

METFORMIN AS A POTENTIAL ANTITUMOR AGENT

Andjela Petrovic¹, Vladimir Markovic¹, Veljko Maric², Nevena Gajovic¹

¹ Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia;

² Department of Surgery, Faculty of Medicine Foca, University of East Sarajevo, Foca, Bosnia and Herzegovina

Received: 27.12.2021.

Accepted: 19.02.2022.

Corresponding author:

Nevena Gajovic, MD, PhD

University of Kragujevac, Faculty of Medical Sciences,
Center for Molecular Medicine and Stem Cell Research,
Svetozara Markovica 69, 34000 Kragujevac, Serbia

Phone: + 381 34 306 800

E-mail: gajovicnevena@yahoo.com

ABSTRACT

Some recent findings suggest that metformin, an oral antidiabetic drug, may have antitumor properties. Studies have shown that metformin can alter cell metabolism, both tumor and immune cells, which can greatly influence disease outcome. In this review, we discuss the potential mechanisms in which metformin can directly induce apoptosis of tumor cells as well as mechanisms in which metformin can elicit or enhance antitumor immune response.

Keywords: Metformin, tumor, antitumor immune response.



DOI: 10.2478/sjccr-2022-0023



METFORMIN

Metformin (dimethylbiguanide) is an oral hypoglycemic medication widely used for over 60 years. Metformin has proved itself as a very effective and safe glucose lowering agent. The main mechanism of action of this drug is acute reduction of hepatic gluconeogenesis and enhancing peripheral glucose uptake (1,2). It has been shown that metformin suppresses hepatic glucose production mainly through inhibiting mitochondrial respiratory chain, more specific its complex 1 as well as by inhibiting mitochondrial glycerophosphate dehydrogenase (mtGDP) (3, 4). Mechanisms by which metformin increases glucose uptake in peripheral tissues are not yet fully understood, however, some of the proposed theories are that metformin reduces activity of a lipid phosphatase Src homology 2 domain-containing inositol-5-phosphatase 2 (SHIP2) and potentiate expression of glucose transporter type 4 (GLUT4) by activation of conventional protein kinases C (PKCs) (5, 6). At the moment, metformin is the most commonly used front-line drug for type 2 diabetes (2). In addition, it was also demonstrated that metformin has a significant role in delaying and preventing development of a diabetes type 2 as well as reducing Body mass index, most likely by modulation appetite regulatory center in hypothalamus and gut microbiome, which can make it an interesting candidate for obesity therapy (7, 8, 9). Studies have also shown multiple benefits of metformin in treating patients with liver diseases, such as chronic hepatitis C; cardiovascular diseases such as heart failure and left ventricular hypertrophy and renal diseases like acute kidney injury (AKI) and chronic kidney disease (CKD) (10-14). Metformin can even modulate multiple aging pathways which makes it a potential target for many age-dependent and age-related diseases (15).

Beside its main, antidiabetic role, numerous meta-analyses have shown an association between the use of metformin and significant reduction of incidence of malignancies, especially hepatocellular, pancreas and colon carcinoma (16). These findings can lead to a new, protecting role of metformin. Recent studies also suggest that metformin, besides prevention of tumor development, can also inhibit proliferation and growth of certain types of cancer (16). So far, it has been confirmed that metformin had beneficial effects and improved survival rate in diabetic patients with endometrial, breast and colorectal cancer (17, 18). It was also found that metformin suppressed proliferation of melanoma cells as well as colorectal carcinoma cells (19, 20). This review will try to highlight some of the underlying mechanisms of metformin anti-cancer properties that include metformin induced cytotoxicity and metformin induced immunomodulation. However, the majority of this mechanism(s) has not yet been fully understood.

CYTOTOXIC EFFECTS OF METFORMIN

There are a number of studies which suggest that metformin has a cytostatic and cytotoxic effect on tumor cells and there are many theories about the mechanism of metformin's actions (Table 1). One of most prominent theories is that

metformin activates adenosine monophosphate-activated protein kinase (AMPK). AMPK is a sensor and regulator of cellular energy status and is activated by low concentration of intracellular adenosine triphosphate (ATP) and high adenosine monophosphate (AMP) due to cellular stresses like ischemia, oxidative stress, glucose deprivation and so on (21). Activated AMPK is crucial in the process of phosphorylation and thus inhibition of serine/threonine protein kinase mammalian target of rapamycin (mTOR) (22). It is already known that activation of mTOR promotes cell proliferation and is observed in various types of cancer, but some studies suggest that inhibition of mTOR can have an important role in cancer treatment by inducing apoptosis (23). This was confirmed in a study on gastric adenocarcinoma after cells were treated with 10, 20, 30, 40 and 50mM metformin for 12, 24 and 48h and then cell viability was analyzed (24). The results were conclusive, metformin has time and concentration dependent toxicity to cancer cells (24).

Another study was conducted on MCF-7 breast cancer cells (25). In this study, MCF-7 cancer cells were treated with 10mM of metformin for 24, 48 and 72h. Similarly, metformin increased cell apoptosis and cell necrosis in MCF-7 cells in a time and concentration dependent manner (25). There are also various studies that analyzed if metformin has a synergic effect if it is given with some cytotoxic drugs. Study by Guo *et al.* confirmed that metformin enhances apoptosis induced by cisplatin when given to non-small-cell lung carcinoma (NSCLC) cells, especially in KRAS/LKB1 co-mutated tumors (26). Similarly, metformin given with FTY720, sphingosine-1-phosphate receptor modulator, synergistically increased levels of apoptosis in multiple myeloma (MM) cells (27).

Metformin can also be cytotoxic to cancer cells by promoting non-apoptotic programmed cell death (PCD)- pyroptosis (28). Pyroptosis is an inflammatory cell death pathway that can be activated by numerous caspases, such as caspase-1, caspase-4 and caspase-5 (29). Study on esophageal squamous cell carcinoma (ESCC) showed that metformin induces pyroptosis by targeting the miR-497/PELP1 axis *in vitro*, as well as *in vivo* (28). Another study on human hepatoblastoma cells HepG2, breast cancer cells MCF-7 and colon cancer cell lines HCT-15 and HT-29 confirmed these findings, but in this study, metformin induced pyroptosis by enhancing AMPK/SIRT1 pathway (30).

Several studies have shown that cellular lipids like sphingolipids have a big effect on tumor growth. One of most prominent sphingolipids that regulates cell growth and stimulates proliferation is sphingosine-1-phosphate (S1P). On the other side, ceramides and sphingosine stimulate apoptosis of the cell (31-33). Influencing on ceramide/sphingosine balance we can, in a great way, influence the growth of a tumor. Study that was conducted *in vivo*, on patients with stage III/IV ovarian cancer showed that metformin can block sphingosine kinase 1 (SPHK1) which normally stimulates



formation of S1P. Inhibition of formation of S1P can increase the rates of apoptosis in tumor cells. Interestingly, ovarian cancer cells that had high levels of SPHK1 expression were highly sensitive to metformin's activation of caspase-3. These cells also expressed higher levels of ceramides. These findings suggest a possible target for metformin as a cancer treatment as high level of expression of SPHK1 is found in numerous cancers such as liver cancer, uterine cancer, ovarian cancer, breast cancer, kidney cancer, prostate cancer, lung cancer, gastric cancer, lymphoma, glioblastoma and small bowel cancer (34, 35).

Another mechanism that can explain metformin induced cytotoxicity is related to activation of apoptosis signaling regulating kinase 1 (ASK1). Activation of ASK1 leads to mitochondrial dysfunction and endoplasmic reticulum (ER) stress-induced apoptosis (36). This was confirmed in a study by Ma *et al.* on human ovarian cells where ER stress and mitochondrial damage induced significant cell apoptosis (37).

One of theories that can explain metformin related cytotoxicity is based on the fact that metformin can regulate expression of p53 protein (38). p53 protein is a transcription factor whose main role in the cell is to arrest cell cycle or induce cell apoptosis in response to DNA damage (39). Activated p53 protein further can induce apoptosis via two pathways, intrinsic (mitochondrial pathway) and extrinsic (death receptor pathway) (40). Study on cervical cancer cells by Yudhani *et al.* confirmed that metformin significantly increases the rate of apoptosis in a p53 dependent manner (41). As this study didn't reveal if activation of p53 was AMPK dependent or not, study by Chen *et al.* confirmed that metformin induced apoptosis by phosphorylation and activation of p53 in an AMPK-mediated pathway in human cervical cancer cells (42). When used on p53 mutated breast cancer cells, metformin increased expression of p53 and its target genes, p21 protein and pro-apoptotic protein Bax which led to enhanced apoptosis of cancer cells (43). Metformin induced autophagy and apoptosis by activating p53 were also detected in prostatic cancer cells and human hepatocellular carcinoma HepG2 cells (44,45). Some studies suggest that metformin used in combination with other agents, such as 2-deoxyglucose or valproic acid, on prostate cancer cells can induce synergistic p53 dependent apoptosis (46,47).

Another potential cytotoxic mechanism of metformin is downregulating signal transducer and activator of transcription 3 (Stat 3) and its target protein B-cell lymphoma 2 (Bcl-2) (48). It has been proven that Stat3 promotes proto-oncogenic pathways such as nuclear factor-kappa B (NF-kappa B) and interleukin 6 (IL-6) and activation of anti-apoptotic protein BCL-XL and by that increases proliferation and survival of tumor cells (49). Inhibition of Stat3 protein leads to decreased expression of anti-apoptotic proteins such as Bcl-2 and increasing expression of pro-apoptotic proteins such as Bax which finally leads to induction of apoptosis (50). This possible mechanism of metformin related cytotoxicity was tested in several studies. One of them was conducted on esophageal squamous cell carcinoma (ESCC) *in vitro* and *in*

vivo and results showed that metformin actually had an antineoplastic effect by inducing apoptosis of tumor cells (48). Study on "basal" or "triple-negative" (TN) breast cancer cells showed similar results, metformin inhibited Stat3 and enhanced the rate of apoptosis. TN cancer cells lack the expression of the progesterone (PRs), estrogen (ER) and human epidermal growth factor receptor (HER-2) and tend to be very aggressive with high mortality rates, so these results may be encouraging (51, 52). Results obtained from study on melanoma cells showed that metformin after 24 hours induced cell cycle arrest, after 72 hours induces autophagy and after 96h hours induced significant apoptosis, all while normal human melanocytes were resistant to these effects of metformin (53).

It was previously stated that apoptosis can be induced via two pathways, intrinsic and extrinsic. Intrinsic pathway is mediated by activation of caspase-9 and extrinsic pathway is mediated by activation of caspase-8 (54). Study on pancreatic cell lines (ASPC-1, BxPc-3, PANC-1 and SW1990) showed that metformin itself can activate caspase-8 and caspase-9 which leads to increased apoptosis of tumor cells in a time- and dose-dependent manner (55). This was tested in several more studies on tumor cell lines *in vitro*, and results were similar, metformin increased rates of caspase-dependent apoptosis in rat glioma cell lines (56) as well as in human breast cancer cells, human endometrial cancer cells and human bladder cancer T24 cells (57-59).

Table 1. Summary of proposed cytotoxic effects of metformin

	Mechanism	Effect	Study
Cytotoxic effect of metformin	↓ mTOR	↑ Apoptosis	Lu <i>et al.</i> (2019) ²⁴
	↓ PELP1	↑ Pyroptosis	Wang <i>et al.</i> (2019) ²⁸
	↑ SIRT1	↑ Pyroptosis	Zheng <i>et al.</i> (2020) ³⁰
	↓ SIP	↑ Apoptosis	Hart <i>et al.</i> (2019) ³⁴
	↑ ASK1	↑ Apoptosis	Ma <i>et al.</i> (2019) ³⁷
	↑ p53	↑ Apoptosis	Sun <i>et al.</i> (2016) ⁴⁵
	↓ Stat3	↑ Apoptosis	Feng <i>et al.</i> (2014) ⁴⁸
	↑ Caspases 8, 9	↑ Apoptosis	Wang <i>et al.</i> (2008) ⁵⁵



IMMUNOMODULATORY EFFECTS OF METFORMIN

Beside direct cytotoxic effects, studies have shown that metformin can in many ways modulate and potentiate the immune response against cancer cells (Table 2). This could potentially lead to a better outcome in cancer patients.

One of the ways in which metformin influences immune responses to cancer is by modulating the expression of the programmed death ligand (PD-L1, also known as CD274 or B7-H1) (60). Studies have shown that molecule PD-L1 is expressed at significantly higher levels in various cancer types in which it promotes cell proliferation, migration and invasion while the other studies showed that PD-1 can have immunomodulatory effect (61-64). Role of PD-L1 is to provide an inhibitory signal to cytotoxic T lymphocytes (CTLs) through binding to an inhibitory PD-1 receptors on activated CTLs. Signal from PD-1 receptor inhibits the antitumor activities of CTLs (65). Metformin can change expression of PD-L1 by activation of AMPK. AMPK further phosphorylates S195 of PD-L1 which leads to abnormal glycosylation of PD-L1. Abnormally glycosylated PD-L1 will degrade in endoplasmic reticulum (ER) resulting in reduced expression of molecule PD-L1 on tumor cells and by this enhancing antitumor immune responses (60). Zhang *et al.* showed that metformin can also reduce expression of molecule PD-L1 through Hippo signaling pathway *in vivo* and *in vitro* study on human colorectal cancer (CRC) cells (66). Results obtained from study on endometrial carcinoma cell lines, Ishikawa and RL95-2, showed that metformin-related inhibition of expression of molecule PD-L1 significantly activated T cells and inhibited cancer cell growth (67). These findings indicate that metformin can possibly be used as an addition to immunotherapy in the future.

Various studies of tumor microenvironment showed that tumor-associated macrophages (TAMs) can significantly influence development of a tumor. Alternatively activated, M2 macrophages can facilitate tumor growth, metastasis and resistance to chemotherapy. Cytokines that activate M2 are IL-4, IL-10 and IL-13. Because of that, TAMs have become an interesting new target for immunotherapy (68). Several studies demonstrated that metformin can inhibit M2-like polarization of macrophages (69, 70). Study by Ding *et al.* showed that metformin blocked M2-like polarization of macrophages through activation of AMPK α 1 and as a result suppressed the metastasis of Lewis lung cancer (LCC) (69). Although the number of metastases was significantly reduced, metformin did not affect tumor growth (69). Additional studies, not only confirmed that metformin can directly inhibit M2 phenotype of macrophages, but showed that metformin can also indirectly stimulate M1 phenotype of macrophages most likely by increasing the expression of IFN- γ (71, 72).

Another type of cell that plays an important role in antitumor immunity are CD8⁺ T cells, especially the Tc1 subset (Typical Cytotoxic T Cell). Main role of these cells is killing infected and tumor cells by secreting IFN- γ and granzyme B.

Cytokine that is necessary for their development is IL-12 (73). Recent findings suggest that chronic exposure to antigen leads to reduced production of cytokines such as IL-2, TNF α and IFN- γ , reduced cytotoxicity and higher expression of inhibitory molecular receptors such as PD-1 and Tim-3 (74). This state is known as T cell exhaustion (74). So far, several studies showed that metformin can improve function of exhausted CD8⁺ T cells and in that way can be a useful addition to cancer immunotherapy. In these studies, after metformin treatment, peripheral CD8⁺ T cells increased their production of cytokines and reduced expression of PD-1 and Tim-3 (75-77). Besides stimulating the cytotoxic effect of CD8⁺ T cells, it was noted that metformin also reduced the number and function of CD4⁺ CD25⁺ regulatory T cells (Tregs) in tumor (78). Study by Ohue *et al.* showed that Treg cells in tumor inhibit antitumor immunity by inhibiting activation of CTLs, secreting of inhibitory cytokines and by directly killing the effector T cells (79). Metformin reduces the number and function of CD4⁺ CD25⁺ Tregs by reducing forkhead box P3 (Foxp3) protein (78). Foxp3 is a necessary regulatory gene for development of Treg cells (80). Results from study by Wei *et al.* additionally suggested that metformin can also enhance the effect of tumor vaccine by reducing the proportion of Treg cells in tumor which leads to even stronger anti-tumor effect (81). Study by Pereira *et al.* showed that administered *in vitro*, metformin also significantly increased the number of effector-memory CD8⁺ T cells which quickly differentiated into effector T cells by mechanism not yet fully understood. These effector T cells proved themselves to be very cytotoxic (82, 83). Same study also proved that metformin can be very useful if combined with other anti-tumor medications like rapamycin and sitagliptin. Combined with these medications, metformin significantly reduced the number of metastatic nodules in mice with lung cancer in comparison with treatment with rapamycin or sitagliptin alone (82).

Besides the direct effect on immune cells, metformin can also indirectly affect certain types of cells by affecting cytokines that are necessary for their development. It was found that, among others, metformin affects interleukin 22 (IL-22) by decreasing its levels (84). IL-22 is a member of the interleukin-10 (IL-10) family of cytokines and is important for both Th1 and Th17 immune response (85). By decreasing levels of IL-22, metformin leads to inhibition of development of Th1 and Th17 cells. This effect was proven in a study conducted by Zhao *et al.* on mouse hepatocellular carcinoma model where it was shown that metformin exhibited significant inhibition of tumor growth (84). The potential mechanism is that IL-22 might be involved in development of liver cancer, and by reducing its levels, metformin suppresses development of tumor which can have therapeutic potential (84).

Another way in which metformin exerts its antitumor activity is by depletion or inhibition of activity of myeloid-derived suppressor cells (MDSCs) (86-90). MDSCs are cells that accumulate in a tumor, block the antitumor immune response and promote tumor progression (91-93). It is proven



that metformin suppresses the function of monocytic and polymononuclear MDSCs in patients with ovarian cancer by blocking the expression of CD39 and CD73 on these cells (86), but mechanism by which metformin inhibits the function of granulocytic MDSCs in mice with colon cancer is still unclear (87).

Besides metformin's direct and indirect effect on tumor and tumor related cells, metformin also has a significant effect on cytokines throughout the organism. Cytokines are signaling molecules that regulate both innate and adaptive immune responses and allow the cells to communicate with each other. Cytokines can have pro-tumor or anti-tumor function and have an important role in tumor-antitumor balance in organisms so they are very interesting for exploring because of their possible clinical potential (94). There are several studies that have proven that metformin can influence concentrations of cytokines in cancers and that can influence the outcome. One of the cytokines that can be affected via metformin is midkine (95). Midkine (MK) is a heparin-binding growth factor which is highly expressed in many types of cancer and is involved in development and progression of many malignancies (96). It was already stated that metformin inhibits PI3K and MAPK pathways which are used by MK for promotion of cell proliferation and cell survival. By inhibiting MK pathways, metformin inhibits tumor development (95). Another cytokine influenced by metformin is interleukin 6 (IL-6) (97). IL-6 is a cytokine whose major role in human organisms is regulation of immune responses, but it is also shown to be highly expressed in various types of tumors and that promotes the proliferation and survival of tumor cells (98). Studies have shown that metformin reduces levels of IL-6 mainly via mTOR pathway. This is another way of antitumor role of metformin and it was confirmed in *in vitro* study in human colon cancer cells and in *in vitro* study on human multiple myeloma cells lines (97, 99). Cytokine that has been associated with promoting malignancy similarly like IL-6 is transforming growth factor beta (TGF- β). Based on *in vitro* and *in vivo* studies, TGF- β acts as a suppressor of antitumor immune responses which makes him a potential therapeutic target (100, 101). Several studies suggested that metformin can inhibit binding of a TGF- β to its receptor which consequently promotes antitumor immune response (102, 103). One of studies has shown that metformin suppresses expression of TGF- β by inhibiting AKT/mTOR/ZEB1 signaling pathway (104). Besides AKT/mTOR/ZEB1 signaling pathway, metformin can also inhibit AKT/GSK-3 β signaling pathway which leads to reduction of levels of basic fibroblast growth factor (bFGF). This growth factor is a member of the fibroblast growth factor family and is associated with promotion of tumorigenesis and metastasis. Study by Chengye *et al.* showed that by inhibiting bFGF, metformin inhibited migration and invasion of hepatocellular carcinoma cells (105).

Table 2. Summary of proposed immunomodulatory effects of metformin

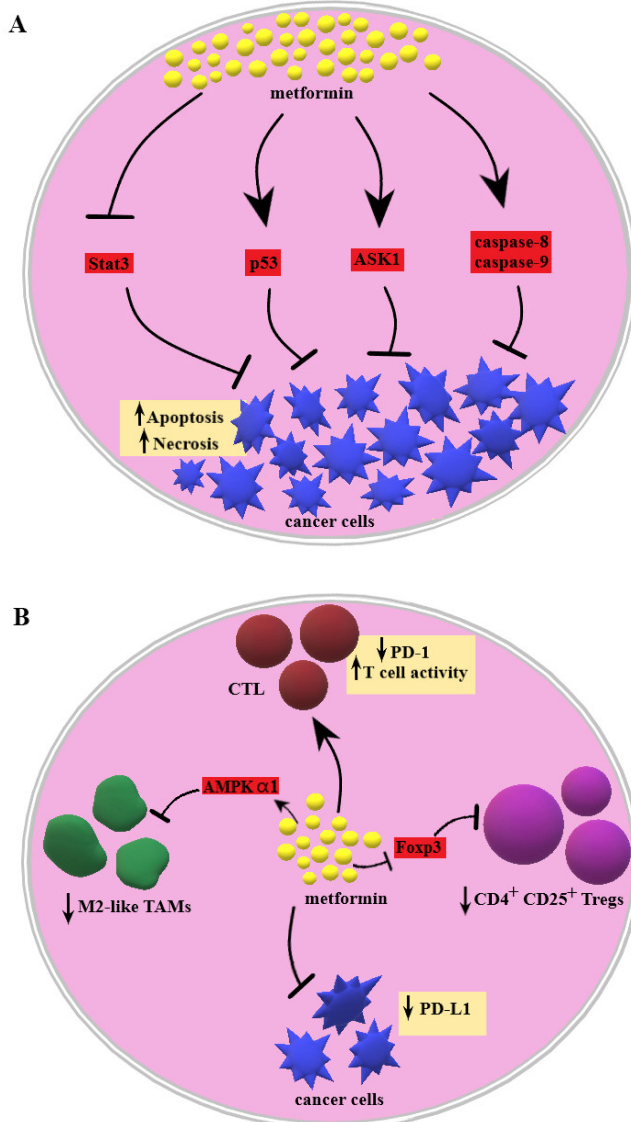
	Mechanism	Effect	Study
Immunomodulatory effects of metformin	↓ PD-L1	↑ T cell activation	<i>Xue et al. (2019)⁶⁷</i>
	↓ M2 / ↑ M1	↓ Metastasis	<i>Ding et al. (2015)⁶⁹</i>
	↓ PD-1, Tim-3	↓ T cell exhaustion	<i>Watanabe et al. (2017)⁷⁵</i>
	↓ Foxp3	↓ Treg	<i>Kunisada et al. (2017)⁷⁸</i>
	↑ Memory CD8 ⁺ T cells	↑ T cell antitumor response	<i>Pereira et al. (2018)⁸²</i>
	↓ IL-22	↓ Tumor growth	<i>Zhao et al. (2015)⁸⁴</i>
	↓ MDSCs	↑ T cell activation	<i>Li et al. (2018)⁸⁶</i>
	↓ MK	↓ Metastasis	<i>Karadeniz et al. (2020)⁹⁵</i>
	↓ IL-6	↓ Cell proliferation and survival	<i>Kang et al. (2018)⁹⁹</i>
	↓ TGF- β	↑ Antitumor immune response	<i>Song et al. (2017)¹⁰⁴</i>
	↓ bFGF	↓ Metastasis	<i>Changye et al. (2017)¹⁰⁵</i>

SUMMARY AND FUTURE PERSPECTIVE

Epidemiological, preclinical and clinical evidence reviewed above have shown that metformin has a great potential as an antitumor agent, both for prevention and therapy. Obtained data *in vitro* and *in vivo* suggested that metformin can in a great way influence various signaling pathways involved in cancer cell proliferation, differentiation and metabolism which can significantly change tumor outcomes. Some of these signaling pathways are shown in Figure 1. However, the exact underlying mechanism(s) of metformin's positive effects are still yet to be fully explained. Besides individual effects, several studies have confirmed that metformin also has synergistic effects with other antitumor agents (26, 46, 47). It is also worth mentioning that besides its positive effects in studies so far, there are more properties of metformin that make it an attractive antitumor agent such as low toxicity, good tolerability by patients and affordability. These observations suggest that metformin should be further tested, both as a single and adjuvant therapeutic pharmaceutical.



Figure 1. Schematic illustration of proposed signaling pathways and cellular effects of metformin.(A)



Metformin activates p53, ASK1, caspase-8 and caspase-9 and inhibits Stat3 which results in increased rates of apoptosis and necrosis of cancer cells. **(B)** Metformin reduces expression of PD-L1 on cancer cells and PD-1 on CTLs which results in enhanced T cell activity. Also, by inhibiting Foxp3, metformin reduces the number and activity of CD4⁺ CD25⁺ Tregs and by activating AMPK α 1, metformin suppresses the number and activity of M2-like TAMs. Overall result of these effects is stimulation of antitumor immune response.

ACKNOWLEDGMENT

This work was supported by grants from the Faculty of Medical Sciences, University of Kragujevac (projects JP 04/15, JP 15/19, JP 14/20), Serbia.

CONFLICTS OF INTEREST

The authors declare no financial or commercial conflict of interest.

REFERENCES

1. Schernthaner G, Schernthaner GH. The right place for metformin today. *Diabetes Res Clin Pract.* 2020;159:107946. doi: 10.1016/j.diabres.2019.107946.
2. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: an overview. *Clin Sci (Lond).* 2012;122(6):253-70.
3. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J.* 2000;348 Pt 3(Pt 3):607-14.
4. Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature.* 2014;510(7506):542-6.
5. Polianskyte-Prause Z, Tolvanen TA, Lindfors S, Dumont V, Van M, Wang H, et al. Metformin increases glucose uptake and acts renoprotectively by reducing SHIP2 activity. *FASEB J.* 2019;33(2):2858-2869.
6. Turban S, Stretton C, Drouin O, Green CJ, Watson ML, Gray A, et al. Defining the contribution of AMP-activated protein kinase (AMPK) and protein kinase C (PKC) in regulation of glucose uptake by metformin in skeletal muscle cells. *J Biol Chem.* 2012;287(24):20088-99.
7. Hostalek U, Gwilt M, Hildemann S. Therapeutic Use of Metformin in Prediabetes and Diabetes Prevention. *Drugs.* 2015;75(10):1071-94.
8. Yerevanian A, Soukas AA. Metformin: Mechanisms in Human Obesity and Weight Loss. *CurrObes Rep.* 2019;8(2):156-164.
9. Seifarth C, Schehler B, Schneider HJ. Effectiveness of metformin on weight loss in non-diabetic individuals with obesity. *Exp Clin Endocrinol Diabetes.* 2013;121(1):27-31.
10. Harris K, Smith L. Safety and efficacy of metformin in patients with type 2 diabetes mellitus and chronic hepatitis C. *Ann Pharmacother.* 2013;47(10):1348-52.
11. Kuan W, Beavers CJ, Guglin ME. Still sour about lactic acidosis years later: role of metformin in heart failure. *Heart Fail Rev.* 2018;23(3):347-353.
12. Mohan M, Al-Talabany S, McKinnie A, Mordi IR, Singh JSS, Gandy SJ, et al. A randomized controlled trial of metformin on left ventricular hypertrophy in patients with coronary artery disease without diabetes: the MET-REMODEL trial. *Eur Heart J.* 2019;40(41):3409-3417.
13. Bell S, Farran B, McGurnaghan S, McCrimmon RJ, Leese GP, Petrie JR, et al. Risk of acute kidney injury and survival in patients treated with Metformin: an observational cohort study. *BMC Nephrol.* 2017;18(1):163.



14. Charytan DM, Solomon SD, Ivanovich P, Remuzzi G, Cooper ME, McGill JB, et al. Metformin use and cardiovascular events in patients with type 2 diabetes and chronic kidney disease. *Diabetes Obes Metab*. 2019;21(5):1199-1208.
15. Valencia WM, Palacio A, Tamariz L, Florez H. Metformin and ageing: improving ageing outcomes beyond glycaemic control. *Diabetologia*. 2017;60(9):1630-1638.
16. Kasznicki J, Sliwiska A, Drzewoski J. Metformin in cancer prevention and therapy. *Ann Transl Med*. 2014;2(6):57.
17. Ko EM, Walter P, Jackson A, Clark L, Franasiak J, Bolac C, et al. Metformin is associated with improved survival in endometrial cancer. *Gynecol Oncol*. 2014;132(2):438-42.
18. Feng JL, Qin X. Metformin and cancer-specific survival among breast, colorectal, or endometrial cancer patients: A nationwide data linkage study. *Diabetes Res Clin Pract*. 2021;175:108755. doi: 10.1016/j.diabres.2021.108755.
19. Li K, Zhang TT, Wang F, Cui B, Zhao CX, Yu JJ, et al. Metformin suppresses melanoma progression by inhibiting KAT5-mediated SMAD3 acetylation, transcriptional activity and TRIB3 expression. *Oncogene*. 2018;37(22):2967-2981.
20. Lan B, Zhang J, Zhang P, Zhang W, Yang S, Lu D, et al. Metformin suppresses CRC growth by inducing apoptosis via ADORA1. *Front Biosci (Landmark Ed)*. 2017;22:248-257.
21. Kahn BB, Alquier T, Carling D, Hardie DG. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab*. 2005;1(1):15-25.
22. Shaw RJ. LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. *Acta Physiol (Oxf)*. 2009;196(1):65-80.
23. Tian, Tian & Li, Xiaoyi & Zhang, Jinhua. mTOR Signaling in Cancer and mTOR Inhibitors in Solid Tumor Targeting Therapy. *International Journal of Molecular Sciences*. 2019;20(3):755.
24. Lu CC, Chiang JH, Tsai FJ, Hsu YM, Juan YN, Yang JS, et al. Metformin triggers the intrinsic apoptotic response in human AGS gastric adenocarcinoma cells by activating AMPK and suppressing mTOR/AKT signaling. *Int J Oncol*. 2019;54(4):1271-1281.
25. Queiroz EA, Puukila S, Eichler R, Sampaio SC, Forsyth HL, Lees SJ, et al. Metformin induces apoptosis and cell cycle arrest mediated by oxidative stress, AMPK and FOXO3a in MCF-7 breast cancer cells. *PLoS One*. 2014;9(5):e98207. doi: 10.1371/journal.pone.0098207.
26. Moro M, Caiola E, Ganzinelli M, Zulato E, Rulli E, Marabese M, et al. Metformin Enhances Cisplatin Induced Apoptosis and Prevents Resistance to Cisplatin in Co-mutated KRAS/LKB1 NSCLC. *J. Thorac. Oncol*. 2018;13(11):1692-1704.
27. Zhao Y, Zhang E, Lv N, Ma L, Yao S, Yan M, et al. Metformin and FTY720 Synergistically Induce Apoptosis in Multiple Myeloma Cells. *Cell. Physiol. Biochem*. 2018;48:785-800.
28. Wang L, Li K, Lin X, Yao Z, Wang S, Xiong X, et al. Metformin induces human esophageal carcinoma cell pyroptosis by targeting the miR-497/PELP1 axis. *Cancer Lett*. 2019;450:22-31.
29. Man SM, Karki R, Kanneganti TD. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. *Immunol Rev*. 2017;277(1):61-75.
30. Zheng Z, Bian Y, Zhang Y, Ren G, Li G. Metformin activates AMPK/SIRT1/NF-κB pathway and induces mitochondrial dysfunction to drive caspase3/GSDME-mediated cancer cell pyroptosis. *Cell Cycle*. 2020;19(10):1089-1104.
31. Shida D, Takabe K, Kapitonov D, Milstien S, Spiegel S. Targeting SphK1 as a new strategy against cancer. *Curr Drug Targets* 2008;9:662-73.
32. Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind S, et al. Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature*. 1996;381(6585):800-3.
33. Maceyka M, Payne SG, Milstien S, Spiegel S. Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. *BiochimBiophys Acta*. 2002;1585(2-3):193-201.
34. Hart PC, Chiyoda T, Liu X, Weigert M, Curtis M, Chiang CY, et al. SPHK1 Is a Novel Target of Metformin in Ovarian Cancer. *Mol Cancer Res*. 2019;17(4):870-881.
35. Wang X, Sun Y, Peng X, Naqvi SMAS, Yang Y, Zhang J, et al. The Tumorigenic Effect of Sphingosine Kinase 1 and Its Potential Therapeutic Target. *Cancer Control*. 2020;27(1):1073274820976664. doi: 10.1177/1073274820976664.
36. Matsuzawa A, Nishitoh H, Tobiume K, Takeda K, Ichijo H. Physiological roles of ASK1-mediated signal transduction in oxidative stress- and endoplasmic reticulum stress-induced apoptosis: advanced findings from ASK1 knockout mice. *Antioxid Redox Signal*. 2002;4(3):415-25.
37. Ma L, Wei J, Wan J, Wang W, Wang L, Yuan Y, et al. Low glucose and metformin-induced apoptosis of human ovarian cancer cells is connected to ASK1 via mitochondrial and endoplasmic reticulum stress-associated pathways. *J Exp Clin Cancer Res*. 2019;38(1):77.
38. Yi Y, Zhang W, Yi J, Xiao ZX. Role of p53 Family Proteins in Metformin Anti-Cancer Activities. *J Cancer*. 2019;10(11):2434-2442.
39. Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell*. 1997;88(3):323-331.
40. Haupt S, Berger M, Goldberg Z, Haupt Y. Apoptosis - the p53 network. *J Cell Sci*. 2003;116:4077-85.
41. Yudhani RD, Astuti I, Mustofa M, Indarto D, Muthmainah M. Metformin Modulates Cyclin D1 and P53 Expression to Inhibit Cell Proliferation and to Induce Apoptosis in Cervical Cancer Cell Lines. *Asian Pac J Cancer Prev*. 2019;20(6):1667-1673.
42. Chen YH, Yang SF, Yang CK, Tsai HD, Chen TH, Chou MC, et al. Metformin induces apoptosis and inhibits



- migration by activating the AMPK/p53 axis and suppressing PI3K/AKT signaling in human cervical cancer cells. *Mol Med Rep.* 2021;23(1):88.
43. Li P, Zhao M, Parris AB, Feng X, Yang X. p53 is required for metformin-induced growth inhibition, senescence and apoptosis in breast cancer cells. *BiochemBiophys Res Commun.* 2015;464(4):1267-1274.
 44. Ahn HK, Lee YH, Koo KC. Current Status and Application of Metformin for Prostate Cancer: A Comprehensive Review. *Int J Mol Sci.* 2020;21(22):8540.
 45. Sun Y, Tao C, Huang X, He H, Shi H, Zhang Q, et al. Metformin induces apoptosis of human hepatocellular carcinoma HepG2 cells by activating an AMPK/p53/miR-23a/FOXA1 pathway. *Onco Targets Ther.* 2016;9:2845-53.
 46. Tran LNK, Kichenadasse G, Butler LM, Centenera MM, Morel KL, Ormsby RJ, et al. The Combination of Metformin and Valproic Acid Induces Synergistic Apoptosis in the Presence of p53 and Androgen Signaling in Prostate Cancer. *Mol Cancer Ther.* 2017;16(12):2689-2700.
 47. Ben Sahra I, Laurent K, Giuliano S, Larbret F, Ponzio G, Gounon P, et al. Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells. *Cancer Res.* 2010;70(6):2465-75.
 48. Feng Y, Ke C, Tang Q, Dong H, Zheng X, Lin W, et al. Metformin promotes autophagy and apoptosis in esophageal squamous cell carcinoma by downregulating Stat3 signaling. *Cell Death Dis.* 2014;5(2):e1088. doi: 10.1038/cddis.2014.59.
 49. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer.* 2009;9(11):798-809.
 50. Nielsen M, Kaestel CG, Eriksen KW, Woetmann A, Stokkedal T, Kaltoft K, et al. Inhibition of constitutively activated Stat3 correlates with altered Bcl-2/Bax expression and induction of apoptosis in mycosis fungoides tumor cells. *Leukemia.* 1999;13(5):735-8.
 51. Deng XS, Wang S, Deng A, Liu B, Edgerton SM, Lind SE, et al. Metformin targets Stat3 to inhibit cell growth and induce apoptosis in triple-negative breast cancers. *Cell Cycle.* 2012;11(2):367-76.
 52. Griffiths CL, Olin JL. Triple negative breast cancer: a brief review of its characteristics and treatment options. *J Pharm Pract.* 2012;25(3):319-23.
 53. Tomic T, Botton T, Cerezo M, Robert G, Luciano F, Puissant A, et al. Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. *Cell Death Dis.* 2011;2(9):e199. doi: 10.1038/cddis.2011.86.
 54. Li J, Yuan J. Caspases in apoptosis and beyond. *Oncogene.* 2008;27(48):6194-206.
 55. Wang LW, Li ZS, Zou DW, Jin ZD, Gao J, Xu GM. Metformin induces apoptosis of pancreatic cancer cells. *World J Gastroenterol.* 2008;14(47):7192-8.
 56. Isakovic A, Harhaji L, Stevanovic D, Markovic Z, Sumarac-Dumanovic M, Starcevic V, et al. Dual anti-tiglioma action of metformin: cell cycle arrest and mitochondria-dependent apoptosis. *Cell Mol Life Sci.* 2007;64(10):1290-302.
 57. Gao ZY, Liu Z, Bi MH, Zhang JJ, Han ZQ, Han X, et al. Metformin induces apoptosis via a mitochondria-mediated pathway in human breast cancer cells in vitro. *Exp Ther Med.* 2016;11(5):1700-1706.
 58. Takahashi A, Kimura F, Yamanaka A, Takebayashi A, Kita N, Takahashi K, et al. Metformin impairs growth of endometrial cancer cells via cell cycle arrest and concomitant autophagy and apoptosis. *Cancer Cell Int.* 2014;14:53. doi: 10.1186/1475-2867-14-53.
 59. Jang JH, Sung EG, Song IH, Lee TJ, Kim JY. Metformin induces caspase-dependent and caspase-independent apoptosis in human bladder cancer T24 cells. *Anticancer Drugs.* 2020;31(7):655-662.
 60. Cha JH, Yang WH, Xia W, Wei Y, Chan LC, Lim SO, et al. Metformin Promotes Antitumor Immunity via Endoplasmic-Reticulum-Associated Degradation of PD-L1. *Mol Cell.* 2018;71(4):606-620.
 61. Yu W, Hua Y, Qiu H. et al. PD-L1 promotes tumor growth and progression by activating WIP and β -catenin signaling pathways and predicts poor prognosis in lung cancer. *Cell Death Dis* 11, 506 (2020).
 62. Jovanovic M, Gajovic N, Lukic M, Popovic A, Jovanovic IP. Synergism of PDL/PD1 and IL33/ST2 axis in tumor immunology. *Ser J Exp Clin Res.* 2019;20(3):223-228.
 63. Jovanovic M, Gajovic N, Jurisevic M, Sekulic S, Arsenijevic N, Jovic M, et al. Anti PD1 therapy activates tumoricidal properties of NKT cells and contributes to overall deceleration of tumor progression in a model of murine mammary carcinoma. *Vojnosanitpregl.* 2021; doi: 10.2298/VSP210126039J
 64. Jovanovic M, Geller D, Gajovic N, Jurisevic M, Arsenijevic N, Jovanovic M, et al. Dual blockage of PD-L/PD-1 and IL33/ST2 axes slows tumor growth and improves antitumor immunity by boosting NK cells. *Life Sciences.* 2022;289:120214. doi.org/10.1016/j.lfs.2021.120214
 65. Han Y, Liu D, Li L. PD-1/PD-L1 pathway: current researches in cancer. *Am J Cancer Res.* 2020;10(3):727-742.
 66. Zhang JJ, Zhang QS, Li ZQ, Zhou JW, Du J. Metformin attenuates PD-L1 expression through activating Hippo signaling pathway in colorectal cancer cells. *Am J Transl Res.* 2019;11(11):6965-6976.
 67. Xue J, Li L, Li N, Li F, Qin X, Li T, et al. Metformin suppresses cancer cell growth in endometrial carcinoma by inhibiting PD-L1. *Eur J Pharmacol.* 2019;15:859:172541. doi: 10.1016/j.ejphar.2019.172541.
 68. Petty AJ, Yang Y. Tumor-associated macrophages: implications in cancer immunotherapy. *Immunotherapy.* 2017;9(3):289-302.
 69. Ding L, Liang G, Yao Z, Zhang J, Liu R, Chen H, et al. Metformin prevents cancer metastasis by inhibiting M2-like polarization of tumor associated macrophages. *Oncotarget.* 2015;6(34):36441-55.



70. Saito A, Kitayama J, Horie H, Koinuma K, Ohzawa H, Yamaguchi H, et al. Metformin changes the immune microenvironment of colorectal cancer in patients with type 2 diabetes mellitus. *Cancer Sci.* 2020;111(11):4012-4020.
71. Chiang CF, Chao TT, Su YF, Hsu CC, Chien CY, Chiu KC, et al. Metformin-treated cancer cells modulate macrophage polarization through AMPK-NF- κ B signaling. *Oncotarget.* 2017;8(13):20706-20718.
72. Ma Q, Gu JT, Wang B, Feng J, Yang L, Kang XW, et al. PlGF signaling and macrophage repolarization contribute to the anti-neoplastic effect of metformin. *Eur J Pharmacol.* 2019; 15;863:172696. doi: 10.1016/j.ejphar.2019.172696.
73. St Paul M, Ohashi PS. The Roles of CD8+ T Cell Subsets in Antitumor Immunity. *Trends Cell Biol.* 2020;30(9):695-704.
74. Blank CU, Haining WN, Held W, Hogan PG, Kallies A, Lugli E, et al. Defining 'T cell exhaustion'. *Nat Rev Immunol.* 2019;19(11):665-674.
75. Watanabe M, Eikawa S, Shien K, Yamamoto H, Shien T, Soh J, et al. Abstract 5592: metformin improves immune function of exhausted peripheral CD8+ T cells derived from cancer patients. *Cancer Res.* 2017; 77(13 Suppl):5592-5592.
76. Eikawa S, Nishida M, Mizukami S, Yamazaki C, Nakayama E, Udono H. Immune-mediated antitumor effect by type 2 diabetes drug, metformin. *Proc Natl Acad Sci U S A.* 2015;112(6):1809-14.
77. Zhang Z, Li F, Tian Y, Cao L, Gao Q, Zhang C, et al. Metformin Enhances the Antitumor Activity of CD8+ T Lymphocytes via the AMPK-miR-107-Eomes-PD-1 Pathway. *J Immunol.* 2020;204(9):2575-2588.
78. Kunisada Y, Eikawa S, Tomonobu N, Domae S, Uehara T, Hori S, et al. Attenuation of CD4+CD25+ Regulatory T Cells in the Tumor Microenvironment by Metformin, a Type 2 Diabetes Drug. *EBioMedicine.* 2017;25:154-164.
79. Ohue Y, Nishikawa H. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? *Cancer Sci.* 2019;110(7):2080-2089.
80. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003;299(5609):1057-61.
81. Wei L, Luo Z, Li J, Li H, Liang Y, Li J, et al. Metformin inhibits proliferation and functions of regulatory T cells in acidic environment. 2019;39(12):1427-1435.
82. Pereira FV, Melo ACL, Low JS, de Castro ÁA, Braga TT, Almeida DC, et al. Metformin exerts antitumor activity via induction of multiple death pathways in tumor cells and activation of a protective immune response. *Oncotarget.* 2018;9(40):25808-25825.
83. Reiser J, Banerjee A. Effector, Memory, and Dysfunctional CD8(+) T Cell Fates in the Antitumor Immune Response. *J Immunol Res.* 2016;2016:8941260. doi: 10.1155/2016/8941260.
84. Zhao D, Long XD, Lu TF, Wang T, Zhang WW, Liu YX, et al. Metformin decreases IL-22 secretion to suppress tumor growth in an orthotopic mouse model of hepatocellular carcinoma. *Int J Cancer.* 2015;136(11):2556-65.
85. Peng Y, Gao X, Yang J, et al. Interleukin-22 Promotes T Helper 1 (Th1)/Th17 Immunity in Chlamydial Lung Infection. *Mol Med* 20, 2014; 109-119.
86. Li L, Wang L, Li J, Fan Z, Yang L, Zhang Z, et al. Metformin-Induced Reduction of CD39 and CD73 Blocks Myeloid-Derived Suppressor Cell Activity in Patients with Ovarian Cancer. *Cancer Res.* 2018;78(7):1779-1791.
87. Xu P, Yin K, Tang X, Tian J, Zhang Y, Ma J, et al. Metformin inhibits the function of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. *Biomed Pharmacother.* 2019;120:109458. doi: 10.1016/j.biopha.2019.109458.
88. Hirayama T, Nagata Y, Nishida M, Matsuo M, Kobayashi S, Yoneda A, et al. Metformin Prevents Peritoneal Dissemination via Immune-suppressive Cells in the Tumor Microenvironment. *Anticancer Res.* 2019;39(9):4699-4709.
89. Qin G, Lian J, Huang L, Zhao Q, Liu S, Zhang Z, et al. Metformin blocks myeloid-derived suppressor cell accumulation through AMPK-DACH1-CXCL1 axis. *Oncoimmunology.* 2018;7(7):e1442167. doi: 10.1080/2162402X.2018.1442167.
90. Uehara T, Eikawa S, Nishida M, Kunisada Y, Yoshida A, Fujiwara T, et al. Metformin induces CD11b+ cell-mediated growth inhibition of an osteosarcoma: implications for metabolic reprogramming of myeloid cells and anti-tumor effects. *Int Immunol.* 2019;31(4):187-198.
91. Filipazzi P, Huber V, Rivoltini L. Phenotype, function and clinical implications of myeloid-derived suppressor cells in cancer patients. *Cancer Immunol Immunother.* 2012;61(2):255-263.
92. Ostrand-Rosenberg S. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. *Cancer Immunol Immunother.* 2010;59(10):1593-600.
93. Gajovic N, Jurisevic M, Pantic J, Radosavljevic G, Arsenijevic N, Lukic ML, et al. Attenuation of NK cells facilitates mammary tumor growth in streptozotocin-induced diabetes in mice. *Endocrine-related cancer* 2018; 25: 493-507.
94. Lee S, Margolin K. Cytokines in cancer immunotherapy. *Cancers (Basel).* 2011;3(4):3856-93.
95. Karadeniz Z, Aynacıoğlu AŞ, Bilir A, Tuna MY. Inhibition of midkine by metformin can contribute to its anticancer effects in malignancies: A proposal mechanism of action of metformin in context of endometrial cancer prevention and therapy. *Med Hypotheses.* 2020;134:e109420. doi: 10.1016/j.mehy.2019.109420.
96. Filippou PS, Karagiannis GS, Constantinidou A. Midkine (MDK) growth factor: a key player in cancer progression and a promising therapeutic target. *Oncogene.* 2020;39(10):2040-2054.
97. Mishra A. K., Dingli D. Metformin inhibits IL-6 signaling by decreasing IL-6R expression on multiple myeloma cells. *Leukemia*, 2019;33(11), 2695-2709.



98. Kumari N, Dwarakanath BS, Das A, Bhatt AN. Role of interleukin-6 in cancer progression and therapeutic resistance. *Tumour Biol.* 2016;37(9):11553-11572.
99. Kang S, Kim BR, Kang MH, Kim DY, Lee DH, Oh SC, et al. Anti-metastatic effect of metformin via repression of interleukin 6-induced epithelial-mesenchymal transition in human colon cancer cells. *PLoS One.* 2018;13(10):e0205449. doi: 10.1371/journal.pone.0205449.
100. Shuchen Gu, Xin-Hua Feng. TGF- β signaling in cancer, *Acta Biochimica et Biophysica Sinica*, 2018;50(10):941-949.
101. Gajovic N, Jovanovic I, Ilic A, Jeremic N, Jakovljevic V, Arsenijevic N, et al. Diabetes mellitus directs NKT cells toward type 2 and regulatory phenotype *Ser J Exp Clin Res.* 2016; 17 (1): 35-41.
102. Xiao H, Zhang J, Xu Z, et al. Metformin is a novel suppressor for transforming growth factor (TGF)- β 1. *Sci Rep.* 2016;6:28597. doi:10.1038/srep28597
103. Leonel C, Borin TF, de Carvalho Ferreira L, Moschetta MG, Bajgelman MC, Vilorio-Petit AM, et al. Inhibition of Epithelial-Mesenchymal Transition and Metastasis by Combined TGF β Knockdown and Metformin Treatment in a Canine Mammary Cancer Xenograft Model. *J Mammary Gland Biol Neoplasia.* 2017;22(1):27-41.
104. Song Y, Chen Y, Li Y, Lyu X, Cui J, Cheng Y, et al. Metformin inhibits TGF- β 1-induced epithelial-to-mesenchymal transition-like process and stem-like properties in GBM via AKT/mTOR/ZEB1 pathway. *Oncotarget.* 2017;9(6):7023-7035.
105. Chengye W, Yu T, Ping S, Deguang S, Keyun W, Yan W, et al. Metformin reverses bFGF-induced epithelial-mesenchymal transition in HCC cells. *Oncotarget.* 2017;8(61):104247-104257.