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ABSTRACT

Despite its position as the first-line treatment for type 2 diabetes mellitus, the mechanisms underlying the plasma glucose level-lowering effects of metformin (1,1-dimethylbiguanide) still remain incompletely understood. Metformin is thought to exert its primary antidiabetic action through the suppression of hepatic glucose production. Furthermore, the discovery that metformin inhibits the mitochondrial respiratory-chain complex 1 has placed energy metabolism and activation of AMP-activated protein kinase (AMPK) at the center of its mechanism of action. However, the role of AMPK has been challenged and might only account for indirect changes in hepatic insulin sensitivity. Various mechanisms involving alterations of cellular energy charge, AMP-mediated inhibition of adenylate cyclase or fructose-1,6-bisphosphatase-1 (FBP1) and modulation of the cellular redox state through direct inhibition of mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH) are proposed for the acute inhibition of gluconeogenesis by metformin. Emerging evidence suggests that metformin could improve obesity-induced meta-inflammation via direct and indirect effects on tissue-resident immune cells in metabolic organs (that is, adipose tissue, the gastrointestinal tract and the liver). Furthermore, the gastrointestinal tract also has a major role in metformin action through modulation of glucose-lowering hormone glucagon-like peptide-1 (GLP-1) and intestinal bile acid pool and alterations in gut microbiota composition.

Key points

• Metformin is the first-line drug for treatment of type 2 diabetes mellitus, with an excellent safety profile, high efficacy on glycaemic control and clear but incompletely understood cardioprotective benefits.

• The pleiotropic properties of metformin suggest that the drug acts on multiple tissues through various underlying mechanisms rather than on a single organ via an unifying mode of action.

• The mitochondrial respiratory-chain complex 1 is a key cellular target of metformin and its mild and transient inhibition is involved in the AMPK-independent regulation of hepatic gluconeogenesis by triggering alterations in the cellular energy charge and redox state.

• Metformin might contribute to improvements in obesity-associated meta-inflammation and tissue-specific insulin sensitivity through direct and indirect effects on various resident immune cells in metabolic organs.

• The gastrointestinal tract has an important role in the action of metformin, which modulates bile acid recirculation and enhances the secretion of the glucose-lowering gut incretin hormone glucagon-like peptide-1 (GLP1).

• The gut microbiota represents a novel target in the mechanisms of metformin action and is involved in both the therapeutic and adverse effects of the drug.

Introduction

Metformin is the first line of treatment in patients with type 2 diabetes mellitus (T2DM) as recommended by clinical guidelines published by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD)[1,2], which are supported by prospective studies[3,4] and recent 2016 meta-analyses[5,6]. These guidelines are based on the improved glycaemic profile and reduction in cardiovascular mortality induced by metformin treatment, without the risk of hypoglycaemia and/or body weight gains that are associated with the use of other antidiabetic drugs[3,4,6,7]. Furthermore, metformin is also the favoured antidiabetic drug because of its good safety profile and minimal cost[8].

The drug metformin is a biguanide derivate (1,1-dimethylbiguanide hydrochloride), which originates from the plant goat's rue or French lilac (*Galega officinalis*)[9]. Originally used in medieval Europe to relieve the symptoms of diabetes mellitus, this plant contains galegine, an isoprenyl guanidine. Although mono- and diguanidine derivatives are toxic, the biguanides (two N-linked molecules of guanidine) synthesized from the reaction between dimethylamine hydrochloride and dicyanodiamide[10] have been used for the treatment of diabetes mellitus since the late 1950s (REF.[9]). Of note, metformin has been approved for usein Europe and Canada since 1957, although it was only introduced into the USA in 1995. The more potent biguanides, phenformin and buformin, were quite popular in the USA and Europe in the 1960s but were removed from the markets in most countries in the late 1970s owing to an increased risk of lactic acidosis[11]. By contrast, the incidence of lactic acidosis associated with metformin treatment is very low (approximately 3 to 10 cases per 100,000 person-years and ~1.5 deaths per 100,000 patient-years.), except in high-risk groups, particularly patients with chronically impaired renal function or in acute kidney disease[8]. The most common adverse effect of metformin is usually gastrointestinal intolerance (BOX 1).

Metformin is described as an anti-hyperglycaemic agent that does not cause clinical hypoglycaemia in patients with T2DM, nor alter glucose homeostasis in non-diabetic individuals[5-7]. Although metformin has been available for over 60 years for the treatment of T2DM, the exact mechanism(s) for its effects on blood glucose levels still remained elusive[12]. However, several actions have been attributed to its ability to lower blood glucose. For example, metformin acts primarily by the suppression of enhanced

basal endogenous glucose production in individuals with T2DM through a 25–40% decrease in the hepatic gluconeogenesis rate as determined by ^{13}C nuclear magnetic resonance (NMR) spectroscopy or tracer infusion[13,14]. Moreover, in some euglycaemic-hyperinsulinaemic clamp studies, a beneficial effect of metformin on insulin sensitivity has been reported in skeletal muscle[15,16], although not in all studies[17]. Metformin treatment is accompanied by insulin-stimulated systemic glucose disposal, which occurs predominantly in skeletal muscle[16,18]. In addition, the drug increases peripheral glucose utilization by the intestine, primarily via non-oxidative metabolism[19]. Emphasis is now given to the importance of metformin actions within the gut[20,21], possibly via alteration of intestinal microbiota[22]. The notion that biguanides decrease mitochondrial oxygen consumption has offered a plausible molecular mechanism of action for a direct suppression of hepatic gluconeogenesis, which occurs via decreases in cellular energy charge and the subsequent activation of AMP-activated protein kinase (AMPK), a cellular energy sensor[23]. However, the contribution of the aforementioned mechanism to metformin's therapeutic effects is a subject of intense debate[12,24]. In addition, the literature is confounded with a plethora of putative mechanisms of action for metformin, which have been elucidated from the use of supra-therapeutic concentrations of metformin in *in vitro* and preclinical studies, as well as the differences reported between acute and chronic metformin administration in human and animal models.

In this Review, we discuss our current understanding of molecular mechanisms that underlying the pleiotropic actions of metformin on metabolism and inflammation. In addition, we focus on the recent research developments on the actions of metformin in the liver and intestine to regulate blood glucose levels in patients with T2DM.

Pharmacokinetics and pharmacogenetics

Metformin is administrated orally and has a low bioavailability (40–60%)[25]. The drug is not metabolized and is excreted unchanged in the urine. After a single oral dose of 0.5 g, metformin is distributed systemically within 6 hours following absorption, which predominantly occurs in the upper small intestine, with negligible absorption in the large intestine[25] The peak plasma metformin concentration occurs 3 hours after administration and leads to concentration of ~3 μg per ml (~18 μM)[26]. Notably, analysis of tissue-specific biodistribution of the drug using ^{14}C-labeled metformin or dynamic

positron emission tomography (PET) with ^{11}C-metformin revealed its substantial accumulation in the gastrointestinal tract, liver and kidney in mice[27,28]. Interestingly, these results have been partially replicated in humans, showing ^{11}C-metformin PET biodistribution mainly in the liver and rapid excretion through the kidneys[29].

Metformin is a strongly hydrophilic compound with an acid disassociation constant (pKa) of 11.5; at physiological pH the drug exists as a monoprotonated cation[23]. Given these chemical properties, the transport of the drug across biological membranes involves uptake via specific transporters. This process is in line with the inverse relationship observed in humans between the dose ingested and the relative absorption rate[25]. The absorption, distribution and excretion of metformin depend mainly on the organic cation transporters (OCTs), multidrug and toxin extruders (MATEs) and plasma membrane monoamine transporter (PMAT). For example, metformin is taken up from the intestinal lumen by PMAT (encoded by *SLC29A4*) and OCT3 (encoded by *SLC22A3*) that are localized at the apical membrane of enterocytes. Subsequently the drug is transported into the bloodstream by OCT1 (encoded by *SLC22A1*), which is localized at the basolateral membrane of enterocytes[30]. The hepatic uptake of metformin depends on OCT1 and possibly OCT3, whereas MATE1 (encoded by *SLC47A1*) contributes to its hepatic elimination[30]. In kidney, the uptake of metformin into renal epithelial cells is primarily facilitated by OCT2 (encoded by *SLC22A2*), whereas MATE1 and MATE2 (encoded by *SLC47A2*) contribute to the renal excretion of the drug into the urine[30]. Importantly, the role of these transporters in the pharmacokinetics of metformin has been recently confirmed by ^{11}C-metformin functional PET imaging in mice deficient in these transporters or treated with specific inhibitors[28].

Some human genetic studies have associated genetic polymorphisms in the aforementioned transporter genes to modifications of metformin pharmacokinetics[30]. However, studies of the effect of these variants altering metformin transport on clinical responses in patients with T2DM have yielded contradictory results, and uncertainty remains[31]. Although all these transporters clearly play a role in metformin pharmacodynamics, they do not appear to be critical to the pharmacodynamics of the drug in diabetic populations. A recent 2017 large-scale meta-analysis reported that polymorphisms in genes encoding tranporters involved in metformin pharmacokinetics (*SLC22A1*, *SLC22A2*, *SLC22A4*, *SLC47A1*, and *SLC47A2*) have no significant effect on glycaemic response to the drug[32], indicating possible transporter redundancy or the

contribution of mechanisms of action that are independent of the systemic exposure to the drug. However, studies of pharmacodynamic genetics revealed that variation in the glucose transporter GLUT2 gene (*SLC2A2*) is associated with a greater glycaemic response to metformin[33]. Moreover, this effect could be linked to the restoration of glucose sensitivity by the drug[34]. Other genetic candidates for metformin response are the genes encoding the subunits of AMPK (*PRKAA1*, *PRKAA2* and *PRKAB2*) and the AMPK upstream kinase LKB1 (*STK11*)[35]. However, additional clinical and mechanistic studies are needed to clearly delineate influences of these genes on the clinical response to metformin.

Targeting mitochondria

The notion that metformin could exert some its action through direct interaction with the mitochondria dates back from the early 1950–1960s, when various biguanides were shown to inhibit mitochondrial oxidative phosphorylation (OXPHOS)[36,37]. However, the hypothesis that mitochondria are involved in the cellular mechanism of action of metformin was conceptually strengthened in the 2000s, when two independent groups reported for the first time that metformin inhibited the mitochondrial respiratory-chain complex 1 (the first and largest enzyme complex of the electron transfer chain)[38,39]. This effect was highly specific but also weak and reversible[38,39], contrasting with the characteristics of canonical mitochondrial respiratory-chain complex 1 inhibitors, such as rotenone and piericidin A[40], which are both uncharged and highly hydrophobic molecules with a very low IC50 ($\sim$2 μM). The inhibition of mitochondrial respiratory-chain complex 1 by metformin leads to a mild decrease in ATP generation by mitochondrial OXPHOS and a concomitant moderate increase in the AMP:ATP ratio in hepatocytes[38,39]. During the last 20 years, the selective inhibition of mitochondrial respiratory-chain complex 1 by metformin has been further confirmed in multiple biological models and species[41,42], including human primary hepatocytes[43] and various cancer cell lines[44-53]. Furthermore, this effect was usually associated with significant decrease in mitochondrial NADH oxidation, a decrease in the proton gradient across the inner mitochondrial membrane and decreases in cellular oxygen consumption rate[42], when these aspects have been studied in the models (**Figure 1**).

Inhibition of mitochondrial complex 1. The exact mechanism by which metformin specifically targets the mitochondria remains unclear and whether the drug inhibits mitochondrial respiratory-chain complex 1 directly or not is still a matter of debate[41,42,54]. Although many studies have shown that metformin accumulates at high concentrations within mitochondria[39,45], others have found that the distribution of metformin in hepatocytes is associated predominantly within the cytosol[55]. The main reasons for this controversy are variations in the concentrations of metformin and the experimental model (for example, intact cells versus isolated organelles) used in *in vitro* studies. Indeed, although clinically relevant drug concentrations of metformin (<100 µM) are sufficient to achieve a dose-dependent and time-dependent *in situ* inhibition of mitochondrial respiratory chain complex 1 in various cell types[38,46,56-58], these concentrations are ineffective on the activity of complex 1 in isolated mitochondria. By contrast, only supratherapeutic concentrations (that is, greater than the therapeutic dose) of the drug (20–100 mM) were reported to directly inhibit mitochondrial respiratory-chain complex 1 in both isolated mitochondria and sub-mitochondrial particles[42].

The low intra-mitochondrial accumulation of metformin in intact cells could primarily be explained by slow transporter-mediated permeation of the drug across the plasma membrane. However, some studies have also suggested the existence of a specific protein-mediated transport system for metformin mitochondrial import[59]. In line with this, experiments performed in *Xenopus laevis* (African clawed frog) oocytes showed that therapeutic concentrations of metformin were able to directly inhibit mitochondrial respiratory-chain complex 1 activity in isolated mitochondria, but only when delivered as a liposomal-encapsulated form. This finding lead to the hypothesis of an endocytic vesicular process that routes the drug from the plasma membrane to the mitochondria[57]. However, the other hypothesis that is currently favored by the majority of the research community is based on the peculiar physicochemical properties of this biguanide. The positive charge of metformin at physiological pH was proposed to account for its slow accumulation within the matrix of energized mitochondria of intact cells, driven by mitochondrial transmembrane electrochemical potential ($\Delta\Psi$)[39,45]. As such, it has been proposed that metformin could accumulate in mitochondria, theoretically reaching millimolar concentrations in the matrix of the organelle despite a cytoplasmic level within the micromolar range[39,41]. This $\Delta\Psi$-driven mitochondrial

import theory might also provide an explanation for the weak inhibitory effect of metformin on mitochondrial respiratory-chain complex 1; as metformin is imported into mitochondria, its progressive accumulation causes a decrease in mitochondrial membrane potential, which limits its own accumulation.

Of note, metformin is known for its metal-binding properties, particularly the binding of the drug with a high affinity to copper. The interaction of metformin with mitochondrial copper ions has been suggested to be involved in the inhibition of mitochondrial respiratory-chain complex 1 by the drug through a mechanism that remains to be fully characterized[60].

Metformin–mitochondrial respiratory-chain complex 1 interactions. The mammalian mitochondrial respiratory-chain complex 1 is a large L-shaped membrane-bound redox enzyme (also known as NADH:ubiquinone oxidoreductase) consisting of 14 core subunits and more than 30 accessory subunits that are encoded by both nuclear and mitochondrial genes[61,62]. The complex couples the transfer of electrons from NADH to the ubiquinone pool with a transfer of four protons from the mitochondrial matrix towards the intermembrane space[61]. Of note, the resolution of the crystal structure of mitochondrial respiratory chain complex 1 showed that it is organized in four functional modules (N, Q, proximal pump [PP] and distal pump [PD]) and exists in two distinct forms: a fully active one and a catalytically inactive so-called 'deactive' D-form (where the enzyme is catalytically inactive)[61,62]. Although research has shown that metformin does not alter the structural integrity of the whole complex[45] and probably binds to one of the phylogenetically conserved core subunits[61], the exact nature of the molecular interactions between the drug and mitochondrial respiratory chain complex 1 remains unknown. A substantial breakthrough came in 2014 from an elegant study, which methodically dissected the effect of metformin at different levels of the catalytic cycle of the enzyme[45]. Altogether, the study proposed that metformin is a reversible non-competitive inhibitor that binds to the Cys39-containing matrix loop of the mitochondrial respiratory-chain complex 1 subunit ND3, which is located within the amphipathic region between the redox and proton-transfer domains of the PP module. This interaction stabilizes the enzyme in an open-loop deactive conformation state[45] (**Figure 1**).

Metformin and hepatic gluconeogenesis

The liver has a central role in the blood glucose lowering effect of metformin in patients with T2DM. Metformin treatment decreases endogenous hepatic glucose production by a mechanism thought to mainly involve inhibition of gluconeogenesis[13,16]. In addition inhibition of hepatic glycogenolysis by metformin has been reported but less consistently[13,14]. After its intestinal absorption, metformin concentration is high in the portal vein, resulting in the accumulation of higher metformin levels in the liver than in other surrounding organs[27]. Furthermore, PET imaging with ^{11}C-metformin confirmed that the drug is primarily taken up in the liver in both humans and mice[28,29,27].

The main hepatic metformin transporter is OCT1. Mice deficient in Oct1 show a decrease in metformin accumulation in the liver and the drug loses its ability to lower plasma glucose levels[28,63]. Similarly, polymorphisms in OCT1 (*SLC22A1*) are thought to decrease the acute effects of metformin on glucose tolerance in humans[64]. However, the influence of OCT1 decreased-function variants on metformin response has not been consistently reported[31,32]. The discrepancy in study results could be explained by key differences in the assessment of metformin response, which is measured either during a dynamic response to an oral glucose challenge[64] or after a prolonged oral metformin use[32]. Although it seems clear that metformin could act via both acute and long term direct and indirect effects to decrease hepatic glucose production, several conflicting mechanisms have been proposed to explain the inhibition of gluconeogenesis by the drug.

Controversy on metformin concentrations. Over the last years, controversy has arisen concerning the use of high concentrations of metformin in preclinical (200–400 mg per kg) and *in vitro* studies (250 μM to 2 mM) using intact cells (specifically primary hepatocytes) when compared to the concentrations achieved in plasma from patients with diabetes mellitus treated with the drug[65]. However, due to a wide interindividual variability in metformin pharmacokinectics, the plasma distribution of the drug at steady-state is highly changeable in patients[66,67], which considerably enlarges the range of clinically relevant metformin concentrations. In rodent studies, the optimal dosage was established on the ability of metformin to induce a relevant glucose-lowering effect, with an effective oral single dose of metformin estimated to be around 200–250 mg per

kg in mice[68-71]. Of note, the high dose of metformin required in rodents compared to humans (humans require about 25-30 mg/kg per day, with patients with T2DM receiving an oral dose of 500 or 850 mg either twice or three times per day, up to a total of 2,550 mg per day) to achieve biological effects might in part result from a faster renal metformin clearance. Indeed, metformin has a mean plasma elimination half-life of 1–2 in mice and 4–9 hours in humans[72,73].

For *in vitro* studies, metformin concentrations above those found in patients with diabetes (40–70 uM, measured in the portal vein) were considered as clinically irrelevant[24,65,74,75]. However, studies in mice treated with oral metformin show an accumulation of a higher concentration of metformin in liver than in hepatic portal vein plasma[27,55]. Although concentrations ranging from 100–250 μM can be viewed as supra-pharmacological concentrations, these concentrations are reached in the liver of mice treated orally with 50 mg per kg metformin (a dose that is generally accepted as falling in the clinical range)[27]. Interestingly, chronic treatment of mice fed a high fat diet (HFD) with 50 mg kg metformin (leading to plasma concentrations of 29 μM) improved metabolic parameters in HFD-fed mice, however, a higher dose of 200 mg per kg was needed to lower glucose in acute metformin tolerance tests[69]. The acute administration of 250 mg per kg metformin to fasted mice lead to metformin concentrations of ~120 μM and ~0.8 μmol per g in plasma and liver, respectively[70]. This concentration metformin accumulating in the liver is comparable to intracellular metformin concentrations observed in measured in primary rat hepatocytes treated with 100 μM metformin for 4 hours[76]. Therefore, the treatment of primary hepatocytes with metformin concentrations above those found in the circulation might not necessarily be above therapeutic concentrations found in the liver.

Of note, the uptake of metformin into hepatocytes is slow, but the drug has the ability to accumulate at high concentrations within the cell and the mitochondrial matrix due to its positive charge, which allows it to interact with the polarized mitochodrial membrane[39,45,77]. Accordingly, the ratio of intracellular to extracellular metformin has been shown to be ~5:1 in primary rat hepatocytes incubated with 100–500 μM metformin[76]. Consequently, the effects of metformin are both concentration-dependent and time-dependent. Moreover, this point has been well illustrated in studies showing inhibition of mitochondrial respiratory-chain complex 1 in rat hepatoma H4IIE cells after incubation with low metformin concentrations (50–100 μM) for a prolonged

period of time (24 and 60 hours)[39]. Similarly, a decrease in glucose production occurs after incubation of mouse primary hepatocytes with 250 µM metformin at 8 hours[68], whereas a separate study showed inhibition of glucose output with lower metformin concentrations (80 µM) after a longer incubation period of 24 hours[24]. Therefore, effects observed with relatively high concentrations of metformin might also occur at lower concentrations after longer incubation. In future studies, it will be interesting to correlate the levels of intracellular metformin accumulation with the impact of the drug on hepatic glucose production.

Metformin-induced AMPK activation. The cellular energy sensor AMPK is a critical regulator of energy homeostasis that is activated by energy stresses that increase cellular ADP:ATP and/or AMP:ATP ratios[23]. In 2001, metformin was reported to stimulate AMPK activation in rat primary hepatocytes[77]. Furthermore, this effect was later attributed to the inhibition of mitochondrial respiratory-chain complex 1 by the drug, which causes an altered cellular energy charge characterized by a decline in intracellular ATP levels concomitant with increase in intracellular ADP and AMP intracellular levels [23,43,68]. An elegant 2010 study confirmed this finding by using cells expressing AMPK mutant insensitive to AMP and showing that metformin-induced AMPK activation is dependent on AMP binding to the regulatory AMPK-γ subunit, even if changes in the levels of intracellular adenine nucleotide levels are technically undetectable[78].

Although it is assumed that AMPK activation is mediated by the canonical energy stress mechanism, evidence is accumulating that glucose starvation can activate AMPK by a non-canonical mechanism that is independent of changes in the AMP:ATP ratio, which involves the formation of a complex comprising AMPK, axin, LKB1, v-ATPase and ragulator at the surface of lysosomes[79,80]. Whether metformin could also activate AMPK via this lysosomal pathway has been examined in mice and primary hepatocytes deficient for axin. Interestingly, metformin-mediated AMPK activation was abolished in liver-specific axin-deficient mice after chronic intraperitoneal administration of 50 mg per kg of metformin and in axin-deficient hepatocytes treated with 70 µM metformin for 3 hours[81]. Nonetheless, the consequence of axin deletion on metformin-induced inhibition of glucose production has not been addressed. These data raise the intriguing possibility that metformin acts at the lysosome surface as well as within mitochondria,

therefore future studies examining the functional link between metformin and the lysosomal pathway are warranted.

An important 2001 study suggested that activation of AMPK was necessary for metformin-induced inhibition of glucose production in rat primary hepatocytes based on the use of the AMPK inhibitor Compound C (also known as Dorsomorphin)[77]. Later, the involvement of AMPK in metformin action was strengthened by a study reporting that deletion of LKB1 (the upstream kinase of AMPK) prevented activation of AMPK and suppressed the glucose-lowering effects of metformin in vivo, in HFD-fed mice[82]. Moreover, this study suggested that activation of the LKB1–AMPK signalling pathway by metformin represses the gluconeogenic transcriptional program through the phosphorylation and nuclear exclusion of the transcriptional coactivator cAMP-response element-binding protein (CREB)-regulated transcription coactivator 2 (CRTC2), which is a key regulator of gluconeogenic gene expression[82] (**Figure 2A**). In addition, other mechanisms induced by metformin that lead to the inactivation of CRTC2 and involving AMPK have also been proposed[83-85].

Importantly a breakthrough 2010 study challenged the aforementioned model of AMPK-dependent transcriptional inhibition[68]. This study demonstrated that metformin-induced inhibition of glucose production was preserved in primary hepatocytes, even when the expression of gluconeogenic enzymes was forced through overexpression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which is a master co-activator of gluconeogenic genes. As such, the findings indicate that metformin inhibits the activity of gluconeogenic enzymes rather than their gene expression[68]. Importantly, metformin still inhibits glucose production and gluconeogenic gene expression in primary hepatocytes from mice deficient in AMPK catalytic subunits or LKB1 expression in the liver, which demonstrates that neither AMPK nor LKB1 are crucial for inhibition of hepatic glucose production by metformin[68]. Furthermore, hepatic AMPK-deficient mice displayed normal glycaemia, pyruvate tolerance and blood glucose-lowering effect of metformin[68,86,87].

To further support the absence of a direct effect of AMPK activation on the regulation of hepatic gluconeogenesis, treatment with the small-molecule direct AMPK activator (A-769662) showed no influence on endogenous hepatic glucose production both *in vitro* in primary hepatocytes[68] and *in vivo* in mice[75]. Similarly, the more potent AMPK activators PF-249 and PF-739 have no impact on the endogenous glucose production after acute

administration in mice[87]. Thus, although metformin activates AMPK by reducing cellular energy charge, the metformin-induced acute suppression of glucose production is seemingly independent of the LKB1–AMPK signalling pathway and transcriptional alterations.

Several studies have suggested that the effects of metformin that are associated with a reduction in cellular energy charge result from supra-pharmacological concentrations of the drug; at therapeutic concentrations, metformin is proposed to act without affecting cellular energy levels[24,74,75,88]. Specifically, low concentrations of metformin have been shown to suppress glucose production in primary hepatocytes via AMPK activation, independently of any detectable changes in AMP:ATP ratio[24].

AMP-dependent mechanisms. It has been proposed that metformin acutely inhibits hepatic glucose production through AMPK-independent mechanisms that involve modulation of cellular energy status and allosteric inhibition of crucial gluconeogenic enzymes. Gluconeogenesis is a highly energy-consuming metabolic pathway, which requires six ATP equivalents (4 ATP and 2 GTP) per molecule of glucose synthesized. As such, the decrease in intracellular ATP levels resulting from the inhibition of mitochondrial respiratory-chain complex 1 could be the simplest explanation to account for the reduction of hepatic gluconeogenic flux by metformin[68]. This model is supported by the strong correlation between decreases in intracellular ATP levels and inhibition of glucose production in mouse primary hepatocytes that are incubated with metformin, which emphasizes the close relationship between hepatic energy status and glucose production[68].

The alteration of cytosolic and mitochondrial NADH:NAD$^+$ ratios by metformin[38,39] could also be involved in the inhibition of glucose production (**Figure 2B**). Furthermore, the mild metformin-induced increase in intracellular AMP levels through compromised hepatic energy status might also exert a major role in the flux control of gluconeogenesis via an allosteric inhibition of fructose 1,6-bisphosphatase (FBP1), which is a key enzyme in gluconeogenesis[68] (**Figure 2B**). Interestingly, in one study the glucose-lowering effect of metformin was shown to be significantly decreased in a mouse model bearing a point mutation in FBP1 that rendered it insensitive to inhibition by AMP[70]. In addition, it has been shown that the increase in intracellular AMP levels induced by metformin decreased glucagon-stimulated gluconeogenesis in liver[71]. Indeed, AMP inhibits the

glucagon-induced activation of adenylate cyclase (the enzyme that catalyzes the cyclization of ATP into cyclic AMP) by binding to an inhibitor site on the enzyme known as site P, which subsequently lowers intracellular cyclic AMP levels, decreases protein kinase A (PKA) activity and decreases the phosphorylation of essential factors that stimulate gluconeogenesis, which include 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1, inositol trisphosphate receptor and CREB-1. These events in turn lead to a decrease of glucagon-stimulated glucose production[71] (**Figure 2C**).

Alteration of redox state. An alternative model of metformin action has been suggested, in which the drug decreases hepatic gluconeogenesis independently of AMPK-mediated and energy charge-mediated effects through a direct inhibition of mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH)[75,88]. The concerted action of the mitochondrial isoform of G3PDH (mG3PDH), which is located on the outer side of the inner membrane, and the cytosolic isoform of G3PDH (cG3PDH) creates the glycerol-phosphate shuttle. This shuttle is one of two shuttles that transfer the cytosolic reducing equivalents (that is, NADH) directly into the mitochondrial electron transport system for their re-oxidation. Importantly, two outcomes of the inhibition of the glycerol-phosphate shuttle by metformin have been proposed: first, a disruption of glucose production from glycerol; second an increase in cytosolic redox potential (NADH:NAD$^+$), which impedes the utilization of lactate for gluconeogenesis[75,88] (**Figure 3A**). This mechanism predicts that metformin suppresses gluconeogenesis from glycerol or reduced gluconeogenic substrates (such as lactate) but not from oxidized substrates (such as alanine or pyruvate).

The aforementioned hypothesis has been experimentally supported by studies *in vivo* and in primary hepatocytes[75,88]. Metformin treatment reduced hepatic glucose production, while increasing cytosolic redox and decreasing mitochondrial redox states in liver[75,88]. These findings were replicated in mG3PDH-deficient mice[75]. Similarly, primary hepatocytes that are deficient in mG3PDH showed inhibited gluconeogenesis, which recapitulates the effects of metformin in control hepatocytes. In mG3PDH-deficient hepatocytes, glucose production was abolished from glycerol or reduced substrates (for example, lactate) but was unaffected from oxidized substrates (for example, alanine or pyruvate)[75]. However, this model of metformin action raises several concerns. The glycerol-phosphate shuttle has an important role in tissues that oxidize

glucose rapidly (such as skeletal muscle and the brain) to regenerate NAD from NADH that is produced by glycolysis[89,90]. However, in the liver, the malate-aspartate shuttle is the primary NADH shuttle[91,92]. Furthermore, as highlighted elsewhere[93], inhibition of the glycerol-phosphate shuttle might not be sufficient to impact gluconeogenesis. In a mouse model, disruption of the glycerol-phosphate shuttle has no effect on glycaemia whereas the disruption of malate-aspartate shuttle lowers fasting glycaemia and increases the cytosolic ratio of NADH:NAD$^+$ (REF.[63]).

Finally, the model of inhibition of mG3PDH by metformin was recently challenged[74]. In this study, researchers found that metformin had no effect on mG3PDH activity in cell-free assays and that the drug inhibited hepatic glucose production from both reduced and oxidized gluconeogenic substrates. In addition, the study reported that pharmacological inhibition of mG3PDH had no effect on glucose production[74]. These findings led to the proposal that metformin increases the cytosolic ratio of NADH:NAD$^+$ independently of the inhibition of mG3PDH or of mitochondrial respiratory-chain complex 1 via mitochondrial membrane depolarization, which is caused by metformin accumulation in mitochondria. In turn, this accumulation then inhibits the electrogenic transporter for aspartate of the malate-aspartate shuttle[74]. However, the same study suggested that metformin inhibits gluconeogenesis by an energy charge-independent and redox-independent mechanism. Metformin-induced inhibition of the malate-aspartate shuttle might stimulate the glycerol-phosphate shuttle, leading to a decrease in glycerol-3P (a potent allosteric inhibitor of PFK1 (phosphofructokinase 1) levels. As a result, gluconeogenesis is inhibited through the partitioning of substrates towards glycolysis[74] (**Figure 3B**).

The alteration of hepatic redox potential caused by metformin probably has an important role in the inhibition of gluconeogenesis. However, concern exists about whether the effect of metformin on the redox state (that is, the ratio of NADH:NAD$^+$) is dissociable from an effect on energy charge (that is, the ratio of AMP:ATP). NADH is the major source of electrons that flow through the mitochondrial electron transport chain to generate ATP. Consequently, whether metformin hampers NADH mitochondrial transfer by mG3PDH inhibition, or alternatively by acting to depolarize the mitochondria membrane, *de facto* cellular energy charge is expected to be impaired. Thus, a model in which metformin modifies redox status without compromising energy charge seems contradictory. Of note, in the studies supporting the metformin redox

state hypothesis, changes in the redox state induced by metformin were concomitant with AMPK activation[74,75,88], which is a very sensitive marker of cellular energy stress.

In addition, in the proposed metformin mechanism of action that involves a mild inhibition of mitochondrial respiratory chain complex 1, both the AMP:ATP and the NADH:NAD$^+$ ratios are altered[38,39]. Altogether, despite intense debate, the relative contributions of energy charge and redox state changes in metformin action still need to be established.

Effects of chronic AMPK activation. Although AMPK activation does not account for the acute glucose-lowering effect of metformin, chronic metformin-induced AMPK activation might indirectly inhibit hepatic glucose production by increasing hepatic insulin sensitivity. For example, pharmacological activation of AMPK has been reported to alleviate hepatic steatosis in fatty liver mouse models[86,94,95] via the inhibitory phosphorylation of acetyl CoA carboxylase (ACC) 1 and ACC2, which results in the inhibition of lipid synthesis and stimulation of fatty acid oxidation (**Figure 2D**). Moreover, the importance of AMPK–ACC pathway in the action of metformin on glucose and lipid metabolism was further demonstrated in mouse models in which ACC1 and ACC2 were rendered insensitive to AMPK phosphorylation[69]. These mouse models are resistant to the lipid-lowering and glucose-lowering effects of metformin, which indicates that the decrease in blood glucose levels in response to metformin depends on the ability of the drug to lower intracellular lipid content. Thus, the ability of metformin to improve insulin sensitivity through the AMPK–ACC pathway might, at least in the long-term, contribute to the suppression of hepatic glucose production in the context of insulin resistance. Importantly, this mechanism might also explain the resistance of HFD-fed LKB1-deficient mice to the glucose-lowering effect of metformin, which was described earlier[82]. However, it is not clear whether this mechanism is relevant in humans owing to the controversy that exists regarding the efficacy of metformin treatment to reduce hepatic lipid content[96].

Metformin and meta-inflammation

The chronic low-grade inflammation associated with obesity, which is also called meta-inflammation or metaflammation, is one of the major contributors to insulin resistance and impaired glucose and lipid homeostasis in metabolic syndrome and

T2DM[97]. The immune system is now well recognized to have a key role in the regulation of whole-body metabolic homeostasis through a variety of innate and adaptive immune cells that are present in various organs, most notably in adipose tissue and the liver[98,99]. In obesity, the oversupply of dietary nutrients (especially fatty acids) triggers enhanced recruitment of monocytes to metabolic organs (that is, adipose tissue and liver), which become polarized towards pro-inflammatory 'M1-like' macrophages. Importantly, M1-like macrophges are believed to mediate, at least in part, tissue-specific insulin resistance[100].

In healthy adipose tissue, a network of type 2 immune cells secretes anti-inflammatory cytokines, which trigger macrophages to become polarized towards an alternatively activated 'M2-like' state. These M2-like macrophages support the resolution of inflammation and tissue-specific insulin responsiveness, this latter effect through mechanism(s) that are currently largely unknown. In obesity and T2DM, this finely-tuned system is altered[101], leading to change in adipose tissue macrophage polarization in favor of classically activated 'M1-like' macrophages, which orchestrate insulin resistance through enhanced secretion of pro-inflammatory cytokines[100]. Similarly, both newly-recruited and resident liver macrophages (Kupffer cells) and neutrophils were shown to play a pathogenic role in insulin resistance in both rodents and humans by increasing hepatic inflammation through the secretion of pro-inflammatory cytokines and elastase, respectively [102-104].

Other immune cells are present in adipose tissue and the liver that are also involved in meta-inflammation, for example CD4$^+$ T helper 1 (T$_H$1) and T helper 17 (T$_H$17) cells, B cells and natural killer T (NKT) cells[98,99]. At the molecular level, both *in vitro* and *in vivo* studies in rodent models of obesity have shown that the pro-inflammatory cytokines produced by macrophages (IL-6, TNFα and IL-1β), T$_H$1 cells (IFNγ) and T$_H$17 cells (IL-17) contribute to the inhibition of the canonical insulin signalling, notably through activation of NFκB and JNK pathways[105]. By contrast, the T$_H$2 cytokines IL-4 and IL-13 have been shown to promote insulin sensitivity and glucose homeostasis, at least in part by triggering the JAK–STAT–PPAR axis in various metabolic organs[106-110]. In addition, the production of IL-10 and TGFβ by regulatory T cells (T$_{reg}$) has been linked with protection against insulin resistance in mouse models of obesity[111,112].

Modulation of immune cell functions. Some clinical studies have suggested beneficial effects of metformin on systemic inflammatory markers, such as serum levels of TNFα, IL-6, PAI-1 and the neutrophil-to-lymphocyte ratio, in patients with type 2 diabetes[113-115] or patients with polycystic ovary syndrome (PCOS)[115] (BOX 2). However, most of the data supporting anti-inflammatory properties of metformin came from *in vivo* studies in HFD-fed obese mice[116-119] and a zebrafish model of obesity[120], or *in vitro* and *ex vivo* experiments with supra-therapeutic concentrations of the drug in various immune cell types, particularly macrophages[115,121-127]. In line with this, metformin was found to inhibit the lipopolysaccharide (LPS)-induced expression of pro-inflammatory cytokines (IL-6 and TNFα) in both mouse peritoneal macrophages and human monocyte-derived macrophages[121,124]. At the mechanistic level, this anti-inflammatory effect was attributed, at least in part, to an AMPK-dependent decrease in NFkB activity that is secondary to either inhibition of JNK signalling pathway or Activating Transcription Factor 3 (ATF3) induction[121,124] (**Figure 4**). Furthermore, metformin has been reported to inhibit monocyte-to-macrophage differentiation in the THP-1 macrophage cell line via an AMPK-mediated inhibition of STAT3 activation, which might contribute to reduce atherosclerosis by lowering the inflammatory environment within the vessel wall[124].

Metformin has also been shown to inhibit LPS-induced IL-1β secretion whilst potentiating IL-10 expression in mouse bone marrow-derived macrophages. However, the mechanism is thought to be independent of AMPK activation and might instead result from the specific inhibition of mitochondrial reactive oxygen species (ROS) production driven by reverse electron transport (RET) at the mitochondrial respiratory-chain complex 1[122]. Of note, this specific inhibition of RET-mediated ROS production has also been proposed to contribute to the decrease in oxidative stress-related cell death induced by metformin[46,56,58] and to its cardioprotective properties.

AMPK, as a central regulator of cellular metabolism, has a prominent role in both myeloid cell polarization and T cell effector responses[128,129]. Indeed, an emerging paradigm suggests that metabolic reprogramming and immune cell functions are intricately linked and that immune cell activation, proliferation and polarization are underpinned by profound changes in core metabolic pathways[130]. For example, pro-inflammatory macrophages and effector T cells mostly rely on glycolysis for the generation of ATP, whereas anti-inflammatory macrophages and T_{reg} depend instead on

AMPK-dependent fatty acid oxidation and mitochondrial OXPHOS[128,129] (Figure 4). So far, the contribution of AMPK-induced changes in cellular metabolism to the anti-inflammatory effects of metformin remains unknown and deserves dedicated investigation. Moreover, since the immunomodulatory properties of metformin have also been proposed to underlie some of its beneficial effects on auto-immune inflammatory diseases[131-134] and cancer[135-138], further studies are required to decipher the immune-mediated mode of action of metformin.

Metformin action in the intestine

Gut as the primary target of metformin. Although it is generally accepted that the anti-hyperglycaemic effect of metformin is primarily attributed to its action on the liver[139], accumulating data have suggested that the metabolic benefits of the drug might also be due to actions in the gut[19,21,140] (**Figure 5**). Early studies provided evidence that intravenous administration of metformin is less effective than oral dosing for inducing its glucose-lowering action[141-143]. In addition, metformin was reported to inhibit intestinal glucose absorption in proximal small intestine, which is seemingly associated with increased glucose utilization by the enterocytes in the intestine and increased lactate production through anaerobic metabolism[19,144-147].

Interestingly, imaging with PET-CT in patients with T2DM treated with metformin revealed enhanced intestinal glucose utilization as demonstrated by diffusely increased ^{18}F-fluoro-deoxyglucose (FDG, a non-metabolized glucose analogue) uptake in the colon and to a minor extent in the small intestine[148]. Of note, metformin is considered to increase the intestinal utilization of glucose that is taken up from the circulation, however, increased uptake of glucose from the intestinal lumen could also be contributing to the metabolic benefits of metformin[19,149]. The mechanism of metformin stimulation of intestinal glucose utilization is debated, but might be related to the redistribution of the glucose transporter 2 (GLUT2) at the apical membrane[34,150,151] of enterocytes or increased expression of the sodium glucose transporter 1 (SGLT1) (REFS[152,153]).

Metformin accumulates at very high concentrations (up to 30–300 times greater than the concentration in plasma) in the small intestine and the large intestine[27,154], suggesting that the gastrointestinal tract probably represents an important site of

metformin action for the management of T2DM. Moreover, studies in rat models and humans with T2DM have linked gastrointestinal metformin exposure in the proximal and distal small intestine to suppression of hepatic glucose production and attenuated glycaemic responses to oral glucose[155,156]. In addition, oral administration of a delayed-release formulation of metformin, which is released slowly in the distal small intestine (ileum) where absorption is low, resulting in low plasma metformin exposure, was shown to be as effective as the standard immediate-release formulation in lowering fasting plasma glucose levels in individuals with T2DM[20,157]. In further support of a dissociation between metformin plasma exposure and gut-restricted metformin action, inhibition of the transporter MATE, which mediates hepatic and renal elimination of metformin, had little effect on blood glucose levels despite an increase in metformin concentrations in plasma in humans[158] or in the liver in mice[28]. Taken together, these findings indicate that the response to metformin is not only related to its systemic exposure and illustrate the quantitative importance of the gastrointestinal tract in the glucose-lowering effect of metformin.

Effects of metformin on GLP1 release. One mechanism proposed to underlie the gut-mediated glucose-lowering effect of metformin relies on incretins. An increasing body of clinical evidence suggests that metformin increases the plasma levels of the gut incretin hormone, glucagon-like peptide 1 (GLP1) in patients with T2DM[159,160]. In addition, metformin has been reported to potentiate the responsiveness to incretin in mice by increasing the expression of pancreatic islet GLP1 receptor[161]. Of note, GLP1 has the capacity to improve blood glucose homeostasis in T2DM via pleiotropic actions, including feedback mechanisms that act through a gut–brain neuronal axis, stimulation of glucose-stimulated insulin secretion, inhibition of pancreatic glucagon secretion, slowing of gastric emptying and reduction in appetite and food intake.

The hormone GLP1 is secreted by intestinal enteroendocrine L cells in response to the presence of nutrients in the intestinal lumen. These cells are located mainly in the distal small intestinal mucosa and the colon. However, evidence suggests that the administration of metformin to the lower and upper gut is not critical to the capacity of the drug to increase plasma GLP1 levels and achieve its glucose-lowering effect in humans[156,162]. Instead, a mechanism has been suggested in which metformin acts to decrease glucose absorption in the upper small intestine, which causes an increase in

glucose concentrations in more distal regions of the gut. In turn, the increase in glucose concentrations then stimulates GLP1 secretion from the large population of enteroendocrine L cells located within the ileum[145]. The contribution of GLP1 signalling to the acute postprandial effect of metformin on plasma glucose levels was confirmed in a 2018 study of patients with T2DM. Infusion of the GLP1 receptor antagonist exendin 9–39. reduced the effect of metformin on postprandial glucose excursions, suggesting that the metformin-induced secretion of GLP-1 contributes to mode of action of the drug[163]. However, pharmacological inhibition or deletion of incretin receptor signalling showed no significant effect on the capacity of metformin to improve oral glucose tolerance in diet-induced obese mice[161]. This finding suggests that in mice, GLP1 secretion is not the predominant mechanism for the glucose lowering effect of metformin. Of note, incretin-based therapies are approved for use as add-ons to the first line metformin monotherapy to further amplify clinical outcomes, which supports the contribution of GLP1-independent mechanisms to the glucose-lowering impact of metformin [164].

Metformin has been hypothesized to increase both fasting and postprandial levels of GLP1 by decreasing its degradation by dipeptidyl peptidase-4 (DPP4) or by directly increasing GLP1 secretion from enteroendocine L cells, although *in vitro* studies have yielded inconsistent results (reviewed in REFS[145,165]). A range of potential mechanisms for acute metformin-induced GLP1 secretion have been proposed from *in vitro* and *ex vivo* studies using intestinal L cell lines or human colonic tissue sections[163]. These mechanisms include a role for muscarinic acetylcholine receptor[166], Wnt signaling[167] or AMPK activation[155].

Metformin has also been reported in *in vitro* studies to protect enteroendocrine GLP1-secreting L cells against lipoapoptosis via a sustained activation of AMPK in these cells[168]. This effect might explain the elevated plasma GLP1 levels that are associated with chronic metformin therapy. However, supporting evidence exists that shows metformin exerting an indirect stimulatory effect by increasing GLP1 secretion in response to the availability of the bile acid pool in the intestine of T2DM patients[160,169]. For example, metformin induces a decrease in the overall absorption of intestinal bile salts by strongly decreasing their absorption from the ileum (via an active process). This effect occurs despite a small increase in absorption of bile acids in the jejunum (via passive non-saturable concentration-dependent diffusion)[170]. Possible mechanisms for

metformin-induced suppression of active bile acid reabsorption in the ileum are inhibition of the apical sodium-dependent bile acid transporter (ASBT)[165] and modulation of the transcriptional activity of farnesoid X receptor (FXR, a bile acid receptor) via an AMPK-mediated mechanism[171] in enterocytes (**Figure 5**). Importantly, this process results in increased luminal concentration of bile acids in the ileum and colon, which increases GLP1 secretion via the stimulation of Takeda G-protein-coupled receptor 5 (TGR5) on enteroendocrine L cells[172-174]. Furthermore, the higher concentration of bile acids now present in the intestinal lumen might simultaneously decrease the activation of FXR in L cells, and hence promote GLP-1 production and secretion[175]. Thus, the combination of these intestinal effects might further stimulate metformin-induced secretion of GLP1 from L cells.

Metformin action on the gut–brain neuronal axis. Emerging evidence suggests the importance of nutrient sensing in the gut for the control of glucose homeostasis, through the activation of a gut–brain–liver negative feedback system to inhibit hepatic glucose production[176]. For example, glucose sensing in the upper small intestine is mediated through a SGLT1-GLP1 receptor (GLP1R)-dependent neuronal axis[153]. As such, the infusion of glucose into the upper small intestine stimulates intestinal GLP1 release, as revealed by increased portal vein active GLP1 levels, however, there is no effect on peripheral circulating GLP1 levels. These findings suggest that GLP1 acts locally in the gut by activating GLP1R on vagal afferent nerves that innervate the gut mucosa[153,177].

The mechanism by which intestinal glucose contribute to the release of GLP1 implicates a role for SGLT1-mediated glucose uptake in enteroendocrine cells[178,179]. To evaluate the contribution of small intestinal glucose-sensing mechanisms to the metabolic benefits of gut-restricted metformin, the impact of intraduodenal metformin infusion was assessed in rodents in response to HFD-induced impairments in intestinal glucose sensing. One-day treatment of metformin that is administrated directly into the upper small intestine of HFD-fed rats was found to restore upper small intestinal SGLT1-dependent glucose sensing, which resulted in the restoration of glucose-stimulated GLP1 release and suppression of glucose production[153]. In treated rats, increased expression of mucosal SGLT1 was dependent on changes in the composition of the upper small intestine microbiota, as transplantation of the microbiota from metformin treated rats into untreated HFD-fed rats restored the activity of the SGLT1-dependent

glucose-sensing pathway[180]. However, future work is warranted to elucidate how metformin affects the composition of the microbiota (further discussed later) and modulates small intestinal glucose sensing.

Interestingly, in an obese diabetic rodent model, infusion of metformin in the upper small intestine activates AMPK in the duodenal mucosa layer to lower hepatic glucose production. This effect is independent of upper small intestinal glucose sensing and acts through a gut–brain–liver neuronal network that triggers a GLP1R-protein kinase A-dependent pathway[155]. The action of intraduodenal infusion of metformin on the inhibition of hepatic glucose production is abrogated by co-infusion with a GLP1R antagonist and through chemical inhibition of neurotransmission via afferent fibers in the gut. Moreover, this study highlights the importance of an intestinal GLP1 dependent mechanism that is relayed by the activation of GLP1R on vagal afferent neurons in mediating the acute effects of intraduodenal metformin[155]. However, the underlying mechanism linking duodenal AMPK activation and GLP1 signalling has not yet been fully elucidated. It remains uncertain whether metformin activates AMPK directly in GLP1 secreting cells or acts indirectly (for example via bile acids accumulation) to stimulate the release of GLP1 for the subsequent stimulation of GLP1R on the vagus afferent nerve (**Figure 5**).

The glucoregulatory role of duodenal AMPK activation was also demonstrated in response to intraduodenal infusion of resveratrol (a natural polyphenolic compound known for its many beneficial effects to human health) or the AMPK activator A-769662, indicating the therapeutic relevance of targeting duodenal AMPK for gut-mediated antidiabetic therapies[155,181,182]. In a recent 2018 study, it was reported that inhibition of mTOR signalling is also required for the glucose-lowering effect of intraduodenal metformin but that mTOR acts independently of AMPK signalling[177], thereby adding a novel potential gut target to potently lower glucose levels in patients with T2DM.

Modulation of gut microbiota. Evidence is accumulating that suggests a causal relationship between intestinal microbiota dysbiosis (defined as an imbalance in the normal populations of microorganisms present in the gastrointestinal tract) and metabolic diseases such as T2DM[183,184]. For example, metagenomic studies have described a significant effect on bacterial diversity present in faecal samples associated with metformin treatment in rodent models[185-187] or individuals with T2DM[22,183,188,189].

These findings suggest that the changes in composition of gut microbiota might be a contributing factor to the glucose-lowering effect of metformin (**Figure 5**). Accordingly, depletion of the gut microbiota by oral intake of antibiotics abrogates glucose-lowering capacity of metformin in rodents[186]. Furthermore, faecal microbiota transplantation from metformin-treated donor rodents (which had improved glucose tolerance) to HFD-fed recipient animals induced similar improvements in glucose tolerance in the recipients[153,189].

Metformin treatment in patients with T2DM was reported to alter the structure of gut bacterial communities by causing a substantial shift in the abundance of specific subsets of bacterial taxa, which partially restores faecal gut dysbiosis that is associated with T2DM[22,160,183,188]. However, these studies analysed the long-term therapeutic outcome in patients on a stable dose of metformin for more than 3 months and could be the result of changes in microbiota composition that are secondary to the subsequent metabolic improvements. However, evidence is now growing that metformin has rapid effects on the composition and function of the faecal gut microbiota, which contribute to the antidiabetic effect of the drug. In a study with treatment-naive individuals with T2DM, a dramatic shift in the composition of the gut microbiota was reported after 2 and 4 months on metformin and was associated with increases in the abundance of *Escherichia* species and decreases in *Intestinibacter* species[189]. Moreover, another study of individuals with newly diagnosed T2DM showed decreased abundance of *Bacteroides fragilis* only 3 days after their first treatment with metformin[180].

Although the aforementioned studies clearly document the effects of metformin on distal gut microbiota, a recent 2018 study indicates that metformin also alters microbiota composition in the upper small intestine[153], where metformin accumulates at a high concentration[27,154]. This effect could also impact on the tolerance to metformin (**BOX 1**). Interestingly, administration of metformin in rodents in the upper small intestine for 1 day was associated with improved glucose sensing and rapid changes in upper small intestinal microbiota composition, with increased abundance of *Lactobacillus* species[153]. Moreover, intestinal microbiota transplantation experiments from metformin treated HFD-fed rats to recipient untreated HFD-fed rats confirmed the key role of changes in the upper small intestinal microbiota in mediating metformin-induced restoration of glucose sensing[153]. Thus, metformin-induced changes of

microbiota in both the upper and lower gastrointestinal tract might concur to regulate glucose homeostasis.

In addition to the modulation of microbiome composition, metformin treatment has been reported to improve metabolic functions of the microbiomeand interactions with host metabolism. This effect is consistent with the enrichment of short chain fatty acid (SCFA)-producing bacteria including *Akkermansia*, *Bifidobacterium, Blautia, Butyrivibrio, Lactobacillus, Megasphaera, Prevotella* or *Shewanella* species in metformin-treated patients with T2DM, which results in increased SCFA in the colon, with potential metabolic benefits for the host[22,187-190]. Interestingly, *in silico* analysis of the prevalent human gut bacterial metabolism using genome-scale metabolic models revealed important commensal and competing behavior following metformin treatment in the production of important SCFAs and their association with host cellular metabolism[191]. An increase in the colonic production of SCFAs in mice, especially butyrate and propionate, is known to trigger intestinal gluconeogenesis and induce metabolic benefits, including a decrease in hepatic glucose production[192]. In addition, manipulation of SCFA production through gut microbial fermentation of prebiotics (nondigestible dietary ingredient that induce the growth or activity of limited number of beneficial bacteria in the colon)[193] or by delivering propionate specifically to the colon was reported to increase plasma GLP1 levels in humans, leading to a decrease in food intake and amelioration of T2DM features (for example, improved glycaemic response)[194,195].

The gut microbiota also plays an important role for bile acid homeostasis, by generating unconjugated and secondary bile acids[196]. Moreover, metformin influences the ability of the microbiota to alter the bile acid pool by decreasing the abundance of *Bacteroides Fragilis* and its bile salt hydrolase activity[180]. These changes are accompanied by increased levels of the bile acid glycoursodeoxycholic acid (GUDCA), which is a potent endogenous antagonist of FXR. Importantly, this effect contributes to elevated GLP1 production via an AMPK-independent mechanism[180]. Notably, metformin-induced improvements of glucose tolerance could be transmitted by transplantation of feces from metformin treated patients with T2DM into antibiotic-treated and microbiota-depleted mice fed a HFD. The action of metformin on a gut *B. fragilis*–GUDCA–intestinal FXR axis was further validated by the abrogation of beneficial effects of metformin effect after supplementation of HFD-fed mice with *B. fragilis* or deletion of intestinal FXR[180]. These data highlight the crosstalk between microbiota and

host cells and the potential therapeutic value of gut microbiota remodelling for the treatment of T2DM and related diseases (such as non-alcoholic fatty liver disease).

A number of studies have highlighted the higher abundance of the mucin-degrading bacteria *Akkermansia muciniphila* in the distal gut of metformin-treated humans with T2DM[185,187-189] and metformin-treated HFD-fed mice[186]. Moreover, *A. muciniphila* has been reported to improve metabolic disorders possibly by increasing the endogenous production of endocannabinoids, which reduce inflammation and promote secretion of GLP1[197]. Furthermore, the beneficial role of *A. muciniphila* on metabolic homeostasis was demonstrated by administration as a probiotic to HFD-fed mice, which resulted in improved glucose tolerance compared to untreated animals[186]. Interestingly, these effects were enhanced by treatment with pasteurized *A. muciniphila* and were partly recapitulated by oral administration of a specific membrane protein isolated from *A. muciniphila*, providing support for its future use in therapy[198,199]. However, recent 2017 data do not support a significant correlation between *A. muciniphila* abundance and decrease in HbA_{1c} in a cohort of treatment-naive individuals with T2DM who received metformin for 4 months[189].

The mechanisms by which metformin selectively shapes the gut microbiota remain to be clarified but could mediated by both direct and indirect action of the drug. The effect of metformin on the abundance of *A. muciniphila* has been attributed to its action on intestinal mucin-producing goblet cells, which favors the production of mucus for the growth of the bacterium[186]. However, research has demonstrated that metformin can also directly promote the growth of *A. muciniphila* as well as *Bifidobacterium adolescentis*[189], and inhibit the growth of *B. fragilis*[189], *Fusobacterium nucleatum* and *Clostridium perfringens*[200] in bacterial cultures. To explain the direct inhibitory effect of metformin on microbial growth, it has been hypothesized that metformin might inhibit bacterial NADH:menaquinone oxidoreductase (NDH-1) complex, which is closely related to the eukaryotic mitochondrial respiratory-chain complex 1, and interfere with ATP production[201]. Interestingly, it was found that metformin impairs folate and methionine metabolism of intestinal microbiota in *C. elegans*[202], via a mechanism that might also contribute to modulate the function and growth of *B. fragilis* in the intestine of patients with T2DM treated with metformin[189]. In a recent 2017 study investigating the direct interactions between metformin and microbiota in a dynamic *in vitro* gut simulator that mimics the human gastrointestinal tract with a stabilized gut microbial community, it

was shown that metformin can regulate many bacterial genes encoding metalloproteins or metal transporters[189], which is possibly related to the metal-binding physicochemical properties of the drug[60]. Overall, these studies suggest that metformin creates an environment that favors the growth of a subset of gut beneficial bacterial species, while directly compromising the survival of potential pathogen-like bacteria to influence the physiology of the host.

Gut microbiota and host immune response. Alterations in gut microbiota are believed to play a role in meta-inflammation, notably through modulation of innate and adaptive immune systems[203]. Specific metabolites are generated by gut commensals, notably SCFAs. These metabolites can affect the local and systemic host immune response and contribute to impairments or improvements in metabolic homeostasis[204]. For instance, increases in the abundance of *Akkermansia muciniphila* in metformin treated obese mice has been associated with increased activity of intestinal goblet cells and activation of adipose tissue-resident T_{reg}, as well as decreased expression of the pro-inflammatory cytokines IL-1β and IL-6 in visceral adipose tissue[186] (**Figure 4**). Although goblet cells have been reported to induce tolerogenic dendritic cells and promote the development of T_{reg}[205], the underlying molecular mechanisms linking modulation of intestinal immune homeostasis to adipose tissue immunoregulatory responses is unclear. However, these preclinical studies suggest that metformin-induced changes in gut microbiota could increase specific bacterial-derived immunomodulatory molecule(s) that can promote regulatory immune responses in both the intestine and in peripheral metabolic organs, ultimately contributing to dampen mucosal inflammation and/or meta-inflammation. Whether this gut–adipose tissue immunoregulatory axis also occurs in patients with T2DM treated with metformin remains to be determined.

Metformin and T1DM

Despite insulin treatment, many patients with type 1 diabetes mellitus (T1DM) do not achieve optimal glycaemic control[206], which increases the risk of microvascular complications and cardiovascular diseases[207]. Furthermore, intensive insulin therapy increases the risk of hypoglycaemia and weight gain. Thus, several adjunctive therapies have been evaluated in combination with insulin in patients with T1DM to improve glycaemic control while decreasing the frequency of adverse events. Based on its

therapeutic benefits in T2DM, metformin is thought to be a good candidate to improve blood glucose levels and decrease insulin requirements in patients with T1DM, especially in those with overweight or obesity[3,4]. Metformin provides a safe oral option with a low risk of hypoglycaemia and a potential to reduce cardiovascular events[3,4].

Several randomized placebo-controlled trials have evaluated the efficacy and safety of metformin therapy in T1DM, notably, the REMOVAL study, the largest and longest trial to date[208]. Unfortunately, as pointed out by a recent meta-analysis[209], these trials concluded that metformin adjunction to insulin therapy in patients with T1DM did not have a sustained effect on glycaemic control, as measured by HbA$_{1c}$ levels[208-210]. Moreover, the REMOVAL study showed that atherosclerosis progression as measured by the carotid intima media thickness was not significantly decreased with metformin compared with placebo[208]. Nevertheless, metformin treatment exhibited minor benefits including decreases in insulin requirement, body weight, total cholesterol and low-density lipoprotein cholesterol[208,209]. Given the absence of metformin effects to improve blood glucose control and its minor beneficial effects, the use of metformin for patients with T1DM is questionable.

Conclusions

Sixty years after its introduction for the treatment of T2DM, the molecular mechanisms of metformin action continue to be a vigorous area of research. Major advances have been made with respect to understanding the direct and indirect effects of the drug on metabolic tissues and the regulation of whole-body glucose homeostasis. Although, it is widely accepted that the main physiological response to metformin is the inhibition of hepatic glucose production via direct action on the liver, evidence exists for indirect actions of the drug by modulation of the glucoregulatory neuronal network and/or enhancement of hepatic insulin sensitivity and meta-inflammation via effects on lipid metabolism and immune cells, respectively. The traditional liver-centered model for the glucose lowering effect of metformin has been also challenged by the key contribution from the gut to increase intestinal glucose utilization, stimulate GLP1 secretion and shape the composition and function of the microbiome. At the molecular level, much work has focused on the inhibition of mitochondrial respiratory-chain complex 1 and the subsequent decrease in cellular energy status and activation of

AMPK. It is now clear that AMPK is dispensable for the direct inhibitory effect of metformin on gluconeogenesis, but it might play an indirect role in the long-term effects of the drug by improving hepatic insulin sensitivity.

Metformin decreases hepatic gluconeogenesis through various molecular mechanisms, including alterations of cellular energy charge, inhibition of adenylate cyclase and FBP1 secondary to mitochondrial-mediated increases in intracellular AMP levels and modulation of the intracellular redox status as a consequence of direct inhibition of mG3PDH and/or mitochondria membrane depolarization. However, it remains to be demonstrated whether these signalling pathways are also relevant in the context of chronic metformin treatment and, more importantly, in patients with T2DM. The mechanisms by which metformin influences the gut microbiota are probably both direct and indirect due to its anti-microbial activities and inhibition of bile acid re-absorption and suggest the potential for the development of novel gut-targeted therapies.

There is considerable variation in the response to metformin at the individual level in terms of glycaemic response and gastrointestinal tolerance (**BOX 1**); thus, a better understanding of the determinants underlying its clinical efficacy may help clinicians to develop a personalized approach in metformin therapy. The use of modified-release or controlled-release formulations have already paved the way[211,212]. Besides its classic application as an anti-hyperglycaemic agent, the use of metformin has been being significantly expanded with the treatment of PCOS (**BOX 2**). The long history and much clinical experience with metformin has also encouraged the repurposing of this drug for further therapeutic applications (**BOX 3**), from cancer to the modulation of age-related diseases, highlighting the vast range of different possible actions of metformin. In addition, moving from the traditional oral use of metformin to different route of administration[213] or drug delivery systems (for example microparticles or nanoparticles)[214,215] might help to accelerate the development of novel therapeutic applications for this multifaceted drug.

Box 1: Metformin intolerance

Gastrointestinal manifestations of abdominal pain, nausea, diarrhoea and vomiting are common adverse effects of metformin when used for the treatment of type 2 diabetes mellitus and polycystic ovary syndrome. These adverse effects occur in up to 20% of patients and limit adherence[216]. The main cause of treatment discontinuation are these initial digestive disorders, with 1.2–5% of all patients stopping therapy due to these effects)[217]. Therapeutic strategies to overcome metformin gastrointestinal intolerance include gradual uptitration of immediate-release metformin or use of extended-release and delayed-release formulations of the drug[217].

A recent 2018 pharmacokinetic study reported the absence of significant differences in the absorption, distribution or elimination of metformin between tolerant and intolerant individuals, suggesting the involvement of local factors within the intestinal lumen or enterocytes[218]. Interestingly, the association between metformin gastrointestinal intolerance with genetic variations in the metformin transporter OCT1 and serotonin transporter SERT supports the concept that metformin induces alterations in gastrointestinal physiology. The receptor variants might result in higher metformin concentrations in the intestine of susceptible individuals, which could cause changes to intestinal motility and peristalsis or gastric-emptying[219,220].

Multiple pathophysiological hypotheses have been proposed to explain functional changes in the gastrointestinal tract that result in nausea and vomiting, including metformin-induced release of serotonin from enterochromaffin cells[165]. However, a clinical study using the serotonin receptor 5-HT3 antagonist ondansetron showed no efficacy in the treatment of metformin-induced side effects [221]. Another potential mechanism by which metformin might cause gastrointestinal disturbances is by decreasing reabsorption of bile acids, leading to elevated colonic bile acid concentrations, thereby causing diarrhoea[160,222]. In addition, metformin-induced changes in the gastrointestinal microbiota have been associated with the increase in abundance of common opportunistic pathogens such as *Escherichia* species and *Shigella* species[22,189,223] that might trigger the pathophysiological gastrointestinal adverse effects. Moreover, in exploratory studies, a better tolerance to metformin was achieved through the consumption of a prebiotic–antioxidant, which modulated the gastrointestinal microbiome[224,225].

Box 2: Metformin in the treatment of PCOS

• Polycystic ovary syndrome (PCOS) is the most common cause of infertility in women, affecting 5–10% women of reproductive age[226] and is often associated with obesity and metabolic syndrome. Although the molecular defect(s) underlying the disease are unclear, PCOS is characterized by enlarged and/or polycystic ovaries, ovulation dysfunction and hyperandrogenism. Patients with PCOS have excessive secretion of luteinizing hormone (LH) by the pituitary gland, which impairs development of follicles, induces chronic anovulation[227] and stimulates ovarian theca cells to produce excessive amounts of androgens that cause some of the most common clinical symptoms of hyperandrogenism (that is, acne, hirsutism and alopecia).

• 50–70% patients with PCOS have insulin resistance[228,229] and the compensatory hyperinsulinaemia observed in most of these women contributes to pathogenesis by several mechanisms: first, by increasing LH pulse frequency in pituitary gland[230]; second, by stimulating ovarian theca cells to produce excessive testosterone[231]; third, by suppressing hepatic sex-hormone-binding globulin (SHBG) production; fourth, by increasing androgen secretion from the adrenal glands[232,233]; and last by acting synergistically with LH to stimulate ovarian theca cells to secrete androgens.

• Metformin is not the first-line treatment for PCOS but the use of this drug is recommended in patients with PCOS and glucose intolerance[234]. Metformin decreases insulin resistance and hyperinsulinaemia, improves dyslipidaemia and meta-inflammation[235], decreases blood pressure and exerts cardioprotective benefits through pleiotropic action on the vascular endothelium[236-238]. In combination with lifestyle intervention, metformin treatment is also associated with lower body weight and improved menstrual cyclicity and fertility potential[239-241] in women with obesity and PCOS[237].

Various underlying mechanisms are proposed for the benefial effects of metformin. For example, metformin increases hepatic production of SHBG, which decreases androgen levels. Alternatively, the drug directly inhibits androgen synthesis in theca cells, which decreases testosterone levels. Although its beneficial role on ovulation is still controversial[242,243], metformin might enhance the efficacy of clomiphene (a selective estrogen receptor modulator inducing ovulation)[239-241] and decrease the risk of gonadotropin-induced ovarian hyperstimulation syndrome in women with PCOS undergoing in vitro fertilization[244].

Box 3: Metformin therapeutic repurposing

• <u>Cancer</u>: Pharmaco-epidemiology has provided several lines of evidence that metformin has utility in cancer prevention and/or treatment[245]. The ability of metformin to inhibit mitochondrial respiratory-chain complex 1 means the drug has potential use for tumorigenesis inhibition[12]. A recent 2019 study reported the benefit of using metformin as an adjuvant therapy in patients with cancer[246]. Many clinical trials have reported encouraging results in protecting patients with T2DM from cancer[245] but the protective effect of metformin remains to be validated in individuals without T2DM.

• <u>Neurodegenerative diseases</u>: There is growing evidence in preclinical and clinical studies for the benefits of metformin to counteract neurodegenerative diseases[247]. The rationale is based on the potential of the drug to act as a neuroprotective agent by targeting neuronal oxidative stress, inflammation and cell death.

• <u>Aging</u>: Early studies have reported that metformin could prolong lifespan in both rodents [248] and *C. elegans* [202], fueling interest in the context of anti-aging drug development. The various mechanisms by which metformin could exert its anti-aging action at the cellular and organismal levels were recently extensively reviewed[249]. The Targeting Aging with Metformin clinical trial (TAME) has been recently designed to investigate the impact of metformin on several age-related diseases in humans[250].

• <u>*Mycobacterium tuberculosis* infection</u>: Evidence has emerged that metformin significant decreased mortality during treatment for *M. tuberculosis* infection[251], suggesting a role for the drug as a host-directed therapeutic adjuvant. Metformin was found to reduce the intracellular growth of *M. tuberculosis* by regulating autophagy and production of ROS[252].

• <u>Lung fibrosis</u>: Idiopathic pulmonary fibrosis is a serious progressive lung disease affecting more than 5 million persons worldwide. A recent 2018 study found that therapeutic concentrations of metformin accelerated the resolution of established fibrosis in a mouse model of lung fibrosis by promoting AMPK activation and apoptosis in fibroblasts[253]. Of note, metformin was also reported to prevent fibroblast differentiation and lung fibrosis via an AMPK-mediated inhibition of TGF-β-induced NADPH oxidase 4 expression[254], suggesting beneficial effects via different mechanisms.

Figure legends:

Figure 1: Action of metformin on mitochondrial respiratory-chain complex 1.

After accumulation into the mitochondria by an as-yet-unclear process, which might be associated with the positive charge of the molecule, metformin inhibits the mitochondrial respiratory respiratory chain complex 1 (C1) in a reversible and non-competitive manner. This inhibition presumably occurs by direct interaction of the drug with the Cys39-containing matrix loop of the respiratory chain subunit ND3, which stabilizes the enzyme in an open-loop deactive conformation state. The inhibition of C1 leads to a decrease in NADH oxidation, a decrease in proton pumping across the inner mitochondrial membrane and a decrease in oxygen consumption rate, resulting in lower proton gradient ($\Delta\psi$) and a decrease in proton-driven ATP synthesis from ADP and inorganic phosphate (Pi). C, cytochrome *c*; C1–C5, mitochondrial respiratory chain complex 1–5; IMS, inner-membrane space; Q, coenzyme Q (ubiquinone).

Figure 2 : Energy-dependent mechanisms of metformin-induced inhibition of hepatic gluconeogenesis.

Metformin is transported into hepatocytes mainly through OCT1 and accumulates in mitochondria. (A–D) In mitochondria, metformin partially inhibits mitochondrial respiratory chain complex 1 (complex 1), resulting in decreased ATP levels and accumulation of AMP. Thus, metformin has a mild effect on the overall cellular energy charge (A) Metformin-induced changes in the AMP:ATP ratio activate AMPK through its phosphorylation by LKB1. Subsequently, AMPK inhibits gluconeogenic gene transcription (*Pck1* and *G6pc*) via the phosphorylation and cytoplasmic sequestration of the transcriptional cofactor CRTC2. This AMPK- dependent mechanism has been challenged by the use of liver AMPK-deficient mice. (B) Gluconeogenesis is a highly energy-consuming metabolic pathway. Therefore, reduction in cellular ATP levels is sufficient to reduce glucose production flux. In addition, elevation of AMP levels contributes to inhibition of glucose production through allosteric inhibition of fructose-1,6-bisphosphatase 1 (FBP1), a key gluconeogenic enzyme. (C) Metformin-induced AMP accumulation inhibits adenylate cyclase and decreases cAMP synthesis, resulting in decreased protein kinase A (PKA) activity and downstream signalling. Gluconeogenesis is suppressed through both the decrease in gluconeogenic enzyme activity and the inhibition of glucagon- induced gluconeogenic gene expression associated with the lack

of phosphorylation of regulators (for example, CREB1 and I3PR). (D) Chronic metformin-induced AMPK activation might indirectly decrease gluconeogenesis by enhancing hepatic insulin sensitivity. AMPK inhibits lipogenesis through phosphorylation and inhibition of acetyl-CoA carboxylase (ACC), thereby decreasing malonyl-CoA synthesis, which activates carnitine palmitoyltransferase 1 (CPT1) and stimulates fatty acid oxidation by increasing the import of acyl-CoA into mitochondria. Over time, metformin-induced AMPK activation decreases hepatic steatosis and improves insulin sensitivity, which in turn inhibits gluconeogenesis. GLUT2, glucose transporter type 2; PFK/FBPase, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase 1.

Figure 3. Redox-dependent mechanisms by which metformin might inhibit hepatic gluconeogenesis. (A) Metformin suppresses gluconeogenesis through a direct inhibition of mitochondrial glycerol-3-phosphate dehydrogenase (mG3PDH), an enzyme involved in the glycerol–phosphate shuttle. Metformin-induced inhibition of mG3PDH disrupts glucose production from glycerol and increases cytosolic redox potential (NADH:NAD+), which impedes the utilization of lactate for glucose production. (B) The accumulation of metformin in mitochondria caused by its positive charge leads to mitochondrial depolarization and inhibition of the electrogenic transporter for aspartate (Asp) of the malate–aspartate (Mal–Asp) shuttle, resulting in an increase in the cytosolic NADH:NAD+ ratio. Metformin-induced inhibition of the malate–aspartate shuttle stimulates the glycerol–phosphate shuttle, leading to a decrease in levels of glycerol-3-phosphate (glycerol-3P), a potent allosteric inhibitor of phosphofructokinase 1 (PFK1). As a result, gluconeogenesis is inhibited through the partitioning of gluconeogenic substrate towards glycolysis. DHAP, dihydroxyacetone phosphate; FBP1, fructose-1, 6-bisphosphatase 1; cG3PDH, cytosolic G3PDH; GLUT2, glucose transporter type 2; LDH, lactate dehydrogenase; OCT1, organic transporter 1.

Figure 4: Metformin and meta-inflammation. Obesity-associated meta-inflammation results partly from pro- inflammatory activation of tissue-resident macrophages, which secrete pro-inflammatory cytokines in metabolic organs (that is, liver and adipose tissue) and contributes to insulin resistance at least partly by inhibiting insulin signalling. Moreover, obesity is often associated with changes in microbiota composition

(dysbiosis) that result in bacterial secretion of various immunomodulatory components (for example, short-chain fatty acids (SCFAs) or lipopolysaccharide (LPS)). Metformin could improve tissue-specific inflammation and insulin sensitivity by inducing regulatory T cells (TReg) and/or macrophages to polarize towards alternatively activated anti-inflammatory macrophages. These effects are thought to occur through direct and indirect pathways that involve changes in the gut microbiota (for example, increases in abundance of *Akkermansia* species) and both AMP-activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms. ATF3, activating transcription factor 3; mTOR, mechanistic target of rapamycin; NF-κB, nuclear factor-κB; OXPHOS, oxidative phosphorylation; TGFβ, transforming growth factor-β; TLR4, toll-like receptor 4; TNF, tumour necrosis factor.

Figure 5: Metformin action in the gut. etformin is taken up from the intestinal lumen in enterocytes by plasma membrane monoamine transporter (PMAT) and OCT3 and transported into the bloodstream by OCT1. Metformin stimulates secretion of the glucose-lowering hormone glucagon-like peptide 1 (GLP1) from enteroendocrine L cells by direct and indirect mechanisms. Of note, GLP1 secretion is directly controlled by muscarinic M3 receptor (M3R), Wnt signalling

and AMPK activation in L cells. The GLP1 secretory effects directly induced by metformin are also mediated indirectly by

modulation of the bile acid pool and gut microbiota composition. For example, the inhibitory effect of metformin on

apical sodium-dependent bile acid transporter (ASBT) reduces the reabsorption of bile acids, leading to an increase in luminal concentrations of bile acids and subsequent stimulation of the bile acid receptor TGR5, as well as a decrease in intracellular bile acid concentrations limiting activation of farnesoid X receptor (FXR). In addition, metformin increases the abundance of short-chain fatty acid (SCFA)-producing bacteria and facilitates SCFA-induced GLP1 secretion via signalling through GPR41 and GPR43 in L cells. Increases in the abundance of *Lactobacillus* species increases release of GLP1 by a glucose–SGLT1-sensing mechanism. Decreases in the abundance of *Bacteroides fragilis* elevate the levels of the bile acid glycoursodeoxycholic acid (GUDCA, a potent endogenous antagonist of FXR) to modulate GLP1 secretion. GLP1 acts locally in the gut by activating a gut–brain–liver neuronal axis that contributes to the regulation of blood

levels of glucose via a reduction in hepatic glucose production. SGLT1, sodium-coupled glucose transporter 1.

Glossary

Lactic acidosis: A medical condition character- ized by excessively low pH in the bloodstream due to excess lactate production by glycolytic tissues, inadequate lactate utilization by gluconeogenic tissues, or varying combina- tions of these two processes.

Pharmacokinetics: The study of the transit of a dosed drug in body fluids and tissues over time, as defined by its rate of absorption, distribution, metabolism and excretion.

Pharmacodynamics: The study of the action of a drug in the body, and its biochemical and physiological effects.

Half-maximal inhibitory concentration (IC50): The concentration of an inhibitor required to decrease the response of the target by 50%.

Pyruvate tolerance: A measure of glycaemic excursion in response to an intraperitoneal or intravenous injection of pyruvate, used to assess hepatic gluconeogenesis.

Cytosolic redox potential: Cytoplasmic oxidation state of the cell, which is assessed by the ratio of reduced to oxidized intracellular metabolite redox couples (for example, lactate/ pyruvate ratio).

Type 2 immune cells: Cells involved in type 2 immune responses, such as type 2 innate lymphoid cells, eosinophils, T helper 2 cells, mast cells, basophils and alternatively-activated macrophages.

Reverse electron transport (RET): The transport of electrons from ubiquinol back to respiratory complex 1, generating a substantial amount of reactive oxygen species.

Incretins: incretins are gut hormones that are secreted after nutrient intake and stimulate glucose- stimulated insulin secretion.

Lipoapoptosis: A non-canonical form of programmed cell death, which is the result of fatty acid over- accumulation that occurs in diseases associated with over- nutrition and ageing.

Short chain fatty acid (SCFA): A fatty acid with fewer than six carbon atoms (for example, acetate, propionate and butyrate) that is the end- product of fermentation of dietary fibres by the anaerobic intestinal microbiota and acts as a signal molecule in the control of mammalian energy metabolism.

Author contributions

The authors contributed equally to all aspects of the article.

Conflict of interest statement

The authors declare no conflict of interest.

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References

1   Davies, M. J. *et al.* Management of Hyperglycemia in Type 2 Diabetes, 2018. A Consensus Report by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetes Care* **41**, 2669-2701, doi:10.2337/dci18-0033 (2018).
2   Davies, M. J. *et al.* Management of hyperglycaemia in type 2 diabetes, 2018. A consensus report by the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetologia* **61**, 2461-2498, doi:10.1007/s00125-018-4729-5 (2018).
3   United Kingdom Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). UK Prospective Diabetes Study (UKPDS) Group. *Lancet* **352**, 854-865 (1998).
4   United Kingdom Prospective Diabetes Study (UKPDS) Group. United Kingdom Prospective Diabetes Study 24: a 6-year, randomized, controlled trial comparing sulfonylurea, insulin, and metformin therapy in patients with newly diagnosed type 2 diabetes that could not be controlled with diet therapy. United Kingdom Prospective Diabetes Study Group. *Annals of internal medicine* **128**, 165-175 (1998).
5   Maruthur, N. M. *et al.* Diabetes Medications as Monotherapy or Metformin-Based Combination Therapy for Type 2 Diabetes: A Systematic Review and Meta-analysis. *Ann Intern Med* **164**, 740-751, doi:10.7326/M15-2650 (2016).
6   Palmer, S. C. *et al.* Comparison of Clinical Outcomes and Adverse Events Associated With Glucose-Lowering Drugs in Patients With Type 2 Diabetes: A Meta-analysis. *JAMA* **316**, 313-324, doi:10.1001/jama.2016.9400 (2016).
7   Sanchez-Rangel, E. & Inzucchi, S. E. Metformin: clinical use in type 2 diabetes. *Diabetologia* **60**, 1586-1593, doi:10.1007/s00125-017-4336-x (2017).
8   Howlett, H. C. & Bailey, C. J. A risk-benefit assessment of metformin in type 2 diabetes mellitus. *Drug Saf* **20**, 489-503, doi:10.2165/00002018-199920060-00003 (1999).
9   Bailey, C. J. Metformin: historical overview. *Diabetologia* **60**, 1566-1576, doi:10.1007/s00125-017-4318-z (2017).
10   Werner, E. & Bell, J. The preparation of methylguanidine, and of ββ-dimethylguanidine by interaction of dicyanodiamide, and methylammonium and dimethylammonium chlorides respectively. *J. Am. Chem. Soc.* **121**, 1790-1794 (1922).
11   Nattrass, M. *et al.* Hyperlactatemia in diabetics with retinopathy during combined sulphonylurea and phenformin therapy. *Diabete Metab* **4**, 1-4 (1978).
12   Foretz, M., Guigas, B., Bertrand, L., Pollak, M. & Viollet, B. Metformin: from mechanisms of action to therapies. *Cell Metab* **20**, 953-966, doi:10.1016/j.cmet.2014.09.018 (2014).
13   Hundal, R. S. *et al.* Mechanism by which metformin reduces glucose production in type 2 diabetes. *Diabetes* **49**, 2063-2069 (2000).
14   Cusi, K., Consoli, A. & DeFronzo, R. A. Metabolic effects of metformin on glucose and lactate metabolism in noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab* **81**, 4059-4067, doi:10.1210/jcem.81.11.8923861 (1996).
15   Hother-Nielsen, O., Schmitz, O., Andersen, P. H., Beck-Nielsen, H. & Pedersen, O. Metformin improves peripheral but not hepatic insulin action in obese patients with type II diabetes. *Acta Endocrinol (Copenh)* **120**, 257-265 (1989).

16   Stumvoll, M., Nurjhan, N., Perriello, G., Dailey, G. & Gerich, J. E. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. *N Engl J Med* **333**, 550-554, doi:10.1056/NEJM199508313330903 (1995).

17   DeFronzo, R. A., Barzilai, N. & Simonson, D. C. Mechanism of metformin action in obese and lean noninsulin-dependent diabetic subjects. *J Clin Endocrinol Metab* **73**, 1294-1301, doi:10.1210/jcem-73-6-1294 (1991).

18   Inzucchi, S. E. *et al.* Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. *N Engl J Med* **338**, 867-872, doi:10.1056/NEJM199803263381303 (1998).

19   Bailey, C. J., Mynett, K. J. & Page, T. Importance of the intestine as a site of metformin-stimulated glucose utilization. *Br J Pharmacol* **112**, 671-675 (1994).

20   Buse, J. B. *et al.* The Primary Glucose-Lowering Effect of Metformin Resides in the Gut, Not the Circulation: Results From Short-term Pharmacokinetic and 12-Week Dose-Ranging Studies. *Diabetes Care* **39**, 198-205, doi:10.2337/dc15-0488 (2016).

- This human study provides strong evidence for a gut-mediated mechanism in the blood glucose-lowering action of metformin.

21   McCreight, L. J., Bailey, C. J. & Pearson, E. R. Metformin and the gastrointestinal tract. *Diabetologia* **59**, 426-435, doi:10.1007/s00125-015-3844-9 (2016).

22   Forslund, K. *et al.* Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature* **528**, 262-266, doi:10.1038/nature15766 (2015).

23   Hardie, D. G. AMPK--sensing energy while talking to other signaling pathways. *Cell Metab* **20**, 939-952, doi:10.1016/j.cmet.2014.09.013 (2014).

24   Cao, J. *et al.* Low concentrations of metformin suppress glucose production in hepatocytes through AMP-activated protein kinase (AMPK). *J Biol Chem* **289**, 20435-20446, doi:10.1074/jbc.M114.567271 (2014).

25   Graham, G. G. *et al.* Clinical pharmacokinetics of metformin. *Clin Pharmacokinet* **50**, 81-98, doi:10.2165/11534750-000000000-00000 (2011).

26   Tucker, G. T. *et al.* Metformin kinetics in healthy subjects and in patients with diabetes mellitus. *Br J Clin Pharmacol* **12**, 235-246 (1981).

27   Wilcock, C. & Bailey, C. J. Accumulation of metformin by tissues of the normal and diabetic mouse. *Xenobiotica* **24**, 49-57 (1994).

28   Jensen, J. B. *et al.* [11C]-Labeled Metformin Distribution in the Liver and Small Intestine Using Dynamic Positron Emission Tomography in Mice Demonstrates Tissue-Specific Transporter Dependency. *Diabetes* **65**, 1724-1730, doi:10.2337/db16-0032 (2016).

29   Gormsen, L. C. *et al.* In Vivo Imaging of Human 11C-Metformin in Peripheral Organs: Dosimetry, Biodistribution, and Kinetic Analyses. *J Nucl Med* **57**, 1920-1926, doi:10.2967/jnumed.116.177774 (2016).

30   Liang, X. & Giacomini, K. M. Transporters Involved in Metformin Pharmacokinetics and Treatment Response. *J Pharm Sci* **106**, 2245-2250, doi:10.1016/j.xphs.2017.04.078 (2017).

31   Todd, J. N. & Florez, J. C. An update on the pharmacogenomics of metformin: progress, problems and potential. *Pharmacogenomics* **15**, 529-539, doi:10.2217/pgs.14.21 (2014).

32   Dujic, T. *et al.* Variants in Pharmacokinetic Transporters and Glycemic Response to Metformin: A Metgen Meta-Analysis. *Clin Pharmacol Ther* **101**, 763-772, doi:10.1002/cpt.567 (2017).

33   Zhou, K. *et al.* Variation in the glucose transporter gene SLC2A2 is associated with glycemic response to metformin. *Nat Genet* **48**, 1055-1059, doi:10.1038/ng.3632 (2016).

34   Ait-Omar, A. *et al.* GLUT2 accumulation in enterocyte apical and intracellular membranes: a study in morbidly obese human subjects and ob/ob and high fat-fed mice. *Diabetes* **60**, 2598-2607, doi:10.2337/db10-1740 (2011).

35   Jablonski, K. A. *et al.* Common variants in 40 genes assessed for diabetes incidence and response to metformin and lifestyle intervention in the diabetes prevention program. *Diabetes* **59**, 2672-2681, doi:10.2337/db10-0543 (2010).

36   Hollunger, G. Guanidines and oxidative phosphorylations. *Acta Pharmacol Toxicol (Copenh)* **11**, 1-84 (1955).

37   Schafer, G. Site-specific uncoupling and inhibition of oxidative phosphorylation by biguanides. II. *Biochim Biophys Acta* **172**, 334-337 (1969).

38   El-Mir, M. Y. *et al.* Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem* **275**, 223-228 (2000).

39   Owen, M. R., Doran, E. & Halestrap, A. P. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *The Biochemical journal* **348 Pt 3**, 607-614 (2000).

- This study together with El-Mir et al. (2000) were the first to report specific inhibition of metformin on the mitochondrial respiratory chain complex 1.

40 Degli Esposti, M. Inhibitors of NADH-ubiquinone reductase: an overview. *Biochim Biophys Acta* **1364**, 222-235 (1998).

41 Fontaine, E. Metformin-Induced Mitochondrial Complex I Inhibition: Facts, Uncertainties, and Consequences. *Front Endocrinol (Lausanne)* **9**, 753, doi:10.3389/fendo.2018.00753 (2018).

42 Vial, G., Detaille, D. & Guigas, B. Role of Mitochondria in the Mechanism(s) of Action of Metformin. *Front Endocrinol (Lausanne)* **10**, 294, doi:10.3389/fendo.2019.00294 (2019).

43 Stephenne, X. *et al.* Metformin activates AMP-activated protein kinase in primary human hepatocytes by decreasing cellular energy status. *Diabetologia* **54**, 3101-3110, doi:10.1007/s00125-011-2311-5 (2011).

- This is the first study showing that metformin inhibits mitochondrial respiratory chain complex 1 and activates AMPK in primary human hepatocytes.

44 Andrzejewski, S., Gravel, S. P., Pollak, M. & St-Pierre, J. Metformin directly acts on mitochondria to alter cellular bioenergetics. *Cancer Metab* **2**, 12, doi:10.1186/2049-3002-2-12 (2014).

45 Bridges, H. R., Jones, A. J., Pollak, M. N. & Hirst, J. Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria. *The Biochemical journal* **462**, 475-487, doi:10.1042/BJ20140620 (2014).

- This elegant study dissects the mechanism by which metformin inhibits the mitochondrial respiratory-chain complex 1.

46 Guigas, B. *et al.* Metformin inhibits mitochondrial permeability transition and cell death: a pharmacological in vitro study. *Biochem J* **382**, 877-884, doi:10.1042/BJ20040885 (2004).

47 Wheaton, W. W. *et al.* Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *Elife* **3**, e02242, doi:10.7554/eLife.02242 (2014).

48 Zannella, V. E. *et al.* Reprogramming metabolism with metformin improves tumor oxygenation and radiotherapy response. *Clin Cancer Res* **19**, 6741-6750, doi:10.1158/1078-0432.CCR-13-1787 (2013).

49 Gui, D. Y. *et al.* Environment Dictates Dependence on Mitochondrial Complex I for NAD+ and Aspartate Production and Determines Cancer Cell Sensitivity to Metformin. *Cell Metab* **24**, 716-727, doi:10.1016/j.cmet.2016.09.006 (2016).

50 Cheng, G. *et al.* Mitochondria-Targeted Analogues of Metformin Exhibit Enhanced Antiproliferative and Radiosensitizing Effects in Pancreatic Cancer Cells. *Cancer Res* **76**, 3904-3915, doi:10.1158/0008-5472.CAN-15-2534 (2016).

51 Boukalova, S. *et al.* Mitochondrial Targeting of Metformin Enhances Its Activity against Pancreatic Cancer. *Mol Cancer Ther* **15**, 2875-2886, doi:10.1158/1535-7163.MCT-15-1021 (2016).

52 Guo, Z. *et al.* Heme Binding Biguanides Target Cytochrome P450-Dependent Cancer Cell Mitochondria. *Cell Chem Biol* **24**, 1314, doi:10.1016/j.chembiol.2017.09.012 (2017).

53 Thakur, S. *et al.* Metformin Targets Mitochondrial Glycerophosphate Dehydrogenase to Control Rate of Oxidative Phosphorylation and Growth of Thyroid Cancer In Vitro and In Vivo. *Clin Cancer Res* **24**, 4030-4043, doi:10.1158/1078-0432.CCR-17-3167 (2018).

54 Fontaine, E. Metformin and respiratory chain complex I: the last piece of the puzzle? *Biochem J* **463**, e3-5, doi:10.1042/BJ20141020 (2014).

55 Wilcock, C., Wyre, N. D. & Bailey, C. J. Subcellular distribution of metformin in rat liver. *J Pharm Pharmacol* **43**, 442-444 (1991).

56 Detaille, D. *et al.* Metformin prevents high-glucose-induced endothelial cell death through a mitochondrial permeability transition-dependent process. *Diabetes* **54**, 2179-2187 (2005).

- This is the first study demonstrating that metformin could reduce oxidative stress through its mitochondrial action.

57 Detaille, D., Guigas, B., Leverve, X., Wiernsperger, N. & Devos, P. Obligatory role of membrane events in the regulatory effect of metformin on the respiratory chain function. *Biochem Pharmacol* **63**, 1259-1272 (2002).

58 El-Mir, M. Y. *et al.* Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons. *J Mol Neurosci* **34**, 77-87, doi:10.1007/s12031-007-9002-1 (2008).

59 Bridges, H. R., Sirvio, V. A., Agip, A. N. & Hirst, J. Molecular features of biguanides required for targeting of mitochondrial respiratory complex I and activation of AMP-kinase. *BMC Biol* **14**, 65, doi:10.1186/s12915-016-0287-9 (2016).

60 Logie, L. *et al.* Cellular responses to the metal-binding properties of metformin. *Diabetes* **61**, 1423-1433, doi:10.2337/db11-0961 (2012).

61   Hirst, J. Mitochondrial complex I. *Annu Rev Biochem* **82**, 551-575, doi:10.1146/annurev-biochem-070511-103700 (2013).

62   Zickermann, V. *et al.* Structural biology. Mechanistic insight from the crystal structure of mitochondrial complex I. *Science* **347**, 44-49, doi:10.1126/science.1259859 (2015).

63   Saheki, T. *et al.* Citrin/mitochondrial glycerol-3-phosphate dehydrogenase double knock-out mice recapitulate features of human citrin deficiency. *J Biol Chem* **282**, 25041-25052, doi:10.1074/jbc.M702031200 (2007).

64   Shu, Y. *et al.* Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest* **117**, 1422-1431, doi:10.1172/JCI30558 (2007).

65   He, L. & Wondisford, F. E. Metformin action: concentrations matter. *Cell Metab* **21**, 159-162, doi:10.1016/j.cmet.2015.01.003 (2015).

66   Christensen, M. M. *et al.* The pharmacogenetics of metformin and its impact on plasma metformin steady-state levels and glycosylated hemoglobin A1c. *Pharmacogenet Genomics* **21**, 837-850, doi:10.1097/FPC.0b013e32834c0010 (2011).

67   Lalau, J. D., Lemaire-Hurtel, A. S. & Lacroix, C. Establishment of a database of metformin plasma concentrations and erythrocyte levels in normal and emergency situations. *Clin Drug Investig* **31**, 435-438, doi:10.2165/11588310-000000000-00000 (2011).

68   Foretz, M. *et al.* Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *The Journal of clinical investigation* **120**, 2355-2369, doi:10.1172/JCI40671 (2010).
   • This paper provides genetic evidence AMPK-independent action of metformin in the inhibition of hepatic glucose production.

69   Fullerton, M. D. *et al.* Single phosphorylation sites in Acc1 and Acc2 regulate lipid homeostasis and the insulin-sensitizing effects of metformin. *Nat Med* **19**, 1649-1654, doi:10.1038/nm.3372 (2013).
   • This paper provides genetic evidence for AMPK-mediated phosphorylation of both ACC1 and ACC2 in metformin regulation of lipid metabolism and insulin sesnsivity.

70   Hunter, R. W. *et al.* Metformin reduces liver glucose production by inhibition of fructose-1-6-bisphosphatase. *Nat Med* **24**, 1395-1406, doi:10.1038/s41591-018-0159-7 (2018).
   • This study provides genetic evidence for meformin inhibition of hepatic glucose production through allosteric inhibition of fructose-1-6-bisphosphatase via metformin-induced increases of intracellular AMP levels.

71   Miller, R. A. *et al.* Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature* **494**, 256-260, doi:10.1038/nature11808 (2013).
   • This paper describes the suppression of hepatic glucagon signalling in response to metformin by inhibition of adenylate cyclase via metformin-induced increases of intracellular AMP levels.

72   Clarke, J. D. *et al.* Mechanism of Altered Metformin Distribution in Nonalcoholic Steatohepatitis. *Diabetes* **64**, 3305-3313, doi:10.2337/db14-1947 (2015).

73   Scheen, A. J. Clinical pharmacokinetics of metformin. *Clin Pharmacokinet* **30**, 359-371 (1996).

74   Alshawi, A. & Agius, L. Low metformin causes a more oxidized mitochondrial NADH/NAD redox state in hepatocytes and inhibits gluconeogenesis by a redox-independent mechanism. *J Biol Chem* **294**, 2839-2853, doi:10.1074/jbc.RA118.006670 (2019).

75   Madiraju, A. K. *et al.* Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature* **510**, 542-546, doi:10.1038/nature13270 (2014).
   • This paper describes a redox-dependent mechanism to account for the inhibition of hepatic gluconeogenesis by metformin.

76   Al-Oanzi, Z. H. *et al.* Opposite effects of a glucokinase activator and metformin on glucose-regulated gene expression in hepatocytes. *Diabetes Obes Metab* **19**, 1078-1087, doi:10.1111/dom.12910 (2017).

77   Zhou, G. *et al.* Role of AMP-activated protein kinase in mechanism of metformin action. *The Journal of clinical investigation* **108**, 1167-1174, doi:10.1172/JCI13505 (2001).
   • This is the first paper showing AMPK activation by metformin.

78   Hawley, S. A. *et al.* Use of cells expressing gamma subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab* **11**, 554-565, doi:10.1016/j.cmet.2010.04.001 (2010).

79   Zhang, C. S. *et al.* The lysosomal v-ATPase-Ragulator complex is a common activator for AMPK and mTORC1, acting as a switch between catabolism and anabolism. *Cell Metab* **20**, 526-540, doi:10.1016/j.cmet.2014.06.014 (2014).
   • This study reports an AMP-independent mechanism for glucose sensing by AMPK through the lysosomal pathway.

80   Li, M. *et al.* Transient Receptor Potential V Channels Are Essential for Glucose Sensing by Aldolase and AMPK. *Cell Metab*, doi:10.1016/j.cmet.2019.05.018 (2019).

81   Zhang, C. S. *et al.* Metformin Activates AMPK through the Lysosomal Pathway. *Cell Metab* **24**, 521-522, doi:10.1016/j.cmet.2016.09.003 (2016).

82   Shaw, R. J. *et al.* The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* **310**, 1642-1646, doi:10.1126/science.1120781 (2005).

83   He, L. *et al.* Metformin and insulin suppress hepatic gluconeogenesis through phosphorylation of CREB binding protein. *Cell* **137**, 635-646, doi:10.1016/j.cell.2009.03.016 (2009).

84   Lee, J. M. *et al.* AMPK-dependent repression of hepatic gluconeogenesis via disruption of CREB.CRTC2 complex by orphan nuclear receptor small heterodimer partner. *J Biol Chem* **285**, 32182-32191, doi:10.1074/jbc.M110.134890 (2010).

85   Caton, P. W. *et al.* Metformin suppresses hepatic gluconeogenesis through induction of SIRT1 and GCN5. *The Journal of endocrinology* **205**, 97-106, doi:10.1677/JOE-09-0345 (2010).

86   Boudaba, N. *et al.* AMPK Re-Activation Suppresses Hepatic Steatosis but its Downregulation Does Not Promote Fatty Liver Development. *EBioMedicine* **28**, 194-209, doi:10.1016/j.ebiom.2018.01.008 (2018).
      - ==This paper provides genetic evidence for AMPK-dependent effect of metformin on lipogenesis inhibition and fatty acid oxidation stimulation.==

87   Cokorinos, E. C. *et al.* Activation of Skeletal Muscle AMPK Promotes Glucose Disposal and Glucose Lowering in Non-human Primates and Mice. *Cell Metab* **25**, 1147-1159 e1110, doi:10.1016/j.cmet.2017.04.010 (2017).

88   Madiraju, A. K. *et al.* Metformin inhibits gluconeogenesis via a redox-dependent mechanism in vivo. *Nat Med* **24**, 1384-1394, doi:10.1038/s41591-018-0125-4 (2018).

89   Ringler, R. L. & Singer, T. P. Studies on the mitochondrial alpha-glycerophosphate dehydrogenase. I. Reaction of the dehydrogenase with electron acceptors and the respiratory chain. *J Biol Chem* **234**, 2211-2217 (1959).

90   Lee, Y. P. & Lardy, H. A. Influence of Thyroid Hormones on L-Alpha-Glycerophosphate Dehydrogenases and Other Dehydrogenases in Various Organs of the Rat. *J Biol Chem* **240**, 1427-1436 (1965).

91   Lin, E. C. Glycerol utilization and its regulation in mammals. *Annu Rev Biochem* **46**, 765-795, doi:10.1146/annurev.bi.46.070177.004001 (1977).

92   Brisson, D., Vohl, M. C., St-Pierre, J., Hudson, T. J. & Gaudet, D. Glycerol: a neglected variable in metabolic processes? *Bioessays* **23**, 534-542, doi:10.1002/bies.1073 (2001).

93   Baur, J. A. & Birnbaum, M. J. Control of gluconeogenesis by metformin: does redox trump energy charge? *Cell Metab* **20**, 197-199, doi:10.1016/j.cmet.2014.07.013 (2014).

94   Cool, B. *et al.* Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. *Cell Metab* **3**, 403-416, doi:10.1016/j.cmet.2006.05.005 (2006).
      - ==This is the first paper describing a small-molecule direct AMPK activator.==

95   Esquejo, R. M. *et al.* Activation of Liver AMPK with PF-06409577 Corrects NAFLD and Lowers Cholesterol in Rodent and Primate Preclinical Models. *EBioMedicine* **31**, 122-132, doi:10.1016/j.ebiom.2018.04.009 (2018).

96   Mazza, A. *et al.* The role of metformin in the management of NAFLD. *Exp Diabetes Res* **2012**, 716404, doi:10.1155/2012/716404 (2012).

97   Hotamisligil, G. S. Inflammation, metaflammation and immunometabolic disorders. *Nature* **542**, 177-185, doi:10.1038/nature21363 (2017).

98   Brestoff, J. R. & Artis, D. Immune regulation of metabolic homeostasis in health and disease. *Cell* **161**, 146-160, doi:10.1016/j.cell.2015.02.022 (2015).

99   Lackey, D. E. & Olefsky, J. M. Regulation of metabolism by the innate immune system. *Nat Rev Endocrinol* **12**, 15-28, doi:10.1038/nrendo.2015.189 (2016).

100   McNelis, J. C. & Olefsky, J. M. Macrophages, immunity, and metabolic disease. *Immunity* **41**, 36-48, doi:10.1016/j.immuni.2014.05.010 (2014).

101   Lumeng, C. N., Bodzin, J. L. & Saltiel, A. R. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *The Journal of clinical investigation* **117**, 175-184, doi:10.1172/JCI29881 (2007).

102   Huang, W. *et al.* Depletion of liver Kupffer cells prevents the development of diet-induced hepatic steatosis and insulin resistance. *Diabetes* **59**, 347-357, doi:10.2337/db09-0016 (2010).

103   Lanthier, N. *et al.* Kupffer cell activation is a causal factor for hepatic insulin resistance. *Am J Physiol Gastrointest Liver Physiol* **298**, G107-116, doi:10.1152/ajpgi.00391.2009 (2010).

104   Talukdar, S. *et al.* Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. *Nat Med* **18**, 1407-1412, doi:10.1038/nm.2885 (2012).

105   Cai, D. *et al.* Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* **11**, 183-190, doi:10.1038/nm1166 (2005).

106   Kang, K. *et al.* Adipocyte-derived Th2 cytokines and myeloid PPARdelta regulate macrophage polarization and insulin sensitivity. *Cell Metab* **7**, 485-495, doi:10.1016/j.cmet.2008.04.002 (2008).

107   Odegaard, J. I. *et al.* Alternative M2 activation of Kupffer cells by PPARdelta ameliorates obesity-induced insulin resistance. *Cell Metab* **7**, 496-507, doi:10.1016/j.cmet.2008.04.003 (2008).

108   Ricardo-Gonzalez, R. R. *et al.* IL-4/STAT6 immune axis regulates peripheral nutrient metabolism and insulin sensitivity. *Proc Natl Acad Sci U S A* **107**, 22617-22622, doi:10.1073/pnas.1009152108 (2010).

109   Stanya, K. J. *et al.* Direct control of hepatic glucose production by interleukin-13 in mice. *The Journal of clinical investigation* **123**, 261-271, doi:10.1172/JCI64941 (2013).

110   Odegaard, J. I. *et al.* Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. *Nature* **447**, 1116-1120, doi:10.1038/nature05894 (2007).

111   Ilan, Y. *et al.* Induction of regulatory T cells decreases adipose inflammation and alleviates insulin resistance in ob/ob mice. *Proc Natl Acad Sci U S A* **107**, 9765-9770, doi:10.1073/pnas.0908771107 (2010).

112   Feuerer, M. *et al.* Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. *Nat Med* **15**, 930-939, doi:10.1038/nm.2002 (2009).

113   Evia-Viscarra, M. L. *et al.* The effects of metformin on inflammatory mediators in obese adolescents with insulin resistance: controlled randomized clinical trial. *J Pediatr Endocrinol Metab* **25**, 41-49, doi:10.1515/jpem-2011-0469 (2012).

114   Fidan, E. *et al.* The effects of rosiglitazone and metformin on inflammation and endothelial dysfunction in patients with type 2 diabetes mellitus. *Acta Diabetol* **48**, 297-302, doi:10.1007/s00592-011-0276-y (2011).

115   Cameron, A. R. *et al.* Anti-Inflammatory Effects of Metformin Irrespective of Diabetes Status. *Circ Res* **119**, 652-665, doi:10.1161/CIRCRESAHA.116.308445 (2016).

   •   This study shows some beneficial anti-inflammatory effects of metformin in human, irrespective of their diabetic status.

116   Lu, C. H., Hung, Y. J. & Hsieh, P. S. Additional effect of metformin and celecoxib against lipid dysregulation and adipose tissue inflammation in high-fat fed rats with insulin resistance and fatty liver. *Eur J Pharmacol* **789**, 60-67, doi:10.1016/j.ejphar.2016.07.012 (2016).

117   Xue, W. *et al.* Alkannin Inhibited Hepatic Inflammation in Diabetic Db/Db Mice. *Cell Physiol Biochem* **45**, 2461-2470, doi:10.1159/000488264 (2018).

118   Shen, C. L. *et al.* Annatto-extracted tocotrienols improve glucose homeostasis and bone properties in high-fat diet-induced type 2 diabetic mice by decreasing the inflammatory response. *Sci Rep* **8**, 11377, doi:10.1038/s41598-018-29063-9 (2018).

119   Jing, Y. *et al.* Metformin improves obesity-associated inflammation by altering macrophages polarization. *Mol Cell Endocrinol* **461**, 256-264, doi:10.1016/j.mce.2017.09.025 (2018).

120   de Oliveira, S. *et al.* Metformin modulates innate immune-mediated inflammation and early progression of NAFLD-associated hepatocellular carcinoma in zebrafish. *J Hepatol*, doi:10.1016/j.jhep.2018.11.034 (2018).

121   Kim, J. *et al.* Metformin suppresses lipopolysaccharide (LPS)-induced inflammatory response in murine macrophages via activating transcription factor-3 (ATF-3) induction. *J Biol Chem* **289**, 23246-23255, doi:10.1074/jbc.M114.577908 (2014).

122   Kelly, B., Tannahill, G. M., Murphy, M. P. & O'Neill, L. A. Metformin Inhibits the Production of Reactive Oxygen Species from NADH:Ubiquinone Oxidoreductase to Limit Induction of Interleukin-1beta (IL-1beta) and Boosts Interleukin-10 (IL-10) in Lipopolysaccharide (LPS)-activated Macrophages. *J Biol Chem* **290**, 20348-20359, doi:10.1074/jbc.M115.662114 (2015).

123   Moiseeva, O. *et al.* Metformin inhibits the senescence-associated secretory phenotype by interfering with IKK/NF-kappaB activation. *Aging Cell* **12**, 489-498, doi:10.1111/acel.12075 (2013).

124   Vasamsetti, S. B. *et al.* Metformin inhibits monocyte-to-macrophage differentiation via AMPK-mediated inhibition of STAT3 activation: potential role in atherosclerosis. *Diabetes* **64**, 2028-2041, doi:10.2337/db14-1225 (2015).

   •   This study shows the inhibition of monocyte-to-macrophage differentiation and atheromatous plaque formation by metformin in ApoE$^{-/-}$ mice though an AMPK/STAT3-dependent mechanism.

125   Yan, Z. *et al.* Metformin suppresses UHMWPE particle-induced osteolysis in the mouse calvaria by promoting polarization of macrophages to an anti-inflammatory phenotype. *Mol Med* **24**, 20, doi:10.1186/s10020-018-0013-x (2018).

126   Buldak, L. *et al.* Metformin affects macrophages' phenotype and improves the activity of glutathione peroxidase, superoxide dismutase, catalase and decreases malondialdehyde concentration in a partially AMPK-independent manner in LPS-stimulated human monocytes/macrophages. *Pharmacol Rep* **66**, 418-429, doi:10.1016/j.pharep.2013.11.008 (2014).

127   Arai, M. *et al.* Metformin, an antidiabetic agent, suppresses the production of tumor necrosis factor and tissue factor by inhibiting early growth response factor-1 expression in human monocytes in vitro. *J Pharmacol Exp Ther* **334**, 206-213, doi:10.1124/jpet.109.164970 (2010).

128   Stienstra, R., Netea-Maier, R. T., Riksen, N. P., Joosten, L. A. B. & Netea, M. G. Specific and Complex Reprogramming of Cellular Metabolism in Myeloid Cells during Innate Immune Responses. *Cell Metab* **26**, 142-156, doi:10.1016/j.cmet.2017.06.001 (2017).

129   Geltink, R. I. K., Kyle, R. L. & Pearce, E. L. Unraveling the Complex Interplay Between T Cell Metabolism and Function. *Annu Rev Immunol* **36**, 461-488, doi:10.1146/annurev-immunol-042617-053019 (2018).

130   Buck, M. D., Sowell, R. T., Kaech, S. M. & Pearce, E. L. Metabolic Instruction of Immunity. *Cell* **169**, 570-586, doi:10.1016/j.cell.2017.04.004 (2017).

131   Negrotto, L., Farez, M. F. & Correale, J. Immunologic Effects of Metformin and Pioglitazone Treatment on Metabolic Syndrome and Multiple Sclerosis. *JAMA Neurol* **73**, 520-528, doi:10.1001/jamaneurol.2015.4807 (2016).

132   Lee, S. Y. *et al.* Metformin Suppresses Systemic Autoimmunity in Roquin(san/san) Mice through Inhibiting B Cell Differentiation into Plasma Cells via Regulation of AMPK/mTOR/STAT3. *J Immunol* **198**, 2661-2670, doi:10.4049/jimmunol.1403088 (2017).

133   Son, H. J. *et al.* Metformin attenuates experimental autoimmune arthritis through reciprocal regulation of Th17/Treg balance and osteoclastogenesis. *Mediators Inflamm* **2014**, 973986, doi:10.1155/2014/973986 (2014).

134   Lee, S. Y. *et al.* Metformin Ameliorates Inflammatory Bowel Disease by Suppression of the STAT3 Signaling Pathway and Regulation of the between Th17/Treg Balance. *PLoS One* **10**, e0135858, doi:10.1371/journal.pone.0135858 (2015).

135   Eikawa, S. *et al.* Immune-mediated antitumor effect by type 2 diabetes drug, metformin. *Proc Natl Acad Sci U S A* **112**, 1809-1814, doi:10.1073/pnas.1417636112 (2015).

136   Kunisada, Y. *et al.* Attenuation of CD4(+)CD25(+) Regulatory T Cells in the Tumor Microenvironment by Metformin, a Type 2 Diabetes Drug. *EBioMedicine* **25**, 154-164, doi:10.1016/j.ebiom.2017.10.009 (2017).

137   Li, L. *et al.* Metformin-Induced Reduction of CD39 and CD73 Blocks Myeloid-Derived Suppressor Cell Activity in Patients with Ovarian Cancer. *Cancer research* **78**, 1779-1791, doi:10.1158/0008-5472.CAN-17-2460 (2018).

138   Pereira, F. V. *et al.* Metformin exerts antitumor activity via induction of multiple death pathways in tumor cells and activation of a protective immune response. *Oncotarget* **9**, 25808-25825, doi:10.18632/oncotarget.25380 (2018).

139   Natali, A. & Ferrannini, E. Effects of metformin and thiazolidinediones on suppression of hepatic glucose production and stimulation of glucose uptake in type 2 diabetes: a systematic review. *Diabetologia* **49**, 434-441, doi:10.1007/s00125-006-0141-7 (2006).

140   Wu, T., Horowitz, M. & Rayner, C. K. New insights into the anti-diabetic actions of metformin: from the liver to the gut. *Expert Rev Gastroenterol Hepatol* **11**, 157-166, doi:10.1080/17474124.2017.1273769 (2017).

141   Bonora, E. *et al.* Lack of effect of intravenous metformin on plasma concentrations of glucose, insulin, C-peptide, glucagon and growth hormone in non-diabetic subjects. *Curr Med Res Opin* **9**, 47-51, doi:10.1185/03007998409109558 (1984).

142   Stepensky, D., Friedman, M., Raz, I. & Hoffman, A. Pharmacokinetic-pharmacodynamic analysis of the glucose-lowering effect of metformin in diabetic rats reveals first-pass pharmacodynamic effect. *Drug Metab Dispos* **30**, 861-868 (2002).

143   Sum, C. F. *et al.* The effect of intravenous metformin on glucose metabolism during hyperglycaemia in type 2 diabetes. *Diabet Med* **9**, 61-65 (1992).

144   Koffert, J. P. *et al.* Metformin treatment significantly enhances intestinal glucose uptake in patients with type 2 diabetes: Results from a randomized clinical trial. *Diabetes Res Clin Pract* **131**, 208-216, doi:10.1016/j.diabres.2017.07.015 (2017).

145   Wu, T. *et al.* Metformin reduces the rate of small intestinal glucose absorption in type 2 diabetes. *Diabetes Obes Metab* **19**, 290-293, doi:10.1111/dom.12812 (2017).

146   Bailey, C. J., Wilcock, C. & Day, C. Effect of metformin on glucose metabolism in the splanchnic bed. *Br J Pharmacol* **105**, 1009-1013 (1992).

147   Penicaud, L., Hitier, Y., Ferre, P. & Girard, J. Hypoglycaemic effect of metformin in genetically obese (fa/fa) rats results from an increased utilization of blood glucose by intestine. *Biochem J* **262**, 881-885 (1989).

148   Gontier, E. *et al.* High and typical 18F-FDG bowel uptake in patients treated with metformin. *Eur J Nucl Med Mol Imaging* **35**, 95-99, doi:10.1007/s00259-007-0563-6 (2008).

149   Ikeda, T., Iwata, K. & Murakami, H. Inhibitory effect of metformin on intestinal glucose absorption in the perfused rat intestine. *Biochem Pharmacol* **59**, 887-890 (2000).

150   Sakar, Y. *et al.* Metformin-induced regulation of the intestinal D-glucose transporters. *J Physiol Pharmacol* **61**, 301-307 (2010).

151   Walker, J. *et al.* 5-aminoimidazole-4-carboxamide riboside (AICAR) enhances GLUT2-dependent jejunal glucose transport: a possible role for AMPK. *Biochem J* **385**, 485-491, doi:10.1042/BJ20040694 (2005).

152   Lenzen, S., Lortz, S. & Tiedge, M. Effect of metformin on SGLT1, GLUT2, and GLUT5 hexose transporter gene expression in small intestine from rats. *Biochem Pharmacol* **51**, 893-896 (1996).

153   Bauer, P. V. *et al.* Metformin Alters Upper Small Intestinal Microbiota that Impact a Glucose-SGLT1-Sensing Glucoregulatory Pathway. *Cell Metab* **27**, 101-117 e105, doi:10.1016/j.cmet.2017.09.019 (2018).
   • This study in rodents shows the impact of metformin on upper small intestinal microbiota and glucose sensing.

154   Bailey, C. J., Wilcock, C. & Scarpello, J. H. Metformin and the intestine. *Diabetologia* **51**, 1552-1553, doi:10.1007/s00125-008-1053-5 (2008).

155   Duca, F. A. *et al.* Metformin activates a duodenal Ampk-dependent pathway to lower hepatic glucose production in rats. *Nat Med* **21**, 506-511, doi:10.1038/nm.3787 (2015).
   • This paper shows studies in rodent models that show that metformin lowers blood glucose levels by inhibiting hepatic glucose production through a neuronal-mediated gut-brain-liver axis.

156   Borg, M. J. *et al.* Comparative effects of proximal and distal small intestinal administration of metformin on plasma glucose and glucagon-like peptide-1, and gastric emptying after oral glucose, in type 2 diabetes. *Diabetes Obes Metab* **21**, 640-647, doi:10.1111/dom.13567 (2019).

157   Henry, R. R. *et al.* Improved glycemic control with minimal systemic metformin exposure: Effects of Metformin Delayed-Release (Metformin DR) targeting the lower bowel over 16 weeks in a randomized trial in subjects with type 2 diabetes. *PLoS One* **13**, e0203946, doi:10.1371/journal.pone.0203946 (2018).

158   Oh, J. *et al.* Inhibition of the multidrug and toxin extrusion (MATE) transporter by pyrimethamine increases the plasma concentration of metformin but does not increase antihyperglycaemic activity in humans. *Diabetes Obes Metab* **18**, 104-108, doi:10.1111/dom.12577 (2016).

159   Mannucci, E. *et al.* Effects of metformin on glucagon-like peptide-1 levels in obese patients with and without Type 2 diabetes. *Diabetes Nutr Metab* **17**, 336-342 (2004).

160   Napolitano, A. *et al.* Novel gut-based pharmacology of metformin in patients with type 2 diabetes mellitus. *PLoS One* **9**, e100778, doi:10.1371/journal.pone.0100778 (2014).

161   Maida, A., Lamont, B. J., Cao, X. & Drucker, D. J. Metformin regulates the incretin receptor axis via a pathway dependent on peroxisome proliferator-activated receptor-alpha in mice. *Diabetologia* **54**, 339-349 (2011).

162   DeFronzo, R. A. *et al.* Once-daily delayed-release metformin lowers plasma glucose and enhances fasting and postprandial GLP-1 and PYY: results from two randomised trials. *Diabetologia* **59**, 1645-1654, doi:10.1007/s00125-016-3992-6 (2016).

163   Bahne, E. *et al.* Metformin-induced glucagon-like peptide-1 secretion contributes to the actions of metformin in type 2 diabetes. *JCI Insight* **3**, doi:10.1172/jci.insight.93936 (2018).
   • This paper presents human studies that analyse the role of GLP1 release in the glucose-lowering effect of metformin.

164   Li, M. *et al.* Efficacy and safety of liraglutide versus sitagliptin both in combination with metformin in patients with type 2 diabetes: A systematic review and meta-analysis. *Medicine (Baltimore)* **96**, e8161, doi:10.1097/MD.0000000000008161 (2017).

165   Bahne, E. *et al.* Involvement of glucagon-like peptide-1 in the glucose-lowering effect of metformin. *Diabetes Obes Metab* **18**, 955-961, doi:10.1111/dom.12697 (2016).

166   Mulherin, A. J. *et al.* Mechanisms underlying metformin-induced secretion of glucagon-like peptide-1 from the intestinal L cell. *Endocrinology* **152**, 4610-4619, doi:10.1210/en.2011-1485 (2011).

167   Kim, M. H. *et al.* Metformin enhances glucagon-like peptide 1 via cooperation between insulin and Wnt signaling. *J Endocrinol* **220**, 117-128, doi:10.1530/JOE-13-0381 (2014).

168   Kappe, C., Patrone, C., Holst, J. J., Zhang, Q. & Sjoholm, A. Metformin protects against lipoapoptosis and enhances GLP-1 secretion from GLP-1-producing cells. *J Gastroenterol* **48**, 322-332, doi:10.1007/s00535-012-0637-5 (2013).

169   Bronden, A. *et al.* Single-Dose Metformin Enhances Bile Acid-Induced Glucagon-Like Peptide-1 Secretion in Patients With Type 2 Diabetes. *J Clin Endocrinol Metab* **102**, 4153-4162, doi:10.1210/jc.2017-01091 (2017).

170   Carter, D., Howlett, H. C., Wiernsperger, N. F. & Bailey, C. J. Differential effects of metformin on bile salt absorption from the jejunum and ileum. *Diabetes Obes Metab* **5**, 120-125 (2003).

171   Lien, F. *et al.* Metformin interferes with bile acid homeostasis through AMPK-FXR crosstalk. *J Clin Invest* **124**, 1037-1051, doi:10.1172/JCI68815 (2014).

172   Hansen, M. *et al.* Effect of chenodeoxycholic acid and the bile acid sequestrant colesevelam on glucagon-like peptide-1 secretion. *Diabetes Obes Metab* **18**, 571-580, doi:10.1111/dom.12648 (2016).

173   Wu, T. *et al.* Effects of rectal administration of taurocholic acid on glucagon-like peptide-1 and peptide YY secretion in healthy humans. *Diabetes Obes Metab* **15**, 474-477, doi:10.1111/dom.12043 (2013).

174   Thomas, C. *et al.* TGR5-mediated bile acid sensing controls glucose homeostasis. *Cell Metab* **10**, 167-177, doi:10.1016/j.cmet.2009.08.001 (2009).

175   Trabelsi, M. S. *et al.* Farnesoid X receptor inhibits glucagon-like peptide-1 production by enteroendocrine L cells. *Nat Commun* **6**, 7629, doi:10.1038/ncomms8629 (2015).

176   Duca, F. A., Bauer, P. V., Hamr, S. C. & Lam, T. K. Glucoregulatory Relevance of Small Intestinal Nutrient Sensing in Physiology, Bariatric Surgery, and Pharmacology. *Cell Metab* **22**, 367-380, doi:10.1016/j.cmet.2015.07.003 (2015).

177   Waise, T. M. Z., Dranse, H. J. & Lam, T. K. T. The metabolic role of vagal afferent innervation. *Nat Rev Gastroenterol Hepatol* **15**, 625-636, doi:10.1038/s41575-018-0062-1 (2018).

178   Kuhre, R. E., Frost, C. R., Svendsen, B. & Holst, J. J. Molecular mechanisms of glucose-stimulated GLP-1 secretion from perfused rat small intestine. *Diabetes* **64**, 370-382, doi:10.2337/db14-0807 (2015).

179   Parker, H. E. *et al.* Predominant role of active versus facilitative glucose transport for glucagon-like peptide-1 secretion. *Diabetologia* **55**, 2445-2455, doi:10.1007/s00125-012-2585-2 (2012).

180   Sun, L. *et al.* Gut microbiota and intestinal FXR mediate the clinical benefits of metformin. *Nat Med*, doi:10.1038/s41591-018-0222-4 (2018).

181   Cote, C. D. *et al.* Resveratrol activates duodenal Sirt1 to reverse insulin resistance in rats through a neuronal network. *Nat Med* **21**, 498-505, doi:10.1038/nm.3821 (2015).

182   Waise, T. M. Z. *et al.* Inhibition of upper small intestinal mTOR lowers plasma glucose levels by inhibiting glucose production. *Nat Commun* **10**, 714, doi:10.1038/s41467-019-08582-7 (2019).

183   Karlsson, F. H. *et al.* Gut metagenome in European women with normal, impaired and diabetic glucose control. *Nature* **498**, 99-103, doi:10.1038/nature12198 (2013).

184   Qin, J. *et al.* A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature* **490**, 55-60, doi:10.1038/nature11450 (2012).

185   Lee, H. & Ko, G. Effect of metformin on metabolic improvement and gut microbiota. *Appl Environ Microbiol* **80**, 5935-5943, doi:10.1128/AEM.01357-14 (2014).

186   Shin, N. R. *et al.* An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut* **63**, 727-735, doi:10.1136/gutjnl-2012-303839 (2014).

- These rodent studies show that metformin induces a profound shift in the faecal microbial community profile in HFD-fed mice, with higher abundance of the mucin-degrading bacterium *Akkermansia* accompanied by an increase in the number of mucin-producing goblet cells and adipose tissue-resident regulatory T cells.

187   Zhang, X. *et al.* Modulation of gut microbiota by berberine and metformin during the treatment of high-fat diet-induced obesity in rats. *Sci Rep* **5**, 14405, doi:10.1038/srep14405 (2015).

188   de la Cuesta-Zuluaga, J. *et al.* Metformin Is Associated With Higher Relative Abundance of Mucin-Degrading Akkermansia muciniphila and Several Short-Chain Fatty Acid-Producing Microbiota in the Gut. *Diabetes Care* **40**, 54-62, doi:10.2337/dc16-1324 (2017).

- These human studies show that metformin shifts gut microbiota composition through the enrichment in short-chain fatty-acid-producing and mucin degrading bacteria.

189 Wu, H. *et al.* Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. *Nat Med* **23**, 850-858, doi:10.1038/nm.4345 (2017).

- This study in humans shows altered gut microbiota mediates some of metformin's antidiabetic effects in treatment-naïve patients with type 2 diabetes mellitus.

190 Tong, X. *et al.* Structural Alteration of Gut Microbiota during the Amelioration of Human Type 2 Diabetes with Hyperlipidemia by Metformin and a Traditional Chinese Herbal Formula: a Multicenter, Randomized, Open Label Clinical Trial. *MBio* **9**, doi:10.1128/mBio.02392-17 (2018).

191 Rosario, D. *et al.* Understanding the Representative Gut Microbiota Dysbiosis in Metformin-Treated Type 2 Diabetes Patients Using Genome-Scale Metabolic Modeling. *Front Physiol* **9**, 775, doi:10.3389/fphys.2018.00775 (2018).

192 De Vadder, F. *et al.* Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell* **156**, 84-96, doi:10.1016/j.cell.2013.12.016 (2014).

193 Gibson, G. R. & Roberfroid, M. B. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr* **125**, 1401-1412, doi:10.1093/jn/125.6.1401 (1995).

194 Cani, P. D. *et al.* Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. *Am J Clin Nutr* **90**, 1236-1243, doi:10.3945/ajcn.2009.28095 (2009).

195 Chambers, E. S. *et al.* Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. *Gut* **64**, 1744-1754, doi:10.1136/gutjnl-2014-307913 (2015).

196 Ridlon, J. M., Kang, D. J. & Hylemon, P. B. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res* **47**, 241-259, doi:10.1194/jlr.R500013-JLR200 (2006).

197 Everard, A. *et al.* Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proc Natl Acad Sci U S A* **110**, 9066-9071, doi:10.1073/pnas.1219451110 (2013).

198 Plovier, H. *et al.* A purified membrane protein from Akkermansia muciniphila or the pasteurized bacterium improves metabolism in obese and diabetic mice. *Nat Med* **23**, 107-113, doi:10.1038/nm.4236 (2017).

199 Depommier, C. *et al.* Supplementation with Akkermansia muciniphila in overweight and obese human volunteers: a proof-of-concept exploratory study. *Nat Med* **25**, 1096-1103, doi:10.1038/s41591-019-0495-2 (2019).

200 Maier, L. *et al.* Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature* **555**, 623-628, doi:10.1038/nature25979 (2018).

201 Vashisht, R. & Brahmachari, S. K. Metformin as a potential combination therapy with existing front-line antibiotics for Tuberculosis. *J Transl Med* **13**, 83, doi:10.1186/s12967-015-0443-y (2015).

202 Cabreiro, F. *et al.* Metformin retards aging in C. elegans by altering microbial folate and methionine metabolism. *Cell* **153**, 228-239, doi:10.1016/j.cell.2013.02.035 (2013).

203 Belkaid, Y. & Harrison, O. J. Homeostatic Immunity and the Microbiota. *Immunity* **46**, 562-576, doi:10.1016/j.immuni.2017.04.008 (2017).

204 Postler, T. S. & Ghosh, S. Understanding the Holobiont: How Microbial Metabolites Affect Human Health and Shape the Immune System. *Cell Metab* **26**, 110-130, doi:10.1016/j.cmet.2017.05.008 (2017).

205 McDole, J. R. *et al.* Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature* **483**, 345-349, doi:10.1038/nature10863 (2012).

206 Weisman, A., Bai, J. W., Cardinez, M., Kramer, C. K. & Perkins, B. A. Effect of artificial pancreas systems on glycaemic control in patients with type 1 diabetes: a systematic review and meta-analysis of outpatient randomised controlled trials. *Lancet Diabetes Endocrinol* **5**, 501-512, doi:10.1016/S2213-8587(17)30167-5 (2017).

207 Wood, J. R. *et al.* Most youth with type 1 diabetes in the T1D Exchange Clinic Registry do not meet American Diabetes Association or International Society for Pediatric and Adolescent Diabetes clinical guidelines. *Diabetes Care* **36**, 2035-2037, doi:10.2337/dc12-1959 (2013).

208 Petrie, J. R. *et al.* Cardiovascular and metabolic effects of metformin in patients with type 1 diabetes (REMOVAL): a double-blind, randomised, placebo-controlled trial. *Lancet Diabetes Endocrinol* **5**, 597-609, doi:10.1016/S2213-8587(17)30194-8 (2017).

209   Meng, H. *et al.* Effect of metformin on glycaemic control in patients with type 1 diabetes: A meta-analysis of randomized controlled trials. *Diabetes Metab Res Rev* **34**, e2983, doi:10.1002/dmrr.2983 (2018).

210   Lund, S. S. *et al.* Effect of adjunct metformin treatment in patients with type-1 diabetes and persistent inadequate glycaemic control. A randomized study. *PLoS One* **3**, e3363, doi:10.1371/journal.pone.0003363 (2008).

211   Scheen, A. J. Will delayed release metformin provide better management of diabetes type 2? *Expert Opin Pharmacother* **17**, 627-630, doi:10.1517/14656566.2016.1149166 (2016).

212   Fujita, Y. & Inagaki, N. Metformin: New Preparations and Nonglycemic Benefits. *Curr Diab Rep* **17**, 5, doi:10.1007/s11892-017-0829-8 (2017).

213   Berstein, L. M. Metformin: not only per os. *Expert Rev Endocrinol Metab* **13**, 63-65, doi:10.1080/17446651.2018.1431123 (2018).

214   Cetin, M. & Sahin, S. Microparticulate and nanoparticulate drug delivery systems for metformin hydrochloride. *Drug Deliv* **23**, 2796-2805, doi:10.3109/10717544.2015.1089957 (2016).

215   Zhao, Y. *et al.* PolyMetformin combines carrier and anticancer activities for in vivo siRNA delivery. *Nat Commun* **7**, 11822, doi:10.1038/ncomms11822 (2016).

216   Bouchoucha, M., Uzzan, B. & Cohen, R. Metformin and digestive disorders. *Diabetes Metab* **37**, 90-96, doi:10.1016/j.diabet.2010.11.002 (2011).

217   Bonnet, F. & Scheen, A. Understanding and overcoming metformin gastrointestinal intolerance. *Diabetes Obes Metab* **19**, 473-481, doi:10.1111/dom.12854 (2017).

218   McCreight, L. J. *et al.* Pharmacokinetics of metformin in patients with gastrointestinal intolerance. *Diabetes Obes Metab* **20**, 1593-1601, doi:10.1111/dom.13264 (2018).

219   Dujic, T. *et al.* Association of Organic Cation Transporter 1 With Intolerance to Metformin in Type 2 Diabetes: A GoDARTS Study. *Diabetes* **64**, 1786-1793, doi:10.2337/db14-1388 (2015).

220   Dujic, T., Zhou, K., Tavendale, R., Palmer, C. N. & Pearson, E. R. Effect of Serotonin Transporter 5-HTTLPR Polymorphism on Gastrointestinal Intolerance to Metformin: A GoDARTS Study. *Diabetes Care* **39**, 1896-1901, doi:10.2337/dc16-0706 (2016).

221   Hoffmann, I. S., Roa, M., Torrico, F. & Cubeddu, L. X. Ondansetron and metformin-induced gastrointestinal side effects. *Am J Ther* **10**, 447-451 (2003).

222   Scarpello, J. H., Hodgson, E. & Howlett, H. C. Effect of metformin on bile salt circulation and intestinal motility in type 2 diabetes mellitus. *Diabetic medicine : a journal of the British Diabetic Association* **15**, 651-656, doi:10.1002/(SICI)1096-9136(199808)15:8<651::AID-DIA628>3.0.CO;2-A (1998).

223   Elbere, I. *et al.* Association of metformin administration with gut microbiome dysbiosis in healthy volunteers. *PLoS One* **13**, e0204317, doi:10.1371/journal.pone.0204317 (2018).

224   Greenway, F., Wang, S. & Heiman, M. A novel cobiotic containing a prebiotic and an antioxidant augments the glucose control and gastrointestinal tolerability of metformin: a case report. *Benef Microbes* **5**, 29-32, doi:10.3920/BM2012.0063 (2014).

225   Burton, J. H. *et al.* Addition of a Gastrointestinal Microbiome Modulator to Metformin Improves Metformin Tolerance and Fasting Glucose Levels. *J Diabetes Sci Technol* **9**, 808-814, doi:10.1177/1932296815577425 (2015).

226   Escobar-Morreale, H. F. Polycystic ovary syndrome: definition, aetiology, diagnosis and treatment. *Nat Rev Endocrinol* **14**, 270-284, doi:10.1038/nrendo.2018.24 (2018).

227   Burt Solorzano, C. M. *et al.* Neuroendocrine dysfunction in polycystic ovary syndrome. *Steroids* **77**, 332-337, doi:10.1016/j.steroids.2011.12.007 (2012).

228   Ovalle, F. & Azziz, R. Insulin resistance, polycystic ovary syndrome, and type 2 diabetes mellitus. *Fertil Steril* **77**, 1095-1105 (2002).

229   Carmina, E. & Lobo, R. A. Use of fasting blood to assess the prevalence of insulin resistance in women with polycystic ovary syndrome. *Fertil Steril* **82**, 661-665, doi:10.1016/j.fertnstert.2004.01.041 (2004).

230   Adashi, E. Y., Hsueh, A. J. & Yen, S. S. Insulin enhancement of luteinizing hormone and follicle-stimulating hormone release by cultured pituitary cells. *Endocrinology* **108**, 1441-1449, doi:10.1210/endo-108-4-1441 (1981).

231   Nestler, J. E. *et al.* Insulin stimulates testosterone biosynthesis by human thecal cells from women with polycystic ovary syndrome by activating its own receptor and using inositolglycan mediators as the signal transduction system. *The Journal of clinical endocrinology and metabolism* **83**, 2001-2005, doi:10.1210/jcem.83.6.4886 (1998).

232   Carmina, E. *et al.* The contributions of oestrogen and growth factors to increased adrenal androgen secretion in polycystic ovary syndrome. *Hum Reprod* **14**, 307-311 (1999).

233   Tosi, F. *et al.* Insulin enhances ACTH-stimulated androgen and glucocorticoid metabolism in hyperandrogenic women. *Eur J Endocrinol* **164**, 197-203, doi:10.1530/EJE-10-0782 (2011).

234   Wild, R. A. *et al.* Assessment of cardiovascular risk and prevention of cardiovascular disease in women with the polycystic ovary syndrome: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society. *The Journal of clinical endocrinology and metabolism* **95**, 2038-2049, doi:10.1210/jc.2009-2724 (2010).

235   Morin-Papunen, L. *et al.* Metformin reduces serum C-reactive protein levels in women with polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism* **88**, 4649-4654, doi:10.1210/jc.2002-021688 (2003).

236   Tang, T. *et al.* Combined lifestyle modification and metformin in obese patients with polycystic ovary syndrome. A randomized, placebo-controlled, double-blind multicentre study. *Hum Reprod* **21**, 80-89, doi:10.1093/humrep/dei311 (2006).

237   Naderpoor, N. *et al.* Metformin and lifestyle modification in polycystic ovary syndrome: systematic review and meta-analysis. *Hum Reprod Update* **21**, 560-574, doi:10.1093/humupd/dmv025 (2015).

238   Orio, F., Jr. *et al.* Improvement in endothelial structure and function after metformin treatment in young normal-weight women with polycystic ovary syndrome: results of a 6-month study. *The Journal of clinical endocrinology and metabolism* **90**, 6072-6076, doi:10.1210/jc.2005-0965 (2005).

239   Nestler, J. E., Jakubowicz, D. J., Evans, W. S. & Pasquali, R. Effects of metformin on spontaneous and clomiphene-induced ovulation in the polycystic ovary syndrome. *N Engl J Med* **338**, 1876-1880, doi:10.1056/NEJM199806253382603 (1998).

240   Lord, J. M., Flight, I. H. & Norman, R. J. Insulin-sensitising drugs (metformin, troglitazone, rosiglitazone, pioglitazone, D-chiro-inositol) for polycystic ovary syndrome. *Cochrane Database Syst Rev*, CD003053, doi:10.1002/14651858.CD003053 (2003).

241   Kashyap, S., Wells, G. A. & Rosenwaks, Z. Insulin-sensitizing agents as primary therapy for patients with polycystic ovarian syndrome. *Hum Reprod* **19**, 2474-2483, doi:10.1093/humrep/deh440 (2004).

242   Legro, R. S. *et al.* Clomiphene, metformin, or both for infertility in the polycystic ovary syndrome. *N Engl J Med* **356**, 551-566, doi:10.1056/NEJMoa063971 (2007).

243   Morley, L. C., Tang, T., Yasmin, E., Norman, R. J. & Balen, A. H. Insulin-sensitising drugs (metformin, rosiglitazone, pioglitazone, D-chiro-inositol) for women with polycystic ovary syndrome, oligo amenorrhoea and subfertility. *Cochrane Database Syst Rev* **11**, CD003053, doi:10.1002/14651858.CD003053.pub6 (2017).

244   Balen, A. H. *et al.* The management of anovulatory infertility in women with polycystic ovary syndrome: an analysis of the evidence to support the development of global WHO guidance. *Hum Reprod Update* **22**, 687-708, doi:10.1093/humupd/dmw025 (2016).

245   Heckman-Stoddard, B. M., DeCensi, A., Sahasrabuddhe, V. V. & Ford, L. G. Repurposing metformin for the prevention of cancer and cancer recurrence. *Diabetologia* **60**, 1639-1647, doi:10.1007/s00125-017-4372-6 (2017).

246   Kurelac, I. *et al.* Inducing cancer indolence by targeting mitochondrial Complex I is potentiated by blocking macrophage-mediated adaptive responses. *Nat Commun* **10**, 903, doi:10.1038/s41467-019-08839-1 (2019).

247   Rotermund, C., Machetanz, G. & Fitzgerald, J. C. The Therapeutic Potential of Metformin in Neurodegenerative Diseases. *Front Endocrinol (Lausanne)* **9**, 400, doi:10.3389/fendo.2018.00400 (2018).

248   Anisimov, V. N. *et al.* Metformin slows down aging and extends life span of female SHR mice. *Cell Cycle* **7**, 2769-2773, doi:10.4161/cc.7.17.6625 (2008).

249   Barzilai, N., Crandall, J. P., Kritchevsky, S. B. & Espeland, M. A. Metformin as a Tool to Target Aging. *Cell Metab* **23**, 1060-1065, doi:10.1016/j.cmet.2016.05.011 (2016).

250   Justice, J. N. *et al.* A framework for selection of blood-based biomarkers for geroscience-guided clinical trials: report from the TAME Biomarkers Workgroup. *Geroscience* **40**, 419-436, doi:10.1007/s11357-018-0042-y (2018).

251   Degner, N. R., Wang, J. Y., Golub, J. E. & Karakousis, P. C. Metformin Use Reverses the Increased Mortality Associated With Diabetes Mellitus During Tuberculosis Treatment. *Clin Infect Dis* **66**, 198-205, doi:10.1093/cid/cix819 (2018).

252   Singhal, A. *et al.* Metformin as adjunct antituberculosis therapy. *Sci Transl Med* **6**, 263ra159, doi:10.1126/scitranslmed.3009885 (2014).

- This study provides evidence for the benefits of metformin treatment as host-adjunctive therapy for improving the effective treatment of tuberculosis.

253   Rangarajan, S. *et al.* Metformin reverses established lung fibrosis in a bleomycin model. *Nat Med* **24**, 1121-1127, doi:10.1038/s41591-018-0087-6 (2018).

254   Sato, N. *et al.* Metformin attenuates lung fibrosis development via NOX4 suppression. *Respir Res* **17**, 107, doi:10.1186/s12931-016-0420-x (2016).