Repurposing Metformin – An old drug with new tricks in its binding pockets

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Abstract
Improvements in healthcare and nutrition have generated remarkable increases in life expectancy worldwide. This is one of the greatest achievements of the modern world yet it also presents a grave challenge: as more people survive into later life, more also experience the diseases of old age, including type 2 diabetes, cardiovascular disease and cancer. Developing new ways to improve health in the elderly is therefore a top priority for biomedical research. Whilst our understanding of the molecular basis of these morbidities has advanced rapidly, effective novel treatments are still lacking. Alternative drug development strategies are now being explored such as the repurposing of existing drugs used to treat other diseases. This can save a considerable amount of time and money since the pharmacokinetics, pharmacodynamics and safety profiles of these drugs are already established, effectively enabling preclinical studies to be bypassed. Metformin is one such drug currently being investigated for novel applications. This review provides a thorough and detailed account of our current understanding of the molecular pharmacology and signalling mechanisms underlying biguanide-protein interactions. It also focuses on the key role of the microbiota in regulating age-associated morbidities and a potential role for metformin to modulate its function. Research in this area holds the key to solving many of the mysteries of our current understanding of drug action and concerted effects to provide sustained and long-life health.

Introduction

The biguanide metformin is the most commonly prescribed drug for type 2 diabetes (T2D) taken by an estimated 150 million individuals worldwide. It is derived from the plant *Galega officinalis* (French lilac), which has a history as a herbal remedy that can be traced back to medieval Europe when it was used to relieve symptoms now attributed to diabetes [1]. In the late 1800s, *G. officinalis* was discovered to be rich in guanidine, a compound that was subsequently shown to have hypoglycaemic properties [2]. However, the toxicity of guanidine precluded its clinical use and attention shifted towards safer analogues. The biguanides, consisting of two N-linked guanidines, were synthesised in the 1920s but their therapeutic potential was initially overlooked due to the introduction of insulin therapy in the same decade. It was not until 1957 that metformin was recommended for the treatment of diabetes following the publication of a successful trial by the French physician Jean Sterne [1]. Unlike the pre-existing diabetes therapies, biguanides had the advantage of being able to reduce blood glucose levels without inducing hypoglycaemia. At first, the more potent biguanides phenformin and buformin received the greatest popularity, however increasing reports of associated lactic acidosis led to these drugs being withdrawn from the markets of most countries during the 1970s [3]. Due to its superior safety profile, metformin eventually became established as the first-line treatment for T2D and it is now featured on the World Health Organisation's list of essential medicines [4].

Interestingly, the therapeutic potential of metformin treatment extends far beyond its prescribed use as an anti-diabetic drug. There is a rapidly growing body of literature demonstrating an effective role for metformin in the treatment of multiple diseases including cancer and cardiovascular disease. Additionally there is evidence to suggest that metformin delays the ageing process and modulates the microbiota to promote health. How metformin is capable of achieving such pleiotropic actions is not yet fully understood. Here, we will review the molecular mechanisms proposed to explain these diverse effects.

Metformin and Type 2 Diabetes

According to the latest global estimates, there are 382 million people are currently living with diabetes [5]. Increasing urbanisation and the accompanying rise in obesity and sedentary behaviour mean that this figure is expected to reach 592 million by 2035, presenting national healthcare systems with an overwhelming challenge. T2D accounts for between 85-95% of diabetes cases and is characterised by hyperglycaemia resulting from insulin resistance or impaired insulin secretion. It is a disorder with a complex aetiology involving interactions between multiple genetic and environmental factors. Strong predictors include family history, increased body-mass index, high blood pressure, physical inactivity, poor diet and advancing age [6]. In the long-term, T2D can give rise to several disabling and life-threatening complications such as cardiovascular disease, neuropathy, retinopathy and nephropathy [7]. The onset of these complications can be prevented or significantly delayed by effective management of blood glucose levels, which is achieved through lifestyle modifications, and in many cases, the use of oral anti-hyperglycaemic agents such as metformin.

Despite being first introduced as a treatment for T2D in 1957, metformin's mechanism of action is not yet fully understood. The drug's anti-hyperglycaemic effect has been partly attributed to increased hepatic insulin sensitivity and elevated uptake of glucose in peripheral tissues, however it is now widely accepted that metformin acts predominantly via suppression of hepatic gluconeogenesis. It has been reported that metformin can lower the rate of gluconeogenesis by as much as 36% in diabetic patients [8]. Nevertheless, the molecular pathways responsible for this reduction of glucose production remain a subject of debate.

Molecular target of metformin

Due to its unusually hydrophilic nature, metformin is unable to passively diffuse through cell membranes and must rely on members of the organic cation transporter (OCT) family for uptake into hepatocytes. Specifically, OCT1 has been shown to be essential for the therapeutic efficacy of metformin, and it has been suggested that genetic polymorphisms in human *OCT1* may contribute to variation in clinical response to the drug [9]. Once it has entered the hepatocyte, metformin accumulates within the mitochondrial matrix. It is likely that this uptake results from the positively charged molecule being driven by the membrane potential of energised mitochondria [10]. Additionally, interactions may take place between metformin's apolar hydrocarbon side chain and the hydrophobic phospholipids of the mitochondrial membrane [11]. It is generally agreed that complex I of the mitochondrial respiratory chain is a key target of metformin. This stems from the work of two independent research groups who reported that metformin selectively inhibits the oxidation of complex I substrates but not complex II or IV substrates [10, 12]. Although these findings were first observed in isolated rat hepatocytes, similar results have since been obtained in numerous cell models including primary human hepatocytes [13].

It is not known exactly how metformin inhibits complex I, although it is possible that the metal-binding properties of the drug may be implicated. It has been shown that the elevated pH of the mitochondrial matrix converts metformin into a deprotonated form with a high affinity for copper ions [14]. These copper complexes have the potential to interfere with the sensitive redox reactions of the respiratory chain. This is consistent with the earlier finding that the cellular effects of metformin depend on its ability to bind copper [15]. An alternative mechanism to account for the inhibition of complex I by metformin has recently been described by Bridges *et al* [16]. They demonstrated that metformin inhibits ubiquinone reduction in a non-competitive manner, possibly by binding at the interface of the hydrophilic and membrane domains and trapping the enzyme in a deactive-like open-loop conformation. It is worth noting that although the inhibitory effect of metformin is largely confined to complex I, other biguanides have been found to inhibit several respiratory chain enzymes and therefore collectively this class of drugs is best considered non-specific [16, 17].

The consequence of complex I inhibition by metformin is a decline in ATP production accompanied by a concomitant increase in ADP and AMP levels. This altered cellular energy charge is detected by the cell's principle energy sensor, AMP-activated protein kinase (AMPK). Whether or not AMPK is a central mediator of metformin action is a controversial issue and both AMPK-dependent and independent models will be outlined here.

AMPK-dependent mechanism

AMPK is a master regulator of cellular energy homeostasis that is activated by the binding of an ADP or AMP molecule to a site on its regulatory γ subunit [18, 19]. This enables the cell to respond to falling energy status by transforming it from an ATP-consuming anabolic state into an ATP-producing catabolic state.

A key role for AMPK in the mechanism of metformin action was established in 2001 following the publication of an influential study by Zhou *et al.* [20]. They demonstrated that metformin stimulates AMPK activation in rat primary hepatocytes and used the AMPK inhibitor compound C to show that AMPK is required for the drug's inhibitory effect on glucose production [20], however it should be noted that this inhibitor was later found to be non-selective. Nevertheless, these initial findings were supported by a subsequent study by Shaw *et al.*, which found that loss of hepatic liver kinase B1 (LKB1), an upstream activator of AMPK responsible for phosphorylating its catalytic α subunit, abolished the glucose-lowering effects of metformin in mice fed a high-fat diet [21].

It was suggested that this LKB1/AMPK pathway, activated by metformin, alters the cell's gluconeogenic programme via inhibition of cAMP response element binding protein (CREB) regulated transcription coactivator 2 (CRTC2) – a pivotal regulator of gluconeogenic gene expression [21]. In its non-phosphorylated state, CRTC2 is located in the nucleus where it associates with CREB to upregulate the transcription of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and its downstream target genes phosphoenolpyruvate carboxykinase and glucose-6-phosphatase. However, AMPK can stimulate the phosphorylation of CRTC2 at Ser^{171} resulting in its nuclear exclusion [21]. Alternatively, the disabling of CRTC2 may be achieved via AMPK-mediated induction of nicotinamide phosphoribosyltransferase and the accompanying increase in hepatic sirtuin 1 (SIRT1). Deacetylation of CRTC2 by SIRT1 leaves it vulnerable to constitutive photomorphogenesis 1-mediated ubiquination and degradation, the consequence of which is inhibition of gluconeogenic gene expression [22]. It has also been shown that AMPK triggers the dissociation of the CREB-CREB binding protein (CBP)-CRTC2 transcription complex via phosphorylation of CBP at Ser^{436} [23].

Despite showing initial promise, AMPK's status as the major mediator of metformin's action was seriously undermined following the publication of work carried out by Foretz *et al.* in 2010. In this study, metformin was found to inhibit glucose production normally in transgenic mice lacking hepatic AMPK catalytic subunits or LKB1 [24]. It was suggested that the discrepancy between this result and the early findings of Shaw *et al.* was due to the fact that in the latter study, the direct effect of metformin on hepatic glucose output was not assessed. Instead they investigated the effect of repeated metformin administration on fasting blood glucose levels. Therefore, these results may actually reflect an indirect effect of the AMPK/LKB1 axis on hepatic glucose production, potentially caused by AMPK's suppression of lipogenesis [24]. A well-known target of AMPK is acetyl-CoA carboxylase (ACC); a rate-limiting enzyme required for the generation of malonyl-CoA, which is both a precursor of lipogenesis and an inhibitor of β-oxidation. The inhibition of ACC by AMPK has been shown to regulate metformin-induced improvements in insulin action in mice [25]. Furthermore, AMPK can down-regulate the expression of multiple lipogenic genes by inhibiting the transcriptional activity of sterol regulatory element-binding protein 1 (SREBP-1) [20] and carbohydrate-responsive element-binding protein (ChREBP) [26]. Therefore, even if AMPK is dispensable for the glucose-lowering action of metformin, it could contribute to the therapeutic effects of the drug in the longer term by inducing favourable modifications of lipid metabolism that help to increase insulin sensitivity.

The prevailing hypothesis is that metformin activates AMPK by increasing ADP/AMP through inhibition of mitochondrial respiration, however, alternative models have been proposed. For example, it has been argued that the metabolic alterations induced by metformin in isolated skeletal muscle cells are not consistent with the interruption of mitochondrial energy supply; instead they better reflect direct inhibition of the enzyme AMP deaminase [27]. This too would result in increased AMP levels and activation of AMPK. However the results of this study have been called into question due to the very high concentrations of metformin used [19]. It has also been claimed that AMPK itself is a direct target of metformin. Specifically, low metformin concentrations can promote the formation of the AMPK αβγ heterotrimeric complex in hepatocytes and when the enzyme is assembled *in vitro*, leading to increased phosphorylation of the catalytic α subunit, although potential metformin binding sites have not been identified [28]. However, this finding contradicts those obtained in previous studies that employed cell-free assays to demonstrate that metformin is not a direct allosteric activator of AMPK [20, 29]. Thus, inhibition of the respiratory chain and the accompanying elevation in AMP levels remains the most plausible mechanism to explain the activation of AMPK by metformin.

AMPK-independent mechanisms

With doubts being cast on AMPK's status as the central mediator of metformin action, several AMPK-independent mechanisms have been proposed. One alternative explanation is that the associated change in cellular energy charge directly modulates glucose output. Gluconeogenesis is energetically demanding process requiring 6 ATP equivalents for every molecule of glucose synthesised [30]. Since metformin treatment causes ATP levels to fall, hepatocytes must respond by reducing glucose production accordingly. Indeed, Foretz *et al.* demonstrated that reduction of ATP content and inhibition of glucose production were strongly correlated in mouse primary hepatocytes incubated with metformin, underscoring the close link between hepatic energy status and glucose output [24]. Additionally, metformin-induced changes in cellular energy status may suppress gluconeogenesis via the allosteric inhibition of essential enzymes. For example, AMP is capable of synergising with fructose 2,6-bisphosphate to inhibit the key gluconeogenic enzyme fructose 1,6-bisphosphatase [31].

The notion that the molecular mechanism of metformin is independent of transcriptional alterations is supported by gene expression studies. Forced expression of PGC-1α, a master co-activator of gluconeogenic genes, does not negate the metformin-induced suppression of glucose production in primary hepatocytes [24]. Moreover, additional studies have found a lack of correlation between gluconeogenic gene expression and hepatic glucose output both in mouse models [32] and in patients with T2D [33]. Taken together, these findings imply that inhibition of gluconeogenic gene expression is not the central determinant of metformin's clinical effects; instead the changes in cellular energy charge and the associated reduction in gluconeogenic flux may be responsible.

An alternative AMPK-independent mechanism of metformin action has recently been put forward by Miller *et al.* that involves antagonism of glucagon signalling. The results of a series of *in vivo* and *in vitro* experiments on mouse primary hepatocytes have shown that metformin and the related biguanide phenformin block the glucagon-induced activation of adenylate cyclase leading to a reduction in cAMP synthesis [34]. This in turn lowers protein kinase A (PKA) activity, abrogating phosphorylation of critical substrates that enhance gluconeogenesis such as 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1, CREB-1 and inositol trisphosphate receptor. It has been proposed that the metformin-associated rise in cellular AMP levels is responsible for the inhibition of adenylate cyclase, possibly due to the direct binding AMP's adenine moiety to an inhibitory 'P-site' [34].

While this finding is undoubtedly significant, an important caveat has been raised. If antagonism of glucagon signalling was the major mechanism of metformin action, it would be reasonable to assume that the use of this drug may lead to hypoglycaemic episodes as seen in glucagon receptor knock-out mice [35]. However, one of the main advantages of metformin treatment is that hypoglycaemia is rare. This implies that either the suppression of glucagon signalling is incomplete in humans or that compensatory mechanisms are at work [36].

It should be noted that the studies on which these AMPK-independent models are based have received criticism for using concentrations of metformin that are considered higher than the maximally achievable therapeutic concentrations found in diabetic patients [37]. Metformin is administered orally and has a maximum recommended dose of 2.5 g per day in humans. It has been demonstrated that the peak plasma concentration reaches approximately 3 μg/ml (equivalent to 18 μM) in individuals who have taken a single dose of 1.5 g [38]. However, evidence from animal studies show that the concentration of metformin in the hepatic portal vein is considerably higher than that of the systemic plasma [39]. Cao *et al.* tested the effect of metformin at low concentrations (≤ 80 μM) in mouse primary hepatocytes. Under these conditions, metformin suppressed gluconeogenic gene expression and hepatic glucose production via AMPK, yet no effect was observed on ATP levels or the AMP/ATP ratio

implying that complex I is not inhibited [40]. However, it is unclear how metformin could activate AMPK without inducing a change in cellular energy charge. Furthermore, it has been shown previously that even if changes in the AMP/ATP ratio are undetectable, the activation of AMPK by metformin is still dependent on AMP binding [41].

Although both Foretz *et al.* and Miller *et al.* have used high concentrations to provide evidence for an AMPK-independent mechanism of metformin action, it does not necessarily mean that their findings should be dismissed as clinically irrelevant. For *in vivo* experiments involving rodents it is common to administer metformin at daily doses between 250 – 350 mg/kg. These values are obtained using a well-established method for converting doses between species based on normalisation to body surface area [42]. According to this formula, a standard therapeutic dose of 20 mg/kg in an adult human (60 kg) is approximately equivalent to a 250 mg/kg dose in a mouse. Moreover, it is known that relatively high concentrations are required in order for the drug to exert its therapeutic effect in diabetic rodents [24, 43]. Additionally, metformin has been shown to accumulate at very high concentrations in several tissues. In mice, a dose of 50 mg/kg can lead to concentrations greater than 250 μM in the liver and those within the millimolar range in the small intestine [39]. Thus the use of concentrations that exceed those typically found in the plasma or portal vein can be justifiable for *in vitro* studies involving primary hepatocytes. Furthermore, it is known that the biological effects of metformin are time-dependent as well as concentration-dependent [20]; a property that likely reflects its capacity to accumulate within the mitochondrial matrix. Therefore it is possible that effects observed at high concentrations may also occur at lower concentrations following more long-term exposure to metformin.

It is also worth mentioning that studies that have used hepatic AMPK knockout models to support an AMPK-independent mechanism of metformin action should be reviewed in light of new evidence that demonstrates the importance of intestinal AMPK in mediating metformin action. Although the liver is considered to be the main site of metformin action, it has recently been demonstrated that activation of intestinal AMPK contributes to the acute glucose-lowering effects of metformin. A study performed on insulin-resistant rats has uncovered the involvement of a gut-brain-liver axis whereby metformin triggers a duodenal AMPK-glucagon-like peptide-1 receptor-Pka-dependent neuronal signalling pathway to reduce hepatic glucose production [44]. This study alludes to the possibility that the activation of AMPK in organs usually under the presence of elevated concentrations of the drug (in the milimolar range) can regulate from a distance the metabolism of tissues directly responsible for glucose production..

A novel mechanism of metformin action has been recently revealed that is independent of both AMPK activation and cellular energy charge. Specifically, metformin has been found to inhibit the glycerophosphate shuttle enzyme mitochondrial glycerophosphate dehydrogenase (mGPD). This prevents glycerol from being directly used as a gluconeogenic substrate. It also leads to an increase in the cytosolic redox state which makes the conversion of lactate to pyruvate unfavourable and thus limits the contribution of lactate to gluconeogenic flux [45]. In addition to revealing a new therapeutic target, this study helps to further underscore metformin's status as a non-specific drug that is capable of interfering with multiple molecular targets to bring about the disruption of gluconeogenesis.

Metformin and Cardiovascular Disease

Cardiovascular disease (CVD) encompasses diseases of the heart and circulation. According to the British Heart Foundation, CVD accounts for more than a quarter of all deaths in the UK with an estimated cost per year of £19 billion. The greatest risk factor of CVD is T2D. There is evidence that metformin can offer protection against CVD in diabetic patients. This is especially pertinent given that CVD is the leading cause of

mortality within this population [46]. In the landmark UK Prospective Diabetes Study (UKPDS), it was reported that metformin treatment lowered the risk of myocardial infarction by 39% when compared to traditional treatments over a period of ten years [47]. Subsequent trials have supported a beneficial role for metformin in protecting against the cardiovascular complications of diabetes, including a ten-year follow-up of the original UKPDS trial [48-50]. Whilst improvements in glucose and lipid metabolism may contribute to some of the positive effects conferred by metformin, numerous alternative mechanisms invoking direct action of the drug on the cardiovascular system have also been discovered.

The results of multiple studies suggest that metformin can protect against atherosclerosis by promoting endothelial integrity and preventing the formation of plaques. For example, it has been reported that the activation of AMPK by metformin limits endothelial cell damage caused by oxidative stress under hyperglycaemic conditions. This occurs via inhibition of the protein kinase C-NAD(P)H oxidase pathway [51]. The resulting reduction in cytosolic reactive oxygen species (ROS) generation halts the initiation of a positive feedback loop involving mitochondrial ROS generation and mitochondrial fission, which in turn prevents the triggering of endothelial apoptosis by the resulting loss of mitochondrial membrane potential [52].

Metformin has also been observed to exhibit anti-thrombotic properties in insulin-resistant models. Specifically, metformin counteracts the stimulatory effect of hyperinsulinaemia on the production of plasminogen activator inhibitor 1 (PAI-1), a negative regulator of fibrinolysis implicated in blood clot formation [53]. It is believed that that this is not mediated by the improvement in insulin sensitivity associated with metformin but rather by the direct inhibition of PAI-1 gene expression [54]. Moreover, metformin treatment has been observed to significantly reduce platelet aggregation in patients with insulin-dependent diabetes [55]. This finding may be explained by the results of a subsequent study in which it was discovered that metformin can enhance Ser^{1179} phosphorylation of endothelial nitric oxide synthase in an AMPK-dependent manner leading to an increase in nitric oxide (NO) bioactivity in the aortas of C57BL/6 mice [56]. NO plays a crucial role in maintaining vascular homeostasis by carrying out multiple functions, including inhibition of platelet aggregation, and therefore helps to protect against the development of coronary artery disease.

In addition to its endothelial actions, metformin affects cardiomyocyte function and has been associated with improvements in diabetic cardiomyopathy in rodent models. This distinct condition is a major cause of heart failure and is characterised by ventricular dysfunction independent of coronary artery disease and hypertension [57]. It was previously suggested that metformin may correct abnormalities in myocyte relaxation via tyrosine kinase-dependent alterations in calcium handling [58]. More recent studies have linked improvements in diabetic cardiomyopathy to AMPK-mediated upregulation of autophagy [59, 60]. Cardiac autophagy is an important homeostatic mechanism known to be supressed in diabetic cardiomyopathy. It has been demonstrated that metformin-activated AMPK protects cardiomyocytes by disrupting the Beclin1-Bcl-2 (B-cell lymphoma 2) complex and inducing a switch from apoptotic to autophagic machinery. Importantly, this restoration of autophagy improves cardiac structure and function in diabetic mice [59].

There is also evidence to suggest that metformin exerts a cardioprotective effect against ischemia reperfusion injury following myocardial infarction. Administration of metformin during the first 15 minutes of reperfusion has been shown to reduce myocardial infarct size in hearts isolated from both diabetic and non-diabetic rats [61]. This was attributed to the activation of the protein kinase B (Akt)/ phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signalling pathway by metformin, which prevents the opening of the mitochondrial permeability transition pore (mPTP) – a critical trigger of cell death during reperfusion. It has since been shown that AMPK activation also inhibits mPTP opening at reperfusion [62]. Moreover, an AMPK-mediated increase in eNOS

activity has also been proposed to explain the positive effect of acute metformin treatment on infarct size [63]. It is interesting to note that a new small-molecule AMPK activator (A-76922) has been shown to act synergistically with metformin to enhance the cardioprotective effects of AMPK activation and therefore may have useful clinical applications during an ischemia reperfusion episode [64].

The effect of chronic metformin treatment on ischemia reperfusion injury has also been investigated, although to a lesser extent. One study has shown that administration of metformin over a 4-week period reduced infarct size in isolated rat hearts regardless of diabetes status [65]. The cardioprotection observed in diabetic hearts was associated with improved mitochondrial organisation, possibly related to increased AMPK activation and PGC-1α expression. Furthermore, long-term metformin treatment post-myocardial infarction may also have a positive impact. It has been demonstrated that rats treated with metformin for 12 weeks following myocardial infarction exhibited enhanced AMPK activation and improved cardiac remodelling, suggesting that metformin could help attenuate the development of heart failure in such a scenario [66]. Ultimately, the evidence suggests that metformin exerts varied positive effects on the cardiovascular system, particularly in diabetic models. Several randomised control trials in non-diabetic individuals are currently underway and data from these will help to establish whether or not metformin can improve cardiovascular outcomes in a wider section of society.

Metformin and Cancer

Cancer is a leading cause of morbidity and mortality worldwide. A recent lifetime risk analysis of the British population found that over 50% of adults below the age of 65 will be diagnosed with the disease at some point in their life [67]. Sobering projections like this have stimulated an enormous amount of investment into cancer research and rational drug design. Despite our best efforts, only 5% of oncology drugs entering phase I trials eventually receive approval and it has been argued that so far, targeted therapies have provided only modest survival benefits [68]. Metformin garnered considerable interest within the field of oncology in 2005 following the publication of an epidemiological report highlighting a link between metformin treatment and reduced cancer risk in diabetic patients [69]. This finding has stimulated a great deal of further research, and numerous observational studies have supported a protective role for metformin against a variety of cancer types including liver, colorectal, pancreas, stomach and oesophagus cancer in diabetics [70]. However, it is important to note that such studies are prone to bias and confounding factors and contrastingly, meta-analyses of randomised controlled trials do not appear to demonstrate a significant effect of metformin on cancer outcomes [70-72]. Furthermore, it is not clear whether or not any positive results could be extrapolated to non-diabetic individuals. Nevertheless, increasing importance is being placed on the role of altered metabolism in cancer and the ability of metformin to interact with several metabolic pathways suggests that it could be effective at preventing the development and progression of this disease.

It is possible that the systemic effects of metformin could be protective against cancer. Both experimental and epidemiological evidence suggests that insulin and insulin-like growth factor 1 (IGF-1) can promote tumourigenesis by stimulating the proliferation of epithelial cells [73]. Metformin may prevent such neoplastic activity by reducing hyperinsulinaemia and lowering levels of these signalling molecules. Metformin can also modify inflammatory processes known to play a role in cancer progression. For example, it has been reported that metformin blocks the activity of the transcription factor nuclear factor-κB (NF-κB) resulting in decreased secretion of pro-inflammatory cytokines by senescent cells [74]. This mechanism may also contribute to the anti-ageing properties of the drug as outlined below. Additionally, metformin has been found to enhance the immune response to cancer cells. A recent study performed

in a mouse model demonstrated that metformin protects CD8+ tumour-infiltrating lymphocytes from apoptosis and functional exhaustion [75]. Encouragingly, it has also been shown that metformin was able to improve the efficacy of an experimental anti-cancer vaccine by promoting the survival of memory T cells [76].

There are several lines of evidence that suggest metformin can exert cancer preventative effects in a cell-autonomous manner. The ability of metformin to inhibit complex I and disrupt oxidative phosphorylation has recently been highlighted as an important requirement for tumourigenesis inhibition. Metabolomic analysis of a breast epithelial cell line undergoing neoplastic transformation has revealed that metformin decreases tricarboxylic acid cycle intermediates which implies impaired complex I activity and therefore reinforces the notion that this enzyme is a key target [77]. Crucially, it has been demonstrated that the anti-tumorigenic effect of both metformin and phenformin is reversed in cancer cells that are forced to express yeast NADH dehydrogenase NDI1 which enables complex I function to be bypassed [78, 79].

AMPK-dependent mechanism

The activation of the LKB1/AMPK pathway may also significantly contribute to the anti-cancer effects of metformin. The AMPK up-stream activator LKB1 is a known tumour suppressor and mutations in this gene are associated with Peutz-Jeghers syndrome, an inherited cancer-predisposing disorder, as well as several sporadic cancers [80]. Additionally, it has been demonstrated that the protective effect of metformin is attenuated by pharmacological inhibition of AMPK and in AMPK-knockdown models [81-83]. A major consequence of AMPK activation is inhibition of mammalian target of rapamycin (mTOR) signalling, a nutrient-sensitive regulator of protein synthesis, cell growth and proliferation. This is achieved via directly phosphorylation of Ser^{1345} and Thr^{1227} on the tumour suppressor protein tuberous sclerosis 2 (TSC2), which forms an mTOR Complex 1 (mTORC1)-inhibitory complex with TSC1[84]. Alternatively, AMPK can prevent mTORC1 activation by phosphorylating its binding partner Raptor [85].

AMPK activation has been associated with suppression of the Warburg effect – a metabolic phenotype adopted by cancer cells characterised by a preference for aerobic glycolysis over oxidative phosphorylation [86]. This transformation is facilitated by hypoxia-inducible factor 1α, a transcription factor that upregulates glycolytic gene expression partly in response to mTORC1 signalling. A related classic metabolomic hallmark of cancer is increased *de novo* fatty acid synthesis and elevated levels of the key lipogenic enzyme fatty acid synthase (FAS) has been described in a wide variety of human cancers [87]. It has been demonstrated that the activation of AMPK reduced FAS expression in prostate cancer cells and was able to diminish their viability [88]. Similarly, experimental work conducted in a mouse model of colon carcinoma has shown that metformin down regulates the expression of FAS and can counteract the stimulatory effect of a high-energy diet on tumour growth [89]. These findings suggest that metformin may exert its anti-cancer effects by inducing alterations in glucose and lipid metabolism that ultimately deprive cancer cells of vital growth substrates.

Recent reports reveal that metformin can act as an anti-folate drug [90, 91] and show that metformin impairs the folate cycle similarly to the action of the antifolate class of chemotherapy drugs. The folate or one-carbon cycle is a major regulator of cell metabolism and an integrator of nutrient status [92]. It assimilates inputs in the form of glucose and amino acids that are processed through chemical reactions and transformed for diverse biological functions. These include cellular biosynthesis, regulation of redox status, regulations of epigenetics through nucleic acid and protein methylation, and genome maintenance through the regulation of nucleotide pools. For example, the folate cycle regulates *de novo* synthesis of nucleotides required for DNA repair and replication and the production of the universal methyl donor *S*-adenosylmethionine, involved in a number of methylation reactions such as DNA methylation [93]. Chemotherapy drugs such as methotrexate, aminopterine and azaserine block folate metabolism and impair

de novo synthesis of both thymidylate and purine nucleotides. We have demonstrated that metformin disrupts folate and methionine metabolism in *E. coli* [90]. Similarly, metformin treatment impairs folate metabolism in several breast cancer cells with concomitant reduction in glutathione and tryptophan metabolites [91]. Thymidine serves as a thymidylate precursor in the pyrimidine salvage pathway and hypoxanthine is an inosine monophosphate precursor in the purine salvage pathway in *de novo* synthesis. In fact, hypoxanthine and thymidine supplementation rescues the cytotoxic effects of metformin on cancer cells independently of LKB1 but requiring the AMPK kinase ataxia telangiectasia mutated kinase (ATM)/APMK axis [91]. In a study by Janzer *et al*., metformin induced a cancer stem cell-specific depletion of nucleotide triphosphates [77].

Aside from these metabolic modifications, there is evidence that the activation of AMPK by metformin may help to regulate the cell cycle via interactions with classical oncogenes and tumour suppressors. For example, it has been demonstrated that metformin downregulates c-MYC in an AMPK-dependent manner in breast cancer cell lines [94]. This is mediated by increased expression of miR-33a, a microRNA that binds to the 3'-UTR region of c-MYC, which in turn results from the upregulation of the RNAse III enzyme Dicer in response to metformin treatment. The modulation of Dicer by metformin is especially relevant given that low levels of this enzyme have been associated with poor prognosis in ovarian, breast and lung cancer [95-97]. AMPK has also been shown to target p53. One study reported that AMPK initiates cell-cycle arrest by phosphorylating Ser^{15} of p53 [98], although it has been argued that this event is not sufficient for p53 activation. Findings from a subsequent study have revealed that AMPK phosphorylates the p53 regulator murine double minute X (MDMX) at Ser^{342} leading to its increased association with 14-3-3 proteins. This suppresses p53 ubiquitylation and enhances p53 stability and activation [99].

Whilst it is apparent that AMPK activation may protect against cancer via multiple mechanisms, it is also understood that during later stages of development the activation of AMPK may actually promote the survival of tumour cells. This is because AMPK enables the cells to adapt to metabolic stress imposed by the solid tumour microenvironment. Support for this hypothesis comes from a study demonstrating that the restoration of activated AMPK in LKB1-deficient lung adenocarcinoma cells conferred protection against glucose-starvation-induced cell death [100]. This suggests that the use of AMPK activators such as metformin may be harmful in some situations. Conversely, metformin and other compounds capable of inducing metabolic stress may be particularly effective against tumours lacking a functional LKB1-AMPK axis. Indeed, it has been shown that in a transgenic mouse model of lung cancer, phenformin selectively induces apoptosis in cells bearing LKB1 mutations [101]. This reflects the inability of these cells to respond appropriately to the decline in ATP production associated with complex I inhibition.

AMPK-independent mechanism
AMPK-independent mechanisms to explain the anti-cancer properties of metformin have also been described. For instance, metformin can protect against DNA damage and mutation by inhibiting ROS generation by complex I [102]. Additionally, metformin can activate mTORC1 in the absence of AMPK in a comparable way to amino acid withdrawal. It achieves this by inhibiting Rag GTPases, which are responsible for inducing the translocation of mTORC1 to cellular compartments containing the mTORC1 activator, Ras homolog enriched in brain (Rheb) [103]. There is also evidence to show that the antiproliferative effect of metformin in a prostate cancer cell line is associated with AMPK-independent inhibition of the critical cell cycle regulator Cyclin D1 [104]. It is thought that this is mediated by a p53-dependent upregulation of REDD1 [105]. Furthermore, AMPK activation may be dispensable for the triggering of cell death pathways induced by metformin treatment. A study conducted on oesophageal

squamous cell carcinoma cells showed that metformin upregulates apoptosis and autophagy resulting in reduced tumour growth [106]. This was attributed to inactivation of the Stat3/Bcl2 pathway, which was only marginally reverted by AMPK knock-down suggesting a limited contribution. Finally, it has been demonstrated that metformin impairs glucose uptake in a lung cancer cell model via direct allosteric inhibition of hexokinase-II [107]. Deprivation of this energy source leads to mitochondrial depolarisation and the subsequent triggering of apoptosis. Similar findings have also been observed in a breast cancer cell line [108].

Anti-cancer polypharmacy therapy with metformin

Although metformin has shown considerable promise as a stand-alone cancer treatment, the effect of metformin in combination with conventional cytotoxic therapies has also been investigated. There is evidence that metformin improves the response of cancer cells to radiotherapy [109]. Metformin has also been found to enhance the sensitivity of multiple cancer cell types to common chemotherapeutic agents including cisplatin, paclitaxel, carboplatin, and doxorubicin [110-112]. However it should be noted that metformin antagonises the cytotoxic effect of cisplatin in glioma, neuroblastoma, fibrosarcoma and leukemia cell lines, reportedly via AMPK-independent upregulation of the Akt survival pathway [113]. Metformin may be particularly effective when used in combination with small molecule kinase inhibitors that suppress glycolysis. In melanoma cells it has been demonstrated that the efficacy of BRAF protein kinase inhibitors is limited by the induction of compensatory oxidative phosphorylation [114]. Accordingly, several inhibitors of oxidative phosphorylation were found to enhance the efficacy of these drugs. Whilst many inhibitors of oxidative phosphorylation are known to be toxic, the favourable safety profile of metformin implies that it may have therapeutic potential here [115].

Metformin and Ageing

The biological basis underlying the ageing process is a central mystery of science, and its resolution is critical for developing means to reduce the harmful outcomes of ageing. Recent years have seen unprecedented progress towards this goal. Genetic, dietary and pharmacological interventions can improve health and increase lifespan in laboratory model organisms: the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the mouse *Mus musculus* [116, 117]. These interventions ameliorate the ageing process and extend healthspan by protecting against age-related pathology and loss of function. These studies provide a key proof-of-principle: that the ageing process can be targeted to achieve wide-ranging protection against ageing-associated physiological decline [116, 117]. Controlled reduction of food intake without malnutrition (dietary restriction, DR) has long been known to increase healthspan in diverse animals [118-120], and drugs that mimic DR to achieve its benefits in humans [121]. In this section we will provide an account for the role of biguanides in ageing as potential DR mimetics.

Biguanides and the longevity of C. elegans

Several studies have been performed in *C. elegans* that support the life and healthspan extending properties of biguanides. An early report shows that administration of buformin at 0.1 mg/ml throughout life (including the larval period) can extend adult mean and maximal lifespan up to 23 and 26%, respectively [122]. Similarly, administration of metformin throughout life at a 50 mM dose can also extend median lifespan by 40% without changes in maximum lifespan [123]. Interestingly, lower and higher doses of metformin (10 and 100 mM, respectively) do not have lifespan extending properties. Recently, two studies showed that effects of 50 mM metformin are independent of its administration during early developmental time, as positive effects

on lifespan are still observed when the drug is administered at a later stage in life [90, 124]. Similarly, phenformin over a range of concentrations such as 1.5, 3 and 4.5 mM can extend adult worm mean lifespan by 5, 21 and 26%, respectively. So how do biguanides extend lifespan in *C. elegans*? Metformin has an intricate and concerted mode-of-action, which potentially accounts for the wide-ranging effects of the drug on host physiology. We have shown that changes in bacterial folate and methione metabolism induced by metformin are responsible for the life-extending properties of the drug [90]. In parallel, other studies [123, 124] as well as ours [90]suggest that metformin acts directly as a metabolic stressor, potentially through inhibition of complex I of the respiratory chain, to activate an oxidative stress and detoxification response involving the metabolic mediators, AMPK and SKN-1 (orthologue of mammalian nuclear factor (erythroid-derived 2)-like 2 or NRF-2). In support of this view, administration of antioxidants such as N-acetyl cysteine, that abrogate ROS signalling, or mutations in AMPK and SKN-1, impair the development of an adequate detoxifying response and abolish the DR-like state induced by the effects of metformin on bacterial metabolism.

Metformin equally displays healthspan properties. Metformin reduces the accumulation of the molecular damage pigment lipofuscin, promotes youthful mobility into late life, decreases fat accumulation [123], attenuates the morphological decline observed in ageing worms [124] and increase survival rate when exposed to long-term anoxia [125].

Biguanides and longevity in insects
While the lifespan extending properties have been well documented for *C. elegans*, the same does not apply to *insects.* Administration of 1, 2.5 and 5 mM metformin has no effect on lifespan on males or females *Drosophila melanogaster* [126]. Intriguingly, higher doses of metformin shortens the lifespan of males (100 mM) and females (25, 50 and 100 mM), despite robust activation of AMPK and reduced lipid stores [126]. This is a striking finding as increased levels of expression and activation of AMPK have been shown to increase lifespan in *C. elegans* [127] and *Drosophila* [128]. A well-described feature of pre-diabetes in human patients is infection-induced hyperglycaemia [129]. In a fly model of obesity, metformin administration at 10 mM reduced the systemic infection burden of the fungi *Rhizopus*, a causing agent of mucormycosis observed in diabetic patients. It led to reduced weight gain, normalised glucose levels due to infection and increased survival rates [130]. Finally, 5 mM metformin had a beneficial effect on age-related changes in fly intestinal stem cells obtained from the midgut as measured by a decrease in the molecular DNA damage markers gH2AX foci and 8-oxodG [131]. Recently, a study performed in the cricket *Acheta domesticus* [132], shows that metformin leads to an extension in mean and maximal lifespan of organisms of both sexes, with increases in 43.7% and 23.2 % in females and males, respectively.

Biguanides and longevity in rodents
Several studies have been performed in rodents suggesting an evolutionarily conserved pro-longevity role for biguanides. Work by Anisimov *et al*. has greatly contributed to such advancement (reviewed in [133]). Overall, metformin treatment of HER-2/neu, SHR, 129/Sv, C57BL/6 and B6C3F1 mice, over a wide-range of doses led to increases in lifespan ranging from 4 to 38%. However, data suggest that effects of metformin on lifespan are dependent on genotype, gender, biguanide type, dose and duration of the treatment. In general, the later the starting of the treatment the less lifespan benefits are observed. This is to some extent similar to the results obtained with metformin treatment on *C. elegans* survival [90, 123, 124]. For example, treatment of SHR mice at 15 months of age with metformin conferred no longevity benefits in comparison to starting of the treatment at 3 months of age (0 vs 38%, respectively) [133]. Likewise, dose plays an important role in lifespan extension in rodents as observed for *C. elegans*.

Administration of 0.1% of metformin in the diet leads to a lifespan extension of 5.83% compared to -14.4% when 1% of metformin is given to C57BL/6 mice. Reduction in lifespan was caused by drug nephrotoxicity [134]. Gender also influences the effect of metformin on lifespan as biguanide treatment extended the lifespan of 129/Sv female (5%) but not male mice (-13%) [133]. Phenformin administration to C3H/Sn mice also led to a lifespan extension of 21%. Interestingly, the effects of biguanides on the lifespan of rats are not as pronounced as the ones observed for mice. Only buformin and not metformin or phenformin extended the lifespan of rats. Given the wide-ranging effects of metformin in host physiology, which includes alteration of body weight, temperature, normalization of serum glucose, insulin, triglycerides and cholesterol levels, regulation of oestrous function and anti-neoplastic effects, confounders are likely to occur and influence lifespan trials with biguanides and therefore need careful consideration. In a recent study by Martin-Montalvo *et al.*, the calorie restriction mimicking effects of metformin could be the underlying mechanism responsible for the effects the drug on lifespan and healthspan [134]. These include improved fitness, increased insulin sensitivity, and reduced low-density lipoprotein and cholesterol levels without a decrease in caloric intake. At the molecular level, metformin alters gene expression similarly to those observed on calorie restriction. Interestingly, metformin increases AMPK activity without concomitant activity changes in the respiratory chain. It activates an antioxidant and anti-inflammatory response leading to reductions in both oxidative damage accumulation and chronic inflammation.

Altogether, the collected data existing from several model organisms show a promising outlook for the potential benefits of using metformin as an anti-ageing strategy. However, further investigations are required for its use in humans as its definite anti-ageing mechanism is still not fully disclosed.

Metformin and longevity in humans

To date no long-term longitudinal survival/mortality studies have been performed in healthy patients with metformin subjected to metformin therapy. A 10-year long randomised clinical trial of metformin therapy in T2D overweight/obese patients (UK Prospective Diabetes Study – UKPDS-O) shows long-term beneficial effects on health and survival [50], reducing cardiac and all-cause mortality of patients on metformin versus usual care. Indeed, the benefits of metformin on host metabolism have been observed even after cessation of treatment, the so-called "legacy effect". However, the molecular mechanisms by which metformin promotes these long-term positive effects on mortality [50], independent of glycaemic control, remain unknown. Interestingly, another long-term UK Prospective Diabetes Study – UKPDS-S (6.6 years), of metformin plus sulfonylurea vs sulfonylurea in a mixed group of non-overweight and overweight/obese T2D patients showed possible harmful effects of metformin (when combined with sulfonylurea) [47]. In a more recent meta-analysis of randomised clinical trials of metformin therapy in individuals with and without diabetes, which include the two UKPDS studies, Stevens *et al.*, showed no effects on all-cause mortality [71]. Overall, based on the information learned from lower model organisms and reviewed here, in order to ascertain the long-term beneficial effects of metformin on human longevity new studies targeting healthy younger patients are warranted.

Metformin and the Microbiota

Living organisms rarely exist in isolation but rather in intimate association with other species, in particular microorganisms [135]. The intestine is home to the majority of the microbes that inhabit the human body and these vast populations of microorganisms collectively designated as the "forgotten organ", the microbiota, is involved in many metabolic processes with important consequences for host physiology and the modulation of metabolic phenotypes [136]. Recent reports provide a link between

alterations to the microbiota in the gastrointestinal tract (caused by changes in diet and environmental conditions such as antibiotics) and nutrition-related syndromes such as obesity, T2D, metabolic syndrome, cancer and ageing [137, 138], suggesting that the gut microbiota could act as a potential biomarker for host health. Therefore, host-targeted drugs, such as metformin, commonly used to treat metabolic diseases associated with microbiota dysfunction may act on the physiology of the bacterial populations to exert their effects on the host. Does the gut microbiota influence the therapeutic potential of metformin and/or explain its side-effects in mammals? To what extent microbiota mediate the effects of metformin on metabolic disease remains an open question. One possibility is that metformin alters the structure and/or function of the microbiota in a way that promotes metabolic health. An increasing number of recent reports support this interpretation and will be discussed in this section.

Metformin alters microbial metabolism and regulates C. elegans physiology

The nematode *C. elegans* establishes a beneficial interaction with *E. coli* bacteria, a relationship that is similar but far simpler than the vast communities of 'healthy' bacteria residing in the human gut. We have demonstrated that metformin exerts DR on *C. elegans* by altering bacterial folate and methionine metabolism [90], providing for the first time a mechanistic explanation for the effects of metformin on the host through action on its microbiota. In *C. elegans*, metformin slows down the ageing process only if bacteria are present and in a dose-dependent manner, an effect which is proportional to its effects on microbial metabolism and the bacterial strain with which the worms are co-cultured [90]. Remarkably, metformin can also cause folate [139] and vitamin B12 deficiency [140] and increase plasma homocysteine levels [141] in humans.

Metformin disrupts intestinal fluid homeostasis in flies

The gut of *Drosophila melanogaster* is recently becoming one of the most well studied organs in fly research. Similarly to humans, flies show an important conservation in gut functions. Fluid retention, absorption, intestinal peristalsis, intestinal transit, defecation rate and nature of excreta are also subject to complex homeostatic regulation [142]. Recent studies show that the *Drosophila* microbiome modulates host development and metabolic homeostasis via insulin signalling [143] and mTOR signalling [144]. However, the precise role of bacteria in gut physiology remains unclear. Given that metformin leads to gastrointestinal upset when administered to diabetic patients, Slack *et al*. investigated if metformin alters intestinal physiology in ageing flies. Using an assay recently developed to evaluate intestinal physiology through examination of fly excreta [145], these authors showed that 100 mM metformin lead to changes in intestinal homeostasis causing fluid imbalances resulting in more concentrated faecal deposits in a possible attempt to preserve water. Possibly, at such elevated concentrations, metformin acting as a metabolic stressor can produce acid in excess that is counterbalanced by the gut. In fact, the hindgut of flies has the ability to adjust the pH of intestinal material before excretion in response to both internal (e.g. reproductive) or external (e.g. nutrients) signals [145]. Unexpectedly, metformin treatment in flies did not lead to increased lifespan extension despite activation of AMPK. Whether lifespan extension in flies is also dependent on metformin sensitivity of gut flora remains to be tested.

Metformin action on gut function in rodents

Studies performed in mice show that metformin accumulates in the intestine at very high concentrations [39], and this could possibly explain effects of metformin on host metabolism. Evidence collected in rodents also suggests that the intestine is a major organ regulating the action of metformin. Data show that metformin not only delays glucose absorption along the intestinal tract [43] but also increases its utilisation through non-oxidative anaerobic metabolism [146]. Lactate as a product of non-aerobic

metabolism can be delivered to the liver through the portal vein and utilised as a substrate for gluconeogenesis. Therefore, the intestine makes an important contribution to the glucose-lowering effects of metformin providing at the same time a safeguard mechanism against hypoglycaemia [147].

Most of the microbes that co-exist within the host also reside in the intestine and are therefore under selective pressure at high concentrations of metformin. T2D and obesity are characterised by structural and functional changes in the microbial populations in the gut, an inflammatory state, and gut barrier disruption also known as leaky gut. There are many similarities between the role of microbiota and metformin effects on obesity and blood glucose regulation. It is therefore plausible to assume that common mechanisms are at play and metformin has a direct or indirect interaction with functions of the microbiota on the host to accomplish its anti-diabetic effects.

Two recent studies by Shin *et al.* and Lee *et al.* show that metformin treatment of mice on a high-fat diet improves markers of metabolic disorders and provokes an alteration in the structure of faecal microbiota towards of that of mice feeding a normal chow diet [148, 149]. Using metagenomic analysis, these studies show that metformin causes a profound shift in specific subsets of bacterial taxa. The microbial change in mice treated with metformin was accompanied by an increase of the genus *Akkermansia* spp. [148, 149]. Metformin led to an increase of goblet cells producers of mucin, a nutrient source for the mucin-degrading bacteria *Akkermansia municiphila*. In fact, probiotic administration of this bacterial strain or metformin is associated with an improved metabolic profile, reducing metabolic endotoxemia, adipose tissue inflammation, and improved glycaemic control insulin resistance [149, 150]. Further studies to determine a causal link between metformin treatment and *Akkermansia* to promote its anti-diabetic effects are therefore warranted. An increase in *Lactobacilli* populations was also observed in high-fat diet mice treated with metformin. *Lactobacillus* is one of the many bacteria that can utilise glucose to produce lactate. Notably, effects of metformin in improving glucose homeostasis in high-fat mice are abolished through a cocktail of broad-spectrum antibiotics [149]. These data suggest that gut microbiota is a target of metformin to produce its effects on host physiology. It is possible that extra lactate production in the intestine by metformin could derive, in addition to its production through non-oxidative metabolism by intestinal cells, from lactate producing bacteria that utilise glucose. Alternatively, increased lactate and other gluconeogenic precursors such as pyruvate and alanine by metformin [151], in addition to sustaining gluconeogenesis during fasting and protect against hypoglycaemia, could modify the structure of the microbiota by providing new energy substrates favouring specific microorganisms. Whether changes in the structure of the microbiota result directly from high concentrations of the drug in the intestine, or indirectly by metabolites resulting from the host upon metformin treatment remains to be elucidated.

In a study performed in rats, Pyra *et al.* showed that the novel combination therapy of prebiotic fibres such as oligofructose and metformin improves the metabolic profile of high-fat diet induced obese rats. The combined action of metformin and oligofructose has an improved effect over each individual treatment alone on body weight, hepatic triglycerides, secretion of glucose-dependent insulinotropic polypeptide and leptin, and AMPK activation. Effects of this therapeutic approach on gut microbiota were also evaluated in this study. As expected, oligofructose treatment increased significantly bifidogenic bacteria such as *Bifidobacterium* spp. [152].

These studies show that metformin administration to rodents on high-fat diets induces significant modifications in the structure of the microbial populations in the gut. This leads to a new understanding of how metformin could affect metabolism to lower glycaemia through targeting the microbiota. However, further studies are required to fully elucidate what effect this has upon the host.

Metformin action on the human intestine and microbiota

Most anti-diabetic drugs have therapeutic doses in the microgram/milligram range to reduce hyperglycaemia in T2D patients. Interestingly, effective metformin doses can reach up to 2.5 grams per day [153]. The intestine is the main tissue for metformin absorption [154] and accumulation [155] in humans and other animals. Absorption time $T_{1/2}$ is estimated to be between 0.9 to 2.6 h, and is affected by food intake which can delay the process by 35 min [156]. Metformin is stable, does not bind to proteins and has a bioavailability of 50-60%. The drug reaches its maximum concentration in peripheral plasma approximately 2 h after oral ingestion, is eliminated from the plasma rapidly with a $T_{1/2}$ of 1.7 to 4.5 h and excreted unchanged either in the urine or in the faeces [157]. Similar to what is observed in rodents, high concentrations of metformin also accumulate in the human intestine [155]. In fact, the concentration of metformin in the jejunum can attain 30-300 fold its concentration in the plasma [38] or other tissues [39]. Intralumenal concentrations in the range of 20 mM after administration of an 850 mg dose of metformin have been observed in the intestine of diabetic patients [158]. Therefore, it is likely that the effects of metformin on the intestinal tissue might be different to some extent in comparision to other tissues and also be responsible for the observed side-effects associated with this drug.

The most common side-effects associated with metformin administration include gastrointestinal upset and lactic acidosis. However, occurrence of lactic acidosis with metformin is an uncommon event (3-9/100,000 patient-years) and very rarely causes death (2-4/100,000 patient-years) [159]. On the other hand, disturbances of the gastrointestinal tract appear soon after the treatment is initiated, diminish over long periods of therapy and stop if the treatment is discontinued. These mainly include diarrhoea and other minor disorders such as abdominal pain with cramps and swelling, vomiting, nausea, dyspepsia, dysgeusia, flatulence and constipation. These combined digestive disorders can affect up to 29% of the patients treated with up to 2.5 g metformin/day, leading to discontinuation of metformin therapy in 10% of these patients [160]. While these gastrointestinal effects are widely observed in patients taking metformin, the molecular mechanisms underlying such responses are still unclear. Only a few reports have addressed this question and some possible mechanisms have been put forward and reviewed by Bouchoucha *et al*. [161]. These include changes in serotonin metabolism, changes in incretin and glucose metabolism, and alteration of bile acid metabolism. However, none fully explains the pathophysiological effects of metformin gastrointestinal side-effects.

Recently, new studies suggest that metformin affects microbial metabolism in the gut. This is a new interesting hypothesis that could possibly explain inter-individual effects of metformin in digestive disorders. Karlsson *et al*. evaluated the structure and function of faecal microbiota of a cohort of 145 70-year old European women with normal, impaired and diabetic glucose control with metformin [162]. Metagenomic analysis of T2D human patients treated with metformin show increased levels of Enterobacteriaceae (e.g. *Escherichia*, *Shigella*, *Klebsiella* and *Salmonella*) and decreased levels of *Clostridium* and *Eubacterium*. Interestingly, there was a significant correlation of *E. coli* levels and glucagon-like peptide levels. Secretion of glucagon like peptide is reduced in obese and T2D patients [163] and metformin increases its levels in the plasma [164]. According to the authors of this study, changes of structure and function of the microbiota in T2D patients treated with metformin might be a consequence of the treatment and increased glucose availability in the intestinal lumen.

Other side-effects associated with long-term metformin therapy that could possibly be explained by the effects of metformin on microbiota are vitamin B12 and folate deficiency [139] and alterations in bile acid metabolism [165]. Bacteria such as *Bifidobacterium* are important suppliers of vitamins to their host. In addition, several anaerobic bacteria are involved in the transformation of bile acids in the intestine, whose main function is to facilitate the metabolism of dietary fat and absorption of fat-soluble vitamins [166]. Recently, Napolitano *et al.* have characterised these interactions

in human patients by relating glycaemic control, bile acid metabolism and microbiome alteration [165]. These authors find important changes in the levels of members of the microbiota (Firmicutes/Bacteriodetes), changes in the entero-hepatic levels of bile acids (e.g. cholic acid) and entero-endocrine hormones (glucagon-like peptide-1 and peptide YY). Altogether, these suggest a mechanistic link between bile acid-induced microbial changes and glycemic control in T2D patients. Interestingly, cholic acid administration to rodents produces similar changes in the relative abundance of Firmicutes and Bacteroidetes [167]. Altogether, new reports support the evidence from earlier studies showing that intravenous administration of metformin whilst attaining therapeutically effective blood concentrations do not improve blood glucose in stark contrast to oral administration of the drug [168, 169]. However, how exactly metformin affects the intestine and the microbiota contained in it is still unclear. Therefore, new studies are required to understand how metformin affects physiology and re-evaluate its mode of action.

Conclusion

Metformin has been used as a safe and effective treatment for T2D for over half a century, yet the precise mechanism of action of this drug still remains elusive. The anti-hyperglycaemic properties of metformin are chiefly mediated by the suppression of hepatic gluconeogenesis and it is generally accepted that this is achieved via inhibition of complex I in the mitochondrial respiratory chain. The subsequent reduction in cellular energy status has been shown to directly impede gluconeogenic flux, interfere with glucagon signalling and promote activation of the major metabolic regulator AMPK, although which of these make the greatest contribution to metformin's therapeutic effects is a subject of debate. Moreover, it is becoming increasingly apparent that complex I is not the only molecular target of metformin; for example, it has recently been confirmed that metformin inhibits mGPD. It is highly probable that additional targets will be identified in the near future. Our work in *C. elegans* serves as a reminder that it is necessary to consider the effect of drugs not only on the individual but also on their microbiota. Very little is currently known about the bacterial targets of metformin and it is possible that the microbiota could regulate some of its effects on host physiology via private mechanisms. This undoubtedly warrants further investigation. Currently, metformin is only approved as a treatment for T2D however in recent years, a vast number of studies have highlighted the therapeutic potential of metformin in the context of other diseases. In particular, metformin has shown promise as a treatment for CVD and cancer. The various mechanisms proposed to account for these beneficial effects have been outlined in this review, although there are a number of outstanding issues that must still be resolved. Ultimately a greater understanding of the molecular pathways involved will help to guide novel applications of metformin.

Acknowledgments

Figure Legends

Figure 1 Proposed mechanisms of metformin action in Type 2 diabetes. Metformin enters the hepatocyte through OCT1 and accumulates in the mitochondria where it inhibits Complex I. This leads to a reduction in ATP and concomitant rise in AMP. Elevated AMP levels lead to activation of AMPK, although metformin may also promote AMPK activation in a direct manner. AMPK inhibits gluconeogenic gene transcription by preventing formation of the CREB:CBP:CRTC2 complex, both directly and via SIRT1.

Furthermore, AMPK inhibits lipogenesis through ACC, ChREBP and SREBP phosphorylation, which helps to improve insulin sensitivity. Several AMPK-independent mechanisms of metformin action also exist. The reduction in cellular energy status can directly inhibit gluconeogenic flux. Additionally, increased AMP has an inhibitory effect on adenylate cyclase leading to decreased cAMP production. This in turn reduces the activity of PKA and its downstream targets, which include CREB. Metformin also inhibits mGPD. This prevents glycerol from contributing to gluconeogenesis and increases the cytosolic redox state, which in turn makes the conversion of lactate to pyruvate unfavourable thus limiting the use of lactate as a gluconeogenic substrate.

Figure 2. Proposed mechanisms to explain the anticancer effects of metformin. At the systemic level metformin may inhibit tumour development by reducing insulin/IGF-1 signalling, preventing the release of proinflammatory cytokines through NF-kB and enhancing the immune response (mediated by natural killer cells (NK cells) and cytolytic T cells (T-cells)) to cancer cells. Metformin also has direct effects in cancer cells, many of which are mediated by AMPK. When activated, AMPK disrupts cancer cell energy metabolism by inhibiting fatty acid synthesis (inhibition of acetyl-CoA carboxylase (ACC)) and suppressing the Warburg phenotype mediated by the action of the hypoxia-induced factor (HIF-1) on the glycolytic enzymes pyruvate kinase (PDK) and pyruvate dehydrogenase (PDH). AMPK may also block folate and methionine metabolism, which is required for nucleotide synthesis. AMPK downregulates the oncogene c-MYC by inducing the expression of Dicer and upregulates the tumour suppressor p53 by inhibiting its negative regulator MDMX. Furthermore, AMPK blocks the mTORC1 signalling pathway by inhibiting Raptor and activating TSC2. AMPK independent mechanisms have also been described. Metformin can protect against DNA damage by inhibiting complex I and suppressing ROS production. Metformin also inhibits hexokinase activity and impairs glucose uptake. Metformin can block mTORC1 signalling in the absence of AMPK via inhibition of Rag GTPases. Additionally, metformin reduces levels of the cell cycle regulator Cyclin D1 in a p53 and REDD1 dependent manner. Finally, metformin promotes apoptosis by downregulating the Stat3/Bcl-2 pathway through release of cytochrome C and also promotes autophagy.

Figure 3. Effects of metformin in gut physiology to regulate type 2 diabetes and survival in model organisms and humans. Metformin increases *C. elegans* lifespan when co-cultured with a sensitive but not a tolerant *E. coli* strain. In *Drosophila*, high concentrations of metformin lead to intestinal dyshomeostasis and increased concentration of faecal output that correlates with an observed shortening of lifespan. Metformin normalises the gut microbiota of rodents on a high-fat diet and improves glucose homeostasis due to effects on the gut microbiota. In T2D diabetic patients, metformin promotes weigh loss and improved glucose tolerance either by reducing gut glucose uptake or by direct alterations of the gut microbiota which can ultimately lead to increased survival.

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Abbreviations

ACC, acetyl-CoA carboxylase; Akt, protein kinase B; AMPK, AMP-activated protein kinase; Bcl-2, B-cell lymphoma 2; CBP, CREB binding protein; cGPD, cytosolic glycerophosphate dehydrogenase; ChREBP, carbohydrate-responsive element-binding protein; CREB, cAMP response element binding protein; CRTC2, CREB regulated transcription coactivator 2; CVD, cardiovascular disease; DR, dietary restriction; FAS, fatty acid synthase; IGF-1, insulin-like growth factor 1; LKB1, liver kinase B1; MDMX, murine double minute X; mGPD, mitochondrial glycerophosphate dehydrogenase; mPTP, mitochondrial permeability transition pore; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; NF-κB, nuclear factor-κB; NO, nitric oxide; OCT1, organic cation transporter 1; PAI-1, plasminogen activator inhibitor 1; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; PKA, protein kinase A; REDD1, regulated in development and DNA damage response 1; ROS, reactive oxygen species; SIRT1, sirtuin 1; SREBP-1, sterol regulatory element-binding protein 1; Stat3, Signal transducer and activator of transcription 3; T2D, type 2 diabetes; TSC1, tuberous sclerosis 1; TSC2, tuberous sclerosis 2; UKPDS – UK Prospective Diabetes Study.
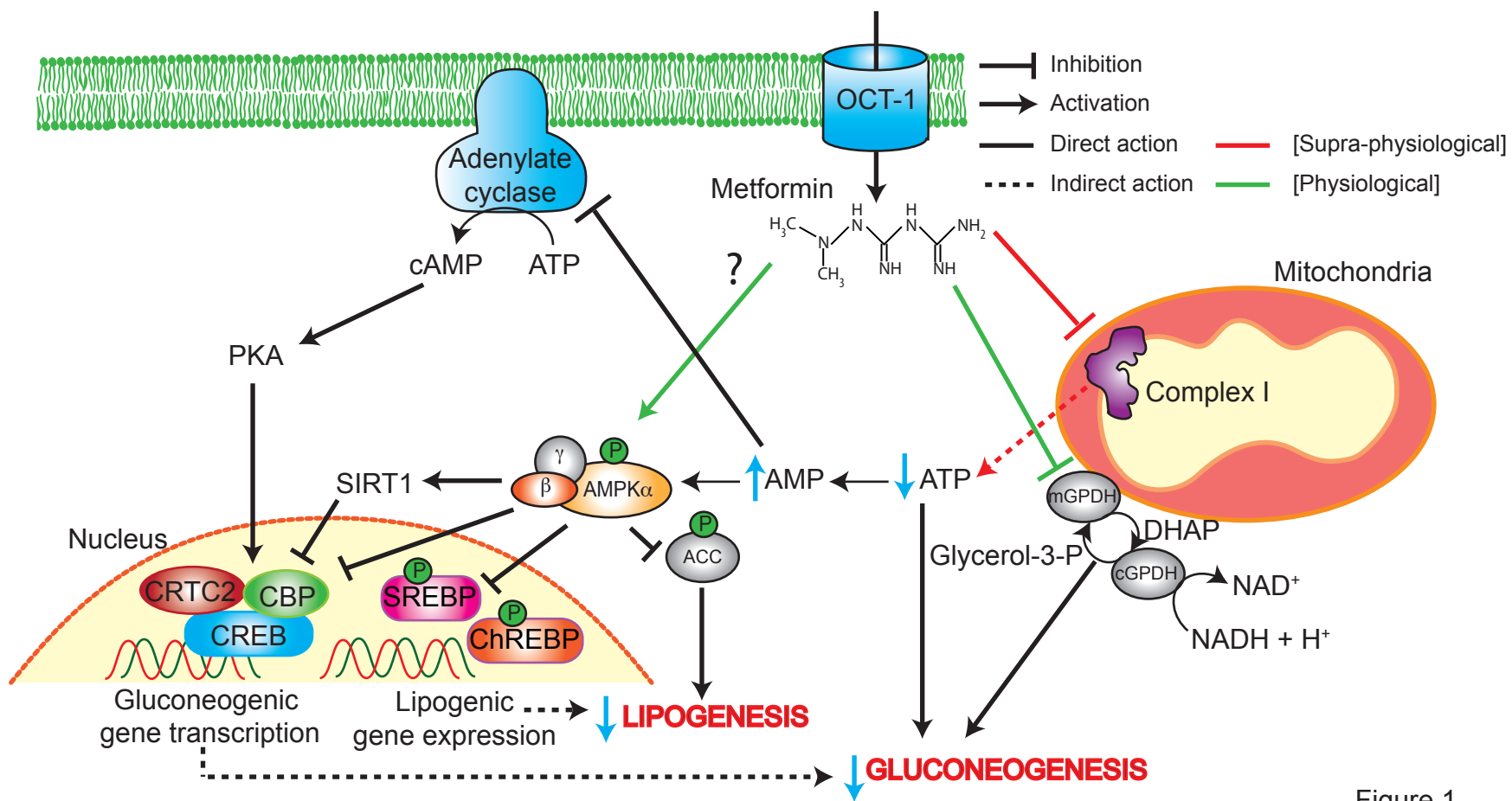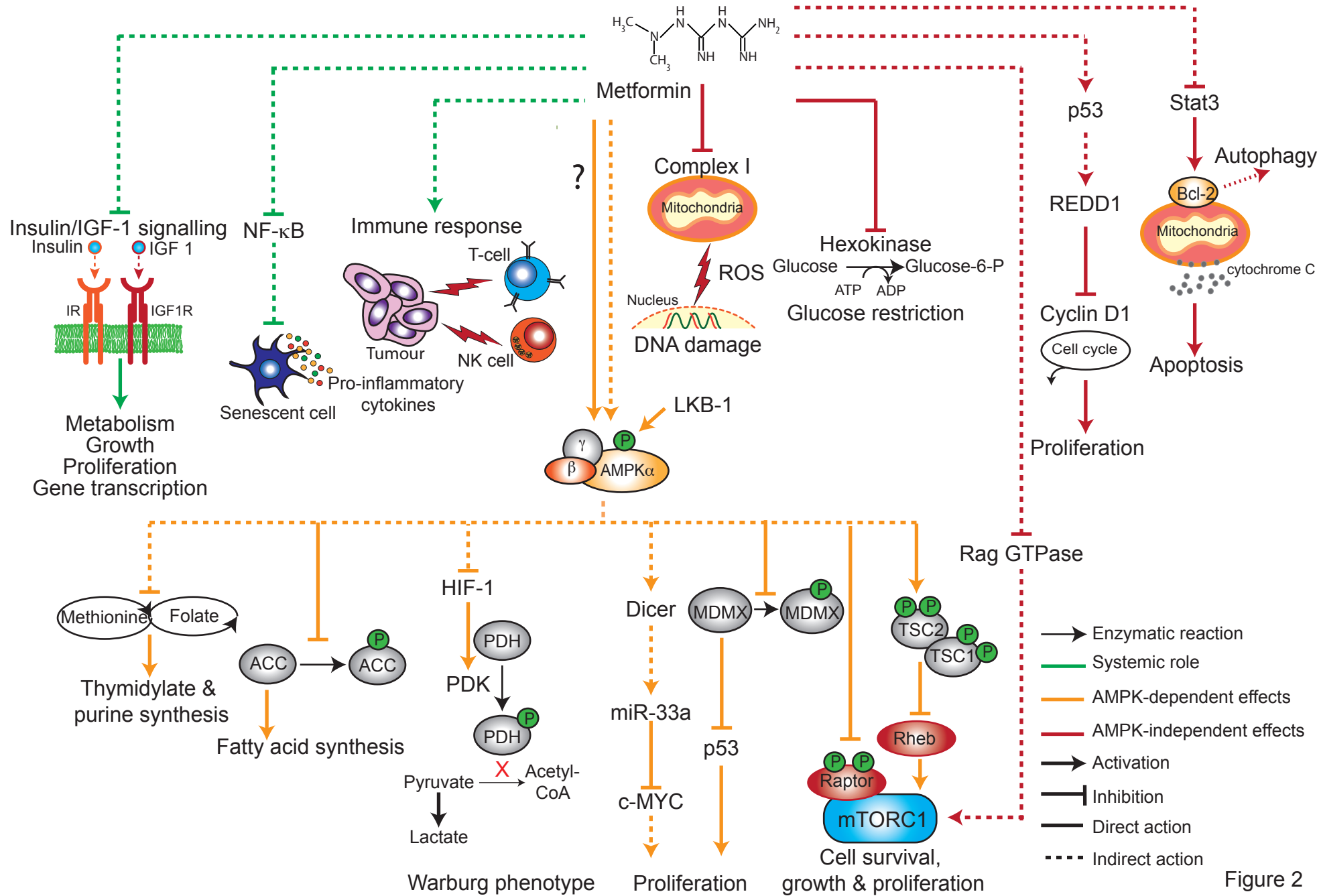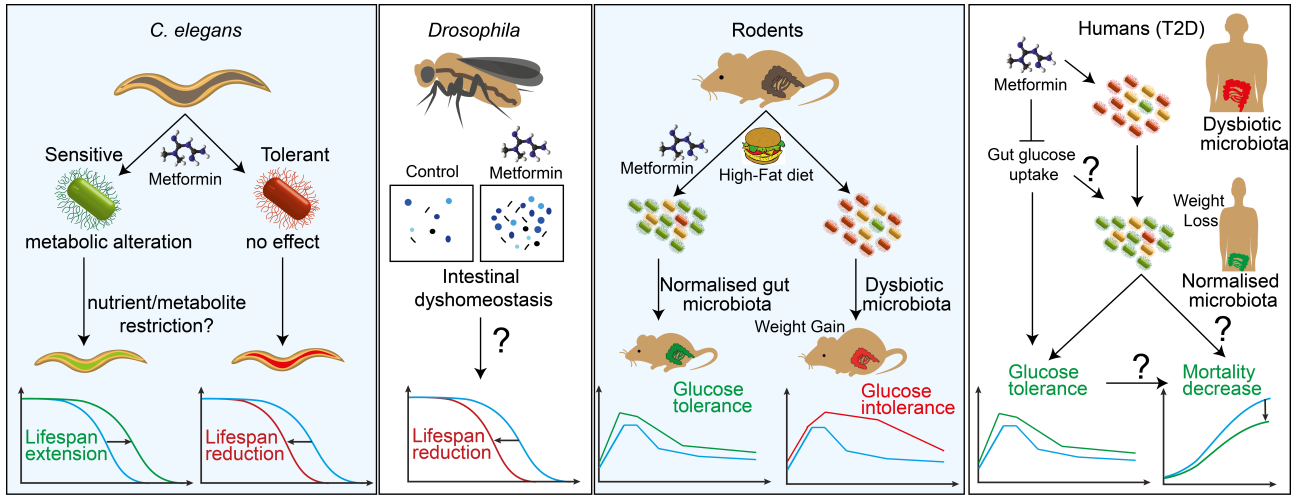
Figure 1

Figure 2

Figure3