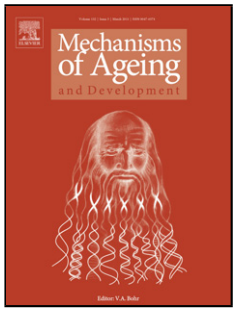

IS METFORMIN A GEROPROTECTOR? A PEEK INTO THE CURRENT CLINICAL AND EXPERIMENTAL DATA

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IS METFORMIN A GEROPROTECTOR ? A PEEK INTO THE CURRENT CLINICAL AND EXPERIMENTAL DATA

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Highlights

- Metformin positively affect the course of age-related diseases
- Metformin-users are at a lower risk of developing certain types of cancer
- Metformin increases lifespan in model organisms
- Metformin modulates molecular hallmarks of ageing

ABSTRACT

Nowadays we observe a growing scientific interest and need to develop novel research approach that target ageing. Metformin, apart from its proven efectiveness as a glucose-lowering agent, was found to exert multidirecional effects because of its cardioprotective, anti-inflammatory and anti-cancer activity. Recently, metformin has become a subject of interest of many researchers as a promising drug with anti-aging properties; however, its impact on clinical ageing features is still hypothetical. Nevertheless, results of cellular experiments and animal studies confirm that metformin has advantageous effects on ageing. Additionally, a number of clinical trials prove positive effects of metformin on the prevalence of age-related diseases (ADR), including cardiovascular disease or carcinoma. We have observed a significant advancement in human research since a few randomised clinical trials evaluating the impact of metformin on aging were launched.

Here, we present an investigation on anti-ageing properties of metformin, and provide the explanation of mechanisms and pathways implicated in this function. We also analyse available clinical evidence on healthspan extension, all-cause mortality and ADR. Finally, we discuss currently conducted randiomized clinical trials which aim to explore metformin potential as an anti-ageing drug in humans.

Key words: metformin, biguanides, senescence, geroprotector, ageing

1. INTRODUCTION

Over the previous few decades, life expectancy has risen dramatically and resulted in a higher population of elderly people across all developed countries (Skirbekk et al., 2019). In 2015, globally, there were 617.1 million (9%) people who were 65 or older. Within next 10 year the older population will reach about 1 billion which constitute 12% of the projected total world population (Roberts et al., 2018). The main factors contributing to the increased human longevity are as follows: implementation of vaccination, disinfectants, and antibiotics significantly reducing the incidence of infectious diseases, improvement in healthcare, nutrition and technology, and raising awareness of preventative actions, including exercise and reduction of smoking (Vaiserman et al., 2016). Development of novel technologies and standards in medicine, and education are associated with increased lifespan. However these positive outcomes do not contribute to improved healthspan, which is regarded as the number of years during which people are generally healthy and free from serious or chronic illness (Mercken et al., 2012). Thus, the growing number of elderly, and higher prevalence of ADR such as cancer, diabetes (type two diabetes mellitus, T2DM), cardiovascular disease are frequently found in most of developed countries. They all pose an extensive socio-economic challenge (Beard and Bloom, 2015; Vaiserman et al., 2016).

Ageing stems from a permanent interplay between single genetic makeup and environment contributing to accumulation of cellular damage over time. It finally leads to disease promotion and death (Gurău et al., 2018). Oxidative stress plays an important role in ageing because oxidative damage leads to cellular hallmarks of ageing which then lead to various ADR (Luo et al., 2020; Lopez-Otin and Kroemer, 2019). Taking into consideration oxidative-related hypothesis of ageing, and positive effects of observational studies, numerous clinical trials examining antioxidants have been carried out to investigate the potential of antioxidants for the prevention and treatment of age-related morbidity and mortality. However, the results of randomized clinical trials showed that antioxidant supplementation does not affect ARD (Luo et al., 2020).

Therefore, investigation of novel interventional strategies, aiming at improving health span, is a current first concern in biomedical research. Traditionally, pharmacological approaches have gained special attention in the field of new discipline known as biogerontology (Campbell et al., 2017; Vaiserman et al., 2016, Vaiserman and Lushchak, 2017). There are several molecules targeting primary ageing pathways, including calorie restriction mimetics, and autophagy inducers. Also senolytic drugs (agents selectively inducing apoptosis of senescent cells), and telomerase activators are now under investigation (Vaiserman et al., 2016; Vaiserman and Lushchak, 2017). Current doubts regarding efficiency of antioxidants supplementation have contributed to an increased interest in other healthspan-promoting options, including calorie-restriction (CR)-based strategy (Vaiserman et al., 2016). Although the beneficial effect of CR on healthspan is incontestable, the applicability of this strategy is difficult in humans. To overcome obstacles, the scientists are attempting to develop novel molecules to mimic the CR state without restricting a diet (Lee and Min, 2013).

One drug which has been a subject of extensive research as a geroprotector is an anti-diabetic drug - metformin, N,N-dimethylbiguanide hydrochloride (Bailey, 2017). Metformin is one of the most frequently administered drugs in T2DM. The anti-hyperglycemic activity of the drug stems from its inhibition of gluconeogenesis and glycogenolysis, and increase in tissue sensitivity to insulin and tissue glucose utilization (Mahmood et al., 2013). Importantly,

metformin was found to be effective in polycystic ovarian syndrome (PCOS) and metabolic syndrome (Knowler et al., 2002).

Given the history of metformin administration in pharmacotherapy presented inFigure 1, the greatest breakthrough was a result of the United Kingdom Prospective Diabetes Study (UKPDS). This study confirmed that the therapy with metformin contributes to 42% reduction of diabetes-related death and a 36% decline in all-cause mortality (UKPDS Group, 1998). In addition, metformin decreases CVD incidence in subjects with T2DM (Soukas et al., 2019). The positive properties of the drug regarding the cardiovascular system result from its beneficial influence on endothelium, protection from oxidative stress, and reduction of proliferation of smooth muscle cells (SMCs) (Nesti and Natali, 2017).

Apart from its glucose-lowering properties, metformin has retained interest due to its pleiotropic effects and activity in various tissues, including muscles, adipose tissue, vascular endothelium, and brain (Foretz et al. 2014; Novelle et al., 2016). Metformin reduces food intake, and body weight through direct action on the hypothalamic center which control satiety and feeding (Novelle et al., 2016). Metabolic effects of metformin have been briefly reviewed by Piskovatska et al. (2019). Additionally, metformin affects metabolic and cellular processes associated with the development of ADR, including inflammation, oxidative damage, protein glycation, cellular senescence, apoptosis, and growth of certain types of cancer (Novelle et al., 2016; Piskovatska et al. 2019).

Another aspect of metformin which makes it specifically encouraging for further studies on its geroprotective potential is the fact that the drug has already been widely used in humans for several decades. Therefore, metformin safety profile, and its potential contraindications are well characterised (Campbell et al., 2017). These characteristics make the drug substantially more straightforward to be implemented as a therapy for ageing than clinically unapproved drugs.

This review presents investigation on the application of metformin as a potential geroprotector. We outline state of the art data regarding anti-ageing activity of metformin, and provide molecular mechanisms and pathways engaged in this function. We also analyse available clinical evidence on healthspan extension, and currently conducted clinical trials which aim to explore metformin capacity as an anti-ageing drug in humans. Next, we provide the experimental and pre-clinical evidence on anti-ageing properties of metformin. Finally, the review shows new favourable circumstances relating to the translational potential of metformin.

2. THE MECHANISM OF METFORMIN ACTION

2.1. The gut – stimulation of hormone secretion

Despite long clinical experience with metformin and increased scientific attention into its pleiotropic activity, the exact way of metformin activity remains unclear. Metformin is active in humans only when administered orally. A typical dose of classical formulation is usually two g per day. Approximately, half of the dose (ca. 6 mmol) is absorbed, and then excreted via kidneys. The other half of the drug is not absorbed, and excreted in the faeces (Graham et al., 2011). It has been estimated that the colon is exposed to the drug at concentrations reaching 40 mM (Glosmann and Lutz, 2019).

Previously, it was hypothesised that metformin exerts its action mainly in liver. Recently, it also has been claimed that the drug is also active in intestine (Glossmann and Lutz, 2019) and these effects in gastrointestinal tract are responsible for the pharmacological properties of the drug (Wu et al., 2017). This statement has been confirmed by Buse et al. (2016), who found separation of the glycemic effect from plasma exposure to the drug with gut-restricted delayed-release formulation. It has also been reported that metformin concentration in the jejunum can be 300-fold higher than that measured in blood (Thomas and Gregg, 2017). Actually, typical side effects of metformin associated with the alimentary tract may be regarded as an indicator of therapeutic efficacy (Thomas and Gregg, 2017). During the last decade, many scientific teams have commenced to explore the drug's effect on the intestine in more detail, since this is a major site of drug concentration. For instance, metformin has a specific influence on the composition of the intestinal microbiome independently on its glucose-lowering properties (Forslund et al., 2015). The authors examined the microbiome of 784 patients, and reported that the metformin-specific effect was associated with an increase in *Escherichia* species proportionally to the blood metformin level. Furthermore, the analysis of gut microbiome of metformin-treated T2DM subjects showed great similarity to the controls, and not to the T2DM subjects. This finding may indicate a rescue from dysbiosis associated with T2DM. The authors concluded that metformin participates in partial gut microbial mediation of both therapeutic and adverse effects. However, further validation is required to identify causality and to clarify how such mediation might occur. In addition, this study highlights the need to disentangle specific disease dysbioses from effects of treatment on human microbiomes (Forslund et al., 2015). These important conclusions were further proved by a study of Bryrup et al. (2019) who reported that metformin intake alters the gut microbiota composition in non-diabetic men, and claimed that this effect does not depend on the dysbiosis triggered by diabetes. Another randomised clinical trial embracing fourty non-treated subjects suffering from T2DM who were using placebo or metformin for four months found an elevation in abundance of *Escherichia spp*. and *Bilophila wadsworthia* along with a reduction in *Intestinibacter spp*. and *Clostridium spp*. (Wu H. et al., 2017). Furthermore, it was found that metformin-altered microbiota mediated some anti-diabetic effects of the drug (Wu H. et al., 2017). As reviewed by Soukas et al. (2019), metformin might also increase the number of bacteria producing short-chain fatty acids that lead to weight loss and anti-inflammatory effect in T2DM subjects. A comprehensive analysis of the effects of metformin on human microbiom can be found in a review of Prattichizzo et al. (2018). So far, the effect of metformin on microbiota and the related anti-aging activity have been underestimated. As summarized by Prattichizzo et al. (2018), metformin reshapes intestinal microbiota, and fosters the growth of bacterial species producing short-chain fatty acids (SCFAs) which increase the barier function of the intestinal epithelium. As a consequence, lower levels of immune system stimulating agents, including LPS and flagellin, get into circulation, which may improve the balance between factors counteracting and promoting inflammation (Prattichizzo et al., 2018).

Metformin might also act through the incretin axis. It has been known for several years that metformin therapy elevates both fasting and postprandial levels of the satiety-promoting incretin hormone, glucagon-like peptide 1 (GLP-1) (DeFronzo et al., 2016; Prattichizzo et al., 2018). Metformin affects postprandial GLP-1 secretion in direct and AMPK mediated effects (Bahne et al., 2018). Furthermore, metformin administration might also lead to the significant increase in peptide YY (PYY) (DeFronzo et al., 2016) and growth differentiation factor 15 (GDF15) (Glosmann and Lutz, 2019). GDF-15 is produced in the intestine, cardiomyocytes and endothelial cells via the "integrated stress response", and is a member of the transforming growth factor beta (TGF-β) superfamily (Glosmann and Lutz, 2019; Adela et al., 2015). It was found that GDF-15 is a biomarker for T2DM and CVD (Adela et al., 2015) since GDF-15 levels

are higher in individuals with heart failure (HF), and coronary artery disease where its plasma levels might be regarded as prognosis of the disease (Natali et al., 2019). These possible associations between metformin administration and its cardiovascular effects were analyzed by Natali et al. (2019) and Gerstein et al. (2017). According to their results, administration of metformin in diabetics contributed to 40% elevation of GDF-15 plasma level (Natali et al., 2019). Therefore, according to authors GDF-15 levels might be a biomarker for the use of metformin (Gerstein et al., 2017). It has been suggested that possible explanation for the association of GDF-15 with metformin therapy, and also with HbA1c, could be the fact that GDF-15 reflects the function of mitochondria (Natali et al., 2019).

2.2. Mitochondrial Complex I Inhibition

A metformin molecule is positively charged at pH 7.4 (99.9% of the molecule exists in ionized form in blood) (Graham et al., 2011) which predisposes the biguanide to concentrate in negatively charged organelles, such as mitochondria (Prattichizzo et al., 2018). Mitochondrial accumulation is frequently considered as the primary target of metformin (Prattichizzo et al., 2018; Hardie et al., 2012). In 2000, metformin was discovered to suppress mitochondrial complex I, but not complexes II, III, and IV (El-Mir et al., 2000; Owen et al., 2000). Metformin was found to induce depolarization of the mitochondrial membrane potential, elevate the AMP/ATP and lactate/pyruvate ratios, and decrease glucose production (Kim and You, 2017). Interestingly, the degree of gluconeogenesis inhibition is related with the extent of suppression of the respiratory chain. These observations confirm that cellular energy depletion induced by metformin results in incomplete flux of ATP which is important to commence gluconeogenesis in the liver (El-Mir et al., 2000).

The molecular mechanism of metformin interaction with complex I has not been fully discovered. It was proved that the drug suppresses NADH oxidation by complex I isolated from several species, including bovine heart mitochondria, yeast *Pichia pastoris*, and bacterium *Escherichia coli*, implying that metformin interacts to the conserved subunits of complex I (Kim and You, 2017). Bridges et al. (2014) found that metformin suppresses a rate-limiting step coupled to ubiquinone reduction, but does not competitively attach to the ubiquinone-binding site in complex I. It is worth pointing out that it has not yet been confirmed whether or not complex I is the only mitochondrial target of metformin. Importantly, it is still not determined whether the biguanide suppresses respiration directly or indirectly (Fontaine, 2014).

Apart from hypoglycaemic effect, related to inhibition of complex I, metformin was also found to inhibit cancer cell growth through its action on this target (Andrzejewski et al., 2014; Birsoy et al., 2014). Activation of the energy sensor AMPK is another effect associated with complex I. However, it will be discussed later in the next chapter of this manuscript.

Hunter et al. (2018) revealed thatinhibition of fructose-1-6-bisphosphatase (FBP1) participating in the process of glucose production is another effect of elevated AMP levels. It was found that metformin decreases glucose concentration by allosteric inhibition of FBP1 in mice. These results provide evidence that metformin at therapeutic concentrations in vivo exerts significant effects via adjustment of cellular energy charge (Soukas et al., 2019).

The key function of mitochondria is ATP production through oxidative phosphorylation which result in generation of energy through oxidation of nutrients that create an electron chemical gradient across the mitochondrial inner membrane. Another important activity related to mitochondria is production of reactive oxygen species (ROS), contributing to DNA and cell damage. Impairment of mitochondria is one of principal causes of ageing because ageing

mitochondria lose their ability to provide cellular energy and release high levels of ROS. Impaired mitochondrial function has been linked to insulin resistance in multiple tissues including skeletal muscles, liver, fat, heart and pancreas (Podhorecka et al., 2017).

Beneficial impact of metformin on ROS production are mediated not only by inhibition of the mitochondrial respiratory chain, but also by suppressing nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase (Saisho et al., 2015). The inhibition of the electron transport chain together with the initiation of antioxidant gene expression by the SKN-1/Nrf2 transcription pathway explains how metformin acts as an anti-oxidative agent, thus reducing the production of ROS (Novelle et al., 2016).

The reduction of ROS production might also stem from other mechanisms. For example, Khallaghi et al. (2016) reported that metformin restores the activity of phosphoinositide 3-Kinase/S6 Protein Kinase (P13K/S6K). Besides, metformin may enhance cell survival by improving anti-oxidant systems, particularly glutathione peroxidase (GSH) and catalase (CAT) (Khallaghi et al., 2016). In turn, Batchuluun et al. (2014) revealed anti-oxidative properties of metformin through suppression of protein kinase C (PKC) - NAD(P)H oxidase pathway. The available data suggests that inhibition of nuclear factor κB (NF-κB) by activation of AMPK is crucial for the anti-inflammatory properties of the drug (Saisho, 2015). For instance, Li et al. (2009) showed inhibitory properties of metformin towards nuclear factor κB (NF-κB) activation in the vessel wall. In another paper, Hattori et al. (2006) found that metformin inhibits cytokine-induced NFκB activation *via* AMPK activation in human umbilical vein endothelial cells (HUVEC).

Interestingly, metformin through the effects on AMPK might have an effect on pain in animal models of neuropathy and acute nociception (Melemedijan et al., 2011; Tillu et al., 2012). Russe et al. (2013) reported that metformin-mediated activation of AMPK leads to analgesic effects, similarly to those induced by ibuprofen. As presented by Lihn et al. (2008), AMPK activation might also be associated with decreasing levels of pro-inflammatory cytokines, including IL-6 and IL-8 in adipose tissue and skeletal muscle.

2.3. Increase of the Activity of Adenosine Monophosphate-Activated Protein Kinase

AMPK is a fundamental indicator of cellular energy condition that controls metabolic energy equilibrium (Hardie et al., 2012). Generally, stimulation of AMPK is due to increased AMP/ATP and ADP/ATP ratios (Novelle et al., 2016), and this route is known as nucleotide-dependent regulation. However, the activity of AMPK is regulated also by other upstream signals, thus making AMPK a central sensor coordinating the cellular metabolism (Garcia and Shaw, 2017). Importantly, AMPK activation is engaged in the acute release of gut hormones, such as GLP-1 and peptide YY from human mucosal preparations, since the kinase inhibitor prevents the metformin effect (Glosmann and Lutz, 2019). In addition, AMPK allosterically activates IR (insulin receptor) and IRS1 (insulin receptor substrate 1) thus increasing insulin sensitivity (Bahrambeigi et al., 2019).

Metformin activates AMPK in two independent manners. The first one is the ‘canonical’ pathway which is nucleotide-dependent (increase in the [ADP/ATP] ratio and phosphorylation by upstream liver kinase B1 [LKB1]). The second possible way of AMPK activation (‘noncanonical’, AMP-independent) is a lysosomal pathway caused by a decrease in the fructose 1,6-bisphosphate level (Glosmann and Lutz, 2019). The importance of AMPK in glucose-lowering properties of metformin was confirmed in a study of Shaw et al. (2005), who reported that the ablation of LKB1 in the liver disturbed antihyperglycemic effects of metformin

in a high-fat diet. The activation of AMPK by metformin results in the following effects, (i) phosphorylation of acetyl-CoA carboxylase (ACC)1 and ACC2, resulting in an elevated fatty acid uptake and β-oxidation, thus improving insulin sensitivity and (ii) activation of 3,5-cyclic phosphodiesterase 4B (PDE4B), thus reducing cAMP and indirectly inhibiting the activity of cAMP-dependent protein kinase A (PKA). It finally leads to glucose consumption and decreased glucose output (Prattichizzo et al., 2018). The effects on ACC are also related with lipid-lowering properties of metformin (Novelle et al., 2016). However, the drug decreases also the levels of sterol regulatory element-binding protein 1 (SREBP-1), a major lipogenic transcription factor, through direct phosphorylation by AMPK (Novelle et al., 2016).

The biological effects of metformin do not only stem from AMPK activation (Bahrambeigi et al., 2019). For instance, a study of Foretz et al. (2010) conducted on liver and primary hepatocytes from knockout models for both AMPKα1/α2 catalytic subunits and the upstream activating kinase LKB1 showed that neither AMPK nor LKB1 are important for metformin suppression of glucose production in the liver. However, one of more recent studies revealed that low doses of metformin effectively inhibit glucose production via AMPK activation regardless of the increased level of the AMP/ATP ratio (Cao et al., 2014). As mentioned above, metformin is able to stimulate AMPK indirectly, secondary to the inhibition of the mitochondrial respiratory chain complex 1, contributing to ATP reduction and an escalation of AMP levels (Foretz et al., 2014). It has been suggested that changes in the intracellular ATP levels, but not direct AMPK activation, are responsible for the influence of metformin on hepatic glucose output (Foretz et al., 2014). Recently another possible mechanism of metformin action has been found by Madiraju et al. (2014). The authors found that the inhibition of gluconeogenesis by metformin might stem from a direct effect on the activity of mitochondrial glycerophosphate dehydrogenase (mGPD). Suppression of mGPD pauses the glycerophosphate shuttle, contributing to the arrest of gluconeogenesis from glicerol (Foretz et al., 2014).

AMPK was also found to adjust mTORC1 signalling (mechanistic target of rapamycin complex 1) which is responsible for the process of ageing, carcinoma and neurodegenerative diseases (Melnik and Schmitz, 2014). In addition, over-stimulation of mTORC1 signaling by overabundance of food and high amino acid intake leads to T2DM evolution since mTORC1 signaling is engaged in pancreatic β-cell growth, β-cell mass regulation, insulin synthesis and secretion. Metformin was found to suppress mTORC1 through different signalling pathways. One of them is LKB1/AMPK-mediated activation of TSC2 (tuberin) which suppresses mTORC1. Additionally, metformin improves AMPK/TSC2-mediated mTORC1 inhibition by stimulating REDD1 (regulated in DNA damage and development 1) and ATM (ataxia teleangiectasia mutated). Furthermore, it was found that metformin blocks amino acid-mediated activation of RAG (RAS-related GTP-binding protein) GTPases at the lysosomal surface (Melnik and Schmitz, 2014). Decreased mTORC1 activity downregulates S6K1 which contributes to improvement in glucose level control, through AKT-mediated glucose uptake and inhibition of FoxO1-mediated gluconeogenesis. The positive effects of mTORC1 suppression are not limited to metabolic benefits, but are also related to other mTORC1-driven diseases, including PCOS, atherosclerosis and CVD, cancer, and neurodegenerative diseases (Melnik and Schmitz, 2014).

Both AMPK and mTOR signalling pathways have been proposed as mediators of caloric restriction (CR) (Lee and Min, 2013). During lack of energy state, LKB phosphorylates and activates AMPK, which subsequently stimulates the processes to generate ATP. It has been proved that worms overexpressing AMPK (aak-2) lived longer than controls, and glucose restriction increased aak-2 activity (Schulz et al., 2007). The function of AMPK activation in lifespan extension was also proved in the *Drosophila* model (Funakoshi et al., 2011). Due to

the advantageous influence of metformin on AMPK, mTOR and insulin/IGF-1 signalling pathways, the drug has been identified as downstream-type calorie restriction mimetic (CRM) (Shintani et al., 2018). Metformin was found to possess a CR-related longevity advantage mediated by the activation of AMPK in several animal models (Lee and Min, 2013). Results of these studies will be discussed in the following parts of this paper.

Recently, it has also been found that prolonged metformin therapy is associated with increased levels of the microRNA‐processing protein DICER1 in mice as well as humans, and subsequently increases the expression of a subset of microRNAs (miR-20a, miR-34a, miR-130a, miR-106b, miR-125, and let-7c) which are related with senescence (Hooten et al., 2016).

Finally, one more molecular target of metformin, and signalling pathway of AMPK activation has been identified (Zhang et al., 2016). It has been found that metformin can interact with v-ATPase (lysosomal vacuolar ATPase) to promote the translocation of AXIN/LKB1 (AXIN – a scaffold protein) onto the surface of lysosomes to form a complex with v-ATPase-Ragulator. It ultimately leads to AMPK activation. Binding of metformin to V-ATPase forces the Ragulator/V-ATPase complex to undergo a conformation change from the 'nutrient-rich' to the 'starvation conformation', which finally is associated with the recruitment of LKB1 and prevents mTORC1 activation even during nutrient-rich state (Kim and You, 2017).

2.4. Novel targets of action

In the previous chapter, we have described the potential interaction between metformin and vATPase which could imply that late endosome/lysosome could be another target of metformin. Additionally, it has recently been found that it is possible for metformin to modulate endosomal trafficking to lysosomes by affecting eNHE (Na^+/H^+ exchangers). This suggests that the drug might be engaged in control of the cellular endocytic cycle (Kim and You, 2017).

Another target for metformin is lipid phosphatase Src homology 2 domain-containing inositol-5-phosphatase 2 (SHIP2), which is upregulated in diabetic rodent models and inhibits insulin signalling by decreasing Akt activation. This in turn leads to insulin resistance and reduced glucose uptake (Lehtonen, 2019). It has been found that metformin directly binds to purified recombinant SHIP2 and blocks its activity, while an in vivo subsequent effect of SHIP2 inhibition includes increased insulin sensitivity (Polianskyte-Prause et al., 2019). The authors list potentially beneficial effects of SHIP2 suppression which are as follow, improved glucose metabolism, attenuation of insulin resistance and hyperglycemia (Polianskyte-Prause et al., 2019).

Although metformin is not a newly developed drug, new mechanisms of action continue to be discovered. Indded, recent studies have supplied us with a long record of possible molecular targets which are as follow: NF-κB inhibition, inflammasome inhibition, increased expression of nuclear pore complex (NPC) and acyl-CoA dehydrogenase family member-10 (ACAD10), increased expression of the peroxiredoxin PRDX-2, Nrf2 activation, and folate metabolism (Prattichizzo et al., 2018).

Recently, it has also been found that metformin can enhance autophagy which is a cellular mechanism responsible for degradation of cytoplasmic constituents, preserving cellular homeostasis through elimination of impaired proteins and organelles (De Santi et al., 2019). Metformin enhances autophagy through AMPK activation and subsequent phosphorylation of unc-51-like kinase (ULK-1) and Beclin 1 (Hur and Lee, 2015). However, Song et al. (2015) reported improvement of hepatic steatosis by metformin through autophagy activation via sirtuin 1 pathway, not AMPK. Autophagy is also engaged in nutrient supply during energy

insufficiency, and is also important for the proper function of mitochondria and the ER. Due to the fact that AMPK is an inducer of intracellular energy equilibrium, the activation of AMPK by metformin implies that autophagy induction might be another mechanism responsible for metabolic improvement related with metformin therapy (Hur and Lee, 2015). Importantly, metformin was also found to prevent cell tumorigenesis through autophagic cell death (De Santi et al., 2019).

Mechanisms of metformin anti-ageing activity have been summarized in Figure 2. Certainly, the above-mentioned mechanisms of metformin action will widen in the future, leading to greater insight of the molecular mechanisms responsible for pleiotropic activity of the drug. Moreover, discovery of new targets for metformin will aid in the search for novel anti-diabetic molecules with improved safety profiles.

3. CLINICAL DATA ON METFORMIN ANTI-AGEING PROPERTIES

Clinical studies have confirmed that metformin reduces the prevalence of diabetes in high risk subjects (Knowler et al., 2002). Later research of Knowler et al. (2015) also confirmed the advantageous properties of metformin in prevention of HbA_{1c}-defined diabetes. There are also plenty of observational studies that provide evidence on geroprotective properties of metformin in humans which have been previously reviewed (Piskovatska et al., 2020). Within this chapter, we briefly review the current literature particularly emphasising all-cause mortality, age-related diseases, including cancer as well as cardiovascular disease.

3.1. All-cause mortality

Based on the survey of currently available clinical outcomes we can distinguish two types of studies focusing on influence of metformin on all-cause mortality. The first group of studies compares diabetic individuals using metformin to the general population or non-diabetic subjects (Bannister et al., 2014; Berard et al., 2014; Bo et al., 2012; Claesenet al., 2016), while the second group of studies compares metformin-treated diabetic subjects to other T2DM patients taking other medications used in management of diabetes, such as insulin (Ekstrom et al., 2012; Ghotbi et al., 2013), sulphonylurea (Evans et al., 2006; Kahler et al., 2007; Sullivan et al., 2011; Wang et al., 2014) or observing a diet (Bo et al., 2012; Sullivan et al., 2011).

Results of the studies comparing metformin-treated diabetic patients with non-diabetics showed that the mortality rate is significantly lower in metformin users than in those who did not use the drug. For instance, Bannister et al. (2014) confirmed that T2DM patients, being administered metformin monotherapy, demonstrated a longer survival than matched, non-diabetic controls. The authors found also that sulphonylurea therapy was associated with a reduced survival compared with controls and metformin monotherapy (Bannister et al., 2014). In turn, Bérard et al. (2014) evaluated a fourteen-year risk of all-cause mortality according to hypoglycemic exposure at baseline in the general population, and found that the hazard ratio for all-cause mortality was lower in the metformin-treated group (HR 2.28) that in the untreated diabetic subjects (HR 3.22).

Ekstrom et al. (2012) evaluated the influence and safety of metformin therapy in T2DM subjects, and found that the biguanide treatment compared with insulin treatment contributed to a decreased risk of CVD, serious infection and all-cause mortality. Importantly, metformin use was associated with lower all-cause mortality in comparison with other hypoglycaemic agents (Ekstrom et al., 2012). In another study, Evans et al. (2006) assessed the risk of

cardiovascular events in T2DM subjects newly using with metformin or sulfonylureas. The most prominent observation of this study was the fact that individuals newly treated with sulfonylureas alone, or with sulfonylureas combined with metformin, were at higher risk of adverse cardiovascular effects than those treated only with metformin. Importantly, metformin treatment was related to a lower cumulative mortality rate in comparison with sulfonylurea therapy (Evans et al., 2006). A decrease in all-cause and cardiovascular mortality linked with metformin treatment compared with sulfonylurea monotherapy was also reported by Johnson et al. (2002). In turn, Kahler et al. (2007) found no significant drug effect on all-cause mortality for all oral treatment cohorts, including metformin relative to sulfonylurea oral monotherapy. Another study of Wang et al. (2014) showed that among older veterans suffering from T2DM without concomitant frailty-related disorders, metformin treatment, compared to sulfonylurea, contributed to a 30% decrease in the mortality risk. On the other hand, metformin appeared to have no effect on the mortality rate in the patients with frailty-related markers (Wang et al., 2014).

To summarize, one of recents meta-analyses of Campbell et al. (2017) revealed that diabetic subjects using metformin demonstrate importantly lower all-cause mortality than healthy people not using this biguanide (HR = 0.93, 95%CI 0.88–0.99). Metformin therapy also appears to be more beneficial regarding all-cause mortality in comparison to other therapies, including insulin or sulfonylurea therapies.

3.2. Age-related diseases

In this chapter, we will concentrate mainly on the relationship between metformin and the occurrence of cancer, cardiovascular disease and neurodegenerative diseases.

3.2.1. Cancer

Anti-cancer properties of metformin were confirmed for the first time in 2005 when Evans et al. (2005) published outcomes of a clinical trial, carried out on 11,867 patients. The authors reported that T2DM individuals using metformin had a lower cancer-related mortality rate than those who did not use metformin. Since then, numerous systematic investigations and meta-analyses have been published. They aim to determine the association between metformin use and cancer incidence or survival outcomes (Campbell et al., 2017; Yu et al., 2019).

Several clinical trials have reported that chronic use of metformin may contribute to decrease in progression of breast cancer and mortality due to this ailment (Pizzuti et al., 2015; Col et al., 2012; Hadad et al., 2011: Goodwin et al., 2011). For example, Bodmer et al. (2010) reported in a nested case-control study that chronic treatment with metformin is significantly related with a reduced risk of breast cancer in T2DM patients. Metformin also appeared to be beneficial in newly diagnosed, untreated, non-diabetic breast cancer patients (Niraula et al., 2012). However, not all studies report advantageous effects of metformin on cancer incidence or outcomes. For instance, Bonanni et al. (2012) did not confirm statistically significant effects of metformin on breast cancer proliferation in non-diabetic women. Metformin was also found to positively affect the incidence of metastases in breast cancer since after 5-years follow-up, 9.2% of patients treated with metformin, and 12.3% subjects not using the drug developed metastases (Jacob et al., 2016).

Ambiguous results were observed for metformin and its effects on endometrial cancer. Becker et al. (2013) and Luo et al. (2014) did not find any effects of metformin on the risk of

endometrial cancer. Also Al Hilli et al. (2016) reported that the effect of diabetes and metformin on clinical outcomes is insignificant in risk-adjusted endometrial cancer groups. On the other hand, Tseng (2015) observed that metformin treatment in diabetic females is associated with an overall essentially lower risk of endometrial cancer with dose-response relationship. Also the results of three studies investigating the potential of metformin on the growth of pancreatic carcinoma in T2DM subjects did not provide the unequivocal answer, since Bodmer et al. (2012) reported that metformin was associated with a reduced risk of pancreatic cancer in women only. On the other hand, Lu et al. (2015) and Walker et al. (2015) did not find any important relationship between metformin and pancreatic cancer.

The advantageous effetcs of metformin on cancer incidence, mortality and prognosis was also confirmed in various types of gastrointestinal cancers. For instance, Van de Voorde et al. (2015) published that metformin therapy contributed to a significantly better distant metastasis-free survival rate and overall survival rate. According to Lee et al. (2011), metformin utilization is related with a significantly decreased risk of incidence of total cancer, colorectal, liver and pancreatic cancer. Metformin was also found to decrease the risk of progression of hepatocellular carcinoma and reduce liver-related death in diabetic patients with HCV cirrhosis (Nkontchou et al. 2011). Positive effects of metformin regarding cancer incidence were also found ina metaanalysis conducted by Campbell et al. (2017). Its authors estimated that metformin therapy is associated with a decreased risk of colorectal and breast cancer. There are also other studies reporting beneficial effects of metformin on the prevalence of various types of cancers, including head and neck cancer (Rego et al. 2015), prostate cancer (Preston et al., 2014) or lung cancer. However, negative results should also be taken into consideration, such as obtained for bladder (Goossens et al., 2015) or thyroid cancer (Tseng, 2012). The summary of the results of the above studies is enclosed in Table 1. A valuable summary of the anti-cancer properties of metformin are also presented by Pitskovatska et al. (2019).

3.2.2. Cardiovascular diseases

The past several years have brought strong evidence proving the favourable influence of metformin on the function of the cardiovascular system (Nesti and Natali, 2017). These beneficial effects may result from the improvements of endothelium function, reduction of proliferation of smooth muscle cells, and anti-inflammatory properties of the drug (Nesti and Natali, 2017). Within this chapter, we focus on clinical outcomes of the influence of metformin on the cardiovascular system.

Apart from UKPDs study (1998), also other studies confirmed advantageous effects of metformin with respect to the cardiovascular system. For instance, Kooy et al. (2009) found that metformin treatment contributed to a reduction of macrovascular end point after a follow-up period of 4.3 years. In another study (SPREAD-DIMCAD trial), metformin administration in patients with the T2DM and cardiovascular disease contributed to a 46% reduction of recurrent cardiovascular events when compared to glipizide (Hong et al., 2013). In turn, Ekstrom et al. (2012) evaluated the risk of CVD in 51,675 individuals with T2DM on continuous anti-hyperglycemic therapy or insulin, and found that metformin-treated patients showed a lower risk of CVD in comparison to patients using insulin. Also Ghotbi et al. (2013) found that metformin therapy of T2DM individuals was related to a lower risk of primary outcome event (POE), and lower mortality, which implies that the drug decreases the risk of CVD. These beneficial effects were not confirmed by results of the BARI2D trial performed in T2DM subjects who were eligible for coronary artery revascularization (Group BDS, 2009). Nevertheless, this study did not confirm a direct effect of metformin, because two therapeutic

strategies including insulin sensitizing (metformin and thiazolidinediones) versus insulin providing (sulfonylureas and insulin) drugs were applied in this study.

There are also studies evaluating effects of metformin on the prevalence of stroke. Floyd et al. (2016) examined the prevalence of stroke in metformin users in comparison to other T2DM subjects non treated with metformin. The investigators found that the use of metformin is connected with a lower risk of stroke compared with other T2DM therapies. On the other hand, metformin was not found to decrease the risk of myocardial infarction (Floyd et al., 2016). In turn, Jansson et al.(2014) found that the incidence of cumulative cardiovascular disease and myocardial infarction were significantly lowered after implementation of metformin treatment. A meta-analysis of these studies reported an important decrease in the stroke incidence among patients using metformin (Campbell et al., 2017). However, there is also a study that does not confirm the efficacy of metformin in preservation of left ventricular ejection fraction in patients without diabetes presenting with ST-segment elevation myocardial infarction (STEMI) (Lexis and Horst, 2014). Also another study failed to demonstrate the benefits of metformin on the carotid intimal medial thickness in non-diabetic subjects (Preiss et al. 2014).

Several studies identified influence of metformin on the incidence of HF. For instance, Hartung et al. (2005) compared various anti-diabetic therapies and found that metformin as opposed to thiazolidinedione was not associated with an elevated risk of hospitalization due to HF. On the other hand, Koro et al. (2005) reported an increase, yet non-significant, in the prevalence of congestive HF during the treatment with metformin compared to subjects treated with sulphonylurea. In the next study study, carried out by Nichols et al. (2005), a non-significant reduction in congestive HF in diabetics undergoing a metformin therapy was observed. Therefore, the authors concluded that metformin may offer some protection from the incidence of HF in comparison to sulphonylurea or insulin. Similar conclusions were presented by McAlister et al. (2008), who compared the prevalence of HF in T2DM subjects using metformin to those treated with sulphonylurea, and reported an insignificant decrease in HF in the metformin-treated group. The summary of metformin clinical effects resulting in lifespan extension is presented in Figure 3.

3.2.3. Neurodegenerative disease

The outcomes of several clinical trials give evidence that chronic treatment with metformin could reduce the liability of cognitive decline (Ng et al., 2014). The investigators examined 365 older T2DM subjects (>55 years old) in the population-based Singapore Longitudinal Aging Study. According to the results, metformin use reduced the risk of cognitive impairment (modified Mini-Mental Status Exam score ≤ 23) by 51%, which remained strong to adjustment for vascular and non-vascular risk factors. Furthermore, Ng et al. (2014) did not report any essential interactive effects of metformin therapy with apolipoprotein (APOE-ε4) and depression. In turn, Cheng et al. (2014) presented results of a large observational study which showed that the risk of dementia is weaker in T2DM individuals treated with metformin or sulfonylurea than those using thiazolidinediones for a longer period. The authors presume that potential mechanisms of positive effects of the drug include: improved insulin sensitivity, a decreased risk of metabolic syndrome, and reduced inflammation (Cheng et al., 2014).

Another study proved that a 24-week administration of metformin improves cognitive function in depressed diabetic patients. In addition, metformin was found to significantly reduce depressive sympthoms and change the glucose metabolism in depressed diabetics (Guo et al.,

2014). Herath et al. (2016) examined the effect of diabetes treatment on certain cognitive parameters over four years, and reported that only metformin users demonstrated a better cognitive function including verbal learning, working memory, and executive functions in comparison to patients using other anti-diabetic drugs. Nevertheless, there is also one study reporting that metformin therapy was linked with impaired cognitive performance (Moore et al., 2013). Conflicting evidence regarding metformin effects on cognitive function was also presented by Piskovatska et al. (2019).

3.3. Clinical studies targeting longevity

Owing to the fact that metformin action can target the anti-ageing mechanism, and has a capability to reduce all-cause mortality and prevalence of certain types of cancers, researchers and clinicians have conducted clinical trials on nondiabetic individuals to determine the potential of metformin in extending human life.

One example might be the Metformin in Longevity Study (MILES), which is a double-blind, placebo-controlled clinical study including fourteen patients. The study aims to find associations between 6-week metformin intake and youthful gene expression in elderly people with impaired glucose tolerance (https://clinicaltrials.gov/ct2/show/results/NCT02432287, available on 29.04.2020). Results of the study showed that in older adults, metformin contributes to metabolic and non-metabolic changes, including pyruvate metabolism and DNA repair in the muscle tissue as well as peroxisome proliferator-activated receptors (PPAR) and sterol regulatory element-binding proteins (SREBP) signaling, and mitochondrial fatty acid oxidation in the adipose tissue (Kulkarni et al., 2018).

Quite recently, a large double-blind, placebo-control TAME (Targeting Aging with Metformin) study has been launched. The principal aim of the study is to establish anti-ageing properties of metformin in nondiabetic subjects, and to find out whether metformin can target the ageing process by slowing the sequelae of existing age-related morbidity. It is planned to include 3,000 participants, aged 65 – 79 years. The authors plan to measure the time to the occurrence of new cardiovascular events, cancer, dementia, and mortality. TAME's aim is also to determine significant functional and geriatric end points.Thanks to this study, scientists will know whether treatment with metformin can inhibit age-releated diseases, including cancer, CVD and AD and thus decrease or postpone mortality (Barzilai et al., 2016).

4. PRECLINICAL IN VIVO EVIDENCE OF GEROPROTECTIVE EFFECTS

As presented above metformin is a medication approved by the Food and Drug Administration for the therapy of T2DM but it has also been found to target some ageing-related mechanisms (Nir Barzilai et al. 2016). This aforementioned activity of metformin calls for its use in treatment of ARD and extension of longevity. Within this chapter we provide the results of pre-clinical studies aiming to confirm the anti-ageing properties of the drug and explain its mechanism of action.

4.1. Invertebrate models

The multidirectional mechanism of metformin action has been demonstrated to beneficially affect ARDs. These effects were confirmed in *in vitro* studies targeting mainly molecular mechanisms of ageing, and also *in vivo* studies, including various types of organisms ranging from asimple worm to a mice and rhesus monkeys (Barzilai et al., 2016). Nematode *Caenorhabditis elegans* is an experimental model. It is widely used as it allows to elucidate

molecular mechanisms involved in longevity (Lapierre and Hansen, 2012). Research on the anti-ageing effects of metformin has been extensively conducted over the past decade, but according to some sources (Potempa et al., 2016), the first reports of these properties of metformin appeared 40 years ago. Several studies have found that metformin prolongs lifespan in *C. elegans* (Cabreiro et al., 2013; De Haes et al., 2014; Onken and Driscoll, 2010; Wu et al., 2016). As reported by Onken and Driscoll (2010), the drug administered at a dose of 50 mM increases the mean lifespan of *C. elegans* by about 40%; however, it is not associated with the maximum life span extension. The authors also found that anti-ageing properties of metformin stem from the activation of LKB1-AMPK-SKN1 signalling pathway. Further research has shown that prolongevity effect of metformin in *C. elegans* is related to both v-ATPase-mediated mTORC1 suppression and v-ATPase-AXIN/LKB1-mediated AMPK activation (Chen et al., 2017).

Interestingly, metformin was also showed to increase lifespan in *C. elegans* co-cultured with *Escherichia coli* which has multidirectional effects on the model organism. The mechanism of action included alteration of microbial folate and methionine metabolism. Additionally, metformin differentially influences nematode lifespan, depending on *E. coli* strain, metformin sensitivity and glucose concentration. Bearing in mind that the intestinal microbiome affects human metabolism and health, metformin effects on gut microbiome can contribute to its therapeutic efficacy (Cabreiro et al., 2013). Reduced glucose supplementation prolongs *C. elegans* lifespan through mitohormesis, a biological response in which a lower level of mitochondrial stress improves health and viability (Bárcena et al., 2018). De Haes et al. (2014) showed that metformin prolongs lifespan by means of mitohormesis and found that the mitohormetic signal was transmitted by the hydrogen peroxide scavenger peroxiredoxin (PRDX-2), whose expression was greater after metformin supplemetation. The investigators also stress that due to its evolutionary conservation, the peroxiredoxin pathway might stand for a general principle of prolongevity signalling. In addition, *C. elegans* treated with the drug also demonstrated beneficial morphology for a longer time, which consequently led to their improved health span (De Haes et al., 2014).

Despite the promising results in nematodes, metformin prolongevital effects were not confirmed in *Drosophila,* whose lifespan is affected by AMPK activation (Tohyama and Yamaguchi, 2010). Also in the fruit fly, *Drosophipla melanogaster*, independently on the gender, metformin did not extend the lifespan. Importantly, metformin at high doses (100 mM) was toxic to the flies, probably due to disturbances in intestinal fluid homeostasis (Slack et al., 2012). These outcomes imply that the drug has evolutionarily conserved influence on metabolism but not on lifespan. Nevertheless, the drug was found to suppress age- and oxidative stress- induced DNA damage and delay stem cell ageing in *Drosphila* (Na et al. 2013). The effects of metformin on lifespan were also examined on a silkworm model (Song et al., 2019). Metformin was found to prolong the lifespan of the male silkworm through AMPK-P53-FoxO pathway, increasing stress resistance and anti-oxidative capacity. Interestingly, the survival change was not observed in female silk worms. Thus we can expect that anti-ageing effects of metformin might be gender-related.

In summary, despite the intriguing benefits of metformin in lifespan extension in some nematodes, the underlying mode of action, not yet well explained has become a subject of extensive debate since metformin targets various cellular signaling pathways associated with inflammation, cellular senescence, and stress defense. The researchers claim that metformin prolongs lifespan through mimicking the effects of diet restriction by activating AMPK.

4.2. Vertebrate models

Frequent application of metformin for the treatment of T2DM contributed to the collection of a large amount of data regarding effects of its potential application, pharmacological profile, safety, and mortality. A vast majority of studies on geroprotective effects of metformin utilised a rodent model, mainly various mice strains (Novelle et al., 2016).

A study of Anisimov et al. (2005) was one of the first studies reporting effects of metformin on life span and progression of mammary tumors in mice. The investigators found that long-term treatment of female transgenic HER-2/neu mice with metformin at a dose of 100 mg/kg in drinking water, slightly reduced food intake, slowed down the age-related elevation of blood glucose and triglycerides level. Importantly, it was confirmed that the drug prolonged the mean life span by 8%, and the maximum life span by 1 month in comparison with the control group. In addition, the prevalence and size of mammary adenocarcinomas in mice treated with metformin got decreased and was similar to the one observed in the non-treated group (Anisimov et al., 2005). In another mice strain (outbred SHR mice), the chronic treatment of females with metformin (100 mg/kg in drinking water) reduced the body weight, improved the mean life span by 37.8%, and the maximum life span by 2.8 months in comparison with the control group. However, in this model, the authors did not find any effect of metformin supplementation on blood estradiol concentration and spontaneous tumor incidence (Anisimov et al., 2008). Metformin extends the mean life span, and in combination with melatonin, significantly inhibited the size of transplanted tumors in HER-2/neu mice, thus giving evidence that it may be useful in prevention and treatment of breast cancer (Anisimov et al., 2010a). Interestingly, Anisimov et al. (2011) showed that the prolongevity effects of metformin in female SHR mice depend on the age of the animals at the onset of treatment. A prolongation of the mean life span and the maximum life span was observed when metformin administration was started at the age of 3 months, while no effects were reported when metformin was supplemented to the animals at the age of 15 months (Anisimov et al. 2011).

The geroprotective effects of metformin were also confirmed by Martin-Montalvo et al. (2013), who reported that the chronic supplementation with metformin (0.1% w/w in diet), introduced at middle age, extends the healthspan and lifespan in C57BL/6 mice. The authors reported that metformin acts as CR mimetic, and its beneficial effects include increased insulin sensitivity, and lowered LDL and cholesterol levels without a decrease in caloric intake. Furthermore, metformin improves antioxidant protection, resulting in reductions of both oxidative damage accumulation and incessant inflammation (Martin-Montalvo et al., 2013). The beneficial effects of metformin were also observed in a second strain of male mice (hybrid B6C3F1), with a 4.15% increase in the mean lifespan (Martin-Montalvo et al. 2013). Research conducted by Smith et al. (2010) is one of a few studies examining anti-ageing effects of metformin in the Fisher-344 rat model. However, the authors did not find any evidence of lifespan extension in the metformin treated group. These discouraging effects were attributed to resistance of this strain of rat to calorie restriction (Smith et al., 2010).

Metformin was also reported to exert advantageous effects on neurological disorders. For instance, Ma et al. (2007) found that metformin supplementation (2 mg/mL in drinking water) significantly increased the survival time of male mice suffering from Huntington's disease (HD). A higher dose of metformin (5 mg/mL) did not affect survival. Interestingly, the positive affect of metformin was reported only in male mice, not female. Also Sanchis et al. (2019) reported that metformin relieves motor and neuropsychiatric phenotypes in zQ175 mice with HD indicating delay of HD progression. However, a study of Kaneb et al. (2011) have shown

that metformin does not influence the onset, progression and survival of male mice with amyotrophic lateral sclerosis (ALS). Results of other studies are summarized in Table 2.
Beneficial properties of metformin were also confirmed with respect to pathological hallmarks of AD. For example, Chen et al. (2016) determined the effect of metformin in β-amyloid (Aβ) transport across the blood-brain barrier (BBB), and found the drug essentially reduced the influx across the BBB via the receptor for advanced end glycation product (RAGE) expression and intra-arterial infusion of ^{125}I–Aβ(1–40) in diabetc male db/db mice. In another study, Li et al. (2012) reported that metformin improves AD-like neuropathology in obese, leptin-resistant mice. However, there are also studies showing adverse effects of metformin regarding the liability of developing AD. Chen et al. (2009) demonstrated that metformin administration in a triple transgenic mouse model of AD results in an increase in the expression of BACE1, being one of the two enzymes that cleave amyloid precursor protein (APP) to generate Aβ, which was associated with an increase in Aβ production and small plaque formation. In addition, the investigators found that the drug can be harmful toward viability of neurons through its AMPK-mediated mechanism (Chen et al., 2009).
Anti-inflammatory properties of metformin were also confirmed in an animal model. Oliveira et al. (2016) observed that diabetic mice treated with metformin demonstrate reduced levels of the expression of inflammation markers (IL-1 and vascular endothelial growth factor (VEGF)), accompanied by enhanced levels of p-AMPK and nitric oxide synthase 3 (eNOS). Anti-oxidative potential of metformin was also examined in a mouse model with carbon tetrachloride (CCl_4)- induced oxidative liver injury (Dai et al., 2014). Supplementation with metformin markedly reduced the level of serum aminotransferases and attenuated hepatic histological abnormalities. Ma et al. (2015), by using a rat model of painful diabetic neuropathy, demonstrated that metformin exerts beneficial effects on malondialdehyde (MDA) and glycation end product levels in blood, as well as increases superoxide dismutase activity, suggesting that the drug suppresses diabetes-induced oxidative stress. In addition, metformin was found to act neuroprotectively through enhancing autophagy and inhibiting the inflammation after a spinal cord injury (SCI) (Wang et al., 2016).

Metformin was also found to be effective in other ARDs including cancer. Numerous pre-clinical reports have confirmed anti-cancer properties of the drug, and discovered plausible mechanisms explaining the molecular mechanism of its action in cancer. This observation has been a subject of many review papers (Rizos and Elisaf, 2013; Yu et al., 2019; Pizutti et al., 2015; Febbraro et al., 2014), so we will focus only on just a few examples. For instance, Gotlieb et al. (2008) demonstrated cytotoxic properties of metformin towards ovarian cancer cells, which has later been supported by a few papers (Wu et al., 2012; Lengyel et al., 2014). Initial experiments identified the molecular mechanism of metformin action, with AMPK, and its downstream targets responsible for anti-cancer activity (Wu et al., 2012). In xenograft mouse models of ovarian cancer, metformin reduced tumor burden, decreased tumor weight, and improved the cisplatin cytotoxicity (Wu et al., 2012; Lengyel et al., 2014; Rattan et al., 2011). Metformin was also found to significantly reduce the risk of pancreatic ductal adenocarcinoma incidence and tumor weights in transgenic mice (Mohammed et al., 2013). Moreover, the authors observed essential inhibition of carcinoma spread in the pancreas. Molecular studies have shown that the pancreatic tissue of mice, fed with metformin, exhibited a significant suppression of mTOR, extracellular signal-regulated kinases (ERK), phosphorylated extracellular signal-regulated kinases (pErk), and insulin-like growth factor 1 (IGF-1) (Mohammed et al., 2013). On the other hand, Cheng and Lanza-Jacoby (2015) suggested that metformin decreases pancreatic cancer cell survival by reducing ROS production through down-regulation of NADPH oxidase 4 (NOX4) protein expression.

In summary, on the base of the analysis of the above data collected in different animal models, metformin seems to be an encouraging geroprotector. The summary of the current knowledge on the metformin effects in various organisms in presented in Figure 4. In addition, growing evidence in preclinical studies suggests advantageous effects of metformin in the treatment of ARD, including cancer and neurodegenerative diseases. Significant is the fact that the drug presents a good safety profile and is well tolerated. However, there are still some discrepancies between results of some studies regarding the effectiveness of metformin. Thus, further studies are required to clarify both the mechanisms and biological properties of metformin.

5. EXPERIMENTAL IN VITRO EVIDENCE OF GEROPROTECTIVE EFFECTS OF METFORMIN

Within this chapter, we provide a brief overview of beneficial properties of metformin obtained in *in vitro* studies which might be valuable in the treatment of selected ADR.

5.1. Anti-proliferative effects

Before discussing the role of metformin as suppressor of cancer cell viability, one should look at the effective concentration of the drug. The efficacy of metformin as an anti-neoplastic drug stems from the sensitivity of certain tissues to the drug, and cellular transport. Based on the number of transporters engaged in cellular uptake of metformin into different tissues, including plasma membrane monoamine transporters (PMAT), organic cation transporters (OCTs), multidrug and toxin extrusion (MATE), we presume that the presence and function of transporters, and interactions between them may influence the uptake of metformin into tumor cells. This may result in different anticancer potential of the drug (Markowicz-Piasecka et al., 2019). Most of the available studies report anti-proliferative properties of metformin at concentrations reaching 5-50 mM which are much higher than those corresponding to therapeutic concentrations applied in T2DM treatment (plasma concentrations between 10–40 μM). However, it should be noted that the concentration of metformin is highly different in various organs (Foretz et al., 2014). For instance, metformin concentration in the colon was found to reach 40 mM (Glosmann and Lutz, 2019).

An overview of current literature shows that anti-cancer properties of metformin are based on several mechanisms, including activation of LKB1/AMPK pathway, and suppression of mTOR, induction of cell cycle arrest or apoptosis, inhibition of protein synthesis, and improvement of the immunity (Franciosi et al., 2013). Favourable inhibitory effects of metformin on cell growth have been described for various cancer cell lines. These outcomes may be divided into two categories – studies presenting the improvement of chemotherapy during metformin treatment and studies confirming metformin cytotoxicity (Rizos and Elisaf, 2013).

For instance, two independent studies (Dong et al., 2012; Hanna et al. 2012) reported that metformin improves the response of endometrial cancer cells to cisplatin and paclitaxel. The synergistic effect between metformin and cisplatin with respect to cytotoxic effect was also found for breast cancer cells (Liu et al., 2012), ovarian cancer cells and metastatic nodules in the lung (Rattan et al., 2011). In turn, Song et al. (2012) reported that metformin elevated the radiosensitivity of human breast cancer cells and mouse fibrosarcoma cells.

The effectiveness of metformin as a cytotoxic agent has been reviewed comprehensively (Rizos and Elisaf, 2013). Metformin was reported to diminish the viability of several types of cancer cells, including lung, gastric or endometrial cancer cells (Rizos and Elisaf, 2013). Other studies proved that metformin blocks cellular transformation and selectively kills cancer stem cells in four types of breast cancer (Hirsch et al., 2013) as well as hepatocellular carcinoma cells (Bhalla et al., 2012). Other highly valuable cytotoxic properties of metformin have been presented in Table 3.

Researchers usually examine both effects and associated mechanisms of metformin action. For example, Buzzai et al. (2007) established the effects of metformin on paired isogenic colon cancer cell lines HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$. The authors found that metformin treatment selectively inhibited growth of HCT116 $p53^{-/-}$ by induction of apoptosis, and concluded that the drug exerts selective toxicity towards p53-deficient cells. Thus, metformin might be valuable in the treatment of patients with harboring p53-deficient tumors which are frequently resistant to traditional radiotherapy or chemotherapy (Buzzai et al. 2007). In turn, Zakikhani and co-workers (2006) reported that metformin suppresses breast and glial cancer cell growth in AMPK-dependent manner. They proved the results using small interfering RNA against AMP kinase, which prevented metformin-induced antiproliferative effect towards breast cancer cells (Zakikhani et al. 2006). Interesting findings were also collected by Ben Sahra et al. (2008), who revealed AMPK-mediated anti-proliferative effects of metformin on the human prostate cancer cells model. However, the scientists did not observe inhibition of anti-proliferative metformin action after using siRNA against the two catalytic subunits of AMPK, which means that the drug has another anti-neoplastic mechanism of action. The authors found that metformin inhibits ribosomal protein S6 kinase beta-1 (p70S6 kinase, S6K1) phosphorylation which is connected with downregulation of the mTOR pathway. The authors reported that metformin antiproliferative activity was due to reduced expression of cyclin D1 protein leading to cell cycle arrest at the G0/G1 phase (Sahra et al., 2008). Another interesting results were presented by Queiroz et al. (2013), who evaluated the anti-proliferative potential and mechanism of action of metformin in MCF-7 cancer cells. Metformin decreased the viability of MCF-7 cells, induced cell cycle arrest at the G0-G1 phase and increased cell apoptosis and necrosis. The authors identified also a molecular mechanism of the anti-proliferative properties of metformin which was linked with AMPK, and its downstream effectors including p38, Akt and ERK 1/2 (pro-inflammatory phosphokinases). The final effect of metformin treatment was stimulation of FOXO3a, being a transcription factor attenuating cancer by promoting cell cycle arrest (Queiroz et al. 2014).

5.2. Antioxidant properties

The process of ageing is closely connected with an onset of several diseases including cancer, T2DM, CVD and neurodegenerative diseases. It has been postulated that one of the causes leading to these diseases is oxidative stress. The main mechanism of anti-oxidative properties of metformin stems from inhibition of the mitochondrial respiratory chain. However, metformin has also been shown to reduce the production of ROS in mouse embryonic fibroblasts independently of AMPK activation (Algire et al., 2010). In turn, Bonnefont-Rousselot et al. (2003) found that metformin at pharmacologically relevant concentrations has the potential to scavenge hydroxyl free radicals. The authors found a decrease, yet nonsignificant, in ROS-induced luminescence in polymorphonuclear cells (PMN) stimulated by phorbol myristate acetate (PMA), or formyl methionine leucyl phenylalanine (fMLP). Thus, considering these

results, the authors concluded that metformin could directly remove ROS or act indirectly by modulating the intracellular synthesis of superoxide anion (Bonnefont-Rousselot et al., 2003).

Vascular diabetic complications are associated with the production of advanced end glycation products (AGEs). Ruggiero-Lopez et al. (1999) was one of the first authors who reported that metformin affects the formation of AGEs through interaction with α-dicarbonyl compounds, including methylglyoxal and glyoxal. Thus, it may be stated that metformin reduces carbonyl stress which can result in the prevention of vascular diabetic complication *in vivo* (Ruggiero-Lopez et al. 1999). Anti-oxidative properties of metformin were also confirmed by An et al. (2016), who assessed the influence of metformin on fluctuating glucose-induced endothelial dysfunction. This *in vitro* study showed that metformin has protective properties towards endothelial cells against oxidative stress. The beneficial effects of metformin included recoupling eNOS (endothelial nitric oxide synthase) through upregulation of GTPCH1 (guanosine 5′-triphosphate cyclohydrolase 1) and BH4 (tetrahydrobiopterin) levels, and attenuation of upregulation of p47-pox subunit in NADPH oxidase in FG-treated HUVECs. It was found that the protective effect of metformin resulted from inhibition of NADPHoxidase via an AMPK-dependent pathway. Additionally, metformin acted through typical activation of AMPK signalling pathway which inhibited generation of ROS and accelerated production of NO (An et al., 2016). Another study, conducted on colorectal cancer cells (Nguyen et al., 2019), proved that the drug decreases ROS production through inhibition of NADPH oxidase activity. Additionally, metformin suppressed NF-κB signalling and blocked interleukin-8 (IL-8) up-regulation induced by lithocholic acid (LCA). These outcomes led to a conclusion that metformin might prevent endothelial cell proliferation and tubelike formation (Nguyen et al., 2019).

5.3. Anti-inflammatory effects

Inflammation constitutes an important part of the pathogenesis of ageing-related diseases, including T2DM, as well as Alzheimer's disease (AD) (Verdile et al., 2015). For instance, multiple data have proved that the development of T2DM is related with elevated levels of inflammatory markers and mediators, including C-reactive protein (CRP) and interleukin 6 (IL-6) (Pradhan et al., 2001). In the course of AD, the degree of inflammation is associated with a cognitive decline (Parachikova et al., 2007) and brain atrophy (Cagnin et al., 2002). Inflammation, including NF-κB signalling, is recognized as an important contributing factor to ARDs, and a few previous experiments have reported that metformin inhibits NF-κB, also in vascular tissue (Isoda et al., 2006) and in hepatocytes (Woo et al., 2014). The effects of NF-κB inhibition in human endothelial cells (ECs) and smooth muscle cells (SMCs) included a reduced release of cytokines, such as interleukin-6 (IL-6) and interleukin-8 (IL-8), and attenuated activation potential of pro-inflammatory phosphokinases (p38, JNK, and Erk and Akt), induced by IL-1 (Isoda et al., 2006). Also Cameron et al. (2016) found that the biguanide in primary hepatocytes inhibits tumor necrosis factor-α–dependent IκB degradation and expression of pro-inflammatory mediators, including IL-6, IL-1β, and CXCL1/2 (C-X-C motif ligand 1/2). In addition, in macrophages, metformin specifically decreases a release of pro-inflammatory cytokines, without blocking M1-macrophages and M2-macrophages differentiation or activation (Cameron et al., 2016).

Anti-inflammatory properties of metformin were confirmed in colon cancer cells (COLO205) as well. The drug was found to disrupt the activation of NF-kB and phosphorylation of inhibitor of kappa B. The activity of this mechanism resulted in decreased production of inflammatory interleukines (IL-8 and IL-1α). The authors evaluated also in vivo effects of metformin, and

found that the drug ameliorates inflammation in epithelial cells of mice, inhibits colitis-associated colon tumorigenesis in a murine model, and weakens the severity of intestinal inflammation in IL-10 knockout mice (Koh et al. 2014). The advantageous effects of metformin on intestinal smooth muscle cells were also reported by Al-Dwairi et al. (2018). Cell stimulation with metformin caused a significant inhibition of secretion and expression of tumor necrosis factor-α (TNF-α), IL-1α, macrophage colony stimulating factor (M-CSF) and T cell activation gene-3 (TCA-3). The authors confirmed that the biguanide reduced levels of lipopolysaccharide-induced NF-κB phosphorylation (Al-Dwairi et al. 2018). The anti-inflammatory properties of metformin were also presented *in vitro* using human retinal microvascular endothelial cells (hRVECs). The anti-inflammatory properties of the drug stem from activation of AMPK and related lowered levels of inflammatory molecules such as NF-κB, intercellular adhesion molecule-1 (ICAM-1), monocyte chemotactic protein-1 (MCP-1) and IL-8 in TNF-α -stimulated hRVECs (Han et al. 2018).

In summary, numerous experimental studies have evaluated the potential of metformin in attenuation of inflammation and oxidative stress, and presented promising results in various types of cells. However, further studies conducted on animal models or clinical trials are required to support the results of *in vitro* studies.

5.4. Neuroprotective effects

It has been proved that metformin is able to counteract basic mechanisms of ARD such as cancer, CVD, and neurodegenerative diseases, including AD. According to Rotermund et al. (2018), the drug might affect neuronal longevity mechanisms because its cellular effects, yet not fully elucidated, are well-studied. Additionally, due to the well-known safety profile and multi-directional activity, metformin is an interesting drug for further studies. The main mechanisms supporting a neuroprotective effect of metformin include glucose metabolism improvement, energy sensing, counteracting protein phosphorylation, oxidative stress and neuroinflammation (Rotermund et al., 2018). The potential of metformin as a drug for treatment of neurodegenerative diseases has been studied thoroughly, and is a subject of many reviews (Palleria et al., 2016; Rotermund et al., 2018; Markowicz-Piasecka et al., 2017). Therefore, this article is a presentation of only a few *in vitro* studies on neuroprotective properties of metformin.

Metformin was found to exert neuroprotective properties preventing apoptotis of primary neurons (El-Mir et al., 2008). Studies demonstrated that metformin reduced neuronal damage and ameliorated a lack of oxygen or glucose in neurons. Owing to this activity, the drug prevented etoposide induced-apoptosis, leading to improvement of neuronal cell survival (El-Mir et al., 2008). Moreover, metformin effectively improves impaired glucose uptake in insulin-resistant neuronal cells, and prevents occurrence of molecular and pathological characteristics of AD (Gupta et al., 2011).

Importantly, metformin has the potential to significantly reduce beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) protein expression and activity in cellular model, thereby reducing BACE1 cleavage products and generation of Aβ (Hettich et al., 2014). The decreased BACE1 expression was caused by interfering with MID1 (E3 ubiquitin ligase) complex which subsequently activates PPA2 (protein phosphatase 2A) and suppresses mTOR signalling. Therefore, in the future, the targeting mTOR/PP2A therapy may be a reasonable method of suppression of AD (Hettich et al. 2014). Another cell research also reported that metformin stimulates PP2A activity and reduces tau phosphorylation at PP2A-dependent epitopes. Interestingly, the authors reported that metformin effects on PP2A and subsequent tau

phosphorylation do not appear to depend on AMPK activation (Kickstein et al. 2010). These findings prove a beneficial effect of chronic metformin therapy and arouse the expectation that metformin would have a neuroprotective and protective effect in individuals with susceptibility for AD (Kickstein et al., 2010). According to Zhou and co-workers (Zhou et al., 2016) pretreating rat cerebellar granule neurons (CGN) with metformin significantly enhances cell viability against neurotoxic effects of glutamate.

AMPK signalling appears to be crucial to protect neurons under pathologic conditions. Paintlia et al. (2013) showed that metformin through AMPK activation reduces inflammation and oxidative stress, which results in protection of oligodendrocytes (OLs) in mixed *in vitro* glial cultures, stimulated with lipopolysaccharide. Similar effects were also found in OLs exposed to cytokines. Based on these results, it can be claimed that metformin as a AMPK activator can decrease deficits in multiple sclerosis and associated neurodegenerative disorders (Paintlia et al., 2013). Another interesting results were presented by Wang et al. (2012), who reported that the biguanide supports neurogenesis and enhances spatial memory in a PKC-CBP-dependent (atypical kinase C) manner. As stated by the authors, PKC-CBP pathway is important for the normal genesis of neurons from neural precursors, and due to its activation, metformin promotes rodent and human neurogenesis in culture (Wang et al., 2012).

In summary, the data presented herein imply that metformin may play a crucial role in the treatment of AD due to targeting several pathological hallmarks of AD, including neurodegeneration, tau phosphorylation, and neuroinflammation. Moreover, anti-cholinesterase activity of the drug should also be pointed out. Nevertheless, negative results, especially those linking long-term use of metformin with accumulation of β-amyloid aggregates, should be kept in mind when evaluating the potential of metformin as an anti-AD agent.

5.5. Anti-senescence properties

Organismal ageing is accompanied by metabolic changes at the cellular level. A rising quantity of senescent cells (SCs) induces secretion of pro-inflammatory factors which impair the regeneration capability of stem cells. The chronic state of low grade inflammation is commonly reported in elderly people and is linked with many ARDs (Bielak-Żmijewska et al., 2014). In the body ageing process, accumulation of damage in cells, decreased efficiency of cell repair systems and impaired removal of damaged cells result in accumulation of malfunctioning cells. These impaired cells worsen the functioning of neighboring tissues, increase inflammation, which promotes the ageing of the whole body (Bielak-Żmijewska et al., 2014).

Metformin is one of synthetic and natural compounds, investigated for their anti-senescence properties, in *in vitro* and animal models. As reviewed by Barzilai et al. (2012) and Gurău et al. (2018), metformin presents beneficial effects on a number of ageing-related processes such as inflammation, autophagy, cell viability, and protein synthesis. In addition, the drug modulates the expression of receptors for cytokines, insulin, and IGF-1, and promotes mTOR inhibition (Gurau et al., 2018).

Metformin was found to significantly attenuate vascular senescence and HFD (high fat diet) induced atherosclerosis in mouse model. Furthermore, metformin increased the expression level of superoxide dismutase-1 (SOD1) in aortas of mice, leading to a decreased level of ROS (Forouzandeh et al., 2014). In turn, Moiseeva et al. (2013) reported that metformin suppresses the expression of genes coding for multiple inflammatory cytokines during cellular ageing. The investigators observed that metformin was able to prevent the translocation of NF-κB to the

nucleus and stopped the phosphorylation of IκB and IKKα/β, processes needed for activation of the NF-κB pathway. These observations can explain the anti-ageing and anti-neoplastic effects of metformin. Metformin was also proposed to be a SASP (senescence-associated secretory phenotype) inhibiting molecule in senescent fibroblasts (Moiseeva et al., 2013; Sultuybek et al. 2019). Interestingly, metformin did not reduce the expression of anti-cancer cytokines (e.g. interferon) in senescent cells, implying that the drug modulates SASP by reducing its inflammatory potential but retaining its anti-cancer properties (Moiseeva et al., 2013). Noren Hooten et al. (2016) discovered that long-term treatment with metformin upregulated DICER1 and increased microRNA both in mice and humans with T2DM. In addition, the authors found that the drug reduced p16 and p21 protein levels and also other inflammatory oncogenes being the hallmarks of SASP. On the base of these results, the authors hypothesised that upregulation of DICER1 levels may become a new approach for ageing-related diseases (Noren Hooten et al., 2016). Metformin up-regulated the expression of microRNAs in human pancreatic, prostate, lung cancer cells (Li et al. 2012; Avci et al. 2013; Dong et al. 2020) and also in endometrial epithelial cells of patients with PCOS (Zhai et al. 2019) in a dose-dependent manner.

Anti-aging effects of metformin cannot be discussed without describing its effects on SIRT1. SIRT are a group of proteins which have nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase activity or ADP-ribosyltransferase activity. The sirtuin family is responsible for supporting mammalian health, regulating various cell functions, perhaps modulating the ageing process including extension of viability and forming responses to stressors. In mammals, there are seven sirtuin homologues of which SIRT1 is the most comprehensively examined for its significance in the vascular ageing process (Kida et al., 2016). The mechanism of SIRT1 action is linked with deacetylation of transcription factors (such as FOXO1, 3 and 4, p53, NF-κB, PGC-1 and HSF-1), histones and DNA repair proteins (Giblin et al., 2014). Metformin was found to act agonistically on SIRT1 by improving its catalytic efficacy (Cuyàs et al., 2018). Importantly, improvement in SIRT1 activity protects cells against apoptotic death by induction of p65 deacetylation, which provokes the inhibition of NF-κB factor (Lee et al., 2009).

6. DOES METFORMIN MEET ALL CRYTERIA FOR A GEROPROTECTOR?

The scientific area of discovery of geroprotectors is a very dynamic discipline. Currently, there is approximately 200 substances which possess anti-ageing properties, including slowing ageing or increasing lifespan in a variety of organisms (e.g. yeasts, nematodes or rodents) (Geroprotectors.org database) (Moskalev et al., 2015). In the previous chapters, we presented the multidirectional action of metformin in preserving youth. However, one should approach this topic critically and consider whether metformin meets all the most important criteria that are the basis to become a geroprotector.

Nowadays, there is no single definition of geroprotectors in the scientific literature (Moskalev et al., 2016). Another important issue are the differences in the study protocols, including methodology, research methods or model organisms and genetic background within species which impede to compare the results and make conclusions. For this reason Moskalev et al. (2016) introduced the concept of geroprotector and developed the criteria for classifying a substance as a geroprotector. In general, a certain substance is regarded as geroprotector when it increases lifespan (Moskalev et al., 2016). The criteria for being a geroprotector have been

divided into primary and secondary groups. Increased lifespan, amelioration of ageing biomarkers, acceptable toxicity, and improving health-related quality of life are listed in the first group. Secondary selection criteria are as follows, evolutionary conservatism of target or mechanism of action, reproducibility of geroprotective effects on different model organisms, increase in stress resistance, and simultaneous influence on several ageing-associated causes of death in mammals (Moskalev et al., 2016). In the case of metformin, these criteria are not always met. Although some studies report increase in longevity in several model organisms: *C. elegans* (Cabreiro et al., 2013), *D. melanogaster* (Slack et al., 2012), and *M. musculus* (Martin-Montalvo et al., 2013), there are also reports showing no effect of metformin in rats with normal genetics (Smith et al., 2010). Importantly, metformin is regarded as a safe drug with acceptable acute toxicity – mouse LD_{50} oral 1450 mg per kg (Tomasulo, 2002), and is well tolerated (Giugliano et al., 1993). The effects of metformin on mice lifespan depend on its dose since it was found that smaller doses of the drug (0.1% w/w in diet) slightly prolonged the lifespan by 5.83%, but higher doses (1% w/w) led to the shortening of mice lifespan due to renal dysfunction (-14.4%) (Martin-Montalvo et al., 2013). Toxic effects of metformin given at 1% (w/w) were also confirmed in 2-year-old mice (Alfaras et al., 2017). The authors examined the effects of 1% metformin given according to the different regimens, every-other week (EOW) or two consecutive weeks per month (2WM) on the survival of mice. During the first few weeks a decrease in body weight was observed; however, the lifespan of mice in both groups (EOW and 2WM) was comparable with non-treated animals. The differences in the action of metformin on metabolic markers between the EOW and 2WM groups, with EOW metformin conferring greater benefits were found. It was concluded that the absence of adverse outcomes associated with chronic, intermittent use of 1% metformin in old mice has clinical translatability into the biology of ageing in humans (Alfaras et al., 2017). Rarely occurring but serious adverse effects include lactic acidosis, respiratory disease (due to inadequate oxygenation of tissues), and impaired renal function (Moskalev et al., 2016). According to Espada et al. (2019) the pro-longevity effects of metformin depend also on the time of metformin supplementation since the investigators found that late life metformin treatment limits cell survival and shortens lifespan of *C. elegans* by triggering an aging-associated failure of energy metabolism. In addition to above-mentioned data, metformin was found to not slow down the epigenetic clock (Quach et al., 2017). It was also found that metformin negatively affects the hypertrophic response to resistance training in healthy older subjects (Walton et al., 2019). Metformin was also reported to inhibit mitochondrial adaptation to aerobic exercise in the elderly (Konopka et al., 2019). Thus, it implies that prior to prescribing metformin as an anti-ageing agent, additional studies are required to understand the mechanisms that elicit positive and negative responses to metformin with and without exercise (Konopka et al., 2019). Among unfavourable effects associated with long-term metformin administration are those related with decreased level of vitamin B_6, B_{12} and folate in the body, which can cause anemia, homocysteinemia (increased risk of atherosclerosis), neuropathy, impaired memory or even cognitive dysfunctions (Aroda et al., 2016; Glossmann and Lutz, 2019; Roy et al., 2016).

To summarize, the benefical effects of metformin on lifespan in humans were reported mainly on people with specific diseases, including T2DM or hypertension. However, those favourable effects were not confirmed in healthy subjects since the causes of reduced mortality in sick people do not necessarily slow down the ageing of healthy individuals. Similar comparison can be done with relation to age because most of the available studies were conducted in elderly patients but we still lack the knowledge how metformin acts in young people.

7. CONCLUDING REMARKS

In summary, metformin is a safe and effective drug for treatment of T2DM. It targets multiple mechanisms involved in senescence at the molecular level. Numerous cellular studies have revealed that metformin exerts anti-oxidative effects, reduces inflammatory markers, suppresses NF-κB, and mTOR signalling pathways, and thus reduces DNA damage. Importantly, mounting evidence in preclinical invertebrate and vertebrate models suggests advantageous contribution of the drug to lifespan extension and reduction of the risk of ageing-related diseases, including cancer and neurodegeneration. These valuable properties of metformin attracted scientists' and clinicians'attention who perceive metformin as a promising geroprotector in humans.

A lot of human studies indicate that individuals using metformin demonstrate a decreased risk of developing any cancer compared with the general population. In addition, metformin-users have a lower probability of developing colorectal, breast or lung cancer compared with T2DM subjects who have been administered non-metformin therapies. However, not all clinical studies report beneficial effects of metformin regarding the incindence of cancer. Therefore, the currently available data is not sufficient to support the direct anti-cancer properties of metformin. Hopefully, ongoing trials, determining the long-term treatment with metformin as a therapy in prostate, colorectal or pancreatic cancer, will help to elucidate the potential of metformin as an anti-cancer drug. As far as we are concerned, the valuable influence of metformin on the prevalence of cancer might be evident over a longer period of time or in more specific treatment groups, including insulin users. Certainly, continuous progress in discovering molecular targets of metformin action will help to understand and continue research on antitumor activity of the drug.

Currently ongoing clinical trials aim to determine effects of monotherapy of metformin or in combination with lifestyle changes on clinical and molecular hallmarks of ageing. For instance, NCT03451006 trial aims to check whether metformin can improve longevity of the cell, reduce ageing-related biochemical parameters and thereby strengthen physical performance, measured by the short physical performance battery test. In turn, the aim of NCT03713801 is to see whether metformin can boost the immune response to the pneumococcal conjugate vaccine (PCV-13) in older adults, and if this effect is mediated by microbiome. While writing this paper, at the beginning of May 2020, the register of clinical trials (www.clinicaltrials.gov) includes six recruiting clinical studies which aim at determining effects of metformin on ageing.

Bearing in mind both the promising results of clinical studies, and neutral or ambiguous data regarding metformin anti-cancer effects, we strongly believe that current clinical studies, including TAME, could provide more accurate information on metformin potential as an anti-ageing agent. Definite data is needed to confirm the role of metformin as a geroprotector.

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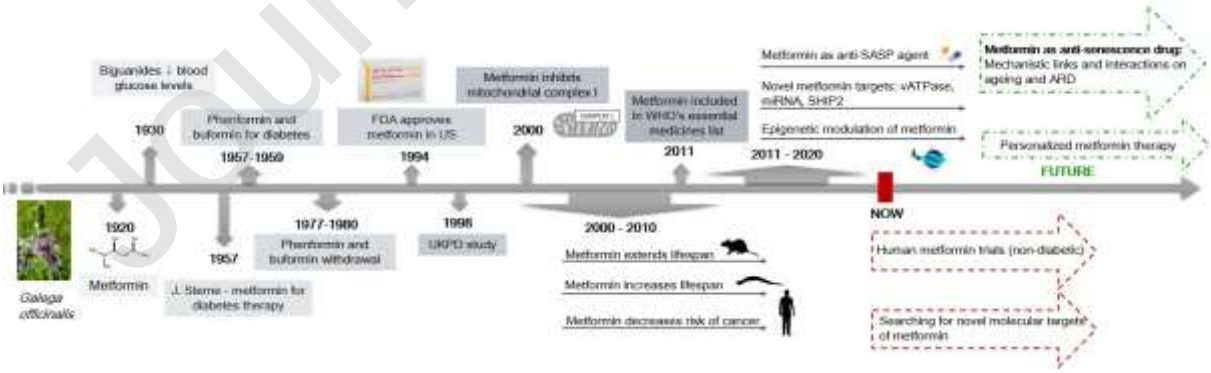

Figure 1. *Schematic review of the history of metformin application and future trends.*

In medieval times Galega officinalis herb was used to treat diabetes like sympthoms. Metformin was synthesized in 1922, and its glucose-lowering properties were studied in animal model in the mid-1920s, and in humans in 1930s. Owing to studies conducted by Dr Jean Sterne, metformin was approved for the treatment of diabetes in Europe in the 1950s. Knowledge about metformin has increased significantly over the past 20 years. New metformin properties have been discovered, including antioxidant and anti-cancer effects. Research into the anti-aging properties of metformin is also underway.

Abbreviations: FDA – Food and Drug Administration; UKPDS – United Kingdom Prospective Diabetes Study; WHO – World Health Organization; SASP - senescence-associated secretory phenotype; SHIP2 - Inositol Polyphosphate Phosphatase-Like Protein 1; ARD – ageing related diseases; vATPase – vacuolar ATPase.

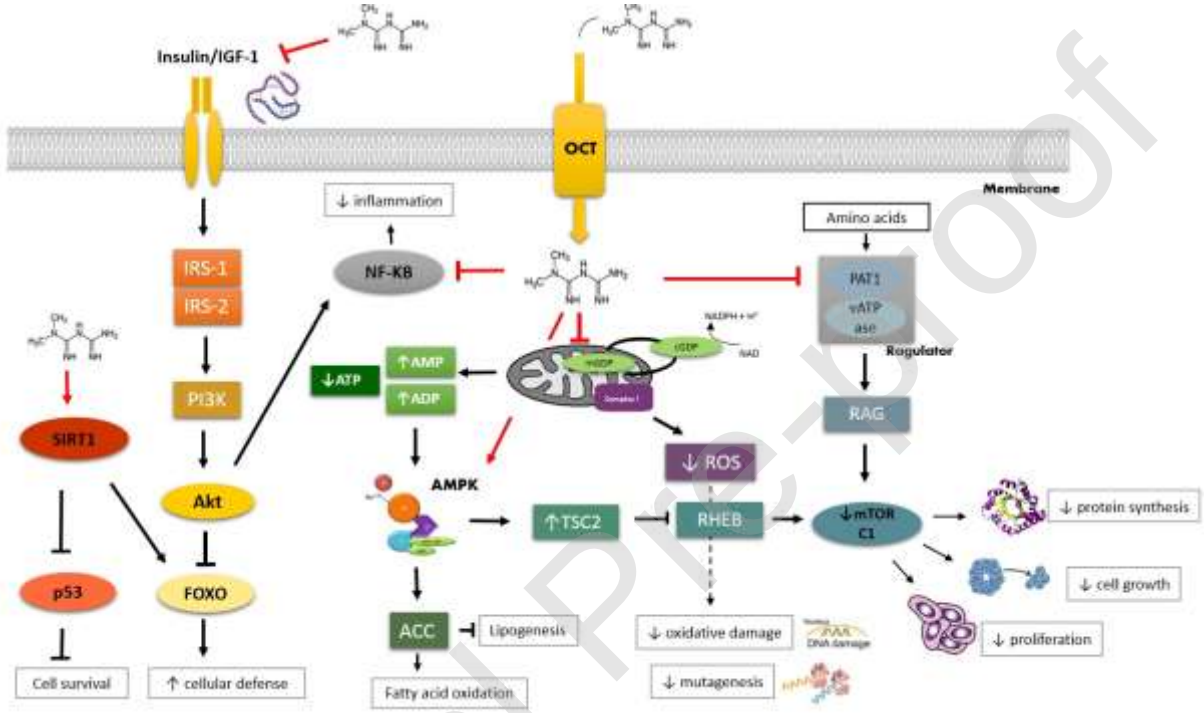

Figure 2. *Mechanisms of metformin action related to ageing.*
The figure shows schematically the most important intracellular targets of metformin. Outside the cells metformin has been found to affect the receptors for insulin and IGF-1 that are activated with ageing. Metformin is transported into the cells via organic cation transporters (OCTs) in which metformin inhibits mitochondrial complex I, and activates AMPK. Therefore, metformin suppresses mTOR, which appears to be a major target to modulate aging. Metformin through inhibition of complex I decreases ROS level, and through inhibition of NF-KB decreases inflammation. Modulation of SIRT1 activity leads to removal of senescent cells. All the processes affect cell proliferation, cellular survival, stress defense, autophagy, protein synthesis, and inflammation which are strongly associated with longevity.
The effects of metformin on its primary targets are marker with red arrows or lines. The figure does not include all the cross-talk between individual factors.

Abbreviations: IGF-1 – insulin growth factor 1; IRS-1/2 – insulin receptor substrate 1/2; PI3K - phosphoinositide 3-kinases; Akt – protein kinase B; FOXO – transcription factors (Forkhead box); SIRT1 – sirtuin 1; NF-KB - nuclear factor kappa-light-chain-enhancer of activated B cells; ACC - Acetyl-CoA carboxylase; TSC2 - tuberous sclerosis complex 2; ROS – reactive oxygen species, RHEB

- Ras homolog enriched in brain; mTOR - mammalian target of rapamycin; RAG - RAS-related GTP-binding protein; PAT1- proton-coupled aminoacid transporter 1 ; vATPase – vacuolar ATPase.

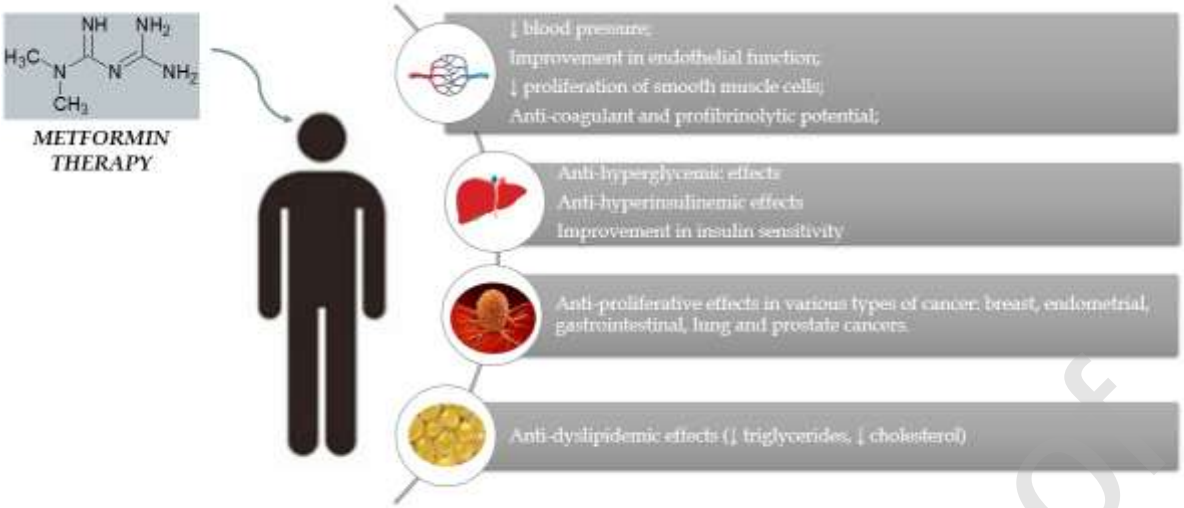

Figure 3. *Potential clinical effects of metformin contributing to its anti-ageing effects. In the ageing patients, metformin may provide many multiple benefits. Metformin basic effects are related with the influence on the liver (e.g. reduction in glucose output), and the peripheral tissues to increase glucose uptake. With regard to cardiovascular system metformin ameliorates hyperglycemia, improves endothelial function, reduces blood pressure and possesses anti-coagulant properties. In addition, metformin reduces insulin resistance and fat redistribution. Accumulating data give evidence on the anti-proliferative role of metformin in several types of cancer. All these beneficial effects may improve physical function (e.g. mobility, endurance), clinical outcomes (e.g. blood pressure, weight and cardiovascular health) leading to improved quality of life and extended lifespan.*

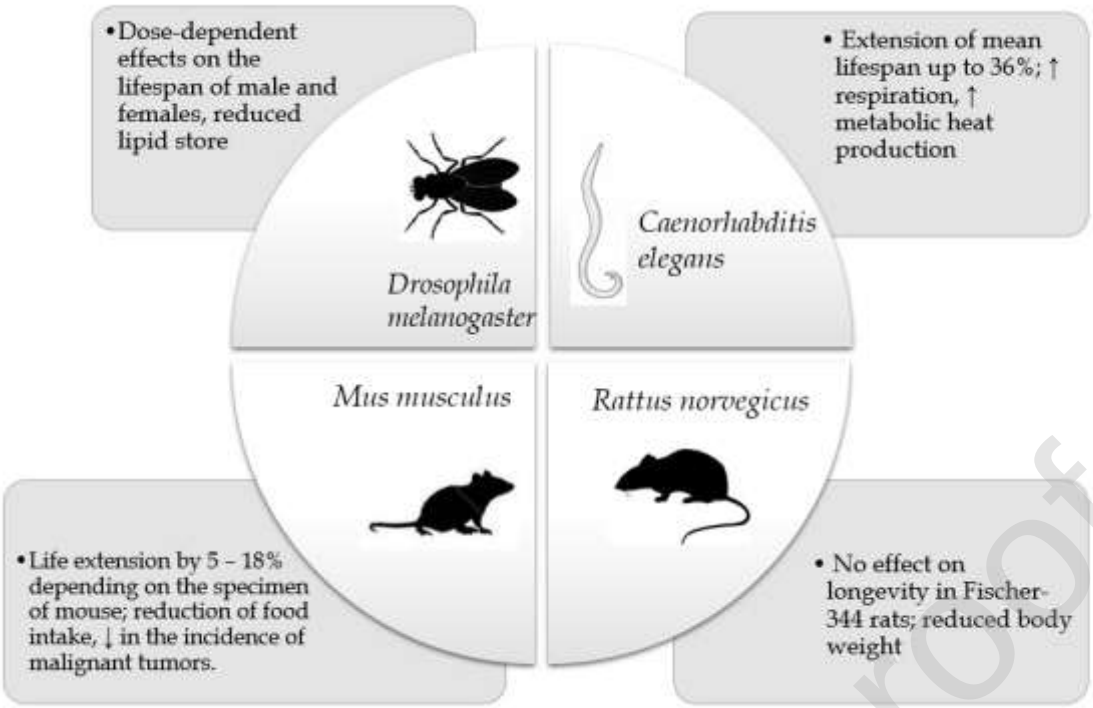

Figure 4. *The summary of lifespan-extending properties of metformin in different animal models (Drosophila melanogaster, Caenorhabditis elegans, mus musculus, Rattus norvegicus). The results are based on the previous findings (Cabreiro et al., 2013; De Haes et al., 2014; Smith et al., 2010; Slack et al., 2012; Martin-Montalvo et al., 2013; Anisimov et al. 2010b). A detailed description of the research results is presented in the text.*

Table 1. *Other clinical studies evaluating the effects of metformin on lung, prostate, bladder, kidney, liver and pancreas cancer.*

Cancer	Study type	Study population	Total participants	Measured outcome	Effect on organs	Results and conclusions	Ref.
Lung	Cohort study	Newly diagnosed lung cancer patients with T2DM	1,443	Lung cancer-specific mortality with metformin treatment	Further clinical studies are required to verify these findings.	Little evidence of a protective association between metformin therapy and cancer mortality in lung cancer subjects.	Menamina et al., 2016
Lung	Nested case-control analysis	New users of oral hypoglycemic drugs from the UK GPRD	115,923	Risk of lung cancer with metformin therapy	No significant change was observed in lung cancer cells.	No beneficial effect of metformin on risk of lung cancer; RR = 0.94.	Śmiechowski et al. 2013
Lung	Retrospective study	Patients with a recorded diagnosis of lung cancer from the UK GPRD	91,301	Risk of lung cancer with metformin treatment	Long-term metformin treatment had preventive effect on subsequent development of lung cancer cells in women only.	Chronic use of metformin was not associated with decreased risk of lung cancer.	Bodmer et al. 2012
Prostate	Meta-analysis	30 cohort studies	1,660,795	Overall survival (OS), PCa-specific survival, recurrence-free survival (RFS)	Further clinical studies are required to verify these findings.	Metformin treatment improves overall survival, and RFS in prostate cancer. Metformin did not decrease PCa-specific survival.	He et al., 2019
Bladder	Retrospective cohort study	Patients with at least one prescription of oral anti-diabetic agents and/or insulin	165,398	Urinary bladder cancer (UBC) risk	No significant change was observed in bladder cancer cells.	No association between metformin use and UBC risk (HR = 1.12) compared with SU only users.	Goossens et al., 2015
Bladder	Meta-analysis	9 retrospective cohort studies	1,270,179	Risk of bladder cancer with metformin treatment	Metformin intake reduced progression of muscle invasive bladder cancer for non-Asians.	Metformin treatment improves survival connected with bladder cancer. Metformin ameliorates RFS, progression-free survival (PFS), and cancer specific survival (CSS). Metformin had no ability to decreased the incidence of bladder cancer.	Hu et al., 2018

Bladder	Retrospective cohort study	Newly diagnosed Taiwanese patients with T2DM	940,708	Risk of bladder cancer with metformin treatment	Metformin revealed potential preventive effect on bladder cancer cells i.e. inhibition the growth and proliferation of cancer cells.	Use of metformin was associated with reduced bladder cancer risk.	Tseng, 2014
Bladder	Retrospective cohort study	Patients with T2DM treated with metformin or sulfonylureas	87,600	Risk of bladder cancer with metformin or SUs treatment.	No significant change was observed in bladder cancer cells.	No significant effect of metformin treatment on prevalence of bladder cancer.	Mamtani et al. 2014
Kidney	Meta-analysis	8 cohort studies and 1 population based study	7,426	Survival rate with kidney cancer after metformin treatment	Metformin treatment decreased progression of kidney cancer in 5 studies.	Metformin use had a protective ability on PFS, CSS and OS. Little evidence of a protective impact of metformin treatment on renal caner survival outcomes. None reached statistical significance.	Nayan et al. 2019
Kidney	Meta-analysis	8 cohorts studies	254,329	Survival rate with kidney cancer after metformin treatment	Reduced risk of cancer cells development in kidney in patients treated with metformin in comparison with non-treated patients.	Metformin treatment improved OS, CSS in patients with renal cell carcinoma (RCC). No beneficial effect of metformin use on DFS and PFS of kidney cancer.	Li et al. 2017
Kidney	Retrospective cohort study	Patients with T2DM and M0 renal cell carcinoma who undergoing nephrectomy.	158	Overall survival, connection between metformin treatment and disease–free.	No significant change was observed in kidney cancer cells.	No positive effect of metformin use on kidney cancer risk.	Nayan et al. 2017
Kidney	Cohort study	Taiwanese patients with type 2 diabetes	247,252	Risk of bladder cancer with metformin treatment	Further clinical studies are required to verify these findings.	Metformin treatment reduced the prevalence of kidney cancer in T2DM subjects in a dose–dependent manner.	Tseng, 2016

Liver	Meta-analysis	Randomized controlled trials, 6 retrospective cohort studies, and case–control studies	13,985	Overall survival (OS) after metformin treatment and other anti-diabetic drugs in hepatocellular carcinoma (HCC) patients with type 2 diabetes	Metformin use led to inhibition of hepatocellular carcinoma cells proliferation.	Beneficial effect of metformin on OS of patients with HCC and T2DM after healing therapy.	Zhou et al. 2020
Liver	Cohort study	Men at the age of 40-89 without renal, chronic liver, or cardiovascular diseases and cancer.	84 433	Risk of hepatocellular carcinoma with metformin treatment	Metformin intake revealed potential preventive effect on hepatocellular carcinoma cells in Hispanics and non‐Hispanic African American only. Thereby, metformin response is probably heritable or dependent on ethnicity disparity.	Metformin use reduced the incidence of HCC by ca. 51% and altered the race/ethnicity disparity.	Wang et al. 2019.
Liver	Meta-analysis	23 studies (observational studies and randomized controlled trials)	17,028,953	Risk of liver cancer with metformin treatment	Further clinical studies are required to verify these findings.	Protective association between metformin treatment and risk of liver cancer. In metformin treated group, the risk of liver cancer was reduced by 48%.	Ma et al. 2017
Liver	Random-effects meta-analysis model	5 studies (2 hospital-based case-control studies, 2 prospective cohort study and 1 retrospective cohort study)	105,495	Risk of liver cancer with metformin treatment	Metformin played a role in chemoprevention of growth of liver cancer cells.	Little evidence of a protective association between metformin use and incidence of liver cancer – reduction by ca. 62%. In four studies, metformin treatment reduced the risk of HCC by ca. 70%.	Zhang et al. 2012
Pancreas	Cohort study	Patients with pancreatic cancer–related diabetes (PCRD) and postpancreatitis diabetes	1,862	Risk of mortalitywith anti-diabetic drugs treatment in patients with PCRD and PPDM	Further clinical studies are required to verify these findings.	No association between metformin therapy and survival in PCRD patients. Beneficial effect on survival in PPDM patients.	Cho et al. 2019

		mellitus (PPDM)					
Pancreas	Meta-analysis	8 studies (randomized controlled trials, cohort studies, or case-control studies)	4,293	Overall survival (OS) of patients with pancreatic cancer with metformin treatment	In Asian patients, metformin treatment was associated with reduced risk of cancer cells development only to small extent.	Little evidence of a beneficial association between metformin therapy and OS. Metformin use caused increase of OS ca. 19%.	Xin et al. 2018
Pancreas	Meta-analysis	17 studies (cohort, observational and case–control)	36,791	Overall survival after metformin adjuvant treatment in patients with pancreatic cancer.	Metformin adjuvant treatment had preventive effect on growth of pancreatic cancer cells, especially in Asian patients at an early tumor stage (I-II).	Association between metformin use and prolonged survival in pancreatic cancer individuals.	Wan et al. 2018
Pancreas	Retrospective cohort study	Patients with T2DM and advanced pancreatic adenocarcinoma (PAC)	516	Survival rate with pancreatic cancer after metformin treatment	No significant change was observed in pancreatic cancer cells.	No clinically important association between metformin treatment and survival in patients with advanced PAC. However, metformin provided survival in PAC in non-metastatic disease.	Hwang et al. 2013

Table 2. *The prolongevity effects of metformin in animal studies.*

Animal model	Metformin dose	Major findings	Reference
129/Sv mice	100 mg/kg in drinking water	Long treatment with metformin increased female mice lives by ca. 5%. This effect has not been noticed among males. Metformin decreased the prevalence of malignant tumors in females.	Anisimov et al. 2010b
Wistar rats fed with high-fat diet	8 week treatment	Metformin acts neuroprotectively against the detrimental effects of Aβ and HFDs on hippocampal synaptic plasticity.	Asadbegi et al. 2016
129/Sv mice	100 mg/kg in s.c. injection at the 3rd, 5th and 7th days of life	Neonatal treatment with metformin increased male mice mean life span by ca. 20% and also extended maximum life span by ca. 3,5%. Nonetheless, such an effect has not been observed in females. Metformin reduced mean life span by ca. 9,1% and maximum life span by ca. 3,8%. Practically half (45%) of male of the control group and 71,8% of the researched group survived up to 800 days of age.	Anisimov et al. 2015
Kras G12D mice	5 mg/ml in the drinking water for 3 or 9 months	Three months treatment with metformin reduced risk of hepatic steatosis development and ability to weight gain in mouse population subjected to HFD. After 9 months treatment, metformin significantly decreased the incidence of pancreatic ductal adenocarcinoma.	Chang et al. 2018
Balb/c female mice	250 mg/kg in the drinking water	Metformin inducted apoptosis in A549 and PANC-1 cell xenografts and decreased growth of K-ras mutant tumors after 21 days of treatment.	Ma et al. 2013
C57BL/6J male mice	50 mg/kg in the drinking water	Metformin weakened enhanced activation of insulin receptor and significantly induced phosphorrylation of AMP kinase leading to considerable reduction of tumor size and growth in HFD mice.	Algire et al. 2008
Female Wistar rats	2 mg/mL in the drinking water	Metformin prevented postmenopausal breast cancer progression. After 8 weeks treatment, mean tumor burden was decreased by ca 86% in the metformin-treated rats.	Giles et al. 2018

Table 3. *The selected studies evaluating cytotoxic potential of metformin in various cell lines.*

Type of cells	IC_{50} value	Major outcome	Reference
The human endometrial carcinoma cell line Hec1A, Ishikawa; the mouse endometrial carcinoma cell line MecPK	IC_{50} = 5.03 mM for Hec1A IC_{50} = 141.12 mM for Ishikawa IC_{50} = 1.39 mM for MecPK	In all cell lines, metformin increased AMPK phosphorylation. Reduction of S6 ribosomal protein (S6rp) phosphorylation, ERK1/2 phosphorylation and increase of apoptosis in K-Ras mutated cells was only observed in Hec1A and MecPK cells. Reduction of AKT phosphorylation influenced PI3K pathway in both cell lines. Metformin demonstrated considerable cytotoxicity effectiveness against tumor with mutation in the K-Ras.	Iglesias et al. 2013
PC-3 (androgen independent phenotype) prostate cancer cells	IC_{50} = 5 mM	Metformin caused up-regulation of 10 miRNAs and down-regulation of 12 miRNAs.	Avci et al. 2013
The human breast cancer cell line MCF-7	IC_{50} = 10 mM	After metformin administration, mTOR phosphorylation was suppressed and led to enhance of AMPK phosphorylation. Metformin also showed the ability to decreased NF-κB level and weakened cyclin D1, IκBα and phospho-IκBα. Significant reduction of ER alpha action was already noticed at a concentration of 2 mM.	Scherbakov et al. 2016
Feline injection-site sarcoma (ISS) line (JB)	IC_{50} = 8 mM	Metformin induced cell death but this effect was not associated with inhibition of mTOR.	Pierro et al. 2017
The Human Glioblastoma Stem Cell GBM1, GBM2, GBM3, GBM4, GBM5, GBM6, GBM7	IC_{50} = 12.96 mM for GBM1 IC_{50} = 12.30 mM for GBM2, IC_{50} = 6.22 mM for GBM3, IC_{50} = 12.65 mM GBM4, IC_{50} = 2.10 mM for GBM5, IC_{50} = 9.12 mM for GBM6, IC_{50} = 6.65 mM for GBM7	Metformin had antiproliferative potency in glioblastoma stem cells (GSCs) by selective inhibition of Chloride Intracellular Channel 1 (CLIC1) - mediated ion.	Barbieri et al. 2018
The human rectal cancer cell lines SW837 and SW1463, the human colon carcinoma cell lines HCT116 and LS513	IC_{50} =1.02 mM for SW837 IC_{50} = 8.75 mM for SW1463, IC_{50} = 34.4 mM for HCT116, IC_{50} = 40 mM for LS513	Metformin showed the ability to regulate chemoresistance in rectal cancer cells by influening molecules acting as oncogenes: the drug stops signal transducer and activator of transcription (STAT3) phosphorylation and transforming growth factor, beta receptor II (TGFBR2)-mediated signalling. Metformin action has oriented	Park et al. 2019

		itself to TGF-β signaling, leading to inhibition the TGF-β1-induced epithelial-mesenchymal transition (EMT).	
The human primary breast cancer cells MBCDF-D5, MBCD3, MBCD23, MBCDF-B3, MBCD25, MBCD17, MBCDF, MBCD4	IC_{50} =44.70 mM for MBCDF-D5 IC_{50} =23.97 mM for MBCD3, IC_{50} =36.55 mM for MBCD23 IC_{50} =52.61 mM for MBCDF-B3, IC_{50} = 10.11 mM for BCD25, IC_{50} = 5.31 mM for MBCD17, IC_{50} = 11.45 mM for MBCDF, IC_{50} = 8.17 mM for MBCD4	After metformin administration, AMPK was activated in all cell lines and led to decrease of STAT3 phosphorylation. The ability of metformin to STAT3 and NF-κB inhibition caused decrease of IL-6-induced epithelial-mesenchymal transition (EMT) expression.	Esparza-López et al. 2019
The human multiple myeloma (MM) cell lines RPMI8226, ARP-1 and OPM2	IC_{50} = 10 mM	Metformin curbed PFKFB3 expression and has a synergistic effect with PFK15.	Liu et al. 2019
Acute Myeloid Leukemia (AML) cell lines HL-60 (AML M3 type) and THP-1 (AML M5 type)	For HL-60: $IC50_{24h}$ = 33.06 mM, $IC50_{48h}$ = 15.15 mM, $IC50_{72h}$ = 10.38 mM. For THP-1 $IC50_{24h}$ = 78.77 mM, $IC50_{48h}$ = 12 mM, $IC50_{72h}$ = 6.386 mM.	Metformin has a synergistic effect with cytarabine (Ara-C) through the suppression of mTORC1/P70S6K pathway. This combination of drugs caused cell cycle arrest (block of S phase).	Yuan et al. 2020