chain [73].

Several studies have shown that metformin not only reduces ROS generation induced by stressors, but also activates endogenous repair systems, preventing ROS toxicity. Indeed, metformin can detoxify ROS as a direct or indirect free radical scavenger, and by AMPK-FOXO3-dependent up-regulation of thioredoxin activity [34]. However, in breast cancer cells, metformin was shown to increase oxidative stress levels [40, 74]. There is therefore no consensus concerning the role of the drug in oxidative stress in breast cancer cells and further studies are warranted.

Antifolate Activity

Folate metabolism is an important target for chemotherapeutic drugs. Antifolate drugs such as methotrexate and pemetrexed exert antiproliferative and cytotoxic effects in several types of tumor cells and are presently used as therapy against several common cancers including breast cancer. The importance of folate metabolism in cancer cells involves its relationship with important processes for tumor cell survival and/or chemotherapy resistance in “one-carbon” metabolism: nucleotide synthesis, the methionine cycle, glutathione synthesis and polyamine metabolism [75].

The relationship between metformin and folate metabolism alterations is based on the observation of an increase in homocysteine and a decrease in folate and B_{12} vitamin levels in patients with T2DM treated with metformin [76–78] (Fig. 2). However, only a few works investigated the

relationship between folate metabolism and the antitumor effect of metformin in breast cancer. Metformin was found to alter folate metabolism in several distinct breast cancer cell lines (MCF-7, BT-474 and MDA-MB-231 cell lines), causing inhibition of de novo synthesis of thymidine and purine nucleotides and an accumulation of homocysteine, but decreased levels of reduced glutathione [79]. In another work, metformin induced an increase in homocysteine levels and a significant decrease in levels of triphosphate nucleotides in breast cancer stem cells [80]. Finally, metformin was shown to cause a decrease in intracellular levels of glutathione in chemotherapy-resistant breast cancer cells (derived from MCF-7, BT-474 and SUM 159 cell lines) [71]. Although specific enzyme inhibition mediated by metformin has not yet been proven in breast cancer cells, inhibition of methionine synthase might be the molecular target of metformin [81].

Angiogenesis

Cancer cells establish important interactions with the surrounding cells, promoting the development of a crucial environment important to their survival, growth and proliferation. This interaction is mediated by the secretion of molecules that promote angiogenesis (pro-angiogenic factors) into the surrounding environment [82–84]. In early stages of tumorigenesis, these molecules (including TNF-α, NF-κB, ICAM-1, VCAM-1 and E-selectin) promote the formation of a large number of vessels that penetrate deep in the tumor in order to suppress its needs [82–84].

In breast cancer, HER2 is commonly overexpressed and is positively correlated with vascular endothelial growth factor (VEGF) [20, 36]. VEGF is associated with high vascularity in solid tumors [84] and is directly regulated by hypoxia inducible factor 1 (HIF-1) [84]. A few works indicate that metformin is able to suppress breast tumor angiogenesis through various effects [84, 85]. The first effect was concluded to involve a mechanism linked to targeting of HER2/HIF-1α/VEGF secretion axis [84]. The second effect involves mTORC1 inhibition through AMPK-dependent mechanisms described above, as HIF-1α production is also stimulated by mTORC1 [86].

JNK and STAT3

JNK and STAT3 are key regulators of many cellular events, promoting cell proliferation, differentiation and apoptosis and, consequently, the progression of cancer. Accordingly, prolonged upregulation of some members of STAT family, such as STAT3, has been associated with some breast cancers [87].

Recent research demonstrated that IL-6 contributes to invasion and growth in breast cancer via JAK and STAT3 signaling pathway [87–89]. Interestingly enough, metformin was reported to inhibit the expression of pro-inflammatory mediators such as IL-6 and Il-17, reducing the activation of NF-kB and consequently tumor development [34]. Metformin can also block the phosphorylation of STAT3, which can induce apoptosis in some breast cancers [79, 87, 88].

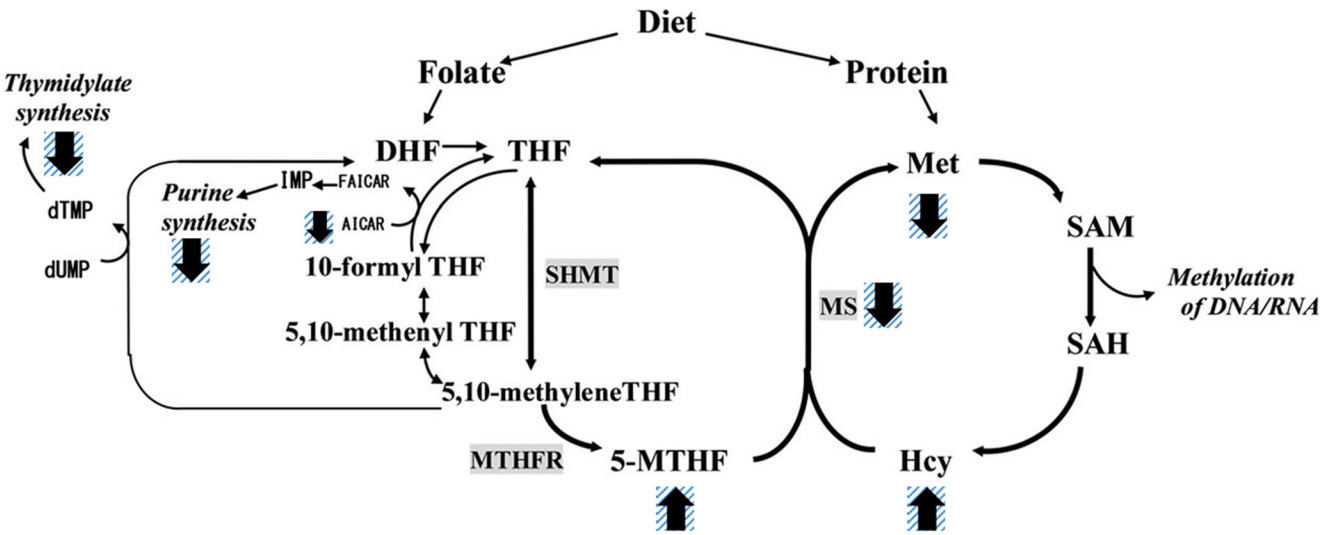

Fig. 2 Effect of metformin on folate and methionine metabolism. Arrows indicate increase and decreased levels of the indicated compounds. DHF = dihydrofolate; THF = tetrahydrofolate; 5-MTHF = 5-methyltetrahydrofolate; 5,10-metyleneTHF = 5,10-methylenetetrahydrofolate; 5,10-methenyl THF = 5,10-methenyltetrahydrofolate; 10-formyl THF = 10-formyltetrahydrofolate; Hcy = homocysteine; Met = methionine; SAM = s-adenosyl methionine; SAH = s-adenosyl homocysteine; dUMP = deoxyuridine monophosphate; dTMP = deoxythymidine monophosphate; AICAR = 5-amino-4-imidazole carboxamide ribonucleotide; FAICAR = 5-formamidoimidazole-4-carboxamide ribonucleotide; IMP = inosine monophosphate; SHMT = serine hydroxymethyltransferase; MTHFR = methylenetetrahydrofolate reductase; MS = methionine synthase

NF-kB

Inflammation contributes to carcinogenesis via stimulation of ROS and NO production. As a result, inflammation causes accumulation of DNA damage and mutations of tumor suppressor genes, which may result in genetic instability and carcinogenesis.

In relation to breast cancer, increased circulating levels of TNFα and IL-6 are found in obese women and have been associated with breast cancer development and progression [90, 91]. TNFα is one of the most potent physiological inducers of the nuclear transcription factor NF-kB, and activation of NF-kB supports progression of breast cancer growth and it is dependent of degradation of IkB-alpha [90, 91].

In in vitro, in vivo, and human studies, metformin was found to reduce inflammation by decreasing the levels of the inflammatory cytokines Il-6 and TNF-α, the activity of COX-2, the proinflammatory transcription factors NF-kB and STAT3 and of Saa1 and Saa2. Stimulation of the SIRT1 signaling pathway seems to be also involved in the anti-inflammatory effect of metformin [34]. In relation to breast cancer cells, metformin was shown to inhibit the inflammatory response in cancer stem cells in breast cancer cell lines, associated with inhibition of NF-KB activation [92].

mTOR

Although AMPK-mediated phosphorylation is the most frequently described mechanism for metformin-induced mTOR inhibition and anticancer effect (see above), AMPK is not mandatory for metformin's anticancer effects [93–95], and alternative mechanisms could be at play in metformin-induced mTOR inhibition and anticancer effect.

An alternative mechanism described in the literature is AMPK-independent but Rag GTPase-dependent metformin-induced mTORC1 inhibition [95]. Rag GTPases mediate amino acid stimulation of mTORC1 signaling by inducing translocation of mTORC1 to a perinuclear intracellular compartment [96].

Wu et al proposed a mechanistic model connecting metformin with mTORC1 through metformin's effect on the Nuclear Pore Complex (NPC), based on data from consecutive genetic screens in *C. elegans*, validated in human cell lines [97]. Biguanides inhibit the mitochondrial respiratory capacity and therefore promote lower ATP levels, leading to impaired transport through the NPC and thus restricting RagA-RagC GTPase transit. Exclusion of RagC from the nucleus prevents it from attaining a GDP-bound state that is necessary for mTORC1 stimulation. In this manner, biguanides are able to inhibit cell growth and proliferation by inhibiting mTORC1 in an AMPK-independent but NPC- and Rag GTPase-dependent way [97].

This AMPK-independent mechanism could be relevant for metformin's action in breast cancer, as in breast cancer cells, RAG GTPase/mTORC signaling was found to promote cancer proliferation via G0/G1 cell-cycle progression by activation of the cyclin D1/Rb/E2F pathway [98].

Another proposed mechanism for metformin-induced AMPK-independent mTOR inhibition is the activation of a chronic energy-depletion response mediated by the tumor suppressor, Regulated in Development and DNA Damage responses (REDD1), described to act in a TSC1/TSC2-dependent manner [99, 100].

Specificity Protein (SP) Transcription Factors 1, 3 and 4

Metformin is associated with down-regulation of SP transcription factors, namely SP1, SP3 and SP4 [101]. These SP proteins regulate the transcription of many genes associated with the 'hallmarks of cancer'(sustained proliferative signaling, replicative immortality, resistance to cell death, avoidance of immune destruction, induction of angiogenesis, invasion and metastasis, and deregulation of cellular energetics) [102] and are further implicated in the regulation of cancer-associated long non-coding RNAs (lncRNAs) [103].

Metformin's effect on the expression of SP transcription factors could be particularly relevant in the context of breast cancer. SP1 expression is increased in breast cancer and associated with a poor prognosis [102]. SP1 and its direct interaction with STAT6 is implicated in the modulation of cell cycle proteins in breast cancer cells, in particular cyclin-dependent kinase inhibitors p21 and p27 [102, 104]. SP1 and SP3 are also implicated in the hormone-dependent regulation of VEGF expression in breast cancer cells [102, 105]. In basal-like breast cancer (BLBC) cells, SP1 can regulate the transcription of FOXF2, which is associated with epithelial-mesenchymal transition and enhanced metastatic ability in this subtype of breast cancer. This effect is impaired by FOXF2 promoter methylation [106].

Caveolin-1

Reduced caveolin-1 mRNA level was shown to be significantly associated with increased tumor size in human breast cancer [107]. Interestingly, metformin has shown promising results in promoting the efficacy of an antibody drug conjugate, trastuzumab emtansine, also known as T-DM1, in the HER-2-positive breast cancer cell line BT-474, and this effect was shown to be partially caveolin-1-dependent. Caveolin-1 is a membrane protein implicated in endocytosis and vesicle trafficking, whose expression levels could be a limiting factor in promoting antibody drug conjugate permeation and efficacy in breast cancer cells. Treatment with metformin induced caveolin-1 up-regulation, leading to an enhanced antibody

drug conjugate internalization and thus to a significantly greater cytotoxic effect on T-DM1-treated breast cancer cells. Metformin, a low cost and safe drug, could therefore be very promising in current and future targeted antibody drug conjugate treatments for breast cancer by enhancing caveolin-1-mediated internalization [108].

Cell-Cycle Regulatory Proteins

Cyclin G2 (CycG2) is negatively regulated by estrogen (E2)-bound ER, and CycG2 overexpression induces a p53-dependent G1-phase cell cycle arrest. Thus, in the presence of E2, ER+ breast cancer cells express lower levels of CycG2, which contributes to their mitotic activity. Interestingly, metformin was found to promote G1 cell cycle arrest in a CycG2-dependent way in the estrogen-sensitive breast cancer cell line MCF7 [109, 110].

Growth factor receptor tyrosine kinase (RTK) signaling pathways trigger hyperactivation of growth promoting PI3K/AKT/mTOR signaling, in turn inducing ER phosphorylation, a modification that promotes ligand-independent ER activity and CycG2 inhibition. Consequently, suppression of RTK signaling or of its downstream PI3K or mTOR signaling, for instance with metformin (through mTOR inhibition), is thus expected to promote higher expression of CycG2, contributing to cell cycle arrest [110].

Metformin's CycG2 dependent effect was further shown to enhance cell cycle arrest when in co-treatment with the selective ER downregulator fulvestrant in MCF7 cells [110]. Metformin upregulation of CycG2 and potentiation of fulvestrant-induced CycG2 expression leading to G1 cell cycle arrest supports the possible application of metformin as an adjuvant in ER+ breast cancer therapy.

Glucose Cellular Uptake

An inhibitory effect of metformin on glucose cellular uptake by breast cancer cells was recently described. Interestingly, this inhibitory effect was verified after a short-term exposure of the cells to the drug, but longer exposures increased the rate of glucose cellular uptake and lactate production, most probably to compensate for the energy depletion associated with inhibition of oxidative phosphorylation [111].

Future Perspectives

While in recent years light has been shed on the mechanisms through which metformin exerts its antitumor effects, a lot remains unanswered or unclarified. In an article by Corominas-Faja et al, those authors proposed that metformin does not inhibit mitochondrial complex I and instead acts as an antifolate drug [79]. In fact, in a phase III randomized clinical trial, it was shown that non-diabetic breast cancer patients in the metformin arm of the study have lower plasma vitamin B12 levels, despite not being associated with clinical vitamin B12 deficiency [112]. These clinical results add consistency to the proposed mechanism of action. However, they do not invalidate the possibility that metformin might have both antifolate effects and mitochondrial complex I inhibitory activity. The fact that metformin's main mechanism of action is not universally accepted warrants further studies to fully clarify what should be the basis of any future research into the antitumor effects of metformin. It is possible that, because of this uncertainty, much information in the literature is contradictory, namely the articles referring to the effects of AMPK on histone acetylation [19, 34, 113]. Given that changes in the epigenetic regulation lead to altered patterns of gene expression, the influence of metformin in epigenetic mechanisms is a research topic that shows much promise in explaining metformin's antitumor effects and thus should be adequately resolved.

Both systemic/indirect and direct (on tumor cell) effects are known to contribute to the antitumor effect of metformin. However, there is a critical gap in knowledge concerning these two types of effects. Indeed, although the effects of metformin in whole-body metabolism are well characterized, less is known concerning the direct effects of metformin on cancer cells. For instance, few in vitro and in vivo studies quantified uptake and intratumor accumulation of the drug. As previously stated, metformin enters the cell through OCT1 and, to a lesser extent, OCT2 and OCT3 [30, 31, 114]. These transporters are primarily expressed in enterocytes, hepatocytes and renal epithelium, having a lower, yet significant expression in other target tissues, such as the breast epithelium or the lung [32, 114]. However, not all breast tumors express these transporters, and metformin's possible antitumor effect in non-OCT-expressing tumors will be severely diminished [114]. Highlighting the importance of these transporters, Lord et al showed that the highest metformin tumor levels were detected in the patient with the highest OCT1 tumor expression [115]. Also, Checkley et al found that the amount of metformin accumulated in mammary tumors in the rat correlated with mammary tumor regression and with tumor expression of OCT2 [116]. So far, most preclinical in vitro studies and clinical trials have not taken these transporters into consideration when evaluating metformin's effects [114], which could explain the lack of agreement between pre-clinical and clinical studies. Therefore, a future possibility would be a clinical trial evaluating and comparing the clinical outcomes of metformin administration to breast cancer patients after screening for OCT expression. Moreover, genetic variants in metformin transporter genes such as OCT1 have been associated with variation in the antidiabetic clinical response to metformin [117]. Therefore, identification of variants within the tumor could also be interesting by helping to predict which patient groups might respond clinically to metformin.

While the dose required for metformin to exert its antidiabetic effects is very well established, the same cannot be said for its antitumor effects [29]. Indeed, most studies using cell lines or animal models use extremely high metformin doses that far exceed the ones used to treat T2DM in clinical practice [31, 115]. It is therefore possible that non-specific or toxic effects of metformin are observed in vitro, because supra therapeutic doses are used. This fact could be a strong argument to explain why most clinical trials have failed to show associations so far; moreover, clinical trials are quite heterogeneous regarding metformin dosage. For example, in the ALTTO phase III trial, the standard, currently administrated dose of metformin as an antidiabetic drug improved the prognosis associated with diabetes in patients with HER-2-positive and hormone receptor-positive breast cancer [24]. However, in a cohort study by Chang et al, in which the relationship between metformin and colorectal cancer occurrence among patients with T2DM was evaluated, it was shown that the risk of colorectal cancer was lower when the dose of metformin employed was higher, expressed as the cumulative defined daily dose and the intensity of metformin use [118]. There seems to be no consensus regarding the ideal, most adequate dose to be used as a neoadjuvant or adjuvant therapy. Therefore, it is important that in vitro, in vivo and clinical trials are performed to clarify such an important parameter that could have serious implications in future clinical trials, not only in terms of metformin's antitumor effects and the occurrence of serious adverse effects, such as lactic acidosis [31], but also in terms of drug interactions.

Another aspect that should be clarified in the future is the efficacy of metformin in relation to breast cancer subtype and/or receptor status. For instance, in vitro studies with the ER+ MCF-7 cell line consistently reported reduced proliferation as well as increased apoptosis in response to metformin. In contrast, studies using the triple-negative MDA-MB-231 cell line have not reported consistent findings [22]. Discrepancies in the in vivo effect of metformin in breast cancers with distinct ER/PR/HER2 status have also been reported. In one study, metformin-treated diabetic patient women compared to nonusers had a significantly lower incidence of grade III tumor, a lower incidence of triple negative and a higher incidence of ER+ and PR+ cancers [119]. In another study, it was concluded that, while the number of years under metformin treatment was not associated with the risk of triple-negative tumors, a negative association with the risk of hormone-receptor+/HER2− breast cancer was observed [120]. Still another study found that long-term use of metformin correlates with molecular subtype of breast cancer in diabetics on metformin in comparison to diabetics not on metformin and patients without diabetes mellitus [121]. All these observations thus reinforce the need to correctly classify breast cancers when studying their association with diabetes.

Another point worth discussing is the reason for the discrepancies found between the in vitro and the in vivo effect of metformin and between distinct epidemiological studies. Besides the reasons already mentioned (distinct effects of metformin in different breast cancer subtypes, distinct accumulation of the drug in different tumors due to transporter variability, the high doses of metformin used in in vitro studies and the heterogeneity in metformin doses used in clinical trials), some other reasons can be advocated. These include distinct basal autophagy and mTOR activity in different cancer cells [122] and the need to better select patients and disease outcome, because multiple and heterogeneous features from both the patients and disease are currently considered.

It is also possible that, to be effective in cancer treatment, new strategies of drug delivery must be employed [19, 123]. For example, in a study by Qian et al, it was shown that a metformin delivery vehicle based on metformin-loaded glyconanoparticles (MGNPs) significantly improved the antiproliferative effect of metformin over standard use against MCF-7 breast cancer cells [123]. This observation could have an impact both in future research, but also in the clinical setting.

To conclude, metformin appears as an effective anticancer drug in relation to breast cancer. Also, drug combination with metformin (e.g. chemotherapy or glycolytic inhibitors) can act as a synergistic association, highlighting the potential of metformin in breast cancer treatment. However, metformin's mechanisms of action have only begun to be clarified and much research is still needed before establishing metformin as a new anticancer drug. It is obvious that complex metabolism-modifying drugs such as metformin have several modes of action, involving multiple and mutually nonexclusive mechanisms. A deeper knowledge about these mechanisms could improve the design of new drugs and the understanding of the potential use of metformin in cancer. The fact that the safety profile and side effects of metformin are thoroughly well established [19, 27] make it a very appealing drug to test in cancer patient settings as it could lead to a new era of personalized medicine and improved treatments.

Compliance with Ethical Standards

Conflict of Interest Authors J. Faria, G. Negalha, A. Azevedo, F. Martel declare that they has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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