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Review

Metformin: Is it a drug for all reasons and diseases?

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ABSTRACT

Metformin was first used to treat type 2 diabetes in the late 1950s and in 2022 remains the first-choice drug used daily by approximately 150 million people. An accumulation of positive pre-clinical and clinical data has stimulated interest in re-purposing metformin to treat a variety of diseases including COVID-19. In polycystic ovary syndrome metformin improves insulin sensitivity. In type 1 diabetes metformin may help reduce the insulin dose. Meta-analysis and data from pre-clinical and clinical studies link metformin to a reduction in the incidence of cancer. Clinical trials, including MILES (Metformin In Longevity Study), and TAME (Targeting Aging with Metformin), have been designed to determine if metformin can offset aging and extend lifespan. Pre-clinical and clinical data suggest that metformin, via suppression of pro-inflammatory pathways, protection of mitochondria and vascular function, and direct actions on neuronal stem cells, may protect against neurodegenerative diseases. Metformin has also been studied for its anti-bacterial, −viral, −malaria efficacy. Collectively, these data raise the question: Is metformin a drug for all diseases? It remains unclear as to whether all of these putative beneficial effects are secondary to its actions as an anti-hyperglycemic and insulin-sensitizing drug, or result from other cellular actions, including inhibition of mTOR (mammalian target for rapamycin), or direct anti-viral actions. Clarification is also sought as to whether data from ex vivo studies based on the use of high concentrations of metformin can be translated into clinical benefits, or whether they reflect a 'Paracelsus' effect. The environmental impact of metformin, a drug with no known metabolites, is another emerging issue that has been linked to endocrine disruption in fish, and extensive use in T2D has also raised concerns over effects on human reproduction. The objectives for this review are to: 1) evaluate the putative mechanism(s) of action of metformin; 2) analyze the controversial evidence for metformin's effectiveness in the treatment of diseases other than type 2 diabetes; 3) assess the reproducibility of the data, and finally 4) reach an informed conclusion as to whether metformin is a drug for all diseases and reasons. We conclude that the primary clinical benefits of metformin result from its insulin-sensitizing and antihyperglycaemic effects that secondarily contribute to a reduced risk of

Abbreviations: ACE2, angiotensin converting enzyme 2; AMPK, AMP-dependent kinase; CAD, coronary artery disease; CIMT, carotid artery intima-media thickness; COVID-19, Coronavirus disease; CREB, cAMP response element-binding protein; CRP, C-reactive protein; CV, cardiovascular; CVD, cardiovascular disease; 2DG, 2-deoxy-glucose; DPP, Diabetes Prevention Program; DPP-4, diphenyl-peptidase-4; EDV, endothelium-dependent vasodilation; EIDV, endothelium-independent vasodilation; eNOS, endothelial nitric oxide synthase; FMD, flow-mediated vasodilation; GDF15, Growth Differential Factor 15; G6PD, glucose-6-phosphate dehydrogenase; GLP-1, Glucagon like Peptide 1; HMGB1, high mobility group box 1 protein; IGF, insulin growth factor; IGFR, insulin growth factor receptor; LKB1, liver kinase B1; MATE, multidrug and toxin extrusion transporter; mGPD, mitochondrial glycerol-3-phosphate dehydrogenase; mTOR, mammalian target for rapamycin; OCT, organic cation transporter; PAI-1, plasminogen activator inhibitor-1; PCOS, Polycystic Ovary Syndrome; PKA, protein kinase A; PMAT, plasma membrane monoamine transporter; PDGFB, platelet-derived growth factor B; PEN2, presenilin-enhancer protein-2; RCT, randomized controlled trials; SARS-CoV-2, severe acute respiratory syndrome–related *coronavirus 2*; *SASP*, senescence-associated secretory phenotype; SGLT2, Sodium-Glucose CoTransporter-2; sICAM-1, soluble intercellular adhesion molecule; TID, type 1 diabetes; T2D, type 2 diabetes; t-PA, tissue type plasminogen activator; UKPDS, United Kingdom Prospective Diabetes Study; VEGF, vascular endothelial growth factor; VCAM-1, vascular cell adhesion molecule-1; vWF, von Willibrand Factor.

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a number of diseases and thereby enhancing healthspan. However, benefits like improving vascular endothelial function that are independent of effects on glucose homeostasis add to metformin's therapeutic actions.

1. Introduction

1.1. Brief history

Metformin, dimethyl biguanide, is a synthetic biguanide that combines two guanidine moieties together into one molecule. Its development as an anti-diabetic drug can be linked to Southern and Eastern European folk medicine knowledge dating back until the 17th Century when extracts from French lilac (*Galega officinalis*) were used to treat people with 'sweet urine'. *French Lilac* is a widely distributed perennial found in temperate regions and is also known by a variety of names including *goat's rue*, *Italian fitch*, and in the USA as *Professor Weed* and has been employed in folk medicine for a wide range of afflictions including diuretic and anti-diabetic actions as well as use in farm animals and humans as a galactogogue [1–3]. Arguably many of the benefits of French lilac, including its effects as a galactogogue, can be attributed to the insulin-sensitizing actions of guanidines. The history of the development of metformin from botanical origins to chemical synthesis has been well documented by others and is summarized in Table 1 [1,4–6].

In brief, the primary active anti-diabetic chemical in the extracts from French Lilac is the alkaloid galegine (isoamylene guanidine); however, galegine is too toxic for chronic use and in the late 19th century German chemists, Adolph Strecker and Bernhard Rathke synthesized guanidine and biguanides. Studies with these synthetic guanidine derivatives provided the stimulus to develop an orally effective and less toxic anti-diabetic drug and guanidine hydrochloride was reported to lower blood glucose levels in rabbits [8]. Metformin was synthesized in 1922 [9] and reports of the ability of metformin and other synthetic guanidines to lower blood glucose in rabbits and dogs were published shortly thereafter [10–12]. Synthalin A (decamethylene diguanide) and Synthalin B (dodecamethylene diguanide) were biguanidines developed by Schering AG to treat diabetes. Synthalin B, with an aliphatic chain with 12 links was claimed to be safer than Synthalin A but reports of liver toxicity led to the withdrawal of Synthalin B from use in most countries in the 1930s and in Germany in the mid-1940s. There was little interest in metformin until the late 1950s when French physician, Jean Sterne, described its benefits in patients with diabetes [15]. However, it was Ciba's more potent biguanide, phenformin (phenethylbiguanide), which was adopted into clinical use and reduced interest in metformin [22,23]. In 1978 as a result of increasing concerns with hepatotoxicity and lactic acidosis phenformin and another biguanide, buformin, were withdrawn from use in most countries. Positive data from the Multicenter Metformin Study in the USA was published in 1995 and provided renewed interest in the role of metformin as well as the importance of blood glucose control. The conclusions of this 1995 study were further enhanced by the results of the larger United Kingdom Prospective Diabetes Study (UKPDS) in 1998 [18,24]. Currently, metformin remains the first-choice drug for most patients with T2D [18,25], and as depicted in Fig. 1 subsequent to the completion of UKPDS in 1998 there has been a steady increase in publications focusing on the use of metformin to treat T2D.

2. Search strategy

In order to evaluate the evidence for and against the putative mechanisms of action of metformin and the potential clinical benefits of the drug, a narrative review was conducted of publications identified through PubMed and Scopus searches and facilitated by a librarian who is also a co-author (RM). Summaries of Scopus searches and the terms used in the searches are provided in Figs. 1, 5-7, 9, and 11–13, and indicate an extensive database on metformin. In this narrative review we focused on critically evaluating representative original studies, and where appropriate review articles that provided evidence either for, or against, a particular cellular mechanism of action, clinical benefit, and/or viewpoint. Summaries that cover all aspects of the history, pharmacology, and putative clinical benefits of metformin have also been provided in in eight tables.

3. Risk-benefits of chronic metformin use

Metformin is off patent, comparatively inexpensive and has proved to be a safe drug for long-term use and, unlike phenformin, its use is associated with a low risk of lactic acidosis, which is minimized when avoided in patients with liver disease, or severely reduced kidney function. The most common side effects are dose-related gastrointestinal (GI)-related (nausea, vomiting, bloating and diarrhea) and with minimal problems with patient compliance estimated at only 5% [26–28]. The usual dose-range for metformin is from 250 to 2550 mg/day with plasma levels ranging from approximately 5 to 20 μM (Table 2). Compliance, however, may be much lower than previously considered as a retrospective cohort study of 15,981 patients indicated 48% became non-adherent within the first year of treatment with metformin [29]. Discontinuance of metformin is primarily attributed either to side effects, or glycemic control being achieved independent of pharmacotherapy [29]. Similarly, a 2018 report indicated that 30% of prescribed doses of metformin were not taken, whereas higher adherence was seen for sulfonylureas, diphenyly-peptide-4 inhibitors (DPP-4 inhibitors, or gliptins) and sodium-glucose co-transport inhibitors-2 (SGLT-2, or gliflozins), but not for glucagon-like peptide-1 receptor agonists (GLP-1 receptor agonists) [29]. In the latter study it was noted that whereas DPP-4 inhibitors were generally well-tolerated, GI side effects were more frequently associated with metformin and attributed to the lower adherence with the biguanide [30]. Based on these data, patient compliance might prove to be a deterrent should metformin be repurposed for prophylactic purposes such as an anti-aging drug. The chronic use of metformin can also result in vitamin B12 deficiency due to malabsorption in from 6% to up to 30% of patients and possibly linked to changes in the microbiota, altered motility, and/or alterations in the calcium-dependent transport via the gastric intrinsic factor glycoprotein [31–34]. Vitamin B12 deficiency could offset putative benefits associated with using metformin for the treatment of neurodegenerative diseases [35]. Concerns over reproductive health in males have also been raised and are discussed in the conclusions section together with the risk of environmental contamination [36]. Metformin is also being increasingly used in gestational diabetes and is considered a safe alternative to insulin [37,38]. Unlike insulin, metformin crosses the placenta and will also be transferred to the newborn via the mother's breast milk and there is evidence that metformin may have effects on postnatal growth as suggested by data from the Metformin in Gestational diabetes (MiG) trial [39]

Additional concerns over promoting the chronic use of metformin are linked to data indicating that lifestyle modification is more effective than metformin in preventing the development of T2D. In the Diabetes Prevention Program (DPP) study of pre-diabetic subjects randomized to receive metformin (850 mg bid), lifestyle intervention (low fat diet and at least 150 min of exercise/week), or placebo, the benefits of exercise were reduced in patients prescribed metformin (DPP, 2002) [40]. A study in 2016 reported that men and women with pre-diabetes who were placed on an exercise protocol for 12 weeks and took metformin alone (2000 mg/day), or exercise plus metformin, or placebo, achieved superior benefits from exercise than metformin in terms of improved

Table 1
History of the discovery and development of metformin to treat diabetes.

Chronology	Brief description of observation	Source
~1600s: Use of herbs in folklore medicine in Medieval Europe and described in *Culpeper's Complete Herbal* of 1653.	Extracts of leaves and seed pods from the perennial herb, French lilac (Galega officinalis, also known as Italian fitch, Goat's rue, Spanish sainfoin, and Professor weed) used to treat diabetes as detected as 'sweet urine' and polyuria. Also used as a galactogogue in cows and goats and a variety of other maladies. Later determined that active chemical was the guanidine, galegine	[Bailey and Day [2,4] Bailey [5] Culpepper [7] Witters [1]
1844–1861 and 1878–1879	1) German chemist Adolph Strecker first described the chemical synthesis of guanidine. 2) The synthesis of biguanides was carried out by German chemist Bernhard Rathke	See Table 1 in Bailey [5]
1918: Guanidine hydrochloride	Glucose-lowering effects of guanidine observed when injected into rabbits	Watanabe [8]
1922: The synthesis of the biguanide dimethyl guanidine (metformin) first described.	Synthesis based on a previous description of producing guanidine thiocynate from ammonium thiocyanate and dicyanodiamide.	Werner & Bell [9]
1926–1928: Description and antihyperglycemic properties of Synthalin A & B.	The link between the ability of guanidine to lower blood glucose and toxicity stimulated the search for guanidines with high anti-hyperglycemic potency and reduced toxicity. Frank, Nothmann and Wagner and also Graham and Linder described the effectiveness of Synthalin (two guanidine groups linked by an aliphatic chain consisting of 10 links) as a promising molecule for the treatment of diabetes. Synthalin (later re-named Synthalin A) was marketed by Schering AG, and less toxic than guanidine. Synthalin B was developed with a longer aliphatic chain with 12 links and claimed to be safer. An accumulation of liver and renal toxicity reports resulted in the withdrawal of Synthalin B from the market in the 1930s and finally in Germany in the mid-1940s.	Frank et al [10] Graham & Linder [11]
1929: Metformin lowers blood glucose	Metformin injected into rabbits lowers blood glucose and determined to be the most potent of a series of compounds tested. Lack of follow up may be linked to discovery on insulin in 1922.	Slotta and Tschesche [12]
1948: Approval of proguanil (chloroguanide) by the FDA to treat malaria and marketed as Paludrine.	Proguanil, a structural analogue of metformin, is a pro-drug that is metabolized by CYP2C19 to the active cycloguanil, Metformin was also tested in the 1940s for use in malaria and interest recently focused on using metformin as an adjunct in combination with anti-malarial drugs.	Vera et al [13]
1950: Metformin used to treat influenza	Metformin under the name of Flumamine	Garcia [14]
1957: Metformin used in humans with diabetes.	Jean Sterne described the effectiveness of metformin in patients with diabetes. However, the more potent phenformin and buformin were preferred until their withdrawal from most markets in the 1970s due to the risk of lactic acidosis.	Sterne [15] Campbell [16]
1958: Toxicity study of phenformin versus Synthalin B.	Comparison of liver toxicity in guinea pigs comparing Synthalin B versus DBI (phenformin). Conclusion: Phenformin a safer drug than Synthalin B.	Creutzfeldt and Moench [17]
1978: Phenformin, the phenethylbiguanide relative of metformin, withdrawn from most markets.	Due to an increasing number of reports of lactic acidosis and resultant high mortality the FDA announced the withdrawal of phenformin on November 15, 1978.	Phenformin and related biguanide, buformin, (1-butyl-bigunanide) are now only available in a few countries.
1998: UKPDS (United Kingdom Prospective Diabetes Study). A landmark randomized, multicentre trial involving 23 sites and 5102 patients with newly diagnosed type 2 diabetes. UKPDS comparing insulin, sulfonylureas and metformin. 1995 the FDA approved metformin for the treatment of type 2 diabetes.	UKPDS involved 5102 patients with newly diagnosed type 2 diabetes. The study, published in 1998, reported the cardiovascular benefits of the use of metformin for diabetes. In the UKPDS 34 subgroup 1704 overweight patients with T2DM were assigned to one of three arms: 1. conventional therapy with diet alone, i 2. intensive therapy with metformin, 3. intensive therapy with first generation sulphonylurea chlorpropamide, and second generation, glibenclamide, or insulin). The results demonstrated a reduction in diabetes-related complications and all-cause mortality for those in the metformin arm of the study compared to the other two arms of the study. Benefits were maintained after an additional 10 years of follow-up.	UKPDS Group. 1998. UKPDS 34 [18] A 2005 Cochrane Review (Saenz et al [19]) confirmed the benefits of metformin monotherapy in overweight patients.
2020: despite the availability of many new drugs, and also formulations of insulin available metformin maintains the position as the first choice drug for most patients diagnosed with T2D.	As concluded in a 2020 review article: *"Until further safety data becomes available for SGLT2i and GLP-1RA use in treatment-naïve individuals, we recommend that not only the efficacy but also the cost and the long-term safety profile should guide decisions in clinical practice and metformin should continue to be used as a first-line therapy for newly diagnosed individuals with T2D. The key message is to avoid therapeutic inertia, as the uptake of these 'newer' GLTs (glucose-lowering therapies) with proven cardiovascular benefits remains generally low and to consider early addition of these agents to baseline metformin therapy when indicated."* In addition: *"Metformin prescribing peaked from 55.4% in 2000 to 83.6% in 2013 among all individuals with T2D who were on at least*	Ahmad et al 2020, [20] Sharma et al., [21] Montvida et al [22]

(*continued on next page*)

Table 1 (*continued*)

Chronology	Brief description of observation	Source
	one medication for their diabetes management in the UK (Sharma et al, 2016). Similarly, in the USA use for metformin increased from 60% in 2005 to 77% in 2016". (Montvida et al., 2018 [20])	

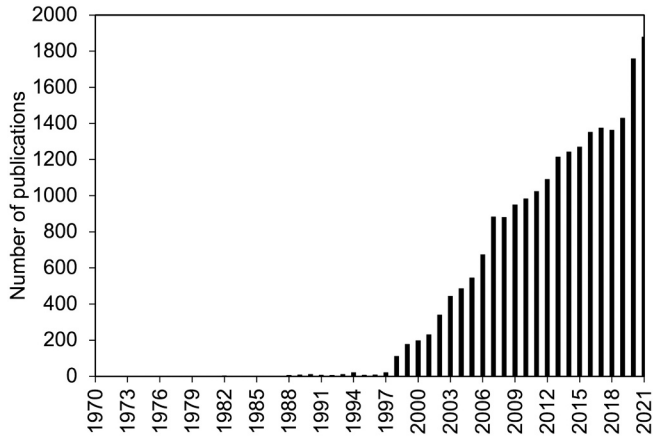

Fig. 1. Growth in publications mentioning metformin and diabetes type 2. Data were obtained from Scopus, 6 February 2022, using this search: (TITLE-ABS-KEY (metformin OR dimethylbiguanidine OR dimethylguanylguanidine OR glucophage)) AND (TITLE-ABS-KEY ("diabetes mellitus type 2" OR "diabetes type 2" OR "type 2 diabetes").

Table 2
Physiochemical and pharmacokinetic properties of metformin.

Properties	References
Metformin is a strongly basic hydrophilic drug with a pKa of approximately 11.5 and at physiological pH it exists predominantly as a cation.	Mucklow et al [53] Pentikäinen et al [54]
Metformin is not metabolized and is excreted unchanged by the kidney.	Sirtori et al [55] Pentikäinen et al [54]
Metformin has an oral bioavailability of 50–60%, plasma levels of ~5 to 20 μM, plasma half-life of approximately 2 to 6 h, a urinary half-life of 9 h, a slower half-life from erythrocytes ~20 h, and a volume of distribution of 70–276 l.	Tucker et al [56] Graham et al [57] Christensen et al [58] Kajbaf et al., 2016 [59,60]
The biguanide phenformin enhances Ca^{2+} uptake into mitochondria and metformin requires Cu^{2+} to activate AMPK kinase suggesting role of metal cations and notably Cu^{2+} in cellular actions of metformin.	Davidoff et al [61,62] Logie et al [63] Repiščák et al [64] Glossmann and Lutz [65]
Metformin utilizes cation transporters to cross cell membranes: the bi-directional Organic Cation Transporter (OCT) 1, 2 and 3 (SLC22A1, A2, A3); Plasma membrane Monoamine Transporter (PMAT; SLC29A4); and Multidrug And Toxin Extrusion protein (MATE) 1 and 2 (SLC47A1, A2), to enter and leave cells with high levels of OCT1 expressed in the liver (see also Fig. 2). Based on the short half-life of metformin it is unlikely that there is significant accumulation in tissues.	Gong et al [66] Chen et al [67] Sirtoli et al [55] Koepsell et al [68] Schmiit & Gorboulev [69]
Metformin transport via OCT transporters is bidirectional (Koepsell et al), and based on ^{11}C-metformin-PET studies tissue (liver) levels passively equilibrate with plasma levels. Conclusion: Cellular/tissue retention of metformin is likely transient and therefore that metformin exerts its clinical actions, including an anti-proliferative effect, via inhibition of mitochondrial respiration.	Koepsell et al [68] Gormsen et al [70] Iversen et al [71]

skeletal muscle insulin sensitivity (90% versus 55%) [41]. In contrast, the combination of exercise and metformin resulted in only a 30% enhancement [41]. In a double-blinded study of the effects of exercise on healthy men and women over the age of 65, the *Metformin to Augment Strength Training Effective Response in Seniors (MASTERS)* trial (NCT02308228), treatment with metformin resulted in the blunting of exercise-induced hypertrophy in skeletal muscle [42]. Other studies have raised similar concerns about metformin negating the benefits of exercise [43,44]. Konopka et al., 2018 [43] noted that metformin reduced the benefit of exercise on mitochondrial adaptations as reflected by inhibition of exercise-induced improvement in mitochondrial respiration and also cardiorespiratory fitness in elderly patients (~60 years of age) with T2D. In the Look AHEAD study Terada and Boulé [39] reported that the addition of metformin to those undergoing Intensive Lifestyle Intervention (ILI) (defined as at least 175 min of moderate exercise/week plus caloric reduction) did not enhance the benefits of ILI on cardiorespiratory fitness and weight loss. Since exercise is considered the 'Gold Standard' for improving cardio-respiratory health these data raise a cautionary red flag for the re-purposing of metformin beyond its current use as an anti-hyperglycemic drug.

Metformin is also being increasingly used in pregnant women with gestational diabetes and although there is an absence of data from large scale studies, the anti-folate effects of metformin that have been reported in the nematode *Caenorhabditis elegans* (*C. elegans*) may also have implications in fetal development during pregnancy [45]. Although the studies with *C. elegans* used very high concentrations of metformin (25, 50 and 100 mM) data from human studies suggest that the use of metformin may negatively impact fetal development during pregnancy and be linked to vitamin B12 deficiency with consequent implications for the treatment of gestational diabetes [46]. Concerns have also been expressed that metformin at the equivalent of comparatively high therapeutic concentrations (50–100 μM) negatively affects pancreatic beta-cell differentiation from human embryonic stem cells resulting in metabolic dysfunction in later life [47,48]. These concerns add to those raised in the study from Denmark linking metformin use in men with genital defects in male offspring [36].

4. Physiochemical and pharmacokinetic properties of metformin

In order to analyze the cellular actions of metformin it is important to emphasize using therapeutically appropriate concentrations of metformin as reflected in the title of a Cell Metabolism paper in 2015 by He and Wondisford: *"Metformin action: Concentrations matter"* [49]. Attention to the concentration and dose used are important and not infrequently in pre-clinical in vitro studies have employed concentrations in excess of x10 to x1000 maximal plasma levels observed in humans. Similarly, very high doses have been used for some in vivo studies in animal models [50]. Although in some instances there may be valid arguments for using such high concentrations/doses caution is needed before justifying the applicability of the data to define the mechanism of action and effects of metformin in humans [51,52]. It is therefore important to consider the physiochemical and pharmacokinetic properties of metformin, which are summarized in Table 2.

Table 3
Metformin and mitochondria function.

Studies in support of metformin mediating cellular actions via inhibition of mitochondrial function.	Evidence of concentration/dose-dependent effects of metformin independent of inhibition of complex 1.
El-Mir et al [77]: Demonstrated that 1, 5 and 10 mM metformin and a 20–30 min incubation inhibited mitochondrial complex 1 in isolated hepatocytes or isolated liver mitochondria from rats. Inhibition not seen in permeabilized hepatocytes or mitochondria. Suggested that since inhibition only seen in intact cells that a signaling process is involved rather than direct inhibition of mitochondrial function.	Schäfer [79]: Reported that metformin has a low binding affinity for mitochondria membranes; however, the binding affinity for the alkyl biguanide derivative, phenformin, was reported as x 50 higher.
Owen et al [78]: a. Exposure for 24 or 60 h with 50 and 100 μM metformin inhibited mitochondria respiration in rat hepatoma (H4IIE) cells permeabilized with digitonin. b. Isolated rat liver hepatocytes required long exposure time at 8C to 10 mM metformin to inhibit NADH-dependent respiration. $K_{0.5}$ for metformin reported as 14.9 mM. c. Hepatocytes were isolated from rats after oral treatment with metformin (50 or 150 mg/kg) for 5 days and the ATP/ADP ratio shown to have dropped by 20–32%. Conclusion: A slow accumulation of metformin driven by mitochondria membrane potential inhibits complex 1.	Wilcock et al [80]: Studied the distribution of ^{14}C-metformin in the rat liver and concluded that 78% of the metformin was associated with the cytosol and only <10% with the mitochondria. Meng et al [81]: Low concentrations of metformin (25–100 μM) activated AMPK in isolated hepatocytes from mice, whereas high concentrations (≥ 500 μM) resulted in inhibition. Concluded that metformin activated via stabilizing the heterotrimeric α, β, γ, complex of AMPK, promoting phosphorylation at Thr-172 through augmenting phosphorylation by the upstream serine-threonine kinase, LKB1.
Stephenne et al [83]: 500 μM and 1000 μM, but not 100 μM metformin, reduced mitochondrial oxygen rate, lowered the ATP/ADP ratio, and activated AMPK in isolated hepatocytes from rats and humans.	Larsen et al [82]: a. No evidence of the inhibition of mitochondria complex 1 was shown in skeletal muscle biopsies taken from patients with type 2 diabetes treated with metformin. b. Threshold for inhibition of complex 1 by metformin in rat skeletal muscle reported to be 1 mM. Ravera et al [84]: The effects of low concentrations (15 and 150 μM) of metformin that reflect (15 μM) therapeutic levels versus a high concentration (1.5 mM) on were investigated in Fanconi Anemia cells and HL60 leukemia cells. Only the low concentrations (15 and 150 μM) of metformin activated oxidative phosphorylation, the oxidative stress response and the AMPK/Sirt1 pathway, whereas 1.5 mM proved toxic.
Chien et al [85]: Based on data using ^{14}C-metformin distribution following a 60 min incubation with 5 μM metformin in HEK cells in which the cation transporter, OCT1, had been overexpressed it was concluded that metformin could be trapped in intracellular organelles including endoplasmic reticulum and mitochondria up to ~200 μM.	Wang et al [86]: The effects of metformin on complex 1 and its subcellular distribution at either 75 or 1000 μM were investigated in murine hepatoma, Hepa 1–6, cells. No inhibition of complex 1 was observed at either 75 or 1000 μM and metformin remained primarily in the cytosol with levels in mitochondria ~70 μM. Venu et al [87]: Describe an important role for the orphan nuclear receptor NR4A1 in mediating the endothelial protective actions of μM concentrations of metformin against hyperglycemia-induced oxidative stress. In additional, and based on Seahorse analyzer (XFe24) assessment of oxygen consumption rates low (10) μM concentrations of metformin enhanced, whereas high (>250) μM inhibited mitochondria complex 1. LaMoia et al [88] piericidin A, an inhibitor of mitochondrial complex 1, did not reduce hepatic gluconeogenesis when infused directly unto the liver. Parallel studies also performed in rat liver slices.

5. Putative mechanisms and sites of action for antihyperglycemic effects of metformin

Early assumptions about the mechanism of action of metformin highlighted the liver as the major site of action as an anti-hyperglyaemic drug. The high expression of the organic cation transporter, OCT1, in the liver facilitates the rapid uptake of metformin (Table 2); however, other sites of action are important including effects in the GI tract prior to its absorption. A hint that metformin's mechanisms of action are complex is provided by a number of observations. First, a benefit for clinical use of metformin is the very low risk of hypoglycemia when used as monotherapy. Second, as reported by Bonora et al. in 1984, intravenous (IV) metformin does not lower blood glucose levels in non-diabetic subjects, and, supported by data from subjects with T2D using a hyperglycemic clamp technique, the acute administration of IV metformin does not reduce hepatic glucose production or affect peripheral glucose disposal [72,73]. These data imply that the anti-hyperglycemic action of metformin requires chronic administration. Further, an important contribution to the antihyperglycemic effects of metformin has been attributed to a pre-absorption effect in the lower GI tract. In this regard, a delayed release formulation of metformin, despite considerably reduced bioavailability (50%), has been shown to have a greater effect on fasting plasma glucose than the intermediate- and extended-release formulations, which are primarily absorbed in the upper GI tract [74,75].

5.1. Role of mitochondria as a target for metformin

Mitochondrial dysfunction has been linked to a number of chronic diseases, including diabetes and neurodegenerative diseases [76] The argument that metformin may mediate its multiple putative benefits via actions on mitochondrial function centers on many of the effects of metformin being linked to the activation of AMP-activated protein kinase (AMPK). The hypothesis was first advanced in 2000 as a result of two independent studies of the effects of metformin on isolated hepatocytes [77,78].

Summaries of these studies are provided in Table 3 and in Fig. 2 where metformin is depicted to act as a weak mitochondrial poison that inhibits complex 1 thereby reducing the ATP/AMP ratio with the activation of AMPK resulting from the elevated levels of AMP [77,78] Furthermore, since metformin exists as a cation at physiological pH and with an estimated potential difference across the inner membrane of approximately 120–150 mV it is argued that metformin would accumulate in mitochondria to between 100 and 300 fold relative to the plasma concentration [89]. While an attractive hypothesis a concern is the concentrations of metformin required to inhibit complex 1. Despite arguments in support of the *"Complex I Hypothesis"* [90] there is a lack of convincing data that with the low μM plasma concentrations seen during clinical use and combined with the short plasma half-life, that metformin can accumulate in mitochondria to sufficient levels to inhibit complex 1. Confounding issues are: 1) the IC_{50} for the inhibition of complex 1 by metformin is reported to be 19.6 mM [80]; 2) Concentrations ≥8 mM are required to impair the respiratory chain and oxidative phosphorylation in isolated mitochondria and even at these concentrations inhibition of hydrogen peroxide production is not observed [91]; 3) piericidin, a potent inhibitor of complex 1 does not inhibit hepatic gluconeogenesis as demonstrated in a protocol where the inhibitor was infused into the livers of rats by an indwelling portal vein catheter [88] and, 4) the in vitro protocols used to demonstrate inhibition of complex 1 frequently use long exposure times and metformin

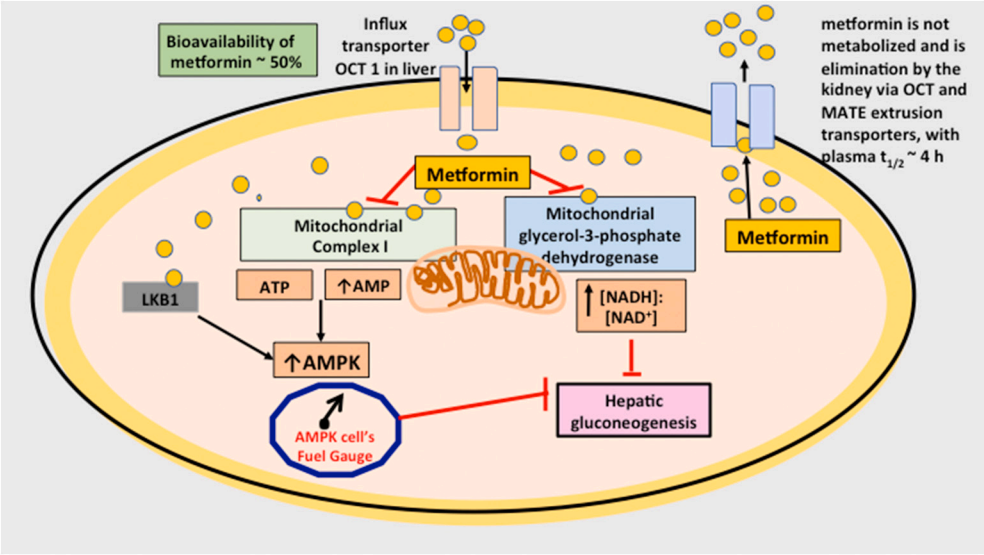

Fig. 2. Summary of the putative effects of metformin on mitochondria function and regulation of gluconeogenic genes.
Metformin, ●, is a strong base and exists as a cation at physiological pH and as depicted in this schematic requires the availability of organic cation transporters to cross cell membranes. Primarily the organic cation transporters, OCT 1, 2 and 3 (SLC22A1, A2, A3) and the plasma membrane monoamine transporter (PMAT, SLCC9A4) transport metformin into cells with the multidrug and toxin extrusion (MATE1/2, SLCC47A1, A2) transporters serving for elimination. OCT1 is the predominant OCT in liver cells. The plasma half-life ($t_{1/2}$) is approximately 4 h. Metformin has a bioavailability of approximately 50%, which, due to absorption-limited pharmacokinetics that results from saturation of intestinal transporters, is reduced at higher doses [105]. With a typical dose range from 250 to 2550 mg/day a considerable amount of metformin remains in the GI tract and a component of metformin's beneficial effects are mediated by gut-based mechanisms, but at the same time metformin in the gut interferes with vitamin B12 absorption.

has a short plasma half-life of approximately 4 h (h) (2–6 h) (Table 2) implying that cellular levels are rapidly lowered [92]. In contrast, low μM concentrations of metformin enhance complex 1 activity [52,79,81,84,86,92–96]. Finally, concentrations of metformin as low as 5 μM inhibit gluconeogenesis in primary hepatocytes from mice without altering the ATP/AMP ratio [97].

In conclusion, an accumulation of data implies that the therapeutic actions of metformin are mediated by signaling pathways that do not depend on the inhibition of complex 1 as evidenced by the concentration/dose-dependent effects of metformin and also reflected in the title of a 2021 publication by Panfoli et al.: *The Hormetic Effect of Metformin: "Less Is More"?* [95]. An exception may be in the intestinal enterocytes where an accumulation of metformin occurs following oral ingestion and lactate production is increased as reported in biopsies of human jejunal mucosa [98].

Nonetheless, if metformin does lower the ATP/AMP ratio, then the increase in cellular levels of AMP would also serve to inhibit adenylyl cyclase activity, reduce c-AMP, and inhibit protein kinase A (PKA) and the cAMP response element-binding protein (CREB)-mediated activation of gluconeogenic genes and subsequent enhancement of hepatic gluconeogenesis. This novel mechanism is supported by data reported by Miller et al. (2013) [99] and implies that metformin would reduce the enhanced effects of glucagon that is a contributor to the hyperglycemia associated with diabetes [100]. Of note is that hyperglycemia increases PKA activity and this results in phosphorylation and inhibition of glucose-6-phosphate dehydrogenase (G6PD), which is the rate-limiting enzyme of the pentose phosphate pathway and also plays an important role in reducing oxidative stress via enhancing the $NADPH/NADP^+$ ratio [101,102]. Theoretically, an increase in cellular AMP would serve not only to reduce hepatic gluconeogenesis, and lower glucagon levels, but also to offset oxidative stress associated with hyperglycemia and thereby enhance healthspan (defined as the period of life spent in good health), as has been demonstrated in mice with enhanced expression of human G6PD [102]. However, contrary to the results from the rodent-based studies of Miller et al. (2013) [87] are data from a randomized, crossover, placebo-controlled, double-blinded study in prediabetic patients indicating that treatment with metformin not only increased insulin sensitivity and glucose tolerance but also increased plasma glucagon and was associated with enhanced endogenous glucose production in individuals with hyperglucagonemia [103]. In addition, the expression of gluconeogenic genes as well as those regulating lipogenesis are inhibited by AMPK [104].

Fig. 2 reflects the mitochondrial basis for metformin's inhibitory effect of hepatic gluconeogenesis that has been attributed to: 1. Inhibition of the electron transport chain of mitochondrial complex 1 that results in a reduction in ATP levels thereby increasing the AMP/ATP ratio; a small increase in AMP promotes the phosphorylation of Thr-172 and the activation of AMPK [77,78] Activation of AMPK can also inhibit gluconeogenesis and has been also shown to enhance the release of GLP-1 from the intestine. 2. There is also evidence that metformin activates AMPK via the serine-threonine liver kinase B1 (LKB1, which is an upstream regulator of AMPK. 3. An alternative site of action for metformin is mitochondrial glycerol-3-phosphate dehydrogenase (mGPD). Inhibition of mGPD results in an increase in the cytosolic redox state ([NADH]:[NAD+] as a result of disrupting the α-glycerophosphate redox shuttle [106–108]. In a 2022 publication LaMoia et al. propose that inhibition of mGPD is indirect and results from inhibition of mitochondrial complex IV [88].

The main argument against inhibition of complex 1 as the basis of metformin's effects on hepatic gluconeogenesis is the high concentration of metformin that is required to inhibit complex 1 and that piericidin A, a complex 1 inhibitor, does not reduce hepatic gluconeogenesis, whereas mGPD is inhibited at low μM concentrations of metformin [88,106–108]. However, the data supporting the argument that metformin is a potent inhibitor of mGPD have been challenged [109–111]. An alternative viewpoint is that the effects of metformin on mitochondrial complex 1 function are indirect via inhibition of reverse-electron transport (RET) and reduces superoxide generation; however, whether RET is a target for metformin when the drug is used clinically remains unproven [112].

Mitochondrial glycerophosphate dehydrogenase (mGPD) is the rate-limiting enzyme of the glycerol phosphate redox shuttle that plays an important role in carbohydrate and lipid metabolism and has also been proposed as a target for metformin. Madiraju et al. (2014) have reported that metformin is a non-competitive inhibitor of mGPD and increases the [GSH]:[GSSG] ratio in the liver [106]. This increased ratio leads to a decrease in the mitochondrial redox state and an increase in the cytosolic redox state, a decrease in the mitochondrial redox state, such that the total NADH/NAD+ ratio remained unchanged with no change in AMPK activity or in downstream targets of AMPK [106]. Both acute (IV)

and the chronic (intraperitoneal) administration of metformin lowered hepatic glucose production in rats. This result was based on dosages of 20 mg/kg and 50 mg/kg respectively that are comparable to the dose range, 500–2550 mg/day, used in patients with T2D and equate to plasma levels of metformin approximating 25–50 μM [106]. Support for mGPD2 as the primary target for metformin was provided using in vivo carbon flux analysis that, within the same concentration range, also negates mitochondrial complex 1 as a target for metformin, and is based on a redox-dependent mechanism that regulates [NADPH]:[NAD] ratio and hepatic gluconeogenesis [107,108]. This hypothesis has been revised such that the target for metformin is complex IV and the inhibition of mGPD2 is indirect resulting from interrupting the glycerolphosphate shuttle as a result of a backlog of the electron transport chain [88]. However, the argument for mGPD2 as the hepatic target for metformin has been critically challenged by data that show low micromolar concentrations of metformin do not reduce lactate-induced glucose output [93,109]. These findings are also discussed in a commentary by Glossmann and Lutz (2019) [110] and of note is that the protocols adopted by Madiraju et al. (2014, 2018) [106,107] describe the effects of metformin on fasting plasma glucose (FPG) levels in non-diabetic rats; but as already discussed it is established that metformin does not affect FPG in non-diabetic humans and neither does acute IV administration of metformin lower plasma glucose in T2D. In addition, a number of studies have failed to demonstrate that metformin has a significant effect on mGPD2 except at mM concentrations that generate high levels of reactive oxygen species (ROS) [93,109]. Finally, MacDonald et al. (2021) have reported that metformin did not inhibit mGPD2 in homogenates, in mitochondria from mouse pancreatic cells, or liver cells [111]. MacDonald et al. (2021) also raise the concern based on the comparative low activity of mGPD2 in the liver compared to many other tissues. For instance, mGPD2 activity is 30 to 60 times higher in pancreatic islet cells than the liver and inhibition of mGPD2 in tissues other than the liver would have significant adverse effects [111]. Finally, there are also concerns over the protocol design in the 2022 study by LaMoia et al. [88] wherein metformin was infused directly into the liver via portal vein catheter at 100 mg[kg/h] for 1 h.

Collectively, these data argue against complex 1 or mGPD2 via inhibition of complex IV as the therapeutic targets of metformin, but do not rule out metformin having other effects on mitochondria function. For instance, metformin treatment of T2D patients reduces plasma glucose and improves endothelium-leukocyte dynamics arguably by reducing their interaction through raising mitochondrial membrane potential, thereby normalizing mitochondrial dynamics and lowering the generation of mitochondrial reactive oxygen species [113,114].

5.2. Role of AMPK in the anti-diabetic actions of metformin

AMPK has been described as the fuel gauge, or fuel sensor, of the cell [115]. AMPK is a key regulator of a number of metabolic functions including enhancing glucose uptake, increasing glycolysis, fatty acid oxidation and mitochondria biogenesis while decreasing gluconeogenesis, glycogen synthesis, protein synthesis and proliferation as well as decreasing fatty acid and cholesterol synthesis. AMPK also activates endothelial nitric oxide synthase (eNOS) via the phosphorylation of Ser1177 thereby providing an explanation for the protective effects of metformin on endothelial function that is summarized in a later section [116]. Zhou et al. (2001) first demonstrated that metformin, at concentrations of 10 and 20 μM, activated AMPK in hepatocytes isolated from rats, thus providing a cellular mechanism for its antiyhperglycemic action via the inhibition of liver gluconeogenesis [117]. Meng et al. (2015) reported that therapeutic levels of metformin stabilize the α, β, γ, complex and activates AMPK independent of the inhibition of complex 1 [81]. It has also been argued that metformin activates AMPK indirectly via LKB1 [118]. In addition, metformin activates AMPK in skeletal muscle of patients with T2D and thereby enhances glucose disposal [119]. However, AMPK and LKB1-independent effects of metformin have also been documented as shown in mice with a liver-specific knockout of AMPKα2 [120].

5.3. Contribution of pre-absorption effects of metformin in the GI tract

In 1998 Lugari reported that in patients with T2D there was an increase in post-prandial GLP-1 via an AMPK-dependent mechanism [121]. Subsequently, a number of studies have confirmed that a significant component of metformin's anti-hyperglycemic action occurs before it is absorbed from the gut and results from the release of GLP-1 via an AMPK-dependent action [74,122–125]. In addition, following an oral dose a significant amount of metformin remains in the gut with concentrations estimated to be 30 to 300 fold higher than in the plasma [98]. Metformin also alters the microbiome by increasing the growth of some bacteria whilst decreasing others, and also enhances the role of a SGLT1-glucose-sensing pathway in the upper small intestine [126–128].

5.4. Contribution of Growth Differential Factor 15 (GDF15) to the effects of metformin

Metformin enhances the release of the novel cytokine, GDF15, a member of the transforming growth factor β superfamily, which is highly expressed in adipocytes, cardiomyocytes, endothelial cells, and macrophages [129]. GDF15 has been linked to positive cardiovascular (CV) outcomes, anti-aging, and anorexic actions that facilitate weight loss, however, the release of GDF15 is not required for the anti-hyperglycemic actions of metformin [130–133]. GDF15 binds to the GDNF (glial-cell-derived neurotrophic factor) family α-like (GFRAL) receptor, which is only expressed in the hindbrain of mice and has been shown to have potent effects on obesity and mediating weight loss [134,135]. The binding of GDF15 to GFRAL facilitates the formation of a complex with the transmembrane tyrosine kinase coreceptor and proto-oncogene, RET (REarranged during Transfection) [136].

Interestingly, GDF15 levels are elevated in a number of, but not all, cancers, and GDF15 has been proposed as a biomarker for digestive system tumors and elevated serum GDF15 in cancer patients is associated with reduced muscle mass and anorexia [137–141]. Clarification is required as to whether the elevation of GDF15 in some cancers, such as cervical cancer, contributes to the growth of the cancer, or is secondary and promotes apoptosis and serves a role as a tumor suppressor and thereby contributes to the putative anti-cancer effects of metformin [141,142].

6. Metformin, T2D and cardiovascular disease

6.1. Clinical studies of metformin in T2D

The results from the UKPDS, a 20-year randomized, multicenter study of patients with T2D, provided convincing evidence that intensive blood-glucose control decreases micro- and macrovascular disease [24,143], and that the use of metformin in patients with T2D, and in particular in overweight patients, significantly reduced diabetes-related death and all-cause mortality over a 10-year period [18]. These conclusions were based on 1704 overweight subjects of whom 342 were treated with metformin, 265 with the first generation sulfonylurea, chlorpropramide, 277 with the second generation, glibenclamide, 409 on an insulin regimen, and 951 as the internal control group [18]. A number of follow up studies have supported the conclusions of UKPDS including that monotherapy with metformin versus monotherapy with a sulfonylurea reduced CV morbidity and mortality [144]. A ten-year follow up of UKPDS reported a continued reduction in microvascular risk, MI and all-cause mortality [145]. A meta-analysis of 40 studies comprising over 1 million patients also supports the conclusion that metformin reduces all-cause mortality, CV mortality, and CV events in patients with coronary artery disease (CAD) and T2D, but not for non-T2D patients with CAD and post MI [146]. Comparable results, but

based on a smaller number of patients in Taiwan, were reported by Jong et al., (2019) [147]. However, although results from the DPP research group provided support for the long-term safety and weight loss benefits of metformin, as previously discussed, the analysis also indicated that metformin reduced the benefits of lifestyle intervention [26,40]. Concerns have also been raised by an earlier meta-analysis that questioned the UKPDS conclusions and a possible risk of bias in the analysis [148]. Furthermore, based on the meta-analysis of 13 randomized trials that included 2079 patients, not all studies have indicated the same level of benefits with metformin as reported by UKPDS and indicate the need for additional trials preferably comparing metformin with newer anti-hyperglycemic drugs [149].

Masson et al. (2021), based on a meta-analysis of studies with SGLT-2 inhibitors and GLP-1 receptor agonists concluded: *"metformin would not be indispensable to obtain positive cardiovascular effects when new anti-diabetic drugs are administered"* [150]. In 2016 Boussageon et al. argued for: *"A big and beautiful trial for glucose lowering drugs in type 2 diabetes"* that would be double-blinded with appropriate follow up for at least 10 years and enroll 5000 to 10,000 participants [151]. Others, however, have commented on the potential beneficial effects of metfomin in countering the development of the serious sequalae of diabetes such as heart failure and arguing for appropriate CV outcome trials to provide evidence of whether, for instance, there are superior benefits to metformin versus SGLT2 inhibitors for the prevention of heart failure secondary to diabetes [152]. Schernhaner et al. (2022) have summarized the current evidence and status of on-going clinical trials to assess the benefits of metformin versus other anti-diabetic drugs and cardiovascular outcomes [153]. For instance, the RCT, SGLT2 Inhibitor or Metformin as Standard Treatment of Early Stage Type 2 Diabetes (SMARTEST) study (NCTO3982381) due for completion in late 2025, compares the CV benefits of metformin versus dapagliflozin in 4300 patients with T2D.

Of note is that comparisons of data derived from different trials versus UKPDS are complicated by the so-called "legacy effect" noted in the UKPDS wherein the CV benefits were not immediately apparent and observed on follow-up only after >10 years [154]. Despite positive clinical trial data concerning the gliflozins and GLP-1 receptor agonists and their CV protective benefits in patients with T2D, an analysis based on data from the UK's Clinical Practice Research Datalink (CPRD) indicated that metformin remained the drug of choice and was prescribed to >70% of the patients with or without CVD [155]. It should also be noted that subsequent to the UKPDS trial second- and third generation sulfonylureas, such as glimepiride and glipizide, that are more potent and considered safer are in use and as supported by a Cochrane systematic review and meta-analysis may reduce non-fatal macrovascular outcomes [156]. Another complication with respect to the long-term treatment of T2D and the benefits of individual drugs is that the UKPDS 49 report [157] concluded that in order to achieve HbAIc below 7.8 mmol/l, 50% of patients within 3 years of diagnosis required more than one anti-diabetic drug. Thus, for many people with T2D it is important to consider not just the risk-benefits of treatment with metformin, but also the effects of a combination of metformin with additional anti-diabetic drugs.

6.2. Metformin and the endothelium

6.2.1. Studies in humans

Endothelial function can be directly assessed in vivo by determining the effectiveness of an endothelium-dependent vasodilator, usually acetylcholine, or measuring flow-mediated vasodilation (FMD). Endothelial dysfunction can also be assessed indirectly by measuring non-specific biomarkers of vascular inflammation such as C-reactive protein (CRP), as well as biomarkers of vascular inflammation including P-selectin, vascular cell adhesion molecule-1 (VCAM-1), von Willibrand Factor (vWF), soluble intercellular adhesion molecule (sICAM-1), plasminogen activator inhibitor-1 (PAI-1), and tissue type plasminogen activator (t-PA). Endothelial dysfunction is considered to be a *'barometer'* for cardiovascular risk and can be defined as a reduction in endothelium-dependent vasodilation (EDV) in response to an endothelium-dependent vasodilator and is considered to be the earliest indicator of the development of cardiovascular disease [158,159]. A wealth of pre-clinical and clinical data provides support for a pleiotropic action of metformin on the endothelium through protecting endothelial cells from hyperglycemia-induced oxidative stress and senescence. There are a number of comprehensive reviews on the endothelium and the effects of metformin [160–163].

Mather et al. (2001) [164] used forearm strain-gauge plethysmography to assess the effects of a three month twice-a-day 500 mg metformin treatment versus placebo on forearm blood flow in metformin naïve subjects with T2D. The comparison of the effects of intra-brachial artery administration of acetylcholine versus endothelium-independent vasodilators such as sodium nitroprusside, or verapamil, revealed that metformin lowered insulin resistance and improved EDV, but not endothelium-independent vasodilation (EIDV). These results indicate that endothelial dysfunction was the primary defect corrected by metformin and the benefits were presumed to be secondary to improved insulin sensitivity [164]. Comparable results were reported by Vitale et al. (2005) for a study of the effects of metformin (500 mg bid) for 3 months on endothelial function in patients with metabolic syndrome where again the improvement in EDV, as determined by FMD of the brachial artery, was linked to a reduction in insulin resistance [165]. The conclusions of the Mather et al. and Vitale et al. studies were confirmed by a larger randomized placebo-controlled study of patients with T2D who were treated for 52 months with metformin and demonstrated lower levels of a number of biomarkers of endothelial dysfunction: PAI-1, siCAM-1, t-PA, and vWF [166].

Benefits of metformin on endothelial function have also been reported in non-diabetic subjects. In a report that preceded the results of UKPDS it was demonstrated that a six month treatment with 850 mg/day of metformin, versus placebo, improved FMD in patients with peripheral artery disease but free of diabetes; no changes in fasting glucose or insulin were noted but treatment did improve the lipid profile by raising HDL and lowering triglycerides in some patients, but not VLDL [167]. Further support is provided by data from a study of acetylcholine-mediated EDV in 31 first-degree relatives of T2D patients with metabolic syndrome, but normal glucose tolerance, that showed that metformin, 850 mg/bid for at least 90 days, lowered BP and improved endothelial function independent of effects on fasting glucose [168]. In addition, in T1D the addition of metformin (850 mg/tid) to the insulin regimen for 6 months improved FMD, but not EIDV, mediated by glyceryl trinitrate [169]. Interestingly, the 6-month treatment with metformin did not change any of the metabolic parameters of the T1D patients, including HbA1c, but paradoxically enhanced plasma PGF2α, a marker of oxidative stress [169]. Jahn et al. (2022) reported that in a 12 week treatment with metformin versus placebo crossover study of patients (~53 years of age) with metabolic syndrome although metformin did not affect basal fasting glucose, improve aortic stiffness, or enhance basal brachial artery FMD, it did increase skeletal muscle insulin sensitivity and microvascular perfusion [170].

Overall, these data suggest that metformin has beneficial effects on vascular function beyond improving glycemic control. However, prospective studies designed to determine whether metformin has CV protective benefits independent of its antihyperglycaemic actions have not always provided positive results [171]. When used to treat T2D, the beneficial effects of metformin have been the attributed primarily to its insulin sensitizing actions that enhance glucose disposition in striated muscle and adipose tissue, reduce hyperglycemia and thereby reduce oxidative stress in tissues like the endothelium. See Fig. 3 for a summary of the CV benefits of metformin.

6.2.2. Pre-clinical studies

Metformin has also been shown to both correct endothelial dysfunction in aortae from non-diabetic spontaneously hypertensive rats

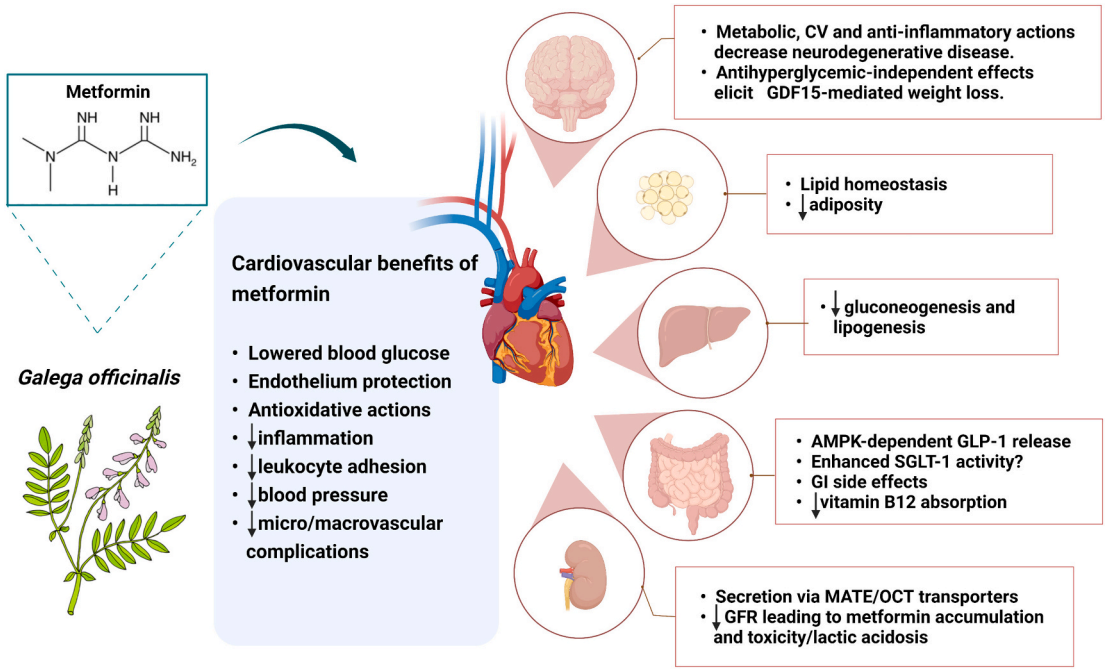

Fig. 3. The cardiovascular benefits of metformin.
The cardiovascular (CV) benefits of metformin result from its effects at muliple sites. At the level of the GI tract prior to absorption metformin enhances the release of glucagon-like peptide 1 (GLP-1). Metformin also has effects on the microbiota as well as the activity of the sodium-glucose co-transporter (SGLT-1) and improves glucose sensing. Overall, the insulin sensitizing action of metformin helps to improve glycemic control via enhancing glucose uptake into striated muscle and adipose tissue. In the liver, metformin, via the activation of AMPK, reduces gluconeogenesis and lipogenesis. Unrelated to its anti-hyperglycemic actions, metformin also promotes the release of the cytokine, growth differential factor 15 (GDF-15) that via effects in the CNS mediates the anorexic, and putative anti-aging actions of the drug. Collectively, these actions reduce the negative effects of hyperglycemia, reduce oxidative stress, and improve endothelium-vascular function thereby contributing to reduced CV morbidity. Improved CV health may also contribute to reduced risk of other diseases, including renal and neurodegenerative diseases. This figure was created with BioRender.com.

(SHR) and to lower blood pressure and the beneficial effects were also apparent in SHR with streptozotocin-induced diabetes [172].

A number of potential cellular targets in endothelial cells other than mitochondrial complex 1 have been proposed as targets for metformin. Sirtuin-1 is an NAD-dependent deacetylase and the protein product of the 'anti-aging' gene, SIRT-1 [173]. Sirtuin-1 is important for the regulation of angiogenesis, protects against oxidative stress, senescence and CVD, and positively regulates via deacetylation the serine-threonine kinase, LKB1 [174–177]. Furthermore, sirtuin-1 also deacetylates lysines 496 and 506 on endothelial nitric oxide synthase (eNOS) and

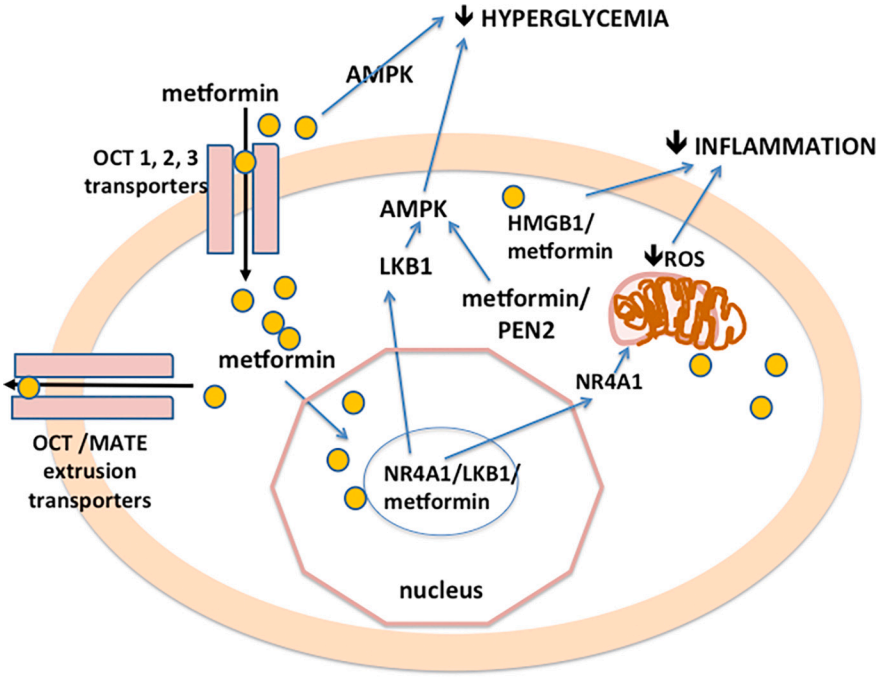

Fig. 4. NR4A1 and other proteins as targets for metformin.
It has been proposed that metformin (●) interacts with multiple proteins including the orphan nuclear receptor NR4A1 to increase AMPK, decrease inflammatory signals, and reduce cellular ROS. Metformin interacts with multiple proteins including orphan nuclear receptor NR4A1 to increase AMPK, decrease inflammatory signals and reduce cellular oxidative stress (ROS). The scheme shows the cellular uptake of metformin via the OCT transporters, and secretion by MATE and OCT transporters. Metformin interacts with targets in the cytosol such as the high mobility group box 1 protein (HMGB1), an alarmin, to inhibit HMGB1-mediated increases in inflammatory signals [180]. Metformin also binds to presenilin-enhancer protein-2 (PEN2) [97] to increase AMPK activity and also inhibit endosomal/lysosomal v-ATPase activity (see also Fig. 14). Metformin enters the nucleus to interact with the resident orphan nuclear receptor, NR4A1. The latter interaction releases NR4A1-bound liver kinase B1 (LKB1) into the cytosol, where it activates AMPK [181]. The metformin-NR4A1 interaction also releases NR4A1 into the cytosol, where it traffics to the mitochondria to suppress the production of ROS. This action has been shown to preserve endothelial function in the setting of hyperglycemia [87]).

thereby enhances NO-mediated EDV [178]. Of particular interest is the requirement for the expression of sirtuin-1 in linking the endothelial-vascular protective effects of metformin and reducing hyperglycemia/oxidative stress-induced endothelial senescence in murine endothelial cells [179].

In vitro studies with isolated murine blood vessels, endothelial cells in culture and in silico modeling have identified the orphan nuclear receptor, NR4A1 (Nur77) as critical for mediating the protective effects of metformin against hyperglycemia-induced endothelial dysfunction independent of metformin's impact on blood glucose Venu et al., (2021 [87]). As depicted in Fig. 4 NR4A1 is among a number of proteins that can bind metformin directly. Remarkably, the endothelial protective effects of metformin are observed in the low micromolar range, 1 to 10 μM, and are associated with protective effects due to the reduction of hyperglycemia-mediated, mitochondrial-generated ROS, without inhibition of complex 1. The data indicate a role for NR4A1/Nur77 in mediating the vascular protective effects of metformin in patients with T2D [87]. Of significance, metformin-NR4A1 interactions regulate the localization of LKB1 that in turn activates AMPK (Fig. 4 and reference [181]). However, the signaling pathway that mediates the protective effects of metformin on endothelial cell function via NR4A1/Nur77 requires further investigation.

Metformin, at a therapeutically appropriate concentration (20 μM), inhibits the pro-inflammatory NF-κβ pathway via blocking PI3K–Akt in human endothelial and vascular smooth muscle cells in culture [182]. Metformin has also been reported to inhibit high glucose induced NF-κB activation that was associated with an increase in AMPK phosphorylation in rat glomerular mesangial cells in vitro, but much higher concentrations of metformin were used in this study in the range 0.5 to 2 mM [183]. Similarly, in 2006 Hattori et al. reported that metformin at 10 mM, a concentration much higher than that found therapeutically in humans, inhibited cytokine-induced activation of NF-κB in human umbilical vein endothelial cells via an AMPK-dependent pathway [184]. Additionally, in the same study, metformin was shown to inhibit the induction of the mRNAs for adhesion molecules including the chemokine monocyte chemoattractant protein (MCP-1), VCAM-1, soluble *E*-Selectin, and sICAM-1 [184].

6.2.3. *Summary, metformin and the endothelium*

In conclusion, evidence from a variety of sources indicates that metformin has endothelial-vascular protective effects independent of the drug's anti-hyperglycemic actions. The direct protective actions of metformin on the endothelium, combined with its effects on cell metabolism result in a reduction of ROS and decreased vasculoinflammation. These effects of metformin combined with actions in the gut prior to absorption, could provide therapeutic benefits that extend beyond T2D. Based on data from both pre-clinical and clinical studies metformin has been investigated to treat atherosclerosis, aging, neurodegenerative diseases as well as broadly to other diseases with an inflammatory component including rheumatoid arthritis, cancer and COVID-19.

7. The repurposing of metformin

There is a long history of interest in using guanidines and biguanides for a variety of diseases [78], with a recent resurgence of attention [185,186]. Specifically, the repurposing of metformin has been investigated for PCOS, cancer, rheumatoid arthritis, neurodegenerative diseases, including cognitive dysfunction and dementia, as an anti-aging drug, for treating parasitic infections such as malaria, use as an antibiotic, and for treating COVID-19. Collectively, these repurposed uses imply that metformin is truly a multi-purpose drug for all diseases [13,187–193]. These highlighted benefits of repurposing of metformin were recognized in the 2017 review article, which had the appropriate title: *"Metformin, the aspirin of the 21st century—"* [194]. However, the question arises *"How strong is the evidence?"* The objectives of the remainder of this review are to: 1) Critically analyze the controversial evidence for metformin's effectiveness in the treatment of diseases other than T2D; 2) Evaluate the putative mechanism(s) of action of metformin; 3) Assess the reproducibility of the data, and, finally, 4) Reach an informed opinion as to whether metformin really is a drug for all diseases and reasons.

7.1. *Type 1 diabetes (T1D)*

The potential benefits of using metformin as an adjunct in combination with insulin to treat T1D were recognized as early as 1985 [195]. As reflected in Fig. 5 and based on the number of publications, interest has rapidly increased with the argument that the insulin-sensitizing effects of metformin would allow the dosage of insulin to be reduced; an argument that is supported by the results of one systematic review [196]. However, this conclusion was not supported by the results of the REMOVAL trial (REducing with MetfOrmin Vascular Adverse Lesions (NCT01483560), a placebo-driven multi-centre international RCT that was conducted over a three year period, 2011–2014, and enrolled 493 patients with T1D of >5 years who were older than 40 years with specified CV risk factors [197,198].

REMOVAL was designed to determine whether the addition of metformin (initially 500 mg bid) to T1D patients treated with insulin could provide vascular protection as measured by common carotid artery intima-media thickness (CIMT), reduce endothelial impairment and improve glycemic control as well as reduce insulin dosing requirements [197,198]. Unfortunately, with the exception of reducing maximal CIMT none of the other specified tertiary outcomes were significantly reduced. REMOVAL was, to date, the largest clinical trial with metformin as adjunct therapy for T1D. The conclusion of a report in the BMJ's Drug and Therapeutics Bulletin in 2018 (dtb.bmj.com): *"Although metformin might limit weight gain and improve lipid levels to a minor extent, this is accompanied by an increased risk of adverse gastro-intestinal effects and biochemical vitamin B12 deficiency. Given such uncertainty over the long-term benefits, we believe that metformin has a very limited role in the management of people with type 1 diabetes"* [199].

A smaller study than REMOVAL with 90 children (mean of 13.6 years) conducted over a 1 year period reported reduced insulin requirements, a beneficial effect on HbA1c, and improved vascular function as determined by brachial artery ultrasound measures of flow-mediated dilatation/glyceryl trinitrate-mediated dilatation, but no effect on CIMT or other CV risk factors [200].

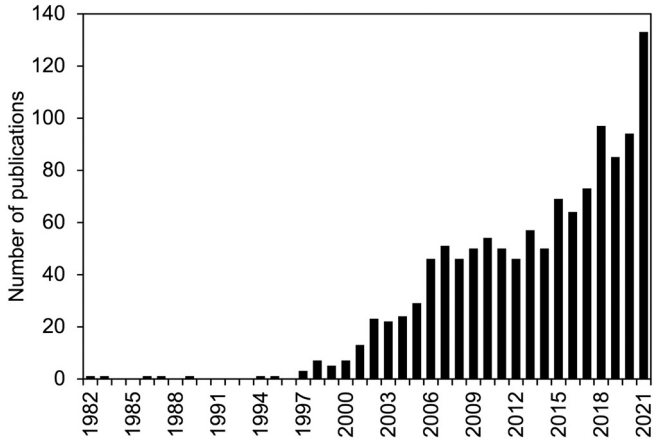

Fig. 5. Growth in publications mentioning metformin and type 1 diabetes. Data obtained from Scopus, 6 February 2022, using this search: (TITLE-ABS-KEY (metformin, OR dimethylbiguanidine, OR dimethylguanylguanidine, OR glucophage)) AND (TITLE-ABS-KEY ("diabetes mellitus type 1", OR "diabetes type 1", OR "type 1 diabetes").

7.2. Polycystic ovary syndrome (PCOS)

Polycystic ovary syndrome (PCOS) is a common multisystem endocrine disorder characterized by both reproductive and metabolic abnormalities. Endocrine features include hyperandrogenism, impaired ovulation and polycystic ovarian morphology. Insulin resistance and elevated levels of insulin along with enhanced signaling through the IGF-1 pathway are known contributors to the development of PCOS and contribute to the reduced ability for the maturation of ovarian follicles and a failure of ovulation [201]. PCOS is associated with insulin resistance and obesity in 40–80% of subjects while also increasing the risk for development of T2D. Of note insulin resistance is present in both lean (75%) and obese (95%) subjects with PCOS [202]. The presence of obesity in PCOS serves to further increase insulin resistance indicating a bidirectional relationship [203].

Approaches to treating PCOS include weight loss through lifestyle intervention, oral contraception and insulin sensitizing agents including metformin. Metformin use in the treatment of PCOS was first described by Valazquez et al. (1994) [204] who reported that treatment led to an improved menstrual regularity, reduced androgen levels and a significant reduction in body weight. Despite success with alternative approaches, such as the use of the selective estrogen receptor modulator, clomiphene, metformin has been extensively used to treat PCOS and justified by the basis of its ability to reduce insulin resistance [205,206] Fig. 6 reflects the maintained interest in the use of metformin as a treatment for PCOS.

Consistent with weight loss and insulin resistance in PCOS being a significant contributor to the efficacy of metformin, GLP-1 receptor agonists (e.g. liraglutide and exanitide) have been reported to be similarly effective. In this regard, recent reviews and meta-analyses have concluded that GLP-1 agonists alone, or in combination with metformin, represent a treatment option in PCOS [207–209]. Naderpoor et al. further reported in a systematic review and meta-analysis that lifestyle interventions plus metformin, compared to lifestyle ± placebo, was beneficial in both weight loss and menstrual cycle regularity [210].

Beyond weight loss and systemic metabolic actions, metformin has been suggested to have effects at the level of the ovary. In addition to metabolic actions within the ovary, metformin has been shown to inhibit in vitro androgen production in isolated human ovarian granulosa cells with this effect being particularly evident in the presence of insulin [211]. This inhibitory in vitro action of metformin on steroidogenesis, however, occurred at concentrations ($<10^{-8}$ M) lower than that achieved in vivo in the treatment of subjects with type 2 diabetes and was not fully supported in all studies [212]. In the latter study it was concluded that the androgen lowering effects of metformin were secondary to decreased circulating insulin levels and subsequent reduced activity of steroidogenic enzymes. Studies have also suggested a significant role for ovarian AMPK as mediating the effects of metformin [213]. In a recent study, glomerulosa cells from patients with PCOS were shown to have a lower level of α1AMPK gene expression while α1AMPK-deficient mice exhibited a PCOS-like phenotype that included irregular cycles, ovulatory dysfunction, altered follicular dynamics and hyperandrogenism [214]. Similarly, silencing of α1AMPK in immortalized human granulosa cells inhibited steroidogenesis [214]

7.3. Metformin and cancer

7.3.1. Epidemiological studies and clinical trials

The association between diabetes, primarily T2D, and an increase in the risk for the development of various, but not all cancers with prostate cancer being one exception, is well established with reports as early as 1932 that noted a strong association between diabetes and cancer [215]. Risk is particularly higher (2-fold) for liver, pancreas, and endometrium [216,217]. The risk is elevated in patients with diabetes who are also obese and is seen for both T1D and T2D [218,219]. Based on the analysis of >9000 cases from the Australian, Danish, Finnish, Scottish and Swedish T1D databases the overall risk is also increased by approximately 7% for women with TID, but no overall increase for men due to a 44% decrease in risk for prostate cancer [220]. An inverse relationship between the risk of prostate cancer has also been reported for T2D [221]; however, interpretation of the data is controversial [220,222].

Dilman and Anisimov predicted that by virtue of its anti-diabetic actions phenformin would protect against the development of age-related diseases, including cancer, and described the anti-mammary tumor effects of phenformin in rats [223,224]. Later studies demonstrated that phenformin enhanced the anti-cancer effects of cyclophosphamide and hydrazine in mice that had been injected with a number of tumors [225]. The anti-aging and anti-cancer potential of metformin was again re-emphasized by Anisimov in 2015 [226]. The results of the Evans et al. 2005 retrospective study from Tayside, Scotland concluded that metformin reduced the risk of cancer in T2D patients with an unadjusted odds ratio of 0.79 (0.67 to 0.93) and heightened interest in the anti-cancer effects of metformin (Table 4) [187].

Although Evans et al. [187] did not provide data on individual cancers as is reflected in Fig. 7 there has been a substantial increase in the number of publications that have investigated whether metformin can be re-purposed to treat cancer. Interest in metformin is further strengthened by its long history of safe use in humans as well as being

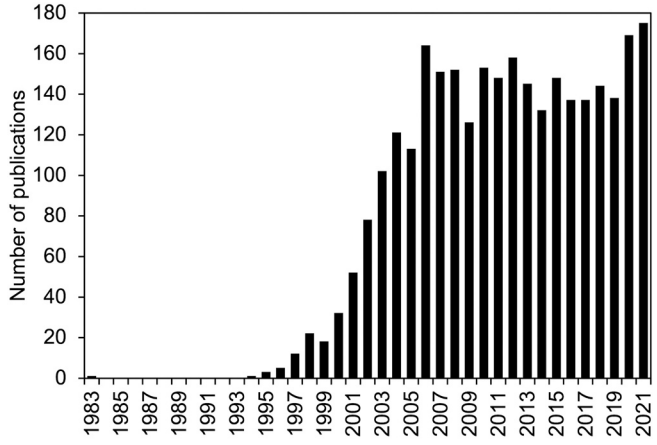

Fig. 6. Growth in publications mentioning metformin and PCOS. Data obtained from Scopus, 6 February 2022, using this search: (TITLE-ABS-KEY (metformin, OR dimethylbiguanidine, OR dimethylguanylguanidine, OR glucophage)), AND (TITLE-ABS-KEY ("polycystic ovary disease", OR "polycystic ovary syndrome").

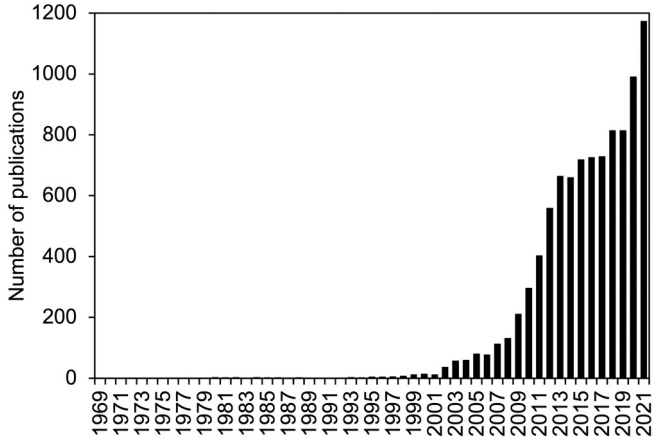

Fig. 7. Growth in publications mentioning metformin and cancer. Data obtained from Scopus, 6 February, using this search: (TITLE-ABS-KEY (metformin, OR dimethylbiguanidine, OR dimethylguanylguanidine, OR glucophage)) AND (TITLE-ABS-KEY (cancer*, OR neoplasm*).

Table 4
Metformin and cancer.

Data in support of anti-cancer effects of metformin.	Data that questions anti-cancer effects of metformin.
Evans et al [187]: Based on analysis of records of 314,127 patients using metformin for type 2 diabetes for the time period 1993–2001 in Tayside, Scotland, it was concluded that metformin reduced the risk of cancer. Furthermore, a potential link was made between the putative anti-cancer effect of metformin and its action to activate AMPK and the role of the upstream serine-threonine kinase, LKB1, a known tumor-suppressor.	Gandini et al. [227]: Systematic review of 65,540 cases of cancer from 47 studies of patients with diabetes who had been treated with metformin (originally 750 studies identified). Although evidence for a reduction in the incidence of cancers was apparent the reduction was modest particularly after adjusting for BMI and time-related bias, and not uniform across all populations. An important conclusion from Gandini et al" *Clinical trials are needed to determine if the observations seen in diabetic populations can be expanded to pre-diabetic or non-diabetic populations and to whom they should be expanded for the best benefit/risk ratio."* See also: Home et al [228]: Analysis of RCTs (ADOPT and RECORD) does not support link between metformin use and a reduction in malignancies. Stevens et al [229]: Based on analysis of 11 RCTs no evidence was found that metformin reduced mortality when compared to other anti-diabetic drugs.
https://clinicaltrials.gov/ct2/results?cond=cancer&term=metformin&cntry=&state=&city=&dist= As of May16th 2022, 398 trials involving metformin were listed, although not all were active	Suissa and Azoulay [230]: Raised concerns over interpretation of reduction in cancer risk with metformin that result from 'time-related biases', (differing exposure times), resulting in immortal-time bias. Mamtani et al [231]: Based on a cohort study of 87,600 patients with T2DM in The Health Improvement Network database of whom 71,472 were initiators of metformin it was concluded that after adjusting for different durations of treatment that metformin did not reduce risk of bladder cancer.
Shaw [232]; Shi et al [233]: The importance of the mammalian target of rapamycin (mTOR) as a central regulator of cell growth has been extensively reviewed. Metformin via activation of AMPK inhibits mTOR, a serine/threonine kinase, via phosphorylation of TSC2 (tuberous sclerosis complex) and the scaffold protein, Raptor, thus providing a mechanistic link for a direct anti-proliferative action for metformin. Shi et al [233], demonstrated AMPK-dependence for inhibition of lymphoma cells in ex vivo protocol. Madera et al [234]; Wu et al [235]; Gutkind et al [236]: Pre-clinical and clinical data supportive of metformin-mediated inhibition of mTOR and inhibition of growth in human oral squamous carcinomas	Varghese et al [237]: The concentration-dependent effects of metformin were studied on the proliferation of two types of Triple Negative Breast Cancer (TNBC) cell lines (MDA-MB—231 and MDA-MB-468) using cell culture protocols in either 25 mM or 5.5 mM glucose. Results: In presence of 25 mM glucose, metformin, 50–500 μM, significantly increased cell proliferation in MDA-MB—231 cells, and with no significant effects on proliferation with 1 to 10 mM metformin. In contrast, when studied in cell culture with 5.5 mM glucose and 250 μM to 10 mM metformin reduced viability of cells was observed. MDA-MB-468 cells were more sensitive to metformin: a. In 25 mM glucose the threshold for inhibition of proliferation was 500 μM, b. In 5.5 mM glucose the threshold was 250 μM. Conclusion: Although supportive that metformin has anti-proliferative effects that are enhanced with lower glucose levels of metformin and effectiveness differs between cell type; however, even in 5.5 mM glucose inhibition of proliferation requires ≥250 μM metformin. To inhibit mTOR a high concentration of metformin (2 mM) was required.
Lee et al [238]: A prospective cohort study based on 800,000 patients from Taiwanese National Health Insurance who were diabetes and cancer free on 1st January 2000. Data analyzed to determine whether use of metformin affected incidence of esophageal, gastric, colorectal (CRC), hepatocellular (HCC), and pancreatic cancers. Results: Cancer incidence density increased by ~2 fold in absence of anti-hyperglycemic therapy, but with metformin use cancer incidence was comparable to incidence in non-diabetics. The effective metformin dose to protect against cancer was ≤500 mg/day.	Yu et al [239]: Based on an umbrella review that included 21 systematic reviews and meta-analyses the use of metformin: a. Strong evidence for decreased incidence of pancreatic cancer. b. Highly suggestive evidence for improved overall colorectal survival. c. Only suggestive evidence for overall survival for all cancers, breast, lung, and pancreatic cancers. d. Only suggestive evidence for reduction in cancer incidence for all cancers, and colorectal and liver cancers. Authors suggest caution due to poor methodological quality and risk of bias of systematic and meta-analysis reviews.
Zhang et al [240]: Demonstrated critical role for the scaffold protein, AXIN, and facilitating docking LKB1 to the lysosomal v-ATPase-Ragulator complex for metformin to activate AMPK, and inactivate mTORC1.	

inexpensive and off patent since 2004 [241].

Evans et al. (2005) [187] further highlighted the possible link between metformin and LKB1 as an explanation for a cellular signaling pathway and importantly mutations and deletions in LKB1 have been associated with inactivation of LKB1, and inactivating mutations have been detected in approximately 17% of non-small cell lung carcinomas (van Veelen et al., 2011) [242]. Such mutations could affect how susceptible cancer cells are to nutrient deprivation as reflected by studies in cell culture when cancer cells are deprived of glucose [243,244].

In 2013 over 100 clinical trials designed to assess the potential benefits of metformin in the treatment of cancer were listed on the NIHH Clinical Trials government web site [245]; as of May 2022 the number now exceeds 380 with breast cancer featuring dominantly but also trials involving the following cancers: endometrial and ovarian, head and neck squamous cell, multiple myeloma, thyroid, and lymphocytic leukemia. Several systematic reviews and meta-analyses have concluded that there was a reduction in risk of mortality and developing cancer in the range of 14 to >30%, with, in some analyses, increases in risk in subjects treated with sulfonylureas, insulin, and alpha glucosidase inhibitors when compared to treatment with metformin [227,235,246–251]. However, as reported by DeCensi et al., (2010) the use of metformin is not always associated with a significant benefit as seen for colon, breast and prostate cancers where there was no evidence for a reduction in risk [247]. In addition, both positive and negative associations have also been reported, as with prostate cancer, where previous year treatment with metformin was associated with an increased risk whereas exposure in the previous 2 to 7 years was associated with a decreased risk [252]. Similarly, for the use of metformin as an adjunct for the treatment of myeloma, where both beneficial anti-cancer and pro-cancer effects have also been reported [253,254]; these findings support the need for well- designed longterm RCTs.

Other limitations are that in many studies the initiation of metformin treatment is frequently at a younger age compared with other diabetic drugs, and secondly, sulfonylureas and insulin have been reported to potentially increase cancer risk [247,255]. Although, as summarized in Table 4, extensive support for the protective effects of metformin has been provided, not all reports are positive and concerns have been expressed over data analysis in observational studies and inherent biases in such analyses [90,227,229–231]. Distortion of the actual benefit resulting from immortal time bias and not using time-dependent

analysis of drug exposure may have resulted in an over-estimation of the effectiveness of the anti-cancer effects of metformin [256,257]. Furthermore, although not without controversy, several database studies indicate that metformin does not reduce the risk of cancer [219,258,259] Interestingly, as reflected in a 2019 publication where adjustments were made for time-related biases in a regression analaysis of cancer risk in 315,890 subjects with diabetes over the period 2002–2012, no association was noted for the use of metformin and reduced risk of cancer, including bladder, breast, colon, lung, pancreas and prostate cancer [260].

Data from prospective clinical trials has also been mixed. NCT01266486, *Effect of Metformin on Breast Cancer Metabolism*, (https://clinicaltrials.gov/ct2/results?cond=cancer&term=metformin&country=&state=&city=&dist=, a Phase II study with 41 participants was completed in 2014 and utilized a PET-CT study with 2-deoxy-2-(18F)-FDG as a marker of glycolysis, and a metabolomic analysis of breast cancer tissue [229]. Metformin lowered serum glucose, insulin, C-peptide, and insulin resistance and a transcriptional analysis indicated an upregulation of pathways involved in mitochondrial metabolism suggesting metformin was targeting mitochondrial function in the tumor [261].

Results from RCTs are not all supportive and frequently contradict conclusions reached from the meta analysis of cohort and case-control and also pre-clinical in vitro studies with metformin as was reported by Thakkar et al. (2013) [248]. Based on a meta analysis of 11 RCTs no evidence of a reduction in cancer risk was associated with metformin [229]. The same negative conclusion was reached based on the analyses of data from the ADOPT (A Diabetes Outcome Progression Trial) and RECORD (Rosiglitazone Evaluated for Cardiovascular Outcomes and Regulation of Glycemia in Diabetes) [228]. No beneficial effects of metformin, or other antidiabetic medications, were reported in the four year RCT, REDUCE, that was designed to compare the effect of dutasteride on prostate cancer [262]. In a 12 week placebo-driven trial of 74 patients with Barrett's Esophagus (ClinicalTrials.gov number, NCT01447927), daily administration of metformin although reducing insulin resistance and serum levels of insulin did not cause major reductions in esophageal levels of the serine/threonine downstream target of mTOR, pS6K1 [263].

Collectively, these findings do not support a role for metformin as a chemopreventive agent for either prostate cancer or Barrett's Esophagus. However, it could be argued that to maximize the beneficial effects of metformin requires longer treatment periods and possibly higher doses of metformin. In contrast, there is pre-clinical and clinical support that metformin via targeting the mammalian target for rapamycin (mTOR) pathway reduces the progression of human head and neck squamous cell carcinomas– see Table 4 for details [234–236]. Results, from the several hundred on-going trials will hopefully provide greater clarity as to whether there are unique anti-cancer effects of metformin, or whether metformin, together with other anti-diabetic medications, variably reduce risk via their positive effects on glucose homeostasis and reduction of insulin resistance.

7.3.2. *Cellular basis of the anti-cancer effects of metformin*

7.3.2.1. AMPK. As summarized in the following sections metformin's anticancer action has been linked, at least in part, to the activation of AMPK and subsequent cellular events that collectively will suppress tumor growth. The cellular mechanisms include reducing hyperglycemia, improving insulin sensitivity and reducing signaling via the IGF receptor (IGFR) pathway, suppressing NF-kB signaling, and a direct antiproliferative actions via inhibition of the mTOR pathway [90,186]. Fig. 6 reflects the 'classic' view that the activation of AMPK is secondary to metformin inhibiting mitochondrial complex 1 and reducing the ATP/AMP ratio [77,78].

As already pointed out, it is unlikely metformin affects complex 1 in patients receiving the usual therapeutic doses of metformin that result in a plasma concentration of about 20 micromolar. However, it is important to note that AMPK-independent effects of metformin have been

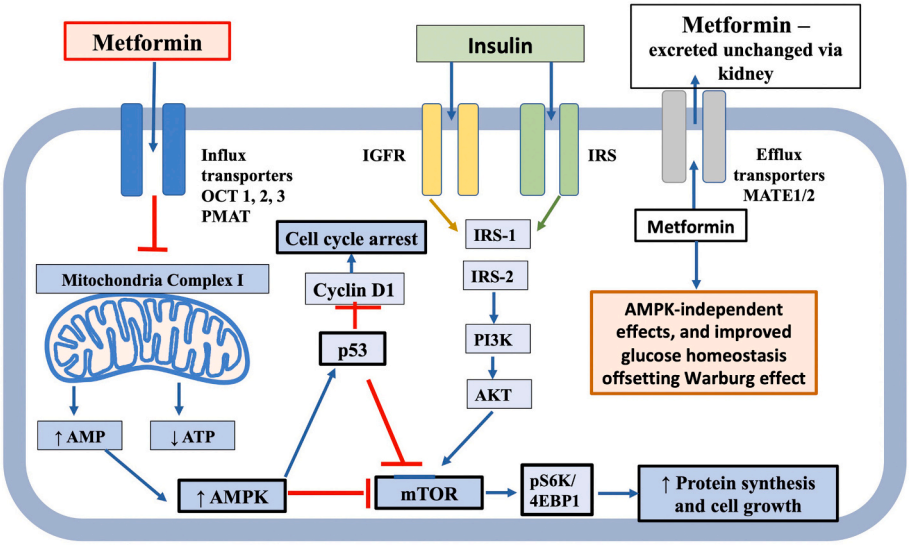

Fig. 8. Putative pathways for the anti-cancer effects of metformin.
Metformin exists as a cation at physiological pH and as depicted in this schematic its absorption and distribution is dependent on the organic cation transporters, OCT 1, 2 and 3 and the plasma membrane monoamine transporter (PMAT). The multidrug and toxin extrusion (MATE1/2) transporter play an important role in transporting metformin out of the cell. Although metformin is thought to mediate most of its cellular effects via activation of AMPK the exact mechanism of activation and the contribution of AMPK-independent effects remain very controversial but in this schematic, metformin inhibits the electron transport chain of mitochondrial complex 1. Of significance and in support of the "Mitochondrial Complex 1 Hypothesis"as an explanation of metformin's cellular actions, targeting the inner membrane of mitochondria by tagging metformin with lipophilic cations, such as triphenylphosphonium (TPP+) enhances the potency of metformin against pancreatic cancer cells [264]. Inhibition of complex 1 results in a reduction in ATP levels, increases the AMP/ATP ratio, and activates AMPK. AMPK activation leading to an inhibition of the mTOR pathway via two mechanisms (not shown in this schematic): 1. Phosphorylation of Raptor, a subunit of the mTOR complex (mTORC1), at serine-792, and 2. Phosphorylation of the tumor suppressor proteins (TSC1/2) at serine-1387, which enhances GTPase activity and turns off Rheb-GTP that lies upstream of the mTORC. As a result of the inhibition of mTOR downstream signaling via pS6K1 and 4EBP1 to repress RNA translation and inhibit protein synthesis. Similarly, AMPK positively regulates the activity of the tumor suppressor p53 via phosphorylation of serine-15, and P-p53, in turn, negatively regulates mTOR. Also depicted in the schematic metformin, via the activation of AMPK, represses the insulin-mediated and insulin receptor (IR), insulin growth factor receptor (IGFR) activation of phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K)/Akt signaling pathways (including, but not shown, ERK1/2 phosphorylation Ras-mitogen-activated protein kinase [MAPK]. In addition, metformin, independent of its effects to modulate mTOR signaling, inhibits protein synthesis and the cell cycle, improves glucose homeostasis and suppresses the proproliferative Warburg effect (for additional details see references [265,266]).

described and these may contribute to the anti-cancer effects [245,267,268].

7.3.2.2. mTOR. Evidence that treatment with metformin inhibits the phosphoinositide *3-kinase/* protein kinase B/mTOR (PI3K/AKT/mTOR) pathway in patients with cancer has been demonstrated by Zhao et al. (2018) [269]. Zhao et al. studied non-diabetic patients with endometrial cancer who were treated for up to 4 weeks with 500 mg metformin (three-times-day; tid) prior to hysterectomy with subsequent immunohistochemical analysis [269]. The data revealed a significant decrease in the phosphorylation of the downstream targets of the mTOR pathway - including PI3K, p-Akt, and downstream of mTOR, p-S6K1, the serine-threonine kinase [269]. p-S6K1 is linked to the modulation of autophagy, and phosphorylation of the translation initiation factor, 4EBP1, thus reflecting a suppression of mRNA translation, as well as a decrease in the expression of Ki-67; Ki-67 is a marker of proliferation for human tumor cells [269]. Previous findings by the same group indicated that with the same intervention protocol treatment of 60 endometrial cancer patients with metformin significantly reduced plasma levels of IGF-1 and increased p-AMPK, and suppressed p-mTOR [270]. In contrast, as already noted in an investigation in patients with Barrett's Esophagus, no evidence for metformin inhibiting the mTOR pathway was seen [263]. Of potential significance is that IGF-1 plays an important role in the brain as a neuroprotective factor with levels decreasing with age but increasing in response to injury and promoting repair; impairment of IGF-1 signaling in the brain has been linked to the development of neurodegenerative diseases including Parkinson's [271].

7.3.2.3. Glycolysis. Metformin by reducing glucose availability and reprogramming metabolic dysregulation via reducing the dependence of tumor cells on aerobic glycolysis – the so-called Warburg effect could reduce cancer cell growth [272]. There is evidence to support this possibility, though much of the data is based on cell culture protocols that have used 1 to 10 mM metformin [237,243,273–276] and linked to activation of AMPK [277] For instance, pro-apoptotic effects of 10 mM metformin have been reported in three breast cancer cell lines (MCF7, SKBR3, and MDA-MB-231) in a cell culture protocol containing 25 mM glucose but the percentage of dead cells was increased approximately three-fold when glucose was reduced to 5.5 mM [237]. Similarly, the concentration of metformin required to promote cell death can be greatly reduced by at least 10-fold when triple negative breast cancer cells, MDA-MB-468, are cultured under a glucose-starved protocol [237,278,279]. Reducing glucose availability by using the glucose analogue and hexokinase inhibitor, 2-deoxy-D-glucose (2-DG), reduces ATP levels and enhances the pro-apoptotic effects of metformin to induce AMPK-dependent cell-death in prostate cancer cells in culture [243,244]. As others have shown [77,78] mM concentrations of metformin will inhibit mitochondrial complex 1, but it is very unlikely that inhibition occurs when metformin is used clinically [84,93,280]. Furthermore, metformin is far less effective in lowering glucose and insulin levels in non-diabetic people despite pre-existing CV risk factors [281]. Nonetheless, these data have promoted studies to investigate the use of 2-DG and derivatives as adjuncts in the treatment of cancer [282].

7.3.2.4. Other targets for the antitumor actions of metformin. Safe et al. (2018) have identified a number of potential novel targets for metformin that may play a role in its antitumor actions [283]. Further, an anti-tumour role for the orphan nuclear receptor, NR4A1/Nur77, with which metformin is now known to interact [87], has recently been summarized [284,285]. It is thus likely that the antitumour effects of metformin may be due to its interaction with multiple effectors.

7.3.2.5. Differential expression of organic cation transporters. Differences in the susceptibility of different cancers to metformin may in part be reflected in cell-specific differences in the expression of the organic cation transporters that regulate cellular transport of metformin into and out of cells (see Fig. 6). Cai et al. (2016) [286] demonstrated the importance of transporter expression levels in determining the anti-proliferative actions of metformin [287]. Significantly higher levels of mRNA for OCT3 were detected in the human breast cancer cell line, MDA-MB-231 and associated with higher metformin-induced phosphorylation of S6K1 [287]. A number of other experimental studies have provided supportive data. For instance, the responsiveness of rat mammary tumors to the anti-proliferative effects of has been linked to the expression level of OCT2 protein and the accumulation of metformin [288]. In addition, metformin accumulation and antiproliferative effects have been shown to be higher in a prostate cancer cell line, LNCaP, which had high OCT3 and low MATE2 expression [288]. As pointed out by the authors a limitation of a number of these studies, is the reliance on mRNA expression levels of the transporters and not quantitative protein data as it is the latter that reflect the levels of functioning transporters. Nevertheless, the authors presented data from mice treated with metformin at a dose, 5–10 mg/ml in drinking water, with plasma concentrations comparable to those seen in patients with T2D who are treated with metformin (9.5–20.8 μM) [288]. This dosing range resulted in the accumulation of metformin in tumors to 20–54 μM [288].

Collectively, these in vitro data suggest that dependent on the expression levels of OCT/MATE transporters some cancer tumors may accumulate sufficient levels of metformin such that it affects mitochondrial function and cell proliferation and thereby enhance the therapeutic efficacy of radio- and chemotherapeutic regimens. However, it remains uncertain whether that when used clinically, and recognizing the short plasma half-life of metformin and that transport via OCT transporters is bidirectional, sufficient accumulation could occur to achieve a sustained ant-cancer effect to the equivalent of that reported with cell culture protocols in vitro at mM metformin concentrations.

7.3.2.6. Anti-angiogenic actions of metformin. Metformin has also been reported to decrease microvessel density and increase vascular cell perfusion via a reduction of signaling via platelet-derived growth factor B (PDGF-B) and it's receptor PDGF-Rβ [289]. Anti-angiogenic effects of metformin in combination with 2-DG have been shown in mouse microvascular endothelial cells that overexpress vascular endothelial growth factor (VEGF) and that form angiosarcomas in mice where exposure to metformin plus 2-DG enhances expression of thrombospondin-1 (TSP-1) and inhibits cell proliferation and tubulogenesis [290]. A limitation of the latter study was that the concentration of metformin required to demonstrate an enhanced expression of TSP-1 and a decrease in proliferation was 2 mM and not seen when micromolar concentrations equivalent to blood therapeutic levels were studied [290].

7.3.2.7. Anti-inflammatory actions of metformin. A number of studies have also linked the putative anti-inflammatory effects of metformin to AMPK-mediated suppression of activation of NF-κB via the phosphorylation of IκB and inhibition of cytokine release [182–184]. Such anti-inflammatory effects also suggest that metformin may prove to be useful for the treatment of immune-mediated diseases and could contribute to the putative benefits in a number of diseases including cancer. A caution, again, is that much of the data from in vitro studies have been generated from protocols using mM concentrations of metformin [291,292]. Metformin may also improve immune cell targeting of cancer cells as supported by both in vitro and in vivo studies reporting that metformin enhances CD8 T-cell memory, but again the in vitro data were based on using 2 mM metformin in vitro and in vivo with injections of 250 mg/kg [293].

7.3.2.8. Metformin and mitochondrial complex 1. A direct link between inhibition of mitochondrial complex 1 and the anti-proliferative effects of metformin has also been implied [268]. The proliferation of human

colon cancer cells (HCT116p53-\-) was inhibited by metformin in the concentration range of 250 μM to 1 mM, and, in the absence of glucose in the culture media, metformin induced cell death with parallel inhibitory effects on oxygen consumption [268]. However, in HCT116P53−/− cells in which the metformin-resistant *Saccharomyces cerevisiae* NADH dehydrogenase, ND11, was overexpressed metformin no longer inhibited proliferation thus suggesting the link to complex 1 [268]. In the same report supportive in vivo data were obtained from nude mice in which human lung cancer (A549) xenografts had been implanted without and with overexpression of the metformin-resistant ND11 cells, demonstrating that metformin was ineffective at inhibiting tumorigenesis in the xenografts overexpressing with ND11 [268]. As indicated in the legend for Fig. 6 tagging metformin with MitoMet that targets mitochondria also enhances the ability of metformin to suppress tumor growth [264]. Collectively, although these data support linking the anti-cancer effects of metformin to the inhibition of mitochondrial complex 1, other data from a study using PET to investigate the distribution and effects of ^{11}C-labelled metformin in tumor bearing mice indicate that the levels of metformin retained in the tumors are not sufficient to inhibit mitochondrial respiration [71]. It has also been argued that metformin can be used in combination with a standard anti-cancer drug regimen and enhance the effectiveness of treatment. However, many such studies have used mM concentrations of metformin as high as 10 mM [294].

In conclusion, although in vitro data provide support for plausible cellular pathways whereby metformin can directly reduce the risk of cancer independent of its anti-hyperglycemic and insulin-sensitizing actions in many instances the data have been generated with protocols using very high concentrations of metformin that if applied systemically would result in significant toxicity. It has been suggested that following the principle of Paracelsus's Law, paraphrased as: *'The wrong dose makes the poison'*, a dose of metformin higher than appropriate for treating T2D could be used for the treatment of a cancer by avoiding the usual oral route and delivering the drug directly to the cancer. This concept formed the basis for suggesting diabetoguanidines as oncoguanidine therapy [295]. It is worthy of note that in the USA French lilac appears on the list of poisonous plants suggesting that similar caution should be applied to the use of the synthetic derivative of galegine, metformin, which although comparatively safe when used in the recommended dose range for T2D (250–2550 mg/day) is a poison when used inappropriately [296].

Furthermore, the data from retrospective analysis, despite considerable debate, has not provided a consistent answer as to whether biases in analysis have greatly over-emphasized the reduction in the risk of cancer that has been attributed to metformin. The significance of conclusions reached by retrospective studies is confused by the variables introduced as a result of the duration of diabetes, the duration of treatment, the therapeutic efficacy of the treatment of metabolic dysfunction, and the contribution of co-morbidities. We therefore conclude that the primary mechanism whereby metformin provides protection against the development of some cancers is likely via its effects to control hyperglycemia, its improvement of insulin sensitivity and reduction of IGF-1 levels and IGFR signaling and possibly via its signaling to the orphan nuclear receptor, nur77/NR4A1 and other proteins (Fig. 4). There is, however, some supportive clinical data that indicates the contribution that via inhibition of the mTOR pathway, metformin has a direct anti-proliferative effect. This possibility requires further investigation from larger RCTs. Comparable conclusions have been reached by others, for instance Heckman-Stoddard et al. (2017) after an extensive review of the available of the data and stated [249]: *"There is biological plausibility for a cancer pre-ventive effect of metformin, given multiple ways that it can interfere with cancer promoting signalling pathways. However, both animal and epidemiological studies have shown somewhat mixed effects."* Anisimov (2022) has offered a similar conclusion [297].

There is support for investigating the use of metformin as an adjunct to be used together with cytotoxic and targeted therapies as has been suggested in a 2021 systematic review for the treatment of lung cancer [298]. Support for using metformin to enhance the effectiveness of cytoxic and targeted anti-cancer drugs and also radiotherapy is provided by pre-clinical data where, using cell culture protocols, metformin has been combined with a number of anticancer drugs including tyrosine kinase inhibitors such as gefitinib [292–303]. A limitation of these studies is the high concentrations of metformin used, ranging from 1 to 5 mM [292,293]. An exception is that Qu et al. (2014) reported that metformin at low as 10 μM resensitized multidrug-resistant MDA-MB-231 breast cancer cells to a number of cytotoxic drugs including 5-fluorouracil [304]. Additional data from in vitro studies using micromolar concentrations of metformin are required. Data from the numerous ongoing trials with metformin should also provide clarification as to whether there is a direct anti-proliferative action of metformin independent of its effects on glucose homeostasis.

If the anti-cancer effects of metformin are primarily linked to its effects on glucose homeostasis then other anti-diabetic drugs should also reduce cancer risk. Comparisons of metformin with other anti-diabetic drugs, in particular the more recently introduced GLP-1 receptor agonists and SGLT2 inhibitors, and the effects on cancer risk are therefore needed. Although, with some exceptions, the available clinical data indicate that the use of GLP-1 receptor agonists and SGLT2 inhibitors are not associated with an enhanced risk of cancer and there is a lack of evidence that they reduce cancer risk [305,306,307]. However, and as seen for metformin, in vitro studies with SGLT-2 inhibitors and GLP-1 receptor inhibitors provide supportive data that these agents also have anti-cancer effects [308–310]. In the case of SGLT-2 inhibitors their anti-cancer effects have been linked to the inhibition of glucose transport and activation of AMPK, inhibition of the mTOR pathway and should presumably be dependent on expression levels of the SGLT-2 transporter [310].

7.4. Anti-Aging effects of metformin

Aging is the most significant risk factor for the development of many diseases including CVD, cancer, diabetes and neurodegenerative diseases and as reflected in Fig. 9 interest has increased in investigating metformin as an anti-aging drug that could not only enhance healthspan, but also increase lifespan.

One target for an anti-aging drug is senescence as senescent cells are pro-inflammatory and they accumulate with age causing tissue dysfunction, including cancer, via the senescence-associated secretory phenotype, or SASP [311]. The pathophysiological sequelae of senescence can be offset by senolytics as supported by a trial (NCT04946383)

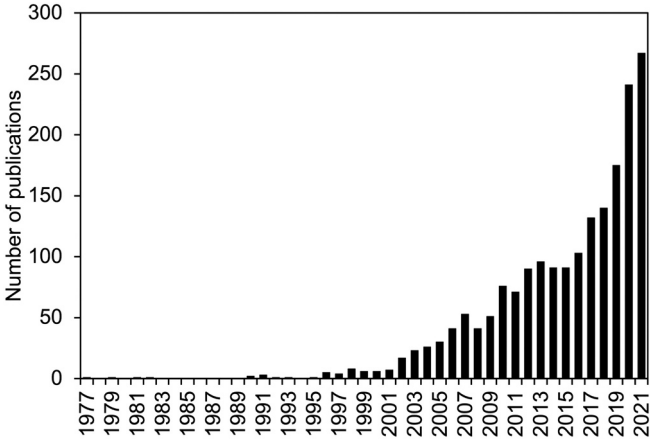

Fig. 9. Growth in publications mentioning metformin and aging. Data obtained from Scopus, 6 February 2022, using this search: (TITLE-ABS-KEY (metformin, OR dimethylbiguanidine, OR dimethylguanylguanidine, OR glucophage) AND (TITLE-ABS-KEY (aging, OR senescence).

with the putative senolytic, dasatinib, which targets tyrosine kinases, in combination with the anti-oxidant plant flavonoid, quercetin [312]. Low μM concentrations (50 μM) of metformin also protect endothelial cells against high glucose-induced senescence [179]. Senescence can also be blocked by gerosuppressants/geroprotectors, such as rapamycin, that target mTOR and suppresses growth [313,314]. The National Institute on Aging Interventions Testing Program has investigated a number of drugs, including rapamycin, to determine whether they prolong lifespan in mice [315]. For instance, rapamycin inhibits mTOR and extends lifespan in several species: *Caenorhabditis elegans* (nematode), *Saccharomyces cerevisiae* (yeast), and *Drosophila melanogaster* (fruit fly) [316–320]. Metformin, like rapamycin, inhibits mTOR signaling and arguably should therefore be a gerosuppressant. Metformin has been shown to reduce inflammatory cytokine activity in senescent cells and also in cancer stem cells by blocking the NF-κB and cytokine expression pathways [321–323]. Of interest is the argument that the inhibitory effect of metformin on the NF-κB expression pathway is linked to the signaling pathway activated by hyperglycemia and therefore not a non-specific anti-inflammatory action [321]. Again, a concern with some of the data derived from in vitro studies is that millimolar concentrations of metformin are required to inhibit the NF-κB pathway [322] and whether comparable anti-inflammatory effects would be observed when metformin is used at appropriate therapeutic levels as reflected by the pharmacokinetic data provided in Table 2.

7.4.1. Anti-aging effects of metformin in non-human species

There is a substantive literature, often controversial, that has investigated the putative benefits of metformin as an anti-aging drug with data derived from a number of species ranging from *C. elegans*, to *Drosophila melangaster,* to rodents and humans. A number of reviews that supports the benefits of metformin are available [189,324–326] as well as several that are more critical of the evidence [280,327–329].

We will not reiterate all of what has been previously reviewed other than key aspects of the data; however, it is worthy to quote a caution stated by Pyrkov et al. (2021) who analyzed a dataset based on >500,000 people from Russia, UK and the USA and the potential for extending lifespan and concluded: *"The proximity of the critical point revealed in this work indicates that the apparent human lifespan limit is not likely to be improved by therapies aimed against specific chronic diseases or frailty syndrome."* [330].

Data from studies in *C. elegans* have been frequently cited to advance the argument that metformin can delay aging and increase life span [45,331,332]. However, when the effects of metformin were studied in *C. elegans* of different ages it was found that metformin reduced life expectancy in the older nematodes and this reduction was linked to lower numbers/function of mitochondria and decreased ATP levels in the older nematodes [333]. In *Drosophila* no survival benefit was observed for male and female flies receiving differing concentrations of metformin [334]. Controversial data has also been published for studies of the effects of metformin on life expectancy in rodents. In male mice chronic treatment beginning in middle age with 0.1% metformin *w*/w supplemented in the diet increased healthspan and lifespan, but a higher dose of 1% was toxic and reduced lifespan by 14.4% [335]. However, 1% metformin given intermittently every other week to late-life mice although not enhancing lifespan did improve several metabolic markers of aging without leading to early mortality [336]. The lifespan of Fischer-344 rats treated with metformin (300 mg/kg/day) was not extended compared to those on a metformin-free regimen [337]. Calorie restriction (CR) has been shown to extend lifespan and delay aging in rodents and other species and linked to a role for the nutrient sensor, enzyme AMPK, and also mTOR [338,339]. Since metformin is known to activate AMPK and also inhibit mTOR one might expect it to mimic CR, but in the study by Smith et al. (2010) although metformin significantly extended early lifespan it did not extend overall average lifespan, concluding that metformin is not a bona fide CR mimetic [337]. Other studies of the effects of metformin on aging in rodents have also concluded the anti-aging benefits are reduced or absent in older animals [340–342] and have been summarized by Mohammed et al. (2021) [280]. The variable effects of metformin in a variety of older organisms, including humans, indicate that caution must be applied when recommending the use of metformin as an anti-aging drug [43,280]. Furthermore, and as previously discussed, a number of studies have provided evidence that compared to metformin exercise is a superior intervention for improving healthspan and that combining metformin with exercise either reduces the exercise-induced gains or provides only minimal additional benefit [41–44] and summarized by Mohammed et al. (2021) [280].

7.4.2. Anti-aging benefits of metformin in humans

Concerns over the healthspan and lifespan benefits of initiating chronic treatment with metformin in older animals stresses the need for data from well-designed clinical trials in humans. An analysis of retrospective observational data from the UK Clinical Practice Research Datalink of the medical records of over 180,000 patients with T2D does provide support and indicates that although metformin-treated diabetic patients were more obese and had more co-morbidities than non-diabetic patients, they had survival rates similar to their matched non-diabetic control group [343]. Similarly, a systematic review of 53 studies that met the selection criteria concluded that metformin may extend both healthspan and lifespan independent of its actions as an anti-diabetic drug thus suggesting metformin meets the criteria of a geroprotective agent [325]. Given the uncertainty over the benefits of the use of metformin in elderly subjects data from appropriately designed clinical trials are required. A summary of the progress in a number of such trials is provided in Table 5. Fig. 10 summarizes some of the controversies concening the benefits/risks of metformin as a potential geroprotective drug.

In conclusion, although metformin is considered a safe drug, caution needs to be expressed over-extending its use beyond the treatment of T2D and in particular to older subjects who potentially will have age-related impairments in renal and liver function and therefore at risk of metformin toxicity. As discussed by Stevens et al. (2019) the risk of metformin side-effects should not be under-estimated particularly in those over 60 years of age [296].

7.5. Metformin and neurodegenerative diseases

Diabetes is associated with an increased risk of neurological disorders that includes stroke, dementia (Alzheimer's and vascular dementia) and Parkinson's disease [346]. Collectively, dementia is the most common neurological disorder and affects >50 million people worldwide and Alzheimer's Disease, based on an association with impaired insulin signaling and glucose metabolism, has been referred to as a brain-specific form of diabetes [347,348]. The results of the *Adult Changes in Thought* study of 2067 participants reported an association between elevated glucose levels and the risk of dementia that extended to those without diabetes thereby by providing support for the potential benefits of metformin [349]. It would therefore be expected that treatment with a drug that reduces insulin resistance in the brain should offset the pathophysiology of Alzheimer's disease and arguably other neurodegenerative diseases. An additional benefit of its anti-hyperglycemic actions is the reduction of the effects of protein glycation and the accumulation of advanced glycation end products (AGEs) on the development of degenerative diseases and accelerating the aging process [350,351].

Metformin crosses the blood-brain barrier and therefore meets the requirements of a centrally active insulin sensitizer, although the levels in the cerebrospinal fluid that have been reported are only about one-tenth of basal plasma levels at about 100 ng/ml [352]. The vascular protective actions, putative anti-aging and anti-inflammatory properties of metformin are additional benefits that arguably should also counter neurodegenerative diseases. Mitochondrial dysfunction has also been

Table 5
Clinical Trials studying effects of metformin on human aging.

Trial Name	Details of Trial	Reference
MILES (Metformin in Longevity Study)	A three-year study initiated in October 2014, was a cross over study wherein 14 elderly patients with impaired glucose tolerance were treated with metformin at a dose of 1700 mg/day and acted as their own controls The data indicates that metformin treatment resulted in transcriptomic changes in pathways associated with aging that included mitochondria pathways, adipose tissue and fatty acid metabolism, and DNA repair mechanisms.	Kulkarni et al. [344] NCT02432287
Role of Metformin on Muscle Health of Older Adults.	A Phase 1 study due for completion in April 2022 and designed to determine whether metformin has benefits to reduce the negative effects of bed rest in the elderly and will monitor: insulin resistance, lipid accumulation, inflammation, and muscle loss	NCT03107884
Metformin for Preventing Frailty in High-risk Older Adults.	A placebo-driven Phase 2 study that involves 120 subjects aged 65 to 90 years old with pre-diabetes with results anticipated in late 2024.	NCT02570672
TAME (Targeting Aging with Metformin).	A double-blinded placebo-controlled multi-center trial that has been designed to determine whether treatment with metformin (1500 mg/day) for 6 years will delay the onset of age-related diseases in 3000 ethnically diverse subjects aged 65–80 The anticipated clinical outcomes include data on the appearance of new age-related chronic diseases; measures of cognitive impairment; biomarkers for inflammation and senescence As of early 2022 TAME is yet to start and is not posted on *https://clinicaltrials.gov*	(Barzilai et al. [324] Justice et al. [345]
The Investigation of Metformin in Pre-Diabetes on Atherosclerotic Cardiovascular Outcomes (VA-IMPACT)	A phase 4, randomized, placebo-driven, multi-center study that aims to test the ability of metformin to reduce mortality and cardiovascular morbidity. The planned enrolment size is much larger than the MILES trial with an anticipated enrollment of close to 8000 participants, however, due to COVID-19, the trial has been on hold since the 17th March 2020.	NCT02915198

strongly associated with the pathophysiology of neurodegenerative diseases, particularly Parkinson's Disease [353]. Novel peptides, such as SS-31 (D-Arg-2′,6′-dimethyl-tyrosine-Lys-Phe-NH2), which bind to the inner mitochondrial membrane and reduce the generation of reactive oxygen species have been reported to be protective in animal models of amyotrophic lateral sclerosis [354]. As discussed previously metformin reduces ROS and, at least theoretically, this also argues for a beneficial effect against elevated oxidative stress. Thus, based on the evidence already presented and discussed in this review, as well as the anticipated benefits via improving metabolic control, metformin should at least slow the development of neurodegenerative diseases and be of particular benefit in those subjects with pre-existing diabetes [355]. Indeed, a substantial amount of the pre-clinical and clinical literature pertaining to the benefits of metformin as a putative neuroprotective drug is available and, for example, has been reviewed by Rotermund et al. (2018) [35]. A number of meta-analyses that report the use of other anti-diabetic drugs, notably DPP-4 inhibitors, sulfonylureas and, variably, thiazolidinediones, were also associated with protection against cognitive decline thus suggesting that an anti-hyperglycemic effect was the common denominator [356–357].

Not all of the published data support a direct protective benefit of metformin and, for example, opposing conclusions have been presented in several systematic reviews. Evidence from the US Veterans Affairs electronic medical record analysis of 5528 patients indicates that elderly veterans with T2D diabetes and treated with metformin for more than two years had a lower rate of neurodegenerative diseases [358]. However, a limitation of this study is that the positive impact of metformin on diabetic patients could be attributed to improved glycemic control [359,360]. The conclusion from a 2016 meta-analysis based on data from nine comparisons out of six studies from a total of 544,093 subjects was that the incidence rate of dementia was reduced with either metformin or thiazolidinediones but only with a marginal trend toward significance [361]. The results from a systematic review published in 2020 that was based on 23 comparisons of 19 studies and over 250,000 subjects concluded that there was no benefit associated with the use of metformin and for Parkinson's and, in fact, metformin may worsen the risk for Parkinson's [362]. In contrast, a 2022 systematic review and meta-analysis of 94,462 metformin users versus 100,330 non-users concluded that longterm ($\geq$ 4 years) treatment with metformin was associated with a lower risk of neurodegenerative disease, and particularly in Asiatic populations [363].

Additional studies that argue either for or against the benefits of metformin in protecting against the development of neurodegenerative diseases are summarized in Table 6. Similarly, the preclinical data are summarized in Table 7. Based on the data available it is not possible to reach a conclusion as to whether metformin provides broad protection against the development of neurodegenerative diseases. Comparable to the conclusions over the putative benefits that metformin delays aging, the antihyperglycaemic and vascular protective effects of metformin argue in favour of metformin as a neuroprotective drug; however, other determinants, such as reduced mitochondrial function, as has been described in *C.elegans*, may offset these benefits.

As was indicated in a 2022 narrative review by Liao et al. [397] of the potential role for metformin in offsetting the development of Alzheimer's disease the evidence of benefits remains ambiguous, despite data from preclinical studies providing potential mechanisms of action for metformin as a neuro-protective drug. Furthermore, the data from clinical studies are not universally positive thus raising concerns over-extending the use of metformin to patients without T2D. There is also lack of data for the effects of metformin on cognitive decline in patients without diabetes, and whether GI side-effects and risk of vitamin B12 deficieny would reduce patient compliance. Finally, other antidiabetic drugs with diverse mechanisms of action, including the DPP-4 inhibitors, sulfonylureas, and thiazolidinediones, have also been reported to have beneficial effects thus suggesting that neuroprotective actions are not necessarily unique to metformin.

7.6. *Metformin, malaria, influenza, COVID-19 and anti-bacterial actions*

In parallel with the development of biguanides to treat T2D there was also early interest in the use of biguanides to treat malaria, influenza, and also as an antibiotic – see Figs. 11 and 12. In 1948, proguanil, also known as chloroguanide, was approved by the FDA to treat malaria and marketed as Paludrine, and in the hunt for other guanidine-based antimalarials, proguanil, was modified to metformin [2]. A Philippine physician described the benefits of 'Flumamine' (metformin) in 30 patients as an analgesic and anti-pyretic drug during an outbreak of viral influenza and also noted its ability to lower blood glucose [14] (Table 8).

However, it should be noted that hyperglycemia enhances oxidative stress and results in a pro-inflammatory state with an increase in NF-κB activation as, for example, has been demonstrated in adipose and also vascular tissue [435–437]. In addition, it is also well established that diabetes is associated with an increased risk of infection including viral respiratory tract infections as has been reported for H1N1 influenza [438,439]. It is therefore not surprising that the influenza vaccine is strongly recommended for subjects with diabetes [440]. Thus, the

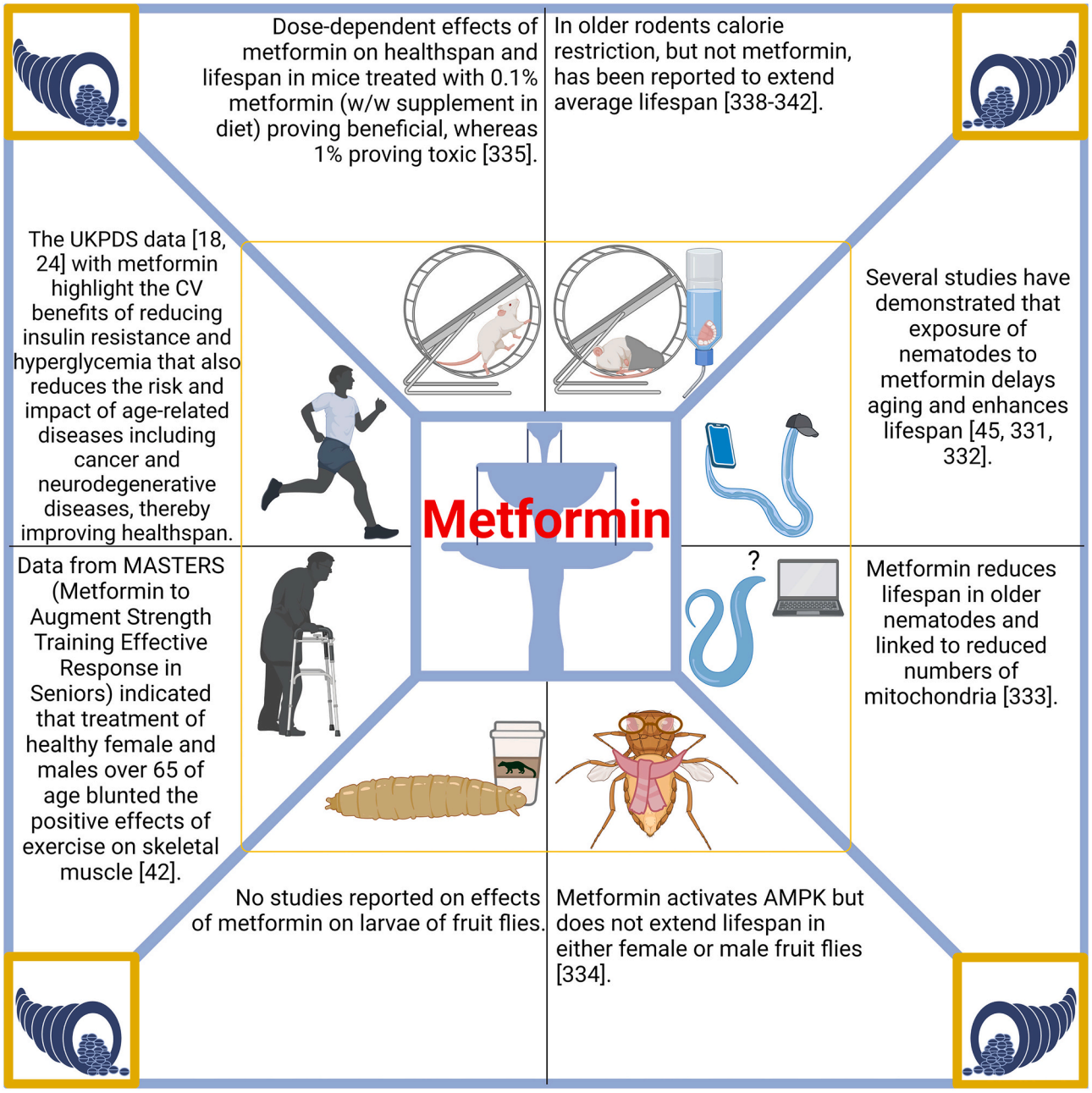

Fig. 10. Metformin as the Fountain of Youth.
It remains controversial whether metformin has beneficial effects as a gerosuppressant/geroprotectant/senolytic drug. Preclinical data as depicted in the schematic for rodents and nematodes (*Caenorhabditis elegans*) indicate age-dependent effects with benefits to offset aging seen only in younger rodents and worms. The age-dependent effects are depicted by the young rodent enjoying the treadmill, whereas the older rodent on the right slumbers. Similarly in young nematodes (as represented by the tech-savvy worm with the reversed baseball cap) metformin extends lifespan, but not so in older nematodes. These age-related differences in the effectiveness of metformin may be linked to declining mitochondria numbers/function. In adult fruit flies (*Drosophila melanogaster*) metformin has been shown to activate AMPK but no effects on lifespan have been reported. No data could be found for the effects of metformin in Drosophila larvae. For humans, there is substantive evidence that the treatment of subjects with type 2 diabetes has long-term benefits to reduce CV morbidity that may thereby enhance healthspan (the period of life when one is healthy). However, studies have also shown that the physiological benefits of exercise in elderly subjects are offset by metformin. Collectively, these data suggest caution for the use of metformin other than for those with type 2 diabetes and, possibly, pre-diabetes. As summarized in Table *5* several clinical trials are underway that have been designed to determine the effects of metformin on age-related morbidities and the results will aid in future decisions regarding extending the clinical use of metformin as a geroprotective drug. For critical reviews of the literature pertaining to metformin and aging see references [280 and 327]. This figure was created with *BioRender.com*.

putative benefits of metformin to treat influenza and other infections may be secondary to its antihyperglycemic and insulin sensitizing properties and predictably, patients with pre-diabetes or diabetes will also be at greater risk of viral-infection related complications [441].

Similarly, in critically ill COVID-19 patients hyperglycemia, regardless of whether associated with diabetes, has been shown to be associated with a higher risk of adverse outcomes [404]. The link between hyperglycemia and the severity of COVID-19 has also been emphasized by a report by Reiterer et al. (2021) [405]. 49.7% of 3854 patients diagnosed with COVID-19 and 91.1% of intubated COVID-19

Table 6
Clinical studies of the effects of metformin on neurodegenerative diseases.

Beneficial effects of metformin	Negative effects of metformin
Koenig et al [352]: Improved executive functioning 8 week randomized placebo controlled study with metformin in 20 non-diabetic subjects with Alzheimer's	Imfeld et al [364]: Analysis based on UK-based General Practice Research Database (1998–2008) of 7086 matched pairs of patients aged older than 65 indicated that subjects prescribed metformin were at greater risk of developing Alzheimer's. Elevated risk was not seen with patients prescribed sulfonylureas, thiazolidinediones, or insulin
Ng et al [365]: Singapore Longitudinal Aging Study of 365 diabetic subjects, aged 55 years or older, followed for 4 years showed improved cognitive function with metformin.	Akimoto et al [366]: Analysis of data from 66,085 subjects over 65 years of age who had volunteered to the FDA Adverse Event Reporting System indicated that patients receiving GLP-I RAs were less likely to develop Alzheimer's than those receiving metformin.
Cheng et al [367]: Results from a 5-year study of 67,731 aged 65 or older indicated that those who took metformin for a longer period of time had a reduced chance of developing dementia.	Kuan et al [368]: A 12-year follow up of >4600 patients with T2DM who received metformin versus the non-metformin cohort indicated that metformin use was associated with an increased risk of neurodegenerative diseases. Authors hypothesize that the increased risk is linked to metformin-induced vitamin B-12 deficiency.
Guo et al [369]: 24 week treatment with of patients with depression indicated that compared to placebo metformin increased cognitive function in patients with T2DM.	A number of studies have linked a beneficial effect of IGF-1 levels in the brain to neuroprotection and reducing the risk of Parkinson's and Alzheimer's diseases (Dore et al [370,371]: Castilla-Cortazar et al [372]; Poor et al [373]. Metformin by lowering IGF-1 may enhance risk of developing neurodegenerative diseases.
Hsu et al [374]: Analysis of a cohort of 800,000 over 50 subjects in Taiwan's National Health Insurance database for the period 2000–2007 indicated that T2D doubled the risk of dementia, but was decreased by 38% those treated with a sulfonylurea or metformin. Wahlqvist et al [360]: Analysis of a cohort of 800,000 over 50 subjects in Taiwan's National Health Insurance database for the period 1996–2007 indicated that T2D increased the risk of Parkinsonism 2.2 fold, and risk was further increased by those treated with a sulfonylurea; whereas combination with metformin was protective. Samaras et al [375] Data from the Sydney Memory and Ageing Study indicated a significantly slower decline in cognitive function in subjects in the age group of 70–90 years with T2D. 123 in study of which 67 received metformin.	Hsu et al [374]: Data infers that protective effect of drug therapy against development of dementia is linked to anti-hyperglycemic actions of drug rather than specific drug. Ping et al [362]: Analysis based on a systematic review and meta analysis of 19 studies and > 280,00 subjects found no significant benefit on incidence of neurodegenerative diseases. Importantly concluded that there was a 66% increase in risk of Parkinson's in patients prescribed metformin versus non-metformin users. Recommendation: Risk-benefit of prescribing metformin should be carefully evaluated in patients at risk of Parkinson's. Antal et al [376] Dataset from UK Biobank of cognitive assessment and neuroimaging of ~1000 subjects with T2D and ~ 19,000 healthy controls revealed no benefit of metformin.

Table 7
Preclinical studies of the putative neuroprotective actions of metformin.

Studies supporting protective effects of metformin against neurodegenerative diseases.	Studies that question unique benefit of metformin to reduce risk of neurodegenerative diseases.
Lennox et al [377] studied the effects of a 20-day treatment with the GLP-1 analogue, Val8)GLP-1(GluPAL), alone or in combination with metformin (300 mg/kg) and reported enhanced learning memory and exploratory behaviour as well as hippocampal expression of mTOR, VEGF, NTRK2, and SIRT1. Allard et al [378] reported that treatment of fat-fed mice with metformin for 6 months enhanced performance in the Morris water maze test suggesting improved hippocampal memory function.	Study by Lennox et al [377] did not include a metformin alone arm. In the study by Allard et al [378] it is reported that chronic treatment with metformin (1% by weight in drinking water) lowered RNA, but not protein, levels for BDNF, NGF and NTF3 as well as Nrf2, the antioxidant gene. The authors suggest caution over long-term use of metformin and that effects in older mice may be detrimental.
Ou et al [379]. APP/PS1 mice are a model of early onset Alzheimer's disease that express a chimeric mouse/human amyloid precursor protein (APP) and a mutant human presenilin 1(PS1). Daily injections with metformin, 200 mg/kg, via an AMPK-dependent process prevented the neuronal death and functional deficits caused by amyloid beta plaques (Aβ) in APP/PS1 mice. Results suggest that metformin can not only promote neurogenesis but also reduce Aβ plaques that have been linked to a variety of neurodegenerative diseases, including Parkinson's disease, and thereby minimize the damage caused by the formation of Aβ plaques. Similarly, Lu et al [380] also with APP/PS1 mice reported that metformin (200 mg/kg/day for 8 weeks) therapy counteracted learning and memory dysfunctions, improved brain uptake of glucose, lowered oxidative stress and enhanced the expression of insulin-degrading enzyme (IDE), and induced the expression of neurotrophic factors such as brain-derived neurotrophic factor (BDNF), synaptophysin (Syp), and nerve growth factor (Ngf).	McNeilly et al [381] demonstrated that a 10-week treatment with metformin improved the metabolic changes associated with a high fat diet but had no beneficial effects on cognitive function in fat-fed rats. Barini et al [382]: Data from in vivo studies involving chronic treatment of the P301S mutant human tau (P301S) transgenic mouse model of tauopathy, with metformin, 2 mg/ml in the drinking water from 4 weeks of age for 4 months, showed reduced tau phosphorylation in the cortex and hippocampus but increased insoluble tau species and exacerbated hindlimb atrophy and leading to the conclusion that the use of metformin in elderly patients with diabetes could increase the risk of tauopathic changes Of significance, is that plasma levels of metformin obtained in the in vivo arm of study reported by Barini et al. (2016) were in the low micromolar range (~1 micromolar) and comparable to the trough levels in T2D patients treated with metformin. Zhang et al [383]: γ-secretase is an important enzyme for the generation of Aβ. Son et al [384]: investigated effects of metformin on Aβ formation via an increase in production of β and γ-secretases. In vivo data from mice injected ip with 200 mg/kg metformin for 9 days showed increases in Aβ plaques. In vitro data was generated with human neuroblastoma cells treated in culture with 2.5, 5 or 10 mM metformin for up to 6 h. Data indicated that metformin results in enhanced β and γ-secretase activity and linked to activation of AMPK-mediated inhibition of mTOR and activation of autophagy and autophagosomes.
Patil et al [385]: The pro-drug MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxin and is used to induce a mouse model of Parkiunson's. A 21 day treatment with metformin, 500 mg/kg/day, resulted in a significant improvement of the locomotor and muscular activities in MPTP-treated mice. Fitzgerald et al [386]: TRAP1 is a mitochondria matrix chaperone protein and important for mitigating α-synuclein-induced mitochondrial	Chen et al [387]: In an in vitro study using N2A neuroblastoma cells metformin at 10 mM enhanced the synthesis of Aβ plaques. Noble et al [388]: Phosphorylated tau is a noted pathology associated with neurodegeneraticve diseases. Gupta et al [389]: Data derived from in vitro studies based on the use of mouse Neuro-2a (N2A) showing reduced tau hyperphosphorylation have used protocols that require 1.6 mM metformin for optimal effectiveness. Such high

(*continued on next page*)

patients had elevated glucose levels (>170 mg/dl; 9.4 mM), that were associated with elevated C-peptide thus indicating insulin resistance as the likely cause of the hyperglycemia [405]. Furthermore, data from hamsters infected with SARS-CoV-2 virus revealed that adiponectin levels were reduced thus inferring adipose tissue dysfunction [405]. The glucose-regulated protein, GRP78, is highly expressed in adipose tissue and has been proposed as a binding partner with ACE2 for the SARS-CoV-2 virus and thereby contributing to the association of obesity with an elevated risk with COVID-19 [441,442]. Early reports, including those from China and Italy, indicated that subjects with diabetes were approximately twice as likely to die from COVID-19 [443–445]. In the report from Wuhan published in May 2020 it was concluded that age,

Table 7 (*continued*)

Studies supporting protective effects of metformin against neurodegenerative diseases.	Studies that question unique benefit of metformin to reduce risk of neurodegenerative diseases.
dysfunction and mutations in PINK1 and is associated with mitochondria dysfunction and early-onset Parkinson's disease in humans. In cell culture protocols with human fibroblasts with the TRAP1 R47X mutation exposure to 10 mM metformin, inhibits complex 1 and rescued the mitochondrial membrane potential suggesting a potential mechanism linking metformin use to a reduced risk for Parkinson's disease.	concentrations are unlikely to be achieved in the brain when metformin is used clinically. In conclusion, data from a number of in vitro that have used mM concentrations of metformin are supportive of the benefits of metformin; however, based on the known pharmacokinetic properties of the drug it is unlikely that such benefits will be seen when metformin is used clinically (Kickstein et al [390] Gormsen et al [70]).
Wang et al [391]; Fatt et al [392]: In adult mouse neuronal stem cells metformin, in a concentration range of 1 to 500 μM, activated the AMPK-atypical protein kinase C (aPKC)-CREB binding pathway and the self-renewal proliferation pathway via the AMPK-independent activation of the putative p53 family member tumor-suppressor transcription factor, TAp73. Supportive data from in vivo study with mice treated with equivalent of 960 mg/day for a 60 kg human. These data suggest that by stimulating two distinct molecular pathways, metformin represents a neuro-regenerative agent capable of extending the adult neural precursor population and also moving them toward neuronal differentiation. Ma et al. [393]: a study with high-fat diet mice fed 250 mg/kg/day metformin has linked the beneficial effects of metformin on learning and memory diet to the microbiota and also shown a positive effect of fecal transplantation from metformin-fed mice.	Miller and Kaplan [394]; Merendez and Vazquez-Martin [395]: Based on advances in our knowledge of stem cell biology there is considerable interest in pursuing whether metformin can differently activate a neuronal repair process via neurogenesis and avoid promoting oncogenesis. de la Monte et al [396]: The strong link between diabetes, obesity and insulin resistance and neurodegenerative diseases suggests that insulin sensitizing drugs including PPAR agonists and not just metformin may be beneficial in reducing risk. In addition, the effects of metformin (and other drugs) on the microbiota and implications for disease modifying require further investigation.

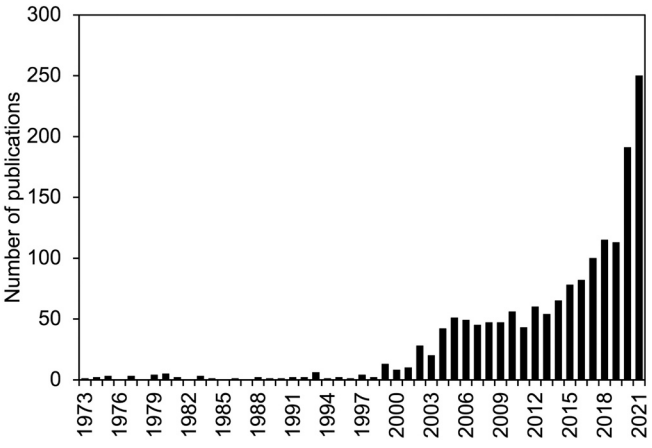

Fig. 11. Growth in publications mentioning metformin as an antibiotic. Data obtained from Scopus, 6 February 2022, using this search: (TITLE-ABS-KEY (metformin, OR dimethylbiguanidin,e OR dimethylguanylguanidine, OR glucophage)) AND (TITLE-ABS-KEY (antibiotic, OR antibacterial).

levels of CRP, and insulin use increased the risk of death. However, the total number of patients, 904, was relatively small and of these, only 136 were confirmed as having diabetes with T2D being dominant [443]. Nevertheless, these findings have been confirmed by analysis of data from much larger numbers of COVID-19 patients [403,445,446].

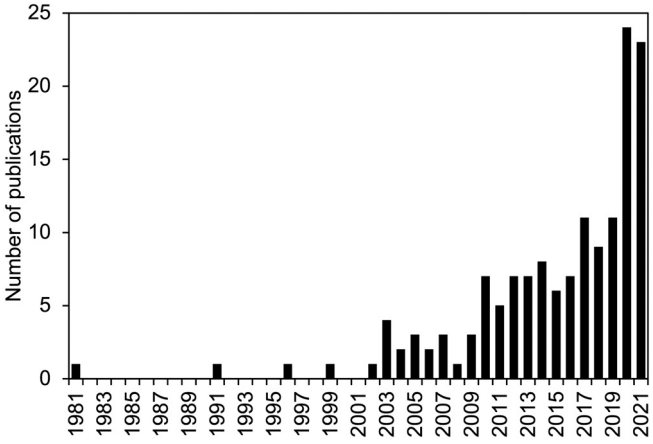

Fig. 12. Growth in publications mentioning metformin and malaria. Data obtained from Scopus, 6 February 2022, using this search: (TITLE-ABS-KEY (metformin, OR dimethylbiguanidine, OR dimethylguanylguanidine, OR glucophage) AND (TITLE-ABS-KEY (malaria, OR plasmodium, OR antimalarial).

Collectively, these data have emphasized the critical importance of intensive blood glucose management in COVID-19 patients [403].

Not surprisingly, the obvious questions that arise from these reports are: *" Of the drugs available to treat T2D is there one choice that provides an added benefit for patients with COVID-19, and secondly, are there anti-diabetic drugs that increase the morbidity and mortality associated with COVID-19?"* Essentially, and based on the currently available evidence, the tentative answer to both questions is "No". However, based on the data from the Reiterer et al. (2021) [358] study, thiazolidinediones, which are known to enhance the release of adiponectin from adipose tissue, might be a treatment option for COVID-19 patients presenting with hyperglycemia and obesity [447]. Drucker (2021) [441] emphasized the importance of critically reviewing the evidence particularly when much of the published data comes from retrospective studies.

Because metformin is the most frequently prescribed drug for patients with T2D attention has been focused on whether COVID-19 patients treated with metformin fare better than patients treated with other anti-diabetic drugs. For the two-year period, 2020–2021 over 500 publications related to this subject have appeared (Fig. 13).

It is first important to point out that based on an interactome evaluation of a large number of potential drugs to be repurposed for treating COVID-19 no evidence was presented that metformin within a concentration range of 10 nM to 100 μM had a direct inhibitory effect on viral growth in Vero E6 cells transfected with SARS-CoV-2 [411]. Clinical data do, however, indicate the benefits of metformin and support its continued use in COVID-19 patients, but subject to kidney and liver function [448]. Data from a retrospective cohort study in Wuhan, China, of 1213 hospitalized COVID patients with pre-existing T2D indicated that metformin treatment reduced heart failure and inflammation although its use was associated with a higher incidence of acidosis and notably in more severe cases of COVID-19 [449]. Data from a cohort study using a large UK primary care dataset analyzed 29,558 subjects with COVID-19 of whom 10,271 who were being treated with metformin concluded that those treated with metformin were not at greater risk for severe outcomes [450]. Several retrospective studies and systematic reviews have reported that the use of metformin reduces mortality in patients with COVID-19 [398–402,451].

The benefits of metformin have been attributed to its previously described anti-inflammatory actions via the suppression of IL6 and TNFα [398,452]. An observational cohort study utilizing the National Diabetes Audit in the UK for people with T2D assessed the risk of different glucose lowering drugs in an impressively large population of >2.8 million people [406]. The conclusion was that there was no clear indication to change the anti-diabetic drug(s) prescribed to the patient with

Table 8
Metformin as an anti-bacterial, anti-viral drug.

Support for anti-viral/anti-SARS-CoV-2 actions of metformin	Alternative explanations
Garcia [14]: Flumamine (metformin) relieved symptoms of influenza. *"Flumamine certainly holds a strong promise as both a antimalarial and bacteriostatic remedy. If it can lower the blood sugar level to the minimum physiological limit, it can destroy the malarial parasites indirectly by attrition. Similarly, if it has some bacteriostatic power, as demonstrated in the rapid recovery of many cases of virus influenza, whether acute or protracted, then its manner of action is probably that of antimetabolite of a certain enzyme or substance, which is present in the body and promotes the growth of the causative agent-a virus in case of influenza"*	Observational study of 30 patients with influenza who were treated with an IM injection of 32.5 mg of metformin. Headache was relieved remarkably quickly 'within 5 min', and anti-pyretic effects were recorded at "1 °C every 18 h". No randomization, or placebo group. Rather than a direct anti-viral action the anti-pyretic action could be attributed to regression to the mean.
Data from retrospective studies and systematic reviews indicate that COVID-19 patients treated with metformin have lower mortality rates (Bramante et al[398]; Lukito et al [399]; Crouse et al [400]; Wargny et al [401]; Zangiabadian et al [402]).	COVID-19 patients with well-controlled blood glucose fared better than those with uncontrolled hyperglycaemia (Zhu et al [403]). The severity of COVID-19 has been linked to the level of hyperglycemia (Mamtani et al [404]; Reiterer et al [405]) and metformin is more likely to be prescribed to younger patients with less severe diabetes (Khunti et al [406]; Wargny et al. [401]). Sulfonylureas were shown to be equally as effective as metformin but have distinct mechanisms of action (Khunti et al [406]). Conclusion: Benefits of metformin result from anti-hyperglyemic actions.
Xun et al [407] report that metformin inhibits hepatitis B replication in human hepatoma cells.	Data is from a cell culture protocol and IC50s are in the mM range (2.75–2.85) and not achievable when used clinically in man without significant toxicity.
Esam [408] hypothesizes that metformin, with a pKa of approximately 12 will accumulate and be 'ion-trapped' in the acidic endosomes and block the pH-dependent endocytotic entry of SARS-CoV-2 and thereby reduce infection. A similar mechanism of anti-viral action has been proposed for hydroxychloroquine that is also a basic drug, but not supported by clinical data as in the NHS RECOVERY study (Krogstad and Schlessinger, [409]; Bansal et al. [410]; Gordon et al [411]). Such actions may contrubute to the effectiveness of hydroxychloroquine as an anti-inflammatory drug for lupus and arthritis (Chen and Geiger [412]). By a similar mechanism it has been proposed that metformin has anti-malarial actions as a result of accumulation in the acidic food vacuole of the Falciparum parasite (Vera et al [13]).	i. Interactome analysis of a large number of drugs with the objective of potential re-purposing for COVID-19 indicates that metformin, in the concentration range 10 nM to 100 μM, shows no anti-viral activity against SARS-CoV-2 as assessed in African green monkey kidney epithelial Vero E6 cells transfected with the virus (Gordon et al [411]). Note: A number of other drugs, including hydroxychloroquine did show anti-viral activity. ii. Unlike hydroxychloroquine that has a very large volume of distribution (V_D) of 200 to 800 l/kg and a very long half-life of ~40 days and does accumulate in endosomes and food vacuoles, metformin has a V_D of no >10 l/kg and a half-life of only 4 to 6 h (Tett et al [413]; Browning, [414]; Liang and Giacomini, [415]). Thus, from a pharmaco-kinetic perspective metformin is very unlikely to accumulate in endosomes and significantly change endosome or food vacuole pH. iii. PET analysis of ^{11}C-metformin indicates that except for accumulation in the GI tract there is no evidence of significant accumulation in other tissues (Gormsen et al [70]; Jensen et al [416]; Iversen et al [71]).
An alternative mechanism whereby metformin raises endosome pH is via inhibition of the Na^+/H^+ exchanger (NHE) and the plasmalemmal V-ATPase as has been reported for *C. elegans* and rat microglia (Labuzek et al [417]; Kim et al [418]; Kim and You, [419]; Zhang et al [213]). Furthermore, such an inhibition would also link to the activation of AMPK and turning off the mTORC1 pathway (Zhang et al [240]; Kim and You [419]). Ma et al [97]: Based on in vivo studies with mice as well primary hepatocytes PEN-2 (presenilin enhancer 2), a regulatory component of the protease γ secretin complex, has been identified as the target for metformin with a Kd of 1.7 μM. Selective knockouts indicate importance of PEN-2 in mediating liver effects of metformin on fat content, and glucose-lowering effects via intestine PEN-2. Metformin-bound PEN2 also shown to inhibit lysosomal v-ATPase and activate AMPK independent of changes in AMP levels. Similarly, anti-aging effects of metformin in *C. elegans* were linked to PEN-2.	Studies with *C. elegans* were performed with 100 mM metformin, and those with rat microglia used 2 mM metformin. As already noted it remains uncertain whether metformin has any significant effect on NHE or V-ATPase during use clinically when plasma levels are in the low μM range [213,370–372]. However, studies by Ma et al [97] were based on a low 5 μM concentration of metformin thus supporting a potential mechanism of action. Replication studies are required.
Simoes e Silva et al [420]; Lei et al [421]: Angiotensin enzyme 2, ACE2, (the cell membrane receptor for the SARS virus) plays an important anti-inflammatory/anti-fibrosis role and offsets endothelial dysfunction. Ursini et al [422]; Sharma et al [423]: Hypothesize that AMPK-mediated phosphorylation of serine-680 on ACE2 will reduce the affinity of the virus for ACE2 and reduce infection.	The contribution of AMPK-mediated changes in the ability of the SARS virus to bind and enter human cells is theoretically possible, but as discussed other anti-hyperglycemic drugs, such as the sulfonylureas are equally as beneficial as metformin in reducing the heightened risk of COVID-19 patients who also have T2D (Khunti et al [406]). In addition, in vitro protocols that have studied the phosphorylation of ACE2 serine-680 have used high concentrations of metformin, 1 and 5 mM (Zhang et al [424]; Shang et al [425]).
In vitro data indicates that metformin has antibacterial effects on T. spiralis, S. aureus, P. aeruginosa and anti-viral actions on hepatitis B (Malik et al [426]; Masadeh et al [192]).	For studies with metformin alone >500 μM was required to see anti-bacterial effects, but in combination with standard antibiotics synergism was observed.
Benefits of metformin in the treatment of tuberculosis (TB) have also been reported both in patients, in mice and also in in vitro assays (Niazi et al [427]; Pan et al [428,429]; Bohme et al [430]; Singhal et al [431]). A systematic review in 2019 (Yu et al [432]) that was based on 12 observational studies and 6980 patients with diabetes concluded that metformin reduced the risk of TB, but also concluded the need for RCTs to provide stronger evidence.	Protocol required 2 mM metformin to demonstrate inhibition of mycobacteria survival in vitro (Singhal et al [431]). Furthermore, there is a strong association between the immunosuppression associated with diabetes and the risk of TB (Berbudi et al [433]; Al-Rifai et al [434]) and thus treatment with an anti-hyperglycemic drug would be expected to reduce the risk of infection and data linking HbA1c levels to the efficacy of different drugs is missing (Pan et al [428] [429]).

COVID-19 [406]. In this study the majority of patients received metformin: 63.1%; with 19.7% a sulfonylurea; 9.3% a SGLT2 inhibitor; DPP-4 inhibitors; 16.8%, and 12.3% insulin, but only 2.1% with a thiazolidinedione and 3.9% a GLP-1 receptor agonists [406]. Furthermore, analysis of the data indicated that those prescribed metformin, SGLT2 inhibitors and sulfonylureas statistically had a lower mortality risk than those prescribed insulin or a DPP-4 inhibitor. However, as the authors stress, metformin is usually the first drug prescribed to newly diagnosed patients with T2D and such patients usually have less severe diabetes, whereas in the UK DPP-4 inhibitors, as reflected in the report, are often reserved for more frail elderly patients who also have reduced renal function, with insulin given to patients with more advanced T2D [406]. Interestingly, the fact that patients prescribed metformin, a sulfonylurea or a SGLT2 inhibitor, drugs with distinct non-overlapping mechanisms of action, fared equally well suggests that the reduced risk is linked to the ability of the drug to control blood glucose levels rather than to pleiotropic actions unrelated to glycemic control. A similar viewpoint has been expressed in editorials [453,454] and also in reports linking

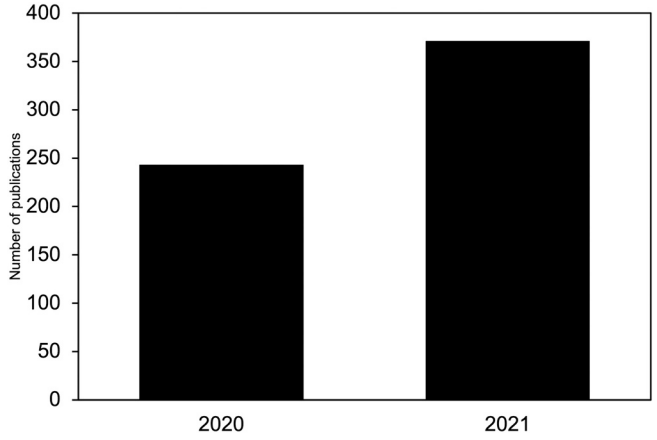

Fig. 13. Publications mentioning metformin and COVID-19. Data obtained from Scopus, 5 February 2022, using this search: (TITLE-ABS-KEY (metformin, OR dimethylbiguanidine, OR dimethylguanylguanidine, OR glucophage)) AND (TITLE-ABS-KEY ("coronavirus pneumonia", OR "COVID-19", OR "2019 novel coronavirus infection", OR "2019-nCoV" OR "SARS-CoV-2").

hyperglcemia to the severity of COVID-19 [405]. Essentially all classes of drugs that are used to treat hyperglycemia and lower blood glucose levels reduce pro-inflammatory markers such as CRP, IL-6 and ferritin [406,441,455–457]. The results from the Coronavirus-SARS-CoV-2 and Diabetes Outcomes (CORONADO) study in France concluded it was the younger patients and those with less severe co-morbidities who were also receiving metformin that fared better [401,458]. A report from Spain also concluded no association between anti-diabetic drug use and adverse outcomes or mortality [459].

Although there are limitations to the interpretation of observational cohort studies the data presented by Khunti et al. are highly suggestive that the primary benefit of treating T2D patients who are infected with COVID-19 is maintaining good glycemic control and a reduction in insulin resistance as was also the conclusion of an earlier study from Wuhan [403,404,406]. Data from ongoing studies may help provide clarification. NCT04510194 is a RCT that is designed to compare benefits of metformin, or ivermectin, or fluvoxamine versus placebo treatment for 14 days in an early outpatient study of 1160 patients with COVID-19; measurements in addition to severity of symptoms include CRP and viral load (https://clinicaltrials.gov/ct2/show/NCT04510194). Results are expected in 2022.

The COVID-19 pandemic has stimulated the publication of a number of reviews discussing the benefits of the use of metformin in patients with COVID-19 and a number have suggested cellular mechanisms that are independent of its anti-hyperglycemic actions [191,193,412,423,460–463] Table 8 and Fig. 14 critically evaluate and summarize some of these putative mechanisms.

In conclusion, the benefits of using metformin in the treatment of bacteria and viral infections, including COVID-19, infections are most likely entirely secondary to its benefits as an anti-hyperglcyemic drug where, as a result of improving glucose regulation, metformin, protects endothelial function and reduces thromboinflammation (likely via mitigating oxidative stress [87]), and enhances the immune response of the patient. The evidence cited that metformin has direct anti-viral/bacterial actions is primarily based on studies that have used supra-pharmacological concentrations and/or are based on incorrect extrapolations of the pharmacokinetic properties of the drug when it is used clinically.

8. Conclusions

Fig. 15 provides a summary of the numerous potential sites of action and putative benefits and potential toxicities of metformin for the management of diabetes, and re-puroposing for the treatment of PCOS,

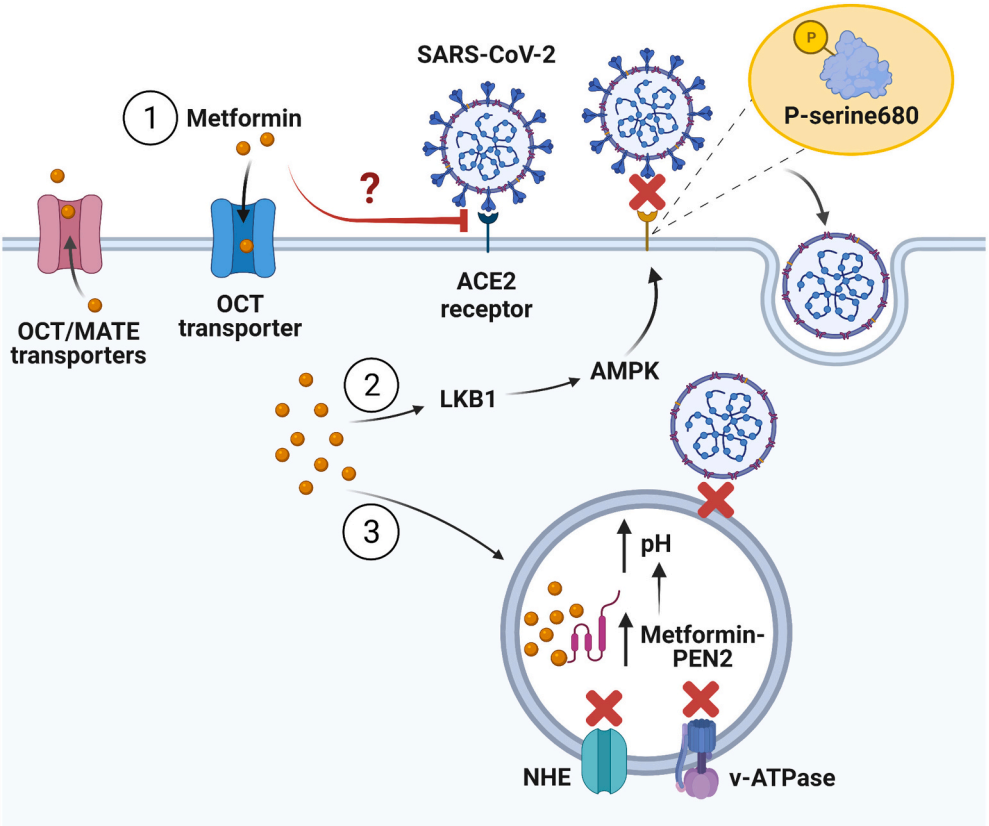

Fig. 14. Putative cellular mechanisms for metformin in the treatment of COVID-19. Clinical data indicates that patients with COVID-19 and exhibit diabetes have a higher mortality, which has been linked to blood glucose levels. Metformin and other anti-diabetic drugs have also been shown to reduce the morbidity and mortality associated with COVID-19. Although no direct anti-viral actions of metformin have been demonstrated, as reflected in this schematic, a number of anti-hyperglycemia-independent effects of metformin that potentially could reduce viral pathology. These actions include AMPK-mediated phosphorylation of serine680 on the host enzyme angiotensin converting enzyme 2 (ACE2) and receptor for the SARS-CoV-2 virus that reduces the ability of the virus to enter the host cell. Inside the host's cell metformin, by virtue of being a cation at physiological pH and with a pKa of approximately 12, will be 'trapped' in the acidic environment of endosomes, thereby raising pH and inhibiting the enzymes critical for viral replication (see Esam, 2020 [408]). The pH of endosomes may also be increased as a result of metformin inhibiting the Na^+/H^+ exchanger (NHE) along with inhibition of the lysosomal v-ATPase as result of metformin binding to presenilin enhancer 2 (PEN2) (see references [97,417–419]). This figure was created with BioRender.com.

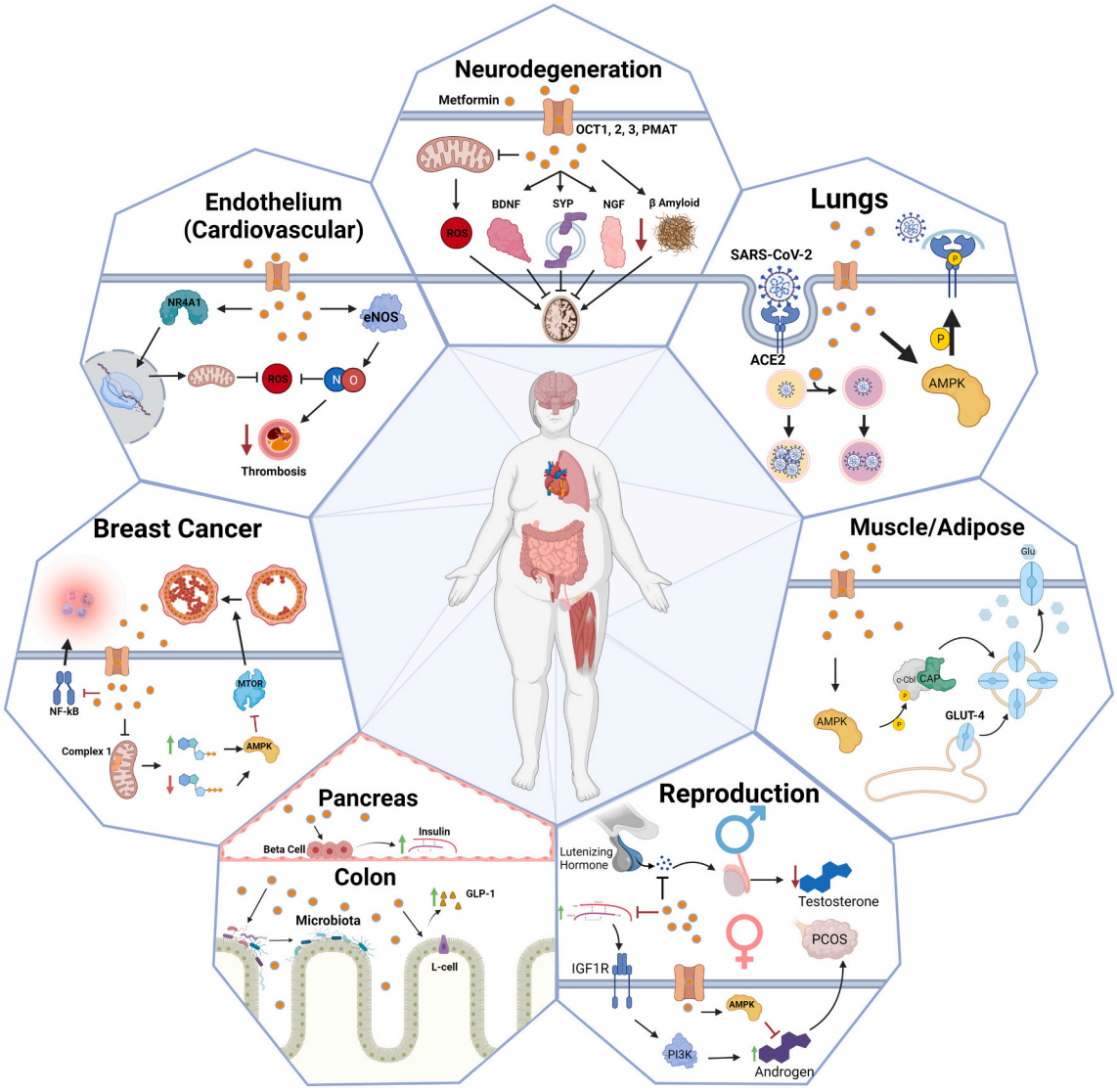

Fig. 15. Summary of putative sites of action of metformin for the treatment of multiple diseases.
Metformin by reducing ROS and ß-amyloid formation and modulating growth factor actions protects against neurodegenerative diseases. In the lungs metformin via activation of AMPK phosphorylates the cellular target, ACE2, for SARS-CoV-2 and reduces the cellular entry of the virus. The effects of metformin to enhance glucose utilization in striated muscle and adipose tissue are indicated with the role of AMPK activation in enhancing the translocation of the glucose transporter, GLUT4, to the plasma membrane. Metformin is used for the treatment of polycystic ovary syndrome (PCOS) with its benefits attributed to improving insulin sensitivity, reducing insulin levels and thereby reducing the activity of steroidogenic enzymes. Conversely, in males the use of metformin has been linked to genital defects in male offspring [36] that may be linked to reports of effects of metformin on human and mouse testicular cells, lowers testosterone levels, and also reports in fish that it is an endocrine disruptor [464–469] The effects of metformin on the microbiota in the gut where the release of GLP-1 from L-cells that in turn enhances the release of insulin from ß-cells in the pancreas. The anti-cancer effects of metformin have been attributed to the activation of AMPK and subsequent inhibition of mTOR. Activation of AMPK also inhibits NFκß resulting in a reduction of inflammatory cytokines. The endothelial-vascular protective effects of metformin are also illustrated. Metformin, via actions involving the nuclear receptor NR4A1 and also via protection of eNOS function, enhances the generation of NO and reduces ROS and thromboinflammation. This figure was created with BioRender.com

cancer, neurodegenerative diseases, and COVID-19.

Although several new classes of anti-diabetic drugs have been introduced in recent years metformin remains the first choice oral anti-hyperglycemic agent for most patients with T2D. The benefits of using metformin include the 60-year history of its use, safety profile and that it is comparatively inexpensive versus the newer drugs available such as the GLP-1 receptor agonists and SGLT-2 inhibitors. Metformin has demonstrated cardiovascular benefits although it is unclear whether metformin is superior to newer agents such as the GLP-1 receptor agonists and SGLT-2 inhibitors. Interestingly the results of a meta-analysis of 19 RCTs with >18,000 subjects with T2D that was published in 2022 concluded that compared to other glucose-lowering drugs and placebo there was no evidence that metformin was clinically superior in protecting against the microvascular complications investigated [470]. However, benefits of metformin in kidney disease, retinal disease, neuropathy, and assessment of quality of life were not included in the studies [470]. There is clearly a need for appropriately designed prospective studies, but it is unlikely that the equivalent of another UKPDS will be launched.

In considering where metformin can be repurposed the evidence is clear that it plays a useful role in the treatment of PCOS. Metformin's role in the treatment of TID is limited and probably only benefits those patients who require high doses of insulin. The apparent low therapeutic efficacy for patients with T1D is perhaps surprising given the extensive evidence that metformin directly protects the endothelium from the effects of hyperglycemia, which arguably would be expected to reduce

morbidity and mortality. Despite a very extensive literature covering data from both pre-clinical and clinical studies the role of metformin in the treatment of cancer remains highly controversial. With respect to the clinical data a couple of concerns relate to the issue of bias in data analysis of retrospective studies, and the issue of time-dependent analysis of drug exposure that collectively may have over estimated the benefits of metformin and several reports indicate no reduction in the risk of cancer. In addition, it remains difficult to separate the indirect benefits of metformin via its anti-hyperglycemic and insulin-sensitizing effects in patients from the putative direct anti-proliferative actions of metformin. For instance, for the latter there is only minimal clinical biomarker data to support a role the inhibition of mTOR. In contrast, there is an extensive pre-clinical database to support a role for metformin in inhibiting the mTOR pathway. Interpretation of much of the in vitro data is problematic because the majority of studies have used cell culture protocols with supra-pharmacological concentrations of metformin and long-incubation times that do not reflect the pharmacokinetics of metformin when used clinically. New data from on-going RCTs may help resolve these uncertainties. Similar concerns exist with respect to the utility of metformin as an anti-aging and neuroprotective agent. Arguably, via its benefits as a widely used anti-hyperglycemic and insulin-sensitizing drug with proven vasoprotective actions it would be expected to provide protection against age-related diseases. However, data indicating that it is less effective than exercise and may, in fact, negate some of the benefits of exercise as well as the therapeutic efficacy of metformin decreasing with age indicate caution is needed in the over-promotion of its use beyond for patients with T2D. Again, new data from appropriately designed RCTs may resolve these questions. It is also uncertain as to whether metformin exerts significant direct anti-inflammatory effects other than via its positive effects on glucose metabolism. In the absence of evidence of a direct anti-viral action the anti-inflammatory and endothelial-vascular protective effects of metformin may prove to be the basis for its use being associated with improving the outcome of patients with COVID-19, particularly because those most severely affected are those who are unhealthily obese individuals with pre-existing diabetes.

The expanded use of metformin may have an environmental impact as metformin has been widely detected in the aquatic environment of many countries, including the Great Lakes in North America, with concentrations ranging from ng/l to mg/l [471–473]. There are no known metabolites of metformin, and it may accumulate and negatively affect numerous organisms as has already reported in species of fish. At levels found in waste water, it is reported to act as an endocrine disrupter [467,468] in *Danio rerio* (zebrafish) and *Daphnia pulex* (water fleas) with behavioural effects seen in zebrafish at concentrations as low as 10 nM [469]. A conservative estimate based on 150 million people taking 1 g/day of metformin is that potentially 150,000 kg/day of the drug are voided in the urine reflecting an increasing concern on the health of numerous species including *Homo sapiens*. Possibly related to the effects of metformin in fish are the data from the nationwide cohort study in Denmark from Wensink et al. (2022), which has provided evidence that treatment of men with metformin is linked to genital birth defects in their male offspring [36]. This finding promoted the comment from Allan Brett, Editor in Chief NEJM Journal Watch: *"This observational study suggests a relation between paternal pre-pregnancy metformin use and birth defects. Without confirmation in another patient population, it would be premature to prohibit metformin use by men of reproductive age who have overt type 2 diabetes. However, clinicians are increasingly giving metformin to men with prediabetes; in my view, these results provide one reason to avoid metformin in such patients when they are in the reproductive age group"* [474]. Clearly, more studies are required to determine the impact of metformin on human reproductive sytems and the environment.

Finally, metformin may still hold secrets as is evident from the evolution from the view that the drug primarily acts in the liver to one where multiple sites of action and signaling pathways are involved, but still not fully resolved including targets such as NR4A1, PEN2, and HMGB1. An expansion of therapeutic benefits of metformin may, however, be offset by the impact of metformin as an endocrine disruptor. Predictably metformin, and in keeping with its botanical association with the perennial flower, *Galega officinalis*, interest will continue to blossom for many years to come.

CrediT authorship contribution statement

CRT was the primary reviewer of the literature, initiated and established the objectives for the manuscript, and finalized the submission. K.B. and I.M. (I. Mohammed) reviewed the literature and prepared first drafts. H.D., I.M. (I. Marei) and K.Y. reviewed the literature and prepared summary figures, 3, 10 14 and 15. R.M. conducted the Scopus analysis and prepared Figs. 1, 5–7, 9, and 11–13 MDH and MAH provided input throughout the preparation of the manuscript and also revisions.

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Declaration of competing interest

All authors declare no conflicts of interest.

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