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Metformin Anti-Tumor Effect and Metabolic Reprogramming in Breast Cancer Cells

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Abstract

Evidence suggests that metformin, an antidiabetic drug, possesses antitumor effects in breast cancer. Although metformin is known to induce metabolic reprogramming, a detailed description of the metabolites altered by metformin in breast cancer cells is limited. In this study, we aimed to investigate metformin's anti-tumor effect on breast cancer cell lines and to conduct an untargeted metabolic analysis in those cell lines post-metformin treatment. Metformin's effect on cell viability of MCF-7 and MDA-MB-231 cell lines was done using a CCK-8 kit, and apoptosis assay was conducted using Annexin V staining. HPLC and LTQ XL Linear ion trap mass spectrometers were used for quantifying metabolites in both cell types post-metformin treatment. Metformin showed a reduction in cell viability in MCF-7 and MDA-MB-231, with IC50s of 14.21±1.33 mM and 12±5.8 mM respectively. It induced 2.1±0.5% and 22.1±2.8% apoptosis in MCF-7 and MDA-MB-231 cells respectively. Both cell lines showed different metabolites in response to treatment. Of 31 identified metabolites in MCF-7, 12 significantly differed with metformin, mostly up-regulated. Of 39 metabolites in MDA-MB-231, 30 significantly differed as compared to untreated cells. Enrichment pathway analysis showed metformin to alter major metabolic pathways. Of interest, was the upregulation of glutathione, sphingosine, and sphingosine-1-phosphate in MDA-MB-231, which could support investigating combining metformin treatment with drugs that can target these pathways. Metformin alters tumor metabolism in a cell-dependent manner in breast cancer cells which can open opportunities for new combination therapies, however, further studies are required to support and understand these findings in depth.

Keywords: Metformin, Breast cancer, Metabolites, LC-MS/MS, Glutathione, Sphingosine

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Introduction

Metformin is one of the most widely described blood glucose-lowering- agents used to treat type 2 diabetes with a high safety profile (Foretz *et al*., 2014; Bailey, 2017). Over the years, studies have shown that the use of metformin reduced cancer incidence in various tumors (Evans *et al*., 2005). Since then, an increasing number of clinical studies have reported the anti-cancer effects of metformin in several cancer types (Suissa & Azoulay, 2012; Margel *et al*., 2013; Niraula *et al.*, 2013; Tang *et al*., 2017). This is not surprising given the cytotoxic effect metformin has on cancer cells, however how the drug reduces tumor growth and the exact mechanism of action remains to be not fully understood (Algarni *et al.*, 2020; Sarami *et al*., 2020).

Recently, more attention has been directed toward understanding metformin's anti-tumor effects, specifically metabolic reprogramming. It is known that energy metabolism is a crucial tumor hallmark that plays a role in cancer development and progression and thus is an attractive target for cancer therapeutics

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(Mohamed *et al*., 2020; Sun *et al.,* 2021). Metformin is known to inhibit mitochondrial electron transport chain (ETC) complex 1, therefore, decreasing cellular respiration. This was seen in human cancer cells, and indeed metformin was able to induce cell death with glucose deprivation, implying that cancer cells would rely on glycolysis in the presence of metformin (Wheaton *et al.,* 2014; Aleidi *et al*., 2022). Metformin's metabolic-effect complexity was further seen in a study that showed that the tumor environment can alter cells' sensitivity to metformin (Gui *et al*., 2016). For example, cells grown in DMEM without Pyruvate were more sensitive to metformin's anti-proliferative effect (Gui *et al.*, 2016). Overall, this was attributed to the fact that environmental cues and metabolites can alter a cell's dependency on complex 1 and cellular respiration.

Despite some of the available metabolic studies for metformin, many questions remain unanswered including which metabolites can be affected by the drug, whether its effect is cell-type dependent, and perhaps what compensatory pathways induced by metformin treatment should be targeted simultaneously.

In this study, we were interested in studying the effect of metformin treatment on two different breast cancer cell lines, the triple-negative breast cancer cell line (TNBC) MDA-MB-231, and the estrogen-receptor-positive MCF-7, and to investigate the effect of the drug on their metabolites (Daivasigamani *et al*., 2022).

Materials and Methods

Drug Preparation

Metformin (Sigma-Aldrich, Zwijndrecht, The Netherlands) was dissolved in PBS to prepare a 1 M solution and stored at -20 ℃ until further use.

Cell Culture

Human breast cancer cell lines MCF-7 (estrogen-receptor-positive) and MDA-MB-231(triple-negative) cells were obtained from the Regenerative Medicine Unit, King Fahad Medical Research Center, SA. All cells were maintained in DMEM (bio sera-France) medium supplemented with 10 % fetal bovine serum (FBS: bio sera-France) and 5% antibiotics (penicillin: bio sera-France), and incubated in 5% CO2 at 37˚C.

Viability Assay

The Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Japan) was used to assess cell viability. Briefly, cells were plated at a density between 4000 and 5000 cells/well in 96-well plates in 200 µl of DMEM supplemented with 10% FBS, and 5% antibiotics (penicillin). After 24 h, cells were treated with various metformin concentrations diluted with a culture medium. Five replicates were prepared for each concentration. Cells were then incubated at 37˚C for 24, 48, and 72 h, and then 5 µl of CCK-8 reagent were added for 2 h at 37˚C. Finally, the optical density was measured using a microplate reader (BioTek-ELx808) at 450 nm. The mean values of five readings were calculated, growth curves were drawn and IC50 was determined.

Apoptosis Assay

MCF-7 and MDA-MB-231 were either treated with the predetermined IC50s of metformin or fed with drug-free media as control cells, for 48 h. As a positive control, cells were treated with 40 µM of Thymoquinone (Bashmail *et al.,* 2018). Cells were harvested and washed twice with PBS, and 3 µl of Annexin V-FITC/PI solution (Abcam Inc., Cambridge Science Park, Cambridge, UK) was added to the cells. Cells were then kept at room temperature in the dark for 5 mins. Three replicates were prepared for each condition. Following incubation, cells were injected via BD FACSAria ™ flow cytometer (BD Biosciences Inc., San Jose, CA, USA) and assessed for FITC and PI fluorescent signals

Stem Cell Detection

To study the effect of metformin on BCSC (CD44+/CD24−), cells were treated with the predetermined IC50s of metformin for 24h, while control cells were cultured with drug-free media. After 24 h, cells were harvested and washed with ice-cold PBS. Cells were then incubated at 4 C for 30 min in the dark with 100µL of 1 µg/mL conjugated anti-CD44 and 1 µg/mL anti-CD24 antibodies (FITC labeled anti-CD44 (Cat. # AB27285) and APC/ Cy7 labeled anti-CD24 antibodies (Cat. #AB197137) (Abcam Inc., Cambridge Science Park, Cambridge, UK) or their isotype controls mouse IgG1, monoclonal [HyblgG1] (FITC)- isotype control (Cat. #Ab1264) and mouse IgG1, kappa monoclonal (APC/CY7)-isotype control (Cat. #AB46739). After incubation, cells were washed three times with ice-cold PBS. Finally, cells (10,000 events per sample) were assessed for FITC and APC/CY7 signals via BD FACSAria ™ flow cytometer (BD Biosciences Inc., San Jose, CA, USA) Cells quantity were analyzed by quadrant analysis using BD FACSDiva Software

Untargeted Metabolomic Experiments

To study the effect of metformin on breast can cell metabolites, 3x106 cells were treated with the predetermined IC50s of metformin for 24h, while negative control cells were cultured with drug-free media. Cells were harvested and washed twice with PBS and frozen immediately as a pellet in liquid nitrogen.

Metabolites were extracted from the cell lines (MCF-7 and MDA-MB-231) treated with metformin and control untreated cells by using the solvent in a ratio of methanol: acetonitrile: water (2:2:1 v/v). One mL of ice-cold solvent was added to the cells to form a cell lysate. The cell lysate was vortexed and then incubated for 1 h at 20° C. Following incubation cells were centrifuged at 13,000 rpm at 4 °C for 15 mins. The supernatant was saved, and the sample was dried in a vacuum concentrator. To remove insoluble debris, the dry extracts were resuspended in 100 µL of acetonitrile: water (1:1, v/v), vortexed, and then centrifuged at 13,000 rpm at 4 °C for 15 mins .10 µL of the sample was injected into an HPLC (Thermo Finnigan Surveyor Autosampler Plus) column (Hypersail gold; 150mm x 4.6 mm, 5 µm) with a flow rate of 0.250 mL/min. The mobile phase (A) of 0.1% Formic acid and (B) 99.9% ACN Formic acid (0.1%, v/v), using a linear gradient program where the component of solution was changed from 5% B to 100% B over 90 min, at a constant flow rate of 0.2 mL/min (95% A from 5% to

30% over 72 min, 30% to 100% over 10 min, and kept at 100% for 5 min at a flow rate of 0.25 mL/min). The column temperature was maintained at 30° C and metabolites were analyzed in LTQ XL linear ion trap instrument (Thermo Fisher Scientific). Full scan scope was chosen from 80 to 1000 m/z. The spray voltage was set at -3.0 kV, the capillary voltage was fixed at 4.0 V. The flow rate of nitrogen (sheath gas) was set at 40 arbitrary units. Further, helium was used as the buffer gas for run 187. The raw data were processed using (XC-MS) data processing software.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism® for Windows, version 8.0 (GraphPad Software Inc., La Jolla, CA, USA). All assays were done in triplicates or more as mentioned. Data were presented as mean ± SD. The differences between groups were tested using a student t-test (unpaired) between two groups, or a one-way ANOVA test when more than two groups. $P<0.05$ was taken as a cut-off value for significance. Statistical analysis of metabolomic results and pathway analysis were performed using Metaboanalyst and metabolite identification using the human metabolome database (HMDB).

Results and Discussion

Effect of Metformin on Cell Viability and Apoptosis of Breast Cancer Cell Lines

To study the anti-tumor effect of metformin on breast cancer cell lines, cell viability post-metformin treatment was determined using the CCK-8 assay. At 72 hours, metformin showed a decrease in cell viability in MCF-7 and MDA-MB-231 with IC50s of 14.21±1.33 mM and 12±5.8 mM respectively **(Figure 1)**.

To determine the proportion of apoptotic cells induced by metformin, both cell lines were treated with the predetermined IC50s for 48 hr. Cells were assessed by Annexin- V/FITC apoptosis detection assay which showed that metformin-induced apoptosis in both cell lines MCF-7 and MDA-MB-231 (2.1±0.5% and 22.1±2.8%s respectively) **(Figure 2)**.

Effect of Metformin on BCSC Population

To study the effect of metformin on tumor-associated stem cells, cells were treated with metformin, and CD44+/CD24- cell clone was assessed using flow cytometry. In MCF-7 cells treated with metformin for 24hr did not significantly alter the percentage of CD44+/CD24- cell clones compared with control untreated cells with a mean of 96.7 ± 0.5 % and 94.9± 1.0% respectively. In MDA-MB-231 cells, however, treatment with metformin appeared to increase CD44+/CD24- cell clone 91.3 ± 0.7% compared to control untreated cells 85.7± 2.8% ($P<0.05$). Expression levels of CD44 and CD24 were plotted as a percentage of total events **(Figure 3)**.

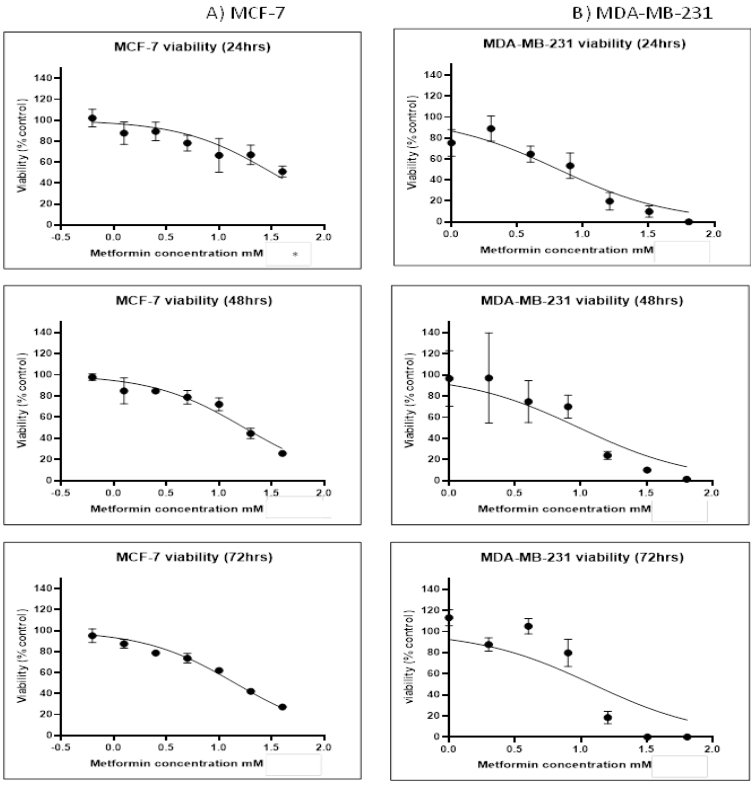


Figure 1. Metformin effect on cell viability in breast cancer cell lines. MCF-7 (Left) and MDA-MB-231 (Right) breast cancer cell lines were exposed to serial dilution of metformin for 24 h, 48 h, and 72 h. Cell viability was determined using the CCK-8 assay kit. Data is expressed as mean ± SD (n =3).

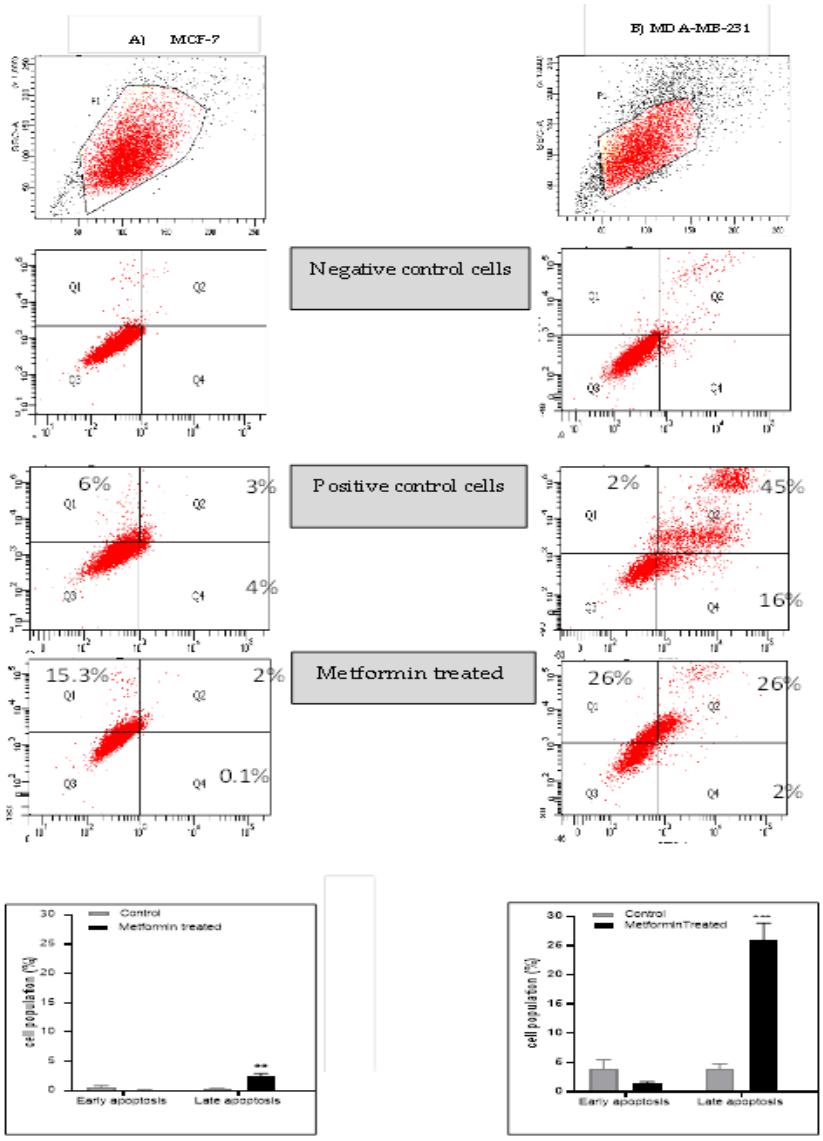
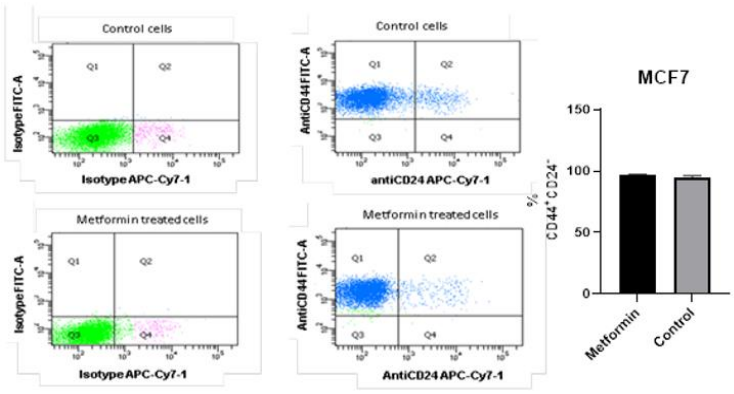
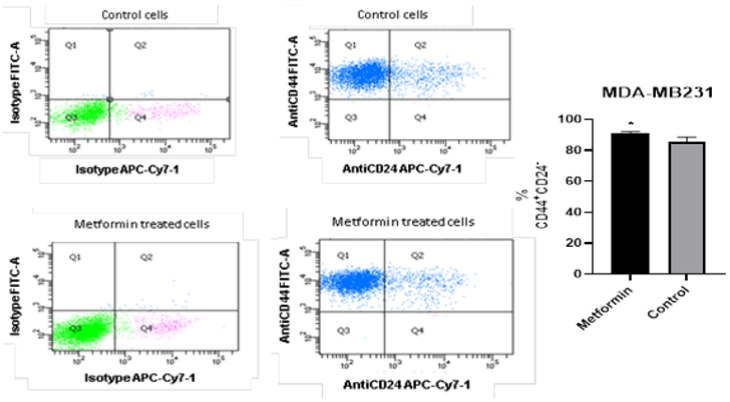


Figure 2. Apoptotic effect of metformin on MCF-7 and MDA-MB-231. The percentage of apoptotic cells following metformin treatment (48h) was measured using the Annexin V-FITC/PI detection kit and flow cytometry analysis on MCF-7 (A) and MDA-MB-231 (B). Data are presented as mean ± SD, n = 3. Significance was tested compared to control untreated cells. (**) P < 0.01; (***) P < 0.001



a) MCF-7



b) MDA-MB-231

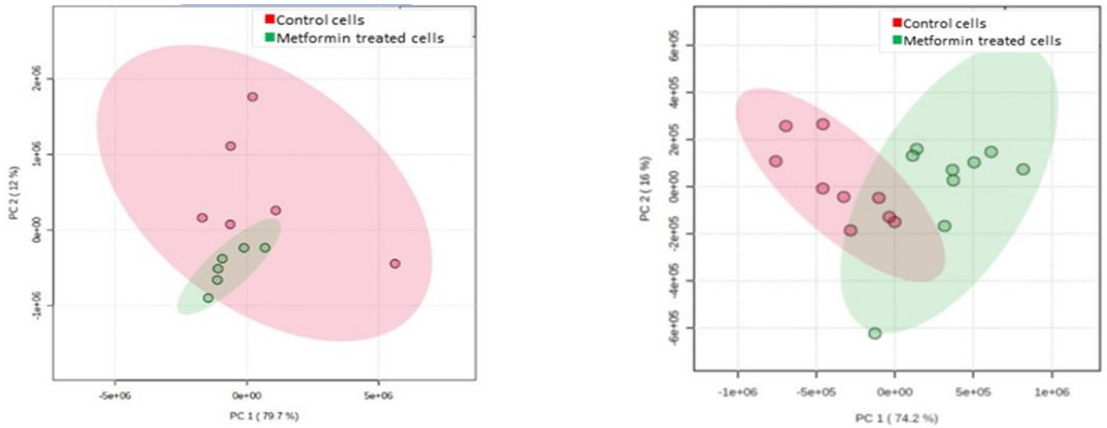
Figure 3. Effect of metformin on the expression of CD44 and CD24 stem cell markers. MCF-7 (a) and MDA-MB-231 (b) cells were treated with metformin for 24 h. Representative flow cytometry dot blot graphs showing the expression levels of CD44 and CD24 plotted as a percentage of total events. Bar graphs represent a statistical summary of dot blots with data expressed as mean ± SD (n =3). (*) $P < 0.01$.

Effect of Metformin on Breast Cancer Cell Lines Metabolites

To study the effect of metformin treatment on MCF-7 and MDA-MB-231 metabolites, both cell lines were either left untreated as control cells or treated with the predetermined IC50s of metformin for 24h. An untargeted metabolite analysis using LTQ XL linear ion trap mass spectrometer LC/MS-based was done. After analyzing the feature peaks (metabolites), 31 and 39 metabolites were detected in MCF-7 and MDA-MB-231, respectively (Bhavyasri *et al*., 2023; Choudhary *et al*., 2023; Nwankwo *et al*., 2023; Sarangi *et al*., 2023).

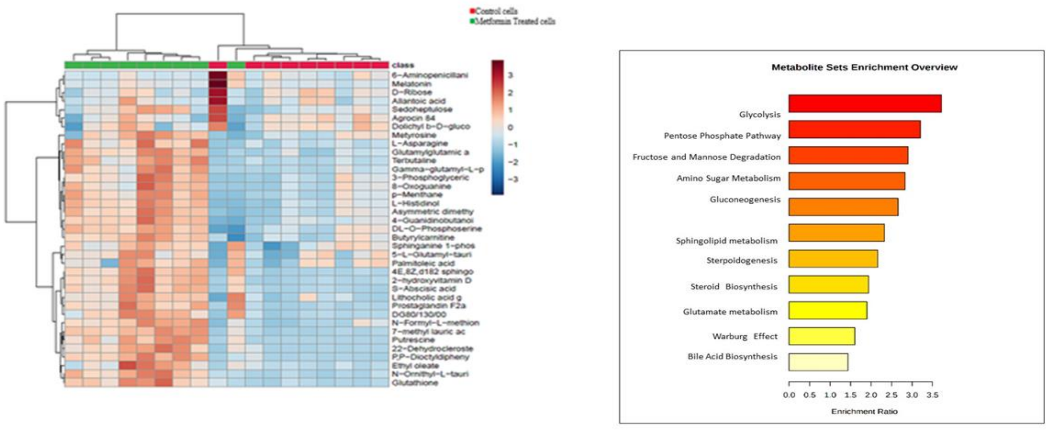
Two-dimensional principal component analysis (PCA) models score plots of all samples showed that all groups of samples were tightly clustered and revealed a significant difference between treated and untreated samples **(Figure 4)**. The heat map further determined that metformin had strong effects, resulting in a metabolic variation on both cell lines used in our study **(Figure 5)**.

Among the 31 metabolites that were detected in MCF-7, 12 metabolites were significantly different when compared to control untreated cells, 10 of them were upregulated while 2 were downregulated **(Table 1)**. Interestingly, different metabolites were detected with MDA-MB-231 cells, and among the 39 detected metabolites, 30 metabolites were significantly different when compared to control untreated cells **(Table 2)**. Interestingly all of those 30 metabolites were upregulated. Using metaboanalyst, the Enrichment Pathway Analysis of metabolites was obtained. The result showed that a total of 11 and 18 metabolic pathways were shown to be enriched in metformin-treated MCF-7 and MDA-MB-231 respectively. These pathways involved mainly carbohydrate metabolism in MCF-7 cells while lipid and amino acid metabolites appeared to be more enriched in MDA-MB-231 cells **(Figure 5)**.

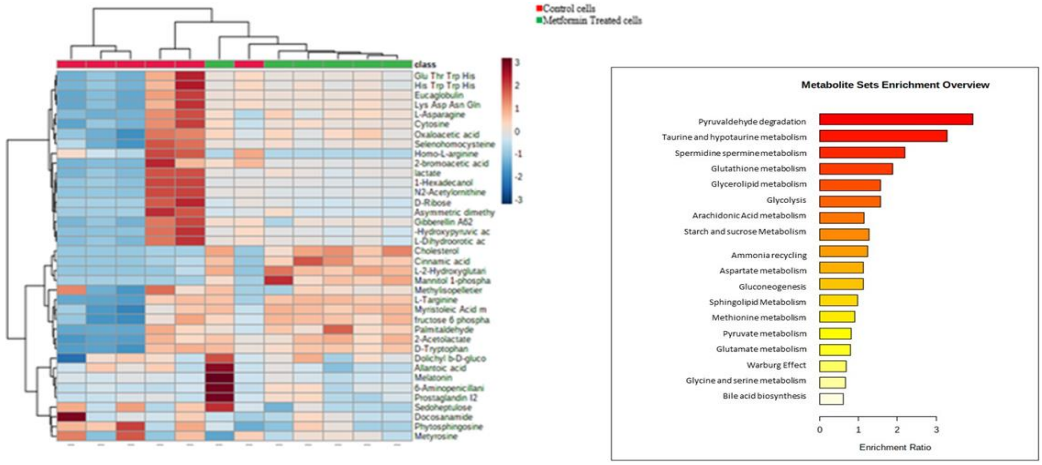


a) MCF-7 b) MDA-MB-231

Figure 4. Two-dimensional PCA score plot of MCF-7 (a) and MDA-MB-231 (b) post-metformin treatment. Green indicates metformin-treated cells; Red indicates control cells. Samples groups were closely clustered together and showed differences between treated and control samples



a) MCF-7



b) MDA-MB-231

Figure 5. Metabolomics of metformin treated breast cancer cell lines. HCA-heatmap analysis showing up-regulated (red) and down-regulated (blue) comparison metabolites of control untreated cell (red) and metformin-treated cell (green). (a) MCF-7; (b) MDA-MB-231. The top enriched pathways of the significantly accumulated metabolites with metformin treatment. 11 pathways in MCF-7 (a) and 18 pathways in MDA-MB-231 (b)

Table 1. The set of upregulated and downregulated metabolites in MCF-7 cells treated with metformin.

S. no.	Metabolites Detected in MCF-7	Regulation (up/down)	Fold	P-value	
1	L-2-Hydroxyglutaric acid	up	16.	2.55E-06	**
2	Cholesterol	up	34.1	0.000119	**
3	Cinnamic acid	up	14.1	0.000378	**
4	Myristoleic Acid methyl ester	up	2.4	0.002108	**
5	Mannitol 1-phosphate	up	231.	0.002757	**
6	L-Targinine	up	2.6	0.003057	**
7	Palmitaldehyde	up	2.7	0.006181	**
8	2-Acetolactate	up	2.2	0.007024	**
9	fructose 6 phosphate	up	2.1	0.010039	**
10	D-Tryptophan	up	2.1	0.015965	**
11	Homo-L-arginine	down	115.8	0.018127	**
12	Phytosphingosine	down	1.7	0.047167	**
13	L-Asparagine	up	1.6	0.18173	◊◊
14	Oxaloacetic acid	up	1.4	0.217267	◊◊
15	Methylisopelletierine	up	1.4	0.291238	◊◊
16	Docosanamide	down	1.5	0.298607	◊◊
17	D-Ribose	down	1.6	0.384076	◊◊
18	Cytosine	up	1.3	0.434602	◊◊
19	Eucaglobulin	up	1.2	0.473795	◊◊
20	Asymmetric dimethylarginine	down	1.4	0.542381	◊◊
21	β-Hydroxypyruvic acid	down	1.2	0.626468	◊◊
22	Lys Asp Asn Gln	up	1.2	0.627541	◊◊
23	Selenohomocysteine	up	1.1	0.63404	◊◊

24	2-bromoacetic acid	down	1.1	0.719644	◊◊
25	1-Hexadecanol	down	1.1	0.769647	◊◊
26	N2-Acetylornithine	down	1.1	0.785538	◊◊
27	Glu Thr Trp His	up	1.0	0.832729	◊◊
28	Lactate	up	1.0	0.876992	◊◊
29	L-Dihydroorotic acid	down	1.0	0.881617	◊◊
30	His Trp Trp His	up	1.0	0.930426	◊◊
31	Gibberellin A62	up	1.0	0.951832	◊◊

Table 2. The set of upregulated and downregulated metabolites in MDA-MB-231 cells treated with metformin.

S. no.	Metabolites Detected in MDA-MB-231	Regulation (up/down)	Fold	P-value	
1	7-methyl lauric acid	up	6.5	2.23E-06	**
2	(4E,8Z,d18:2) sphingosine	up	5.6	5.37E-06	**
3	2α-hydroxyvitamin D3	up	10.2	1.22E-05	**
4	N-Formyl-L-methionine	up	2.3	1.4E-05	**
5	(S)-Abscisic acid	up	4.9	1.95E-05	**
6	Prostaglandin F2a	up	4.8	2.85E-05	**
7	DG(8:0/13:0/0:0)	up	2.4	3.88E-05	**
8	Putrescine	up	401.6	4.08E-05	**
9	22-Dehydroclerosterol	up	3.6	4.16E-05	**
10	P, P-Dioctyldiphenylamine	up	10.9	4.74E-05	**
11	Lithocholic acid glycine conjugate	up	1.8	5.45E-05	**
12	N-Ornithyl-L-taurine	up	4.6	0.000205	**
13	Glutamylglutamic acid	up	3.3	0.000214	**
14	Glutathione	up	4.0	0.00049	**
15	Terbutaline	up	5.3	0.001105	**
16	Gamma-glutamyl-L-putrescine	up	7.2	0.00194	**
17	L-Histidinol	up	2.8	0.002054	**
18	p-Menthane	up	2.9	0.002747	**
19	Alpha-Dihydrodeoxycorticosterone	up	1.3	0.002942	**
20	Asymmetric dimethylarginine	up	2.4	0.003384	**
21	L-Asparagine	up	2.7	0.003775	**
22	Ethyl oleate	up	2.8	0.004357	**
23	4-Guanidinobutanoicacid	up	2.1	0.005013	**
24	Sphinganine 1-phosphate	up	1.4	0.005178	**
25	3-Phosphoglyceric acid	up	3.0	0.007016	**
26	8-Oxoguanine	up	3.2	0.008205	**
27	5-L-Glutamyl-taurine	up	1.5	0.009397	**
28	DL-O-Phosphoserine	up	1.6	0.020931	**
29	Butyrylcarnitine	up	1.4	0.033634	**
30	Palmitoleic acid	up	1.4	0.044087	**
31	Agrocin 84	down	1.3	0.066336	◊◊
32	Metyrosine	up	1.4	0.085476	◊◊
33	Dolichyl b-D-glucosyl phosphate	down	1.2	0.135237	◊◊
34	D-Ribose	down	1.5	0.150891	◊◊
35	6-Aminopenicillanic acid	down	1.2	0.306554	◊◊
36	Prostaglandin I2	down	1.3	0.423168	◊◊
37	Allantoic acid	down	1.1	0.522502	◊◊
38	Sedoheptulose	up	1.1	0.584032	◊◊
39	Melatonin	down	1.3	0.607582	◊◊

In this study, we demonstrated that metformin has an antitumor effect on breast cancer cell lines and that the drug can alter metabolites in a cell-type-dependent manner (Alhuzaim *et al.*, 2021).

Metformin showed a dose-dependent effect on cell viability in both cell lines, MCF-7 and MDA-MB-231, which are ER+ and triple-negative breast cancer cells (TNBC), respectively, indicating that the mechanism is not ER-dependent. The drug appeared to induce apoptosis in both breast cancer cell lines, which was consistent with previous studies demonstrating its apoptotic effect (Zancan *et al.*, 2010; Gao *et al*., 2016).

Given the effect of metformin on metabolism we were interested to study the effect of metformin on breast cancer cell line metabolites. Overall MCF-7 and MDA-MB-231 showed different metabolites and differences in response to metformin. The different metabolic profile between both cell lines before metformin treatment confirms the results of a previous study which showed that MCF-7 and MDA-MB231 showed a distinct metabolic profile from each other and also from MCF-10A the non-cancerous breast epithelial cell line (Nagana Gowda *et al*., 2018). The different metabolites detected in both cell types in our study might indicate that MCF-7 is more dependent on glucose metabolism as a source of energy. This was supported by a previous study, which showed that the glucose consumption rate in MCF-7 is 40% higher than in MDA-MB-231 (Ocana *et al*., 2020). On the other hand, MDA-MB-231 cells appeared more dependent on amino acid (glutamine) and lipid metabolism, and this was shown previously, where MDA-MB-231 cells were not able to survive in the absence of glutamine while they could in the absence of glucose (Xiao *et al*., 2022).

Remarkably, 12 out of 31 and 30 out of 39 metabolites were significantly different with metformin treatment compared to control untreated cells in MCF-7 and MDA-MB-231 cell lines, respectively. Interestingly, metformin appeared to elevate most of these metabolites in both cell lines. Our study sheds light on how different breast cancer types will show different profiles upon treatment with various metabolic drugs. This was confirmed with metformin in our study, however, similar results were also seen with glutaminase inhibitors (Nagana Gowda *et al*., 2018). In this study, glutaminase inhibitor had a more profound effect on MCF-7 metabolic profile than MDA-MB-231, interestingly while it decreased glucose and increased lactate production in MCF-7 it

showed opposite results in MDA-MB-231 (Nagana Gowda *et al*., 2018).

Our study emphasizes the current direction in metabolic characterization of breast cancer, and how identifying metabolic subtypes can lead to major leaps in the selection of treatment strategies. For example, in their recent study, Xio *et al.* were able to characterize triple-negative breast cancer (TNBC) of 330 patient samples into three distinctive metabolic subgroups (Xiao *et al*., 2022). Interestingly, one subgroup together with the luminal androgen receptor (LAR) subtype was characterized by the enrichment of ceramide and sphingolipid-related metabolites. Interestingly, Sphingosine-1-phosphate (S1P) metabolite seemed crucial for LAR development, which made it a therapeutic target potential for this metabolic subgroup (Xiao *et al.*, 2022). Although ceramide is thought to induce apoptosis in cancer cells, S1P which is a product of ceramide hydrolysis can induce proliferation, inflammation, and angiogenesis in cancer cells, rendering it an interesting druggable target over the last years (Hii *et al.*, 2020; Sukocheva *et al*., 2021; Xiao *et al*., 2022). In our study sphingosine was the second-highest metabolite upregulated by metformin in MDA-MB-231, in addition, S1P appeared to be significantly elevated (Chandra & Yadav, 2023). This perhaps can indicate a compensatory pathway induced by metformin treatment which should be targeted simultaneously. Such data offers metabolic information for the investigation of the combination of both metformin and inhibitors of sphingosine metabolism such as PF-543, the potent inhibitor of sphingosine kinase 1, in TNBC. In our study metformin treatment appeared to increase the population of BCSC (CD44+CD24-) in MDA-MB-231 but not in MCF-7 cells. This might be a result of the increase in sphingosine and S1P seen in MDA-MB-231 with metformin treatment since several reports have indicated the importance of this signaling pathway in BCSC proliferation and survival (Hii *et al.*, 2020; Sukocheva *et al.*, 2021). This would further support investigating combining metformin and inhibitors of sphingosine metabolism for targeting this cell population, however, further investigations would be required to confirm this (Bhavyasri *et al*., 2022; Ejikeugwu *et al*., 2022; Hardikar & Patil, 2022; Ofulue *et al*., 2022).

Interestingly, glutathione was significantly upregulated in metformin-treated cells compared to the control untreated MDA-MB-231 cells, which emphasizes that metformin might have antioxidant properties. Glutathione is considered the main endogenous antioxidant in mammalian cells resulting in redox homeostasis (Forman *et al.*, 2009; Ge *et al*., 2020). Thus disturbances in glutathione production and metabolism have been associated with multiple diseases including cancer (Traverso *et al.*, 2013). The increase in glutathione production by metformin could explain the protective role of metformin resulting in the decrease of cancer incidence in diabetic patients treated with the drug. As cancer development progresses tumors would accumulate a significant amount of oxidative stress from high metabolic activity, in addition to drug-induced oxidative stress. Glutathione thus becomes an important element in counteracting oxidative stress and apoptotic responses associated with it (Bansal & Simon, 2018; Kennedy *et al*., 2020). And indeed, several tumors show elevated Glutathione levels and utilize its detoxifying ability to counteract the activity of antineoplastic agents (Bansal & Simon, 2018; Kennedy *et al*., 2020). Targeting Glutathione can therefore be an important mechanism for increasing the efficacy of anticancer therapeutics, specifically with therapeutics that increase metabolic activity. Given the rise of glutathione seen with metformin treatment in MDA-MB-231, it would be interesting to study the metformin effect on cancer cells in combination with glutathione production or function inhibition and look for further synergistic effects which could enhance the overall anti-tumor effect.

In our study, putrescine was significantly elevated in MDA-MB-231 cells as a result of metformin treatment. Polyamines (putrescine, spermine, and spermidine) are metabolic intermediates produced as a result of amino acid arginine catabolism (Lieu *et al*., 2020). However, despite their view as tumorigenic molecules that can support cell proliferation, in a recent study it was concluded that spermidine has an important role in the regulation of cancer-related inflammation through tumor-associated macrophages (TAMs) (Fan *et al.*, 2020). It was found that spermidine increases the production of the M1 phenotype and decreases M2, which results in modulating immunosurveillance at tumor sites and enhancing antitumor immunity (Latour *et al*., 2020). It would be interesting therefore to confirm the exact effect of metformin on this pathway and whether it can play a role in tumor immunity.

Despite its success as an anti-tumor drug in-vitro and in preclinical studies, its use as an antitumor drug in humans remains inconsistent. The variation in results found in the literature was reported by Navdeep in 2014 (Chandel, 2014). According to Navdeep, this variation could be explained by the fact that the wide availability of metformin due to its cheapness and great safety leads to a big variation in the concentrations used in various trials. Finally, it is noticeable that drugs such as metformin, which can alter various metabolic pathways have numerous modes of action and can be affected by several factors such as glucose concentration and the duration of the treatment (Jara & López-Muñoz, 2015). Understanding these mechanisms might help in enhancing the potential use of metformin as an anti-tumor therapy and understanding the potential combinational drugs which could increase the efficacy.

Conclusion

In conclusion, our results provide evidence for the antitumor effect of metformin in human breast carcinoma cells. Metformin appears to modulate cancer cell metabolites in a cell-dependent fashion. Some metabolites can perhaps act as compensatory pathways induced by metformin treatment and thus should be targeted simultaneously. It would be interesting to study in detail all metabolites that are altered as a result of metformin treatment. Understanding the exact effect on metabolism may help to establish new strategies for breast cancer treatment by metformin, which remains to be an attractive cheap, and relatively safe drug.

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