METFORMIN: BEYOND DIABETES

EDITED BY: Frederic Bost, Graham Rena and Benoit Viollet
PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-407-1
DOI 10.3389/978-2-88963-407-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

METFORMIN: BEYOND DIABETES

Topic Editors:
Frederic Bost, INSERM U1065 Centre Méditerranéen de Médecine Moléculaire, France
Graham Rena, University of Dundee, United Kingdom
Benoit Viollet, Université de Paris, Institut Cochin, CNRS, INSERM, Université de Paris, France

Citation: Bost, F., Rena, G., Viollet, B., eds. (2020). Metformin: Beyond Diabetes. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-407-1

Table of Contents

frontiers in Endocrinology

EDITORIAL
published: 06 December 2019
doi: 10.3389/fendo.2019.00851

Editorial: Metformin: Beyond Diabetes

Frédéric Bost[1]*, *Graham Rena*[2]* and *Benoit Viollet*[3]*

[1] Université Nice Côte d'Azur, Inserm U1065, Nice, France, [2] University of Dundee, Dundee, United Kingdom, [3] Université de Paris, Institut Cochin, CNRS UMR8104, INSERM U1016, Paris, France

Keywords: metformin, cancer, diabetes, neurodegenerative disease, mitochondria, gut microbiota, PCOS (polycystic ovarian syndrome)

Editorial on the Research Topic

Metformin: Beyond Diabetes

Metformin, a member of the family of biguanides, is one of the most prescribed medications in the US and Europe and remains the first-line treatment for type 2 Diabetes (T2D) worldwide. It is a low cost medication, relatively well-tolerated, that has been given to millions of patients for more than 60 years in Europe. The literature on metformin is immense and recent discoveries in basic research place metformin on the short-list of the most promising drug for repurposing. Pioneering mechanistic studies demonstrating that metformin inhibits complex I in the respiratory chain of the mitochondria (1, 2) and the work of Zhou et al. showing that metformin activates AMP-activated protein kinase (AMPK) by inducing its phosphorylation at Thr^{172} (3), opened new horizons for maximizing clinical exploitation of metformin. Not only did they spur better understanding of metformin's action in T2D (4–6), but they also provided rational bases for laboratories to study the therapeutic potential of metformin outside of the conventional management of T2D. Twenty years on however, there still remains much debate regarding the key molecular target(s) of metformin. In this Research Topic, the evidence regarding direct effects of metformin on complex I of the electron transport chain and mitochondria are discussed in two focussed reviews (Fontaine; Vial et al.). They address topical research alongside earlier studies on the mechanism of action of metformin on mitochondrial complex I, how metformin modulates reactive oxygen species (ROS) production to prevent mitochondrial-mediated apoptosis and how the drug protects against permeability transition pore (PTP)-induced cell death. These effects are discussed in the context of T2D and cancer.

Metformin is now a well-established disruptor of cellular energy supply that targets the mitochondria [(7); Fontaine; Vial et al.]. The resulting compensatory changes on cellular metabolism to provide alternative sources of ATP and metabolites are detailed in this Research Topic by Andrzejewski et al.: including increased glycolysis, modifications of glutamine metabolism, and increase in PGC-1α [a major regulator of mitochondrial biogenesis also implicated in cancer (8)]. These adaptations are thought to play a central role in the resistance to metformin in cancer cells.

Activation of AMPK has been reported to inhibit the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway frequently activated in cancer cells (9). Furthermore, the tumor suppressor LKB1 was demonstrated to phosphorylate AMPK in response to biguanides (10). What then are the consequences in terms of cancer incidence in patients treated with metformin for decades? Observational evidence suggests that metformin reduces the incidence of cancer in people with diabetes (11). In this Research Topic three articles focus on the action of metformin on cancer

Edited and reviewed by:
Ralf Jockers,
Université Paris-Sorbonne, France

***Correspondence:**
Frédéric Bost
bost@unice.fr
Graham Rena
g.rena@dundee.ac.uk
Benoit Viollet
benoit.viollet@inserm.fr

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *15 November 2019*
Accepted: *21 November 2019*
Published: *06 December 2019*

Citation:
Bost F, Rena G and Viollet B (2019) Editorial: Metformin: Beyond Diabetes. Front. Endocrinol. 10:851. doi: 10.3389/fendo.2019.00851

and more specifically on melanoma (Jaune and Rocchi), leukemia (Biondani and Peyron), and colorectal cancer (Higurashi and Nakajima). These reviews describe in detail the recent advances concerning *in vivo* effects and the different molecular mechanisms underlying the anti-cancer action of metformin (AMPK dependent/independent effects, role of p53 and cellular effects: apoptosis, autophagy, proliferation, and cell migration), and present ongoing clinical trials for the prevention or treatment of various types of cancer.

One of the first reported benefits of metformin in reproductive biology was the increase of fertility in patients with polycystic ovary syndrome (PCOS) (12). This pathology is often associated with insulin resistance; thus, it is perhaps not surprising in hindsight that metformin ameliorates PCOS. Likewise, metformin has beneficial effects on obese male fertility (13). This important aspect of metformin action is addressed in a review that also discusses the potential epigenetic modifications induced by metformin in this context (Faure et al.). Among epigenetic modifications, histone acetylation/deacetylation plays a major role in the regulation of gene expression and metformin via AMPK was shown to regulate the expression of Sirtuin1 (Sirt1), a member of the class III (NAD+-dependent) histone deacetylases (HDACs) (14). An original research article of this collection by the group of J. Menendez, uses a computational approach to identify putative sites of interaction between Sirt1 and metformin (Cuyàs et al.). This is an important issue since metformin similarly to Sirtuins has been reported to expand longevity from yeast to mammals (15).

One of the most surprising effects of metformin found in recent years is its action on the gut microbiota. Indeed, the original discovery made by Oluf Pedersen's lab demonstrated that metformin causes a shift in the composition of microbiota altered during T2D (16). Two examples of the action of metformin on gut microbiota are given in two original research papers of the "Metformin: beyond diabetes" Research Topic (Wang et al.; Ji et al.).

Finally, there is growing evidence showing that metformin may have therapeutic potential in neurodegenerative disease. Rotermund et al. contribute a comprehensive review on the topic. In this article, evidence for effects of metformin on Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease are summarized. Metformin has a protective action on neurons mainly because it protects from oxidative stress and neuroinflammation through mechanisms implicating mitochondria and glucose metabolism. Once again, cellular metabolism is at the forefront.

There are so many pathologies that have been shown to be impacted by metformin that it is a first-class candidate for drug repurposing in the near future. Besides cardiovascular disease (17, 18), tuberculosis (19) and very recently multiple sclerosis (20) may show promise. Future investigations and large-scale prospective clinical trials, some of them currently ongoing, will clarify this fascinating issue.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Research funded by Fondation ARC to FB, BHF PG/18/79/34106 and the Newton Fund to GR.

REFERENCES

1. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem.* (2000) 275:223–8. doi: 10.1074/jbc.275.1.223
2. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J.* (2000) 348(Pt 3), 607–614. doi: 10.1042/bj3480607
3. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest.* (2001) 108:1167–74. doi: 10.1172/JCI13505
4. Foretz M, Guigas B, Viollet B. Understanding the glucoregulatory mechanisms of metformin in type 2 diabetes mellitus. *Nat Rev Endocrinol.* (2019) 15:569–89. doi: 10.1038/s41574-019-0242-2
5. Foretz M, Hebrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, et al. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest.* (2010) 120:2355–69. doi: 10.1172/JCI40671
6. Madiraju AK, Qiu Y, Perry RJ, Rahimi Y, Zhang XM, Zhang D, et al. Metformin inhibits gluconeogenesis via a redox-dependent mechanism *in vivo*. *Nat Med.* (2018) 24:1384–94. doi: 10.1038/s41591-018-0125-4
7. Bost F, Decoux-Poullot AG, Tanti JF, Clavel S. Energy disruptors: rising stars in anticancer therapy? *Oncogenesis.* (2016) 5:e188. doi: 10.1038/oncsis.2015.46
8. Kaminski L, Torrino S, Dufies M, Djabari Z, Haider R, Roustan FR, et al. PGC1alpha inhibits polyamine synthesis to suppress prostate cancer aggressiveness. *Cancer Res.* (2019) 79:3268–80. doi: 10.1158/0008-5472.CAN-18-2043
9. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. *Cell.* (2017) 168:960–76. doi: 10.1016/j.cell.2017.02.004
10. Hawley SA, Boudeau J, Reid JL, Mustard KJ, Udd L, Makela TP, et al. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J Biol.* (2003) 2:28. doi: 10.1186/1475-4924-2-28
11. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ.* (2005) 330:1304–5. doi: 10.1136/bmj.38415.708634.F7
12. Patel R, Shah G. Effect of metformin on clinical, metabolic and endocrine outcomes in women with polycystic ovary syndrome: a meta-analysis of randomized controlled trials. *Curr Med Res Opin.* (2017) 33:1545–57. doi: 10.1080/03007995.2017.1279597
13. Ferreira C, Sousa M, Rabaca A, Oliveira PF, Alves MG, Sa R. Impact of metformin on male reproduction. *Curr Pharm Des.* (2015) 21:3621–33. doi: 10.2174/1381612821666150710150041
14. Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, et al. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. *Nature.* (2009) 458:1056–60. doi: 10.1038/nature07813

15. Martin-Montalvo A, Mercken EM, Mitchell SJ, Palacios HH, Mote PL, Scheibye-Knudsen M, et al. Metformin improves healthspan and lifespan in mice. *Nat Commun.* (2013) 4:2192. doi: 10.1038/ncomms3192
16. Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature.* (2015) 528:262–6. doi: 10.1038/nature15766
17. Mohan M, Al-Talabany S, McKinnie A, Mordi IR, Singh JSS, Gandy SJ, et al. A randomized controlled trial of metformin on left ventricular hypertrophy in patients with coronary artery disease without diabetes: the MET-REMODEL trial. *Eur Heart J.* (2019) 40:3409–17. doi: 10.1093/eurheartj/ehz203
18. Rena G, Lang CC. Repurposing metformin for cardiovascular disease. *Circulation.* (2018) 137:422–4. doi: 10.1161/CIRCULATIONAHA.117.031735
19. Leung CC. Repurposing metformin to prevent and treat tuberculosis. *Respirology.* (2018) 23:974–5. doi: 10.1111/resp.13371
20. Neumann B, Baror R, Zhao C, Segel M, Dietmann S, Rawji KS, et al. Metformin restores CNS remyelination capacity by rejuvenating aged stem cells. *Cell Stem Cell.* (2019) 25:473–485.e478. doi: 10.1016/j.stem.2019.08.015

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 19 July 2018
doi: 10.3389/fendo.2018.00400

The Therapeutic Potential of Metformin in Neurodegenerative Diseases

Carola Rotermund[1], *Gerrit Machetanz*[2] *and Julia C. Fitzgerald*[1,2]*

[1] German Centre for Neurodegenerative Diseases, Tübingen, Germany, [2] Department of Neurodegenerative Diseases, Centre of Neurology and Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany

The search for treatments for neurodegenerative diseases is a major concern in light of today's aging population and an increasing burden on individuals, families, and society. Although great advances have been made in the last decades to understand the underlying genetic and biological cause of these diseases, only some symptomatic treatments are available. Metformin has long since been used to treat Type 2 Diabetes and has been shown to be beneficial in several other conditions. Metformin is well-tested *in vitro* and *in vivo* and an approved compound that targets diverse pathways including mitochondrial energy production and insulin signaling. There is growing evidence for the benefits of metformin to counteract age-related diseases such as cancer, cardiovascular disease, and neurodegenerative diseases. We will discuss evidence showing that certain neurodegenerative diseases and diabetes are explicitly linked and that metformin along with other diabetes drugs can reduce neurological symptoms in some patients and reduce disease phenotypes in animal and cell models. An interesting therapeutic factor might be how metformin is able to balance survival and death signaling in cells through pathways that are commonly associated with neurodegenerative diseases. In healthy neurons, these overarching signals keep energy metabolism, oxidative stress, and proteostasis in check, avoiding the dysfunction and neuronal death that defines neurodegenerative disease. We will discuss the biological mechanisms involved and the relevance of neuronal vulnerability and potential difficulties for future trials and development of therapies.

Keywords: metformin, neurodegeneration, diabetes, Parkinson's disease, Alzheimer's disease, aging, mitochondria

OPEN ACCESS

Edited by:
Frederic Bost, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Hudson Sousa Buck, Faculdade de Ciências Médicas, Santa Casa de São Paulo, Brazil
Guillermo Romero, University of Pittsburgh, United States
Antonio J. Herrera, Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad de Sevilla, Spain

***Correspondence:**
Julia C. Fitzgerald
julia.fitzgerald@uni-tuebingen.de

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *04 April 2018*
Accepted: *27 June 2018*
Published: *19 July 2018*

Citation:
Rotermund C, Machetanz G and Fitzgerald JC (2018) The Therapeutic Potential of Metformin in Neurodegenerative Diseases. Front. Endocrinol. 9:400. doi: 10.3389/fendo.2018.00400

INTRODUCTION

The evolution of genomics has greatly advanced our understanding of the genetic contribution to neurodegenerative diseases and provided an entry point for studying the biological cascades leading to neuronal degeneration. The growing research areas of bioinformatics and systems medicine have also opened up opportunities for better targeted treatments and individualized therapies. However, even for diseases such as Alzheimer's and Parkinson's disease, in which much progress has been made, a clear link between genetics, underlying pathological processes and the resulting clinical phenotype seldom exists. Neurodegenerative diseases are currently incurable, debilitating conditions caused by the progressive degeneration and death of nerve cells and their prevalence is rising in today's society (1).

Therefore, despite substantial advances in the development of symptomatic treatments for Alzheimer's disease (AD) and Parkinson's disease (PD) (**Figure 1**), there is still a major need for novel therapeutic strategies and disease-modifying treatments.

Given the complex and heterogeneous molecular basis of neurodegenerative disease the task can appear overwhelming and the previous decades have seen mostly disappointing clinical trial outcomes and subsequent lack of financial investment.

Gallega officinalis (French lilac) contains glucose-lowering guanidines and has been used for treatment of diabetes for centuries. The derivate metformin is a biguanide which was introduced in Europe in the 1950s and in the United States in the 1990s (2, 3). Metformin has recently been reported to decrease cardiovascular risk, restore ovarian function in polycystic ovary syndrome, reduce hepatic lipogenesis, fatty liver disease, and reduce oxidative stress (3, 4). The mechanisms by which metformin exerts its effects are still not fully defined but it is known that metformin inhibits glucose production in the liver and increases glucose uptake in peripheral tissues thereby lowering blood glucose levels (3, 5, 6). It is also accepted that metformin slows mitochondrial respiration via its direct action on complex I of the respiratory chain of mitochondria.

THE THERAPEUTIC POTENTIAL OF METFORMIN: RATIONALE

Metformin has the potential to interfere with neuronal longevity mechanisms and is therefore an interesting drug since it has already been approved for human use. However, human aging research in general has been slowed down by the lack of good aging models that can be used in the laboratory. Retaining aging signatures in reprogrammed neurons has been made possible by the use of direct reprogramming protocols (7, 8) but this may not be feasible for some research groups and time is needed for the technologies to be established in non-expert laboratories. New, simple and affordable methods to investigate the role of aging in human cells are still greatly needed.

Nonetheless, data from human and animal studies regardless of cell type have shown that dysregulation of insulin function contributes to aging and the development of neurodegenerative diseases (9). Insulin resistance and diabetes are increasingly recognized as a contributor to disease development especially in the field of dementias (10–12). Therefore, the rationale for using metformin is its potential to slow aging processes by acting on mitochondrial metabolism and insulin signaling. Slowing the aging process will be beneficial because quality of life could be improved in old age by delaying disease.

A link between diabetes and neurodegenerative diseases is for the most part accepted, although data is not unequivocal, and the exact mechanisms are unclear. A large body of data on metformin use in humans and animals with neurodegenerative diseases exists but metformin's therapeutic use is not yet accepted since the results are often conflicting. These different outcomes are dependent on disease, model system, species and the underlying biological pathways involved, which are now briefly reviewed.

DEMENTIA

Dementia is a common neurological disease of heterogeneous origin and the most important risk factor is aging. Dementia affects memory and other cognitive functions, interfering with a person's ability to carry out routine daily activities. According to the UN world population prospects, the number of persons aged 60 or over on the globe is estimated to grow approximately four times over the next 30 years (13) bringing the prediction that diagnoses of dementia will also rise. The most common form of dementia is AD but there are other types of dementia including vascular dementia, mixed dementia, frontotemporal dementia, dementia with Lewy bodies, and Parkinson's disease dementia.

Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative disease, with 45 million people worldwide affected (14). AD is characterized by progressive memory loss and decline of cognitive function.

Neurofibrillary tangles (NFTs, composed of abnormal tau protein) and amyloid plaques [composed of extracellular aggregates of amyloid-β (Aβ)] are pathological hallmarks of the AD brain (15–17). The NFT protein tau is associated with microtubules and is responsible for their stabilization (18). Tau pathology and synaptic loss correlates with cognitive impairments in AD patients (19). The amyloid plaque component Aβ derives from the sequential cleavage of the membrane protein APP (Amyloid precursor protein) by β-secretase BACE1 (β-site amyloid precursor protein cleaving enzyme 1) and the γ-secretase complex (20). Dysregulation, abnormal modification, and build-up of these protein structures in the brain are thought to be the major pathologies underlying AD.

From a genetic standpoint, most forms of AD are sporadic and of late onset but familial forms of early onset AD exist and are commonly caused by mutations in APP or presenilin (21–23). The underlying biological mechanisms leading to sporadic forms of AD have still not been defined. Inflammatory response, hormone regulation, mitochondrial dysfunction, and lysosomal dysfunction have been implicated, to name only a few processes. There is also growing genetic evidence for microglial involvement (24–26). Still, the main risk factors for developing AD are aging, genetic risk factors including being an APOE-ε4 allele carrier, variants in TREM2, and several GWAS loci, traumatic brain injury, cardiovascular risk factors, and several environmental risk factors (27–31).

Diabetes and Dementia: Animal Models

Most of the rodent models used to investigate the role of insulin and glucose metabolism in dementia have focused on AD. Insulin signaling and glucose tolerances are altered in APP/PS1 mice fed a high fat diet (32, 33), in partially leptin deficient (db/+)-APP/PS1 mice (34) and APP23-(ob/ob) mice (35). APP load may therefore boost susceptibility to disturbances of energy metabolism.

A high fat diet induces insulin resistance and promotes amyloidosis and memory impairment in both the Tg2576 mouse

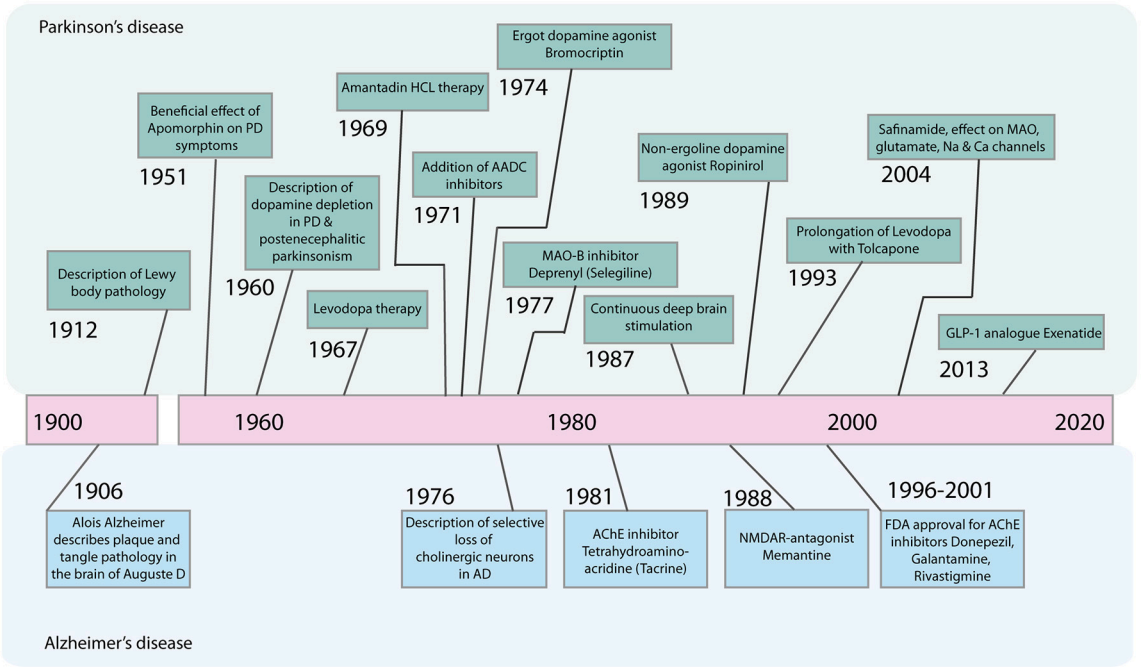

FIGURE 1 | Timeline of major advances in the treatment of Parkinson's disease and Alzheimer's disease in the last century. AADC, Aromatic L-amino acid decarboxylase; AChE, Acetylcholinesterase; GLP-1, Glucagone-like Peptide 1; HCL, Hydrochloride; MAO-B, Monoamine oxidase B; NMDAR, N-Methyl-D-Aspartate Receptor.

model of AD as well as in APP transgenic mice (36, 37). High fat diets or obesity could contribute to memory deficits even in wild type animals. However, some studies reviewed by Agusti and colleagues had no effect on cognition at all (38) leaving the topic still debated because of conflicting data. Diabetic rats show increased levels of APP, Aβ, and phosphorylated tau (39). These data suggest that alteration of energy metabolism via insulin signaling may contribute to Aβ generation and altered tau phosphorylation, two well know biochemical events associated with dementias. The modulation of insulin has been proven to be an effective strategy to protect neurons and synapses against toxic Aβ oligomers and to improve cognition in other AD animal models (40, 41). For example, glucagon-like peptide-1 (GLP-1), insulin-like growth factor 1 (IGF-1) as well as caloric restriction have all been shown to exert neuroprotective effects (42–44).

Metformin and Dementia: Animal Models

Until now only few animal studies have assessed the effect of metformin on cognitive decline and the results are not in line (**Table 1**). There are many different ways in which researchers can modulate energy metabolism in rodents to try to induce cognitive impairments and perhaps this has contributed to the variable data on metformin in this context. Some animals are fed high fat diets, others such as the (db/db) mice have a spontaneous mutation that cause them to be insulin resistant and obese. In three such high fat diet studies, metformin treatment reduces cognitive deficits (57, 58, 60), but one study found no improvement (59). In (db/db) mice, one study found metformin improved memory (53) whereas another study found no effect (55). It should be noted that in one study looking at normal aging in wild type mice, metformin had a detrimental effect on memory impairment (61). In this study activation of AMPK by phosphorylation was not measured and therefore it is not clear whether the metformin diet in these animals was optimal. More studies with proper controls are clearly needed to understand the effect of metformin in normal aging.

It also seems that metformin is capable of simultaneously having both a negative and positive impact on specific biochemical events within the same disease model. For example, in a P301S tauopathy mouse model, metformin treatment reduced tau phosphorylation but promoted tau aggregation (37). The authors suggest that metformin could be beneficial as a dephosphorylating agent but could promote protein aggregation, the latter being unquestionably the more widely accepted neurodegenerative disease pathology. Similarly, short term metformin treatment again reduced tau phosphorylation but had negative effects since it activates APP and BACE-1 (54, 56). Metformin again seems to have positive effects on reducing total tau and tau phosphorylation at serine 236, whereas the sulfonylurea type diabetes drug glibenclamide performed much better in similar tests (53, 55).

Sex may also influence metformin action, which could complicate the interpretation of animal data. Male rodents are often favored and sometimes the sex of the animals used is either overlooked or omitted entirely. In one metformin study already mentioned, male mice showed impaired cognitive function while female mice were improved after treatment (36).

Diabetes and Dementia: Human Studies

Changes in cognition have been reported in type 2 diabetes mellitus (T2DM) patients who have not received a diagnosis of dementia and meta-analyses have found moderate but significant deficits across cognitive domains (64–66). T2DM also seems to increase the risk of conversion from mild cognitive impairment to dementia and the conversion from amnestic mild cognitive impairment to AD (67).

Brain imaging studies in T2DM patients have shown a reduction of whole and regional gray matter volume including hippocampal volume when compared to non-diabetics (68, 69). Taken together, the clinical data mostly shows that T2DM patients have an increased likelihood of developing dementia (10, 70–73). The relationship between diabetes and dementia is further strengthened by reports that reversely, AD patients have an increased risk of developing T2DM or impaired glucose tolerance (74–76). Furthermore, *post mortem* brain pathology in AD shows decreased insulin receptors and IGF protein levels, and insulin levels and markers of insulin signaling are altered in the brain (77–80). Hyperglycemia and hyperinsulinemia have also been positively correlated with AD pathology (75, 81, 82). However, it must be stated that the vast majority of neuropathological studies did not find any association between T2DM or indeed glucose levels and extent of AD pathology (83–87) and two studies even suggest a negative association (88, 89).

One explanation for the discrepancy between clinical and neuropathological studies in AD is the influence of vascular pathology. It is now established that concomitant pathologies in the aging brain are rather the rule than the exception (90). The fact that most studies show no association between T2DM and Aβ deposition therefore seems to hint that there is no major effect of T2DM on Alzheimer's pathology. An additional effect of small vessel disease on cognition in patients with T2DM and Alzheimer's pathology could explain the higher likelihood to develop dementia in this group. This implies that even if T2DM does not have a large impact on Alzheimer's pathology the proper management of diabetes in AD is relevant (67, 91). An interplay between T2DM and Alzheimer's beyond vascular pathology should not be disregarded though especially considering evidence on shared pathophysiological features.

Metformin and Dementia: Human Studies

Results from clinical studies assessing the effect of metformin use on cognitive decline and AD mostly show a positive influence (**Table 2**). Metformin use is associated with significantly lower risk of cognitive impairment in T2DM (102, 107). The incidence of dementia in general is lower in T2DM patients receiving metformin, sulfonylurea or a combination of both drugs compared to those not receiving oral anti-hyperglycemic agents (96). The risk of developing AD was lower in diabetics receiving metformin than in patients receiving sulfonylurea or thiazolidinediones in two studies (97, 101). However, in a single study, long-term use of metformin for T2DM (though not sulfonylureas or thiazolidinediones) was associated with higher risk of developing AD (103). One informative study used latent class analysis to identify groups of men with T2DM receiving metformin who develop different profiles of comorbidities including dementia. They concluded that the effect of metformin may in fact differ depending on the risk-profile of patient receiving the drug (100).

In an interventional study, Luchsinger and colleagues investigated the effect of metformin given daily for 12 months compared to placebo in overweight patients with amnestic mild cognitive impairment. There was improvement in the selective reminding test in the group receiving metformin but not in other cognitive or biomarker outcomes (108). The results were only marginally significant and there was no correction for multiple measurements which at least suggests that the observed improvement must be confirmed in an independent trial. In another interventional, short-term metformin study, nondiabetic patients with mild cognitive impairment or mild dementia due to AD took metformin or a placebo for 8 weeks. Those taking metformin significantly improved in a measure of executive function but not in other cognitive tests or biomarkers. Again, a multitude of test was performed without correction for multiple testing (105).

Although the majority of data on metformin use in dementia with or without T2DM is generally positive, it should be considered that the effect of metformin likely depends on complex underlying pathological processes and may to some extent be related to an effect on vascular rather than neurodegenerative processes. In some instances, metformin could even exert detrimental effects. Prospective interventional studies have not been able to show convincing evidence of a positive effect of metformin in mild cognitive impairment or mild Alzheimer's dementia but were likely underpowered or of too short duration. More, long-term, controlled metformin studies in large, well-defined dementia cohorts are needed.

PARKINSON'S DISEASE

Background

Parkinson's disease (PD) is a common neurodegenerative disease, affecting over 1% of the population above the age of 60 and around 4% older than 85 (109). PD is characterized by bradykinesia and a combination of rigidity, resting tremor, postural instability, and a large range of non-motor symptoms (110). Like other neurodegenerative diseases, PD is clinically and pathologically heterogeneous, with a large variation in disease onset and progression. Progressive loss of dopamine-containing neurons in the *substantia nigra pars compacta*, located in the mid brain, results in a deficit of dopamine in the striatum (111, 112). Insoluble protein inclusions in neurons, termed Lewy bodies, mainly consisting of aggregated α-synuclein (aSyn) are the main neuropathological hallmark of PD (113). Lewy bodies and protein aggregates are found in multiple brain regions and spread with disease progression (114, 115). The exact biological mechanism leading to aSyn aggregation and neuronal loss remains unknown and currently only the symptoms of PD are treated with dopamine-replacement therapy and in some cases deep brain stimulation.

Approximately 5–15% of PD cases can be attributed to disease-causing genetic variants and around 15% of patients have

TABLE 1 | Studies investigating the effect of metformin on neurodegeneration in rodent models.

Study	Mouse strain	Disease model	G	Starting age*	Met dose + Application	Study/Met duration	*n* per Group	Main findings
PARKINSON'S DISEASE								
(45)	B6, Dat-Cre AMPKb1/2 KO	Day 20 + 22: MPTP: 30 mg/kg	M	8–10 weeks	100 mg/kg/day Drinking water	27 days	$n = 6$–10	Met decreases MPTP-induced loss of TH-positive neurons in ST but not SN and reduces astrogliosis Met induces AMPK phosphorylation in WT animals, although effects on TH-positive neurons are independent of AMPK-KO
(46)	C57BL	Day 1+2: MPTP: 10 mg	M	? 20–25 g	150 mg/kg/day Metal probe	7 days	$n = 5$	Met has no effect on MPTP-induced loss of TH-positive neurons in SN but reduces levels of microglia marker Iba1 Reduces amount of dopamine in the striatum
(47)	C57BL/6N	Day 7: MPTP: 15 mg/kg, 4x	M	8 weeks	200 or 400 mg/kg/day, Drinking water	14 days	$n = 3$ /10	Met reduces MPTP-induced loss of TH-positive neurons in SN Met induces pCreb and PGC1α in SN and ST
(48)	C57BL/6	Day 1–7: MPTP: 30 mg/kg	M	10 weeks 20–25 g	200 mg/kg/day Injection	14 days Met: day 8–14	$n = 6$	Met ameliorates MPTP-induced motoric deficits Met decreases MPTP-induced loss of TH-positive neurons in SN and reduces astrogliosis Met induces AMPK and AKT phosphorylation, reduces levels of phosphorylated mTor and induces BDNF in the SN
(49)	C57BL/6	5 weeks: every 3.5 day; MPTP: 20 mg/kg + 250 mg/kg probenecid	M	10 weeks	5 mg/ml Drinking water	5 weeks Met: day 3–35	$n = 4$–5	Met decreases MPTP-induced loss of TH-positive neurons in SN and reduces levels of inflammatory cytokines
(50)	Swiss Albino Mice	Day 1–5: MPTP: 25 mg/kg + 250 mg/kg probenecid	M	? 22–25 g	500 mg/kg/day Oral gavage	21 days	$n = 12$	Met improves regeneration of MPTP-induced motoric deficits Met decreases MPTP-induced loss of TH-positive neurons in SN and induces BDNF expression
(51)	C57BL/6N	None	F	10 weeks	A: 5 g/kg Diet B: 5 g/l Drinking water	A: 1 month B: 6 months	A: $n = 20$ B: $n = 4$	Met reduces protein levels of phosphorylated α-Synuclein in mouse brains
(52)	C57BL/6J	Day 1: MDMA 20mg/kg	M	3 months	200–400 mg/kg/day i.p. injection	3/8 days Met: 400 mg day 1, 200 mg day 2+3	$n = 7$–12	Met reduces MDMA-induced loss of TH-positive neurons in SN and CPu
ALZHEIMER'S DISEASE								
(37)	P301S tau transgenic C57BL/6	Transgenic, tau mutation	m	4 weeks	2 mg/ml Drinking water	4 months	$n = 12$–15	Met reduces Ser262-tau phosphorylation in CX and Hip but increases number of tau inclusions Met induces AMPK phosphorylation and PP2A protein levels and in CX and HIP

(Continued)

TABLE 1 | Continued

Study	Mouse strain	Disease model	G	Starting age*	Met dose + Application	Study/Met duration	*n* per Group	Main findings
(53)	db/db mice (BKS.Cg-m+/+ Leprdb/J)	Transgenic, leptin receptor mutation	M	6 weeks	200 mg/kg/day Oral gavage	6 weeks	$n = 3–10$	Met decreases ^{125}I-Ab$_{1-40}$ influx and RAGE expression at the BBB in db/db mice Met ameliorates memory impairments in db/db mice
(54)	Wildtype	None	?	?	5 mg/ml Drinking water	16–24 days	$n = 6$	Met reduces Ser202- and Ser262-tau phosphorylation in mouse brains
(55)	db/db mice	Transgenic, leptin receptor mutation	m	7 weeks	200 mg/kg/day i.p. injection	18 weeks	$n = 6–11$	Met has no effect on spatial learning and memory Met reduces total tau protein levels as well as Ser396 phosphorylated tau in Hip and decreases JNK phosphorylation
(56)	C57BL/6J	None		5 weeks	2 mg/ml Drinking water	1 week	$n = 4$	Met increases BACE-1 and APP protein levels and induces AMPK phosphorylation in mouse brains
COGNITION								
(57)	C57BL/6	HFD (60% fat)	M	12 months	1% Diet	6 months	$n = 16$	Met attenuates HFD-induced deficits in motor function and memory
(58)	NIH Swiss mice	HFD (45% fat)	M	6-8 weeks	300 mg/kg BW Drinking water	20 days	$n = 10$	Met does not improve HFD-induced cognitive deficits and has no effect on astrogliosis
(59)	Wistar rats	HFD (45% fat)	M	? 125–150 g	144 mg/kg Diet	10 weeks	$n = 16–24$	Met has no effect on HFD-induces deficits in Matching To Position Test
(60)	Wistar rats	HFD (59.28% fat)	M	6 weeks HFD 13 weeks Met	15 mg/kg 2x/day Gavage feeding	9 weeks HFD 3 weeks Met	$n = 8$	Met reduces HFD-induced memory deficits Met reduces HFD-induced mitochondrial dysfunction
(61)	C57BL/6J	None	M	4/11/22 months	2 mg/ml Drinking water	3 months	$n = 16–18$	Met has no effect or even impairs spatial memory
HUNTINGTON'S DISEASE AND AMYOTROPHIC LATERAL SCLEROSIS								
(62)	R6/2- B6CBAF1/J	Transgenic, huntingtin mutation (136-151 CAG repeats)	M/F	5 weeks	2 or 5 mg/ml Drinking water	Around 10 weeks (until death)	$n = 5–9$	Met (2 mg) increases survival time in male mice but has no effect on female mice
(63)	B6SJL-TgNSOD1^{G93A}	Transgenic, SOD1 Mutation (G93A)	M/F	5 weeks	0.5 or 2 or 5 mg/ml Drinking water	Around 16 weeks (until death)	$n = 6–15$	Met has negative effect on start of neurological symptoms and disease progression in female mice and has no effect in males

*BBB, Blood Brain Barrier; BW, Body weight; CPu, Caudate Putamen; CX, Cortex; HFD, High-fat diet; Hip, Hippocampus; KO, Knock-Out; MDMA, 3,4-Methylendioxy-N-methylamphetamine; Met, Metformin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SN, Substantia Nigra; ST, Striatum; TH, Tyrosine Hydroxylase. *If the original article did not contain information about age, body weight was indicated.*

TABLE 2 | Studies evaluating the effect of metformin on incidence and progression of neurodegenerative diseases.

Study	Disease	Characteristics	Result
(92)	PD	Retrospective cohort study, 800,000 individuals of whom 61,166 were diabetics, among the latter 41,003 received OAA therapy	Higher PD incidence for patients with T2DM without (HR 2.18) and with (HR 1.30) OAA compared to controls. HR for treatment with metformin alone was lower (0.95) than for sulfonylurea alone (1.57) and the combination showed the lowest HR (0.78)
(93)	PD	Population-based retrospective cohort study with 93,349 T2DM patients receiving metformin (FU of 657,537 patient years) and 8,346 T2DM patients receiving glitazones with or without metformin (FU of 69,338 patient years)	Incidence of PD significantly lower in T2DM receiving glitazones compared to those receiving metformin (HR 0.72), no incident PD in long-term glitazone users who were still taking glitazones
(94)	PD	Population-based retrospective cohort study with 41,362 patients receiving metformin alone, 316,210 patients receiving simvastatin alone, and 52,311 receiving both, metformin and simvastatin	Lower incidence of PD for patients receiving simvastatin alone (HR 0,64) or in combination with metformin (HR 0.74) compared to metformin alone
(95)	PD/Dementia	Retrospective cohort study, 4,651 patients with T2DM with metformin treatment, 4,651 patients with T2DM with metformin treatment; >21,000 person-years of FU	Higher incidence density for PD (HR 2.27), AD (2.13), and VD (2.30) in the metformin group compared to those in the non-metformin group
(96)	Dementia	Retrospective cohort study, 127,209 dementia-free individuals aged ≥50 years, of which 25,939 w/T2DM, 1,864 w/Metformin only, 9,257 w/Sulfonylureas + Metformin	Higher incidence of dementia in T2DM than controls, higher incidence in T2DM wo/ OAA compared to sulfonylurea (HR 0.85), metformin (HR 0.76), or a combination of metformin and sulfonylurea (HR 0.65)
(97)	Dementia	67,731 non-demented, nondiabetic individuals aged ≥65 years observed for 5 years and observation of onset of T2DM, antidiabetic medication and dementia	Increased risk of dementia onset for new-onset T2DM compared to non-T2DM (HR 1.56), risk to develop dementia was higher for thiazolidinedione users than for sulfonylurea and metformin
(98)	Dementia	189,858 individuals with 122,036 receiving metformin and 67,822 not receiving metformin, dementia incidence rate per 1,000 person-years	Patients with diabetes taking metformin had significantly lower dementia incidence rates than those not taking metformin (21.79 vs. 31.58 per 1,000 person-years, $p < 0.001$)
(99)	Dementia	Meta-analysis including 544,093 participants, risk of dementia in patients with T2DM taking insulin sensitizers	Incidence of dementia reduced with metformin (RR 0.79) compared to those not taking insulin sensitizer but not significant ($p = 0.064$)
(100)	Dementia	Latent class analysis to identify subgroups with differential effect of metformin on risk of age related comorbidities in 41,204 men with T2DM with 8,393 metformin users,	Identified 4 latent classes of patients who showed different effects of metformin on risk to develop ARC including dementia
(101)	Dementia	Retrospective cohort study, 17,200 new metformin users vs. 11,440 new sulfonylurea users aged ≥65 years, average FU 5 years	Individuals <75 years of age on metformin had a lower risk to develop dementia than those on sulfonylurea (HR 0.67, 95% CI 0.61–0.73)
(102)	Cognitive impairment	Longitudinal population-based study, 365 persons aged ≥55 years with T2DM of which 204 received metformin	Metformin use inversely associated with cognitive impairment (OR 0.49), longer use associated with lower risk of cognitive impairment
(103)	AD	Retrospective case-control study, 7,086 AD patients and controls were compared for previous use of metformin/other antidiabetic drugs	Higher risk to develop AD for longterm users of metformin (AOR 1.71) but not sulfonylurea (AOR 1.01), thiazolidinediones (AOR 0.87), or insulin (AOR 1.01) compared to non-users
(104)	AD	71,433 patients newly diagnosed with diabetes and 71,311 nondiabetic controls, follow up of up to 11 years	Higher incidence of AD in diabetic patients compared to non-diabetics (0.48 vs. 0.38%), no positive effect of anti-hyperglycemic treatment on risk
(105)	AD	Randomized placebo-controlled crossover study, 20 nondiabetic patients with MCI or mild dementia and AD received mg metformin or placebo for 8 weeks and then switched to the other treatment for 8 weeks	Metformin was measurable in CSF, in pooled post-hoc analysis significant increase in superior and middle orbitofrontal CBF after 8 weeks metformin exposure in ASL-MRI, significant improvement in Trail making test part B, a measure of executive function
(106)	HD	Observational study; 4325 HD patients, of which 121 had T2DM and received metformin	HD patients on metformin fared better in test for verbal and executive function but not in motor assessments

AD, Alzheimer's disease; AOR, adjusted Odds Ratio; ARC, age related comorbidities; ASL-MRI, Arterial Spin Label Magnetic Resonance Imaging; CBF, Cerebral Blood Flow; FU, Follow-up; HD, Huntington's disease; HR, Hazard ratio; MCI, Mild cognitive impairment; OAA, Oral anti-hyperglycemic agents; PD, Parkinson's disease; T2DM, Type 2 Diabetes; VD, Vascular dementia.

a first degree relative who is also affected (116). The genetic architecture of PD has been well studied but it is complex. 23 loci and 19 genes have so far been associated with familial forms of PD (117). Like in most neurodegenerative diseases, the majority of cases probably result from a complex interplay of risk modifying genetic variation, environmental factors and chance.

Knowledge about the genes involved in PD have allowed insight into the underlying biological pathways. Together with multiple environmental factors and epidemiological data, the genetic data has highlighted several cellular functions and pathways including mitochondrial dysfunction, lysosomal function, inflammation, build-up of aggregation-prone proteins and oxidative stress (118, 119).

Despite large investments in research for neuroprotective compounds for PD, none have so far shown any convincing effects in clinical trials (120).

Diabetes and PD: Animal Studies

Rodent studies have shown that there is a link between insulin resistance and development of PD. A high fat diet enhanced vulnerability to the neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) as measured by increased nigrostriatal neurodegeneration and motor deficits (121, 122). Likewise, in an aSyn mouse model of PD a high fat diet led to an accelerated development of locomotor phenotype and earlier onset of neurodegeneration (123).

Insulin resistance can directly interfere with dopamine signaling. Rats fed a high fat diet exhibit impaired nigrostriatal dopamine function (124) and overweight and diabetic mice show degeneration of dopaminergic neurons (125).

Metformin and PD: Animal Studies

Only a handful of rodent studies have so far assessed the effects of metformin as a neuroprotective agent in PD. These studies have focused mainly on metformin treatment in combination with acute MPTP induced parkinsonism. Although experimental designs in these studies are quite similar, the results are variable, arguing against differences in modeling as the major cause of metformin's variable effects. However, differences in the dose and duration of MPTP and metformin treatments may be important (**Table 1**).

Most studies in rodents find that metformin reduces the damaging effect of MPTP on dopaminergic neurons, shown by tyrosine hydroxylase staining (a marker for dopaminergic neurons) in the *substantia nigra pars compacta* (49, 50), striatum (45), or both (48). Two studies suggest that metformin's protective effect may not be specific. A study by Ismaiel and colleagues however, reported that metformin had no protective effect against MPTP-induced neuronal loss in the SN (46) and Bayliss reported no protective effect on dopaminergic neurons in the SN, only in striatum (45).

Metformin's supposed ability to protect against dopaminergic neuronal death induced by the neurotoxin MPTP correlates in three studies to improvements in the motor function of rodents (48–50). Given that both, MPTP and metformin act on complex 1 of the respiratory chain, a mutual influence of the drugs on mitochondrial survival cannot be excluded. It is possible that in these studies metformin primarily reduced the damaging effects of MPTP itself rather than restoring damaged neurons. Therefore, examination of metformin's action in transgenic mouse models rather than acute toxin models of PD might give better insight about its potential. An interesting first hint comes from a study using healthy non-transgenic mice that showed that metformin could reduce aSyn phosphorylation in the brain (51).

Diabetes and PD: Human Studies

Studies assessing the risk of developing PD in patients with diabetes have very mixed outcomes (126–132). In one meta-analysis comprising 14 case-control studies, PD risk was decreased in T2DM patients (133). Conversely, Cereda and colleagues describe an increased risk for developing PD in diabetics in four prospective cohort studies but not a higher prevalence of diabetes in in patients with PD in five case-control trials (134). It has to be noted that the case-control trials with the largest populations did consistently show a similar of even higher prevalence of diabetes in PD compared to controls. More recently a meta-analysis including seven population-based cohort studies which also found an increased PD risk in patients with diabetes (135). Taken together the meta-analyses seem to hint toward an increased incidence of PD in T2DM. A potential pitfall is the inclusion of vascular PD in some of the studies. T2DM does contribute to cerebral small vessel disease and therefore non-exclusion of patients with vascular lesions may skew the results toward more patients with T2DM exhibiting signs of parkinsonism. This particular problem was addressed in some studies showing an increased incidence of PD in T2DM and therefore cannot sufficiently explain the discrepancies. From a neuropathological view one study describes an association between increased blood glucose levels with increased risk of Lewy body formation in the *substantia nigra pars compacta* and *locus coeruleus* further supporting a role of T2DM in the pathogenesis of PD (136).

Dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD) are common causes of dementia in the elderly (137). PD patients with T2DM are reported to have a greater rate of cognitive decline and lower gray matter and white matter volume, although the group was small (138). PDD patients are more likely to show insulin resistance in an oral glucose-tolerance-test than PD patients without dementia (139). DLB and PDD were less common in patients with diabetes in one study using data from the Swedish Dementia Registry (140), yet T2DM was not significantly associated with PDD in many others (141–144).

Metformin in PD: Human Studies

Clinical studies have not looked solely at metformin, but rather metformin compared to, or in combination with other oral anti-hyperglycemic agents (see **Table 2**). Taken together all the studies look at different medications and are hardly comparable. There is lack of clinical data that suggests a positive effect of metformin on PD risk. Wahlqvist and colleagues tried to determine the effect of sulfonylurea, metformin or a combination of both drugs on the incidence of PD in patients with T2DM. Patients with T2DM receiving sulfonylurea had an increased PD risk compared to those not receiving oral anti-hyperglycemic agents. Metformin alone or in combination with sulfonylurea had no impact, suggesting that metformin might rescue the harmful effect of sulfonylurea (92).

Brakedal and colleagues compared the incidence of PD in patients with T2DM from the Norwegian Prescription Database (NorPD) receiving glitazones with or without metformin or metformin alone. Patients taking glitazones had a significantly lower incidence of PD compared to patients on metformin alone. There was no risk reduction in past users of glitazones, indicating the necessity of long-term or even permanent exposure to glitazones for risk reduction (93). Looking at patients from the NorPD receiving statins, metformin or both showed a lower hazard ratio to develop PD for patients using statins in combination with metformin when compared to metformin alone and the risk was lowest in patients only taking statins (93). The positive effect of statins may come through their anti-inflammatory effect and a reduction of microglial inflammatory response, which has been shown to have a positive effect on striatal dopamine activity (145). The question why metformin seems to have a positive effect when added to sulfonylurea while it has a negative effect when taken together with statins must be addressed. The combination of T2DM and hypercholesterolemia might increase the risk of developing PD more than hypercholesterinemia alone and this risk may not be lowered sufficiently by a combination of statins and metformin. The addition of metformin to sulfonylurea may result in a better control of T2DM than the therapy with sulfonylurea alone thereby reducing effects that promote PD risk. Also, the complex interplay between the different drugs has to be taken into account.

To our knowledge there is no data available on metformin use and disease progression. It is also unclear whether metformin use in individuals without insulin resistance may have a beneficial effect on PD development.

OTHER NEURODEGENERATIVE DISEASES

There are to our knowledge very few or no reports of metformin studies in other, rarer neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Huntington disease (HD), motor neuron disease, or atypical parkinsonian disorders. Here we briefly note relevant studies concerning association with diabetes or use of drugs targeting energy metabolism.

Amyotrophic Lateral Sclerosis

ALS is a progressive neurodegenerative disease that is characterized by degeneration of the first and second motor neuron resulting in spasticity and muscle atrophy. Eventually this results in difficulty speaking, swallowing, and breathing and often leads to death within a few years after diagnosis. Neurochemical imbalance and genetic mutations are known to cause ALS, but most cases are sporadic and old-age is an important risk factor. Most drugs available for ALS relieve symptoms only, although the drug riluzole and more recently edaravone have been shown to slow progression of the disease (146, 147).

A protective effect of diabetes in older patients and an increased risk of developing ALS in younger patients with diabetes has been described which is thought to reflect differences in association of ALS with T1DM and T2DM (148, 149). Most studies have shown a decreased risk for developing ALS in patients with T2DM (150, 151). However, other studies reported no significant effect on ALS risk or progression and even a higher risk of developing ALS in T2DM in patients below 65 years of age (152–154). Nutritional status is negatively associated with ALS severity (155) and hypercaloric nutrition has even been suggested as a potential treatment option for ALS. Two trials with the PPAR-γ agonist Pioglitazone (which reduces insulin resistance) (156, 157) have not shown any benefit in disease progression (158).

Huntington Disease

HD is a progressive neurodegenerative disease that causes choreatic movements, psychiatric symptoms, and cognitive decline. The most common form of the disease is of early onset, usually diagnosed around 30–40 years of age. HD is caused by defects in the gene *HTT*, which encodes the protein huntingtin and the mode of inheritance is autosomal dominant. Expansion of CAG repeats in the *HTT* gene leads to the production of an abnormally long version of the huntingtin protein. This results in the protein being broken down by the cell into small, toxic fragments and these protein fragments aggregate and accumulate in neurons causing the disease.

Altered glucose metabolism and increased rates of T2DM have been reported in patients with HD (159, 160) and a high prevalence of T2DM has been reported in a Chinese family with HD (161). However, other studies were not able to identify differences in oral glucose tolerance test or pancreatic tissue between HD patients and controls (162, 163). HD patients with T2DM receiving metformin had better cognitive test results than HD patients without diabetes not taking metformin. This was in stark contrast to the non-HD control group where people with T2DM taking metformin fared worse in the cognitive test compared to non-diabetic controls (106).

METFORMIN: MECHANISM OF ACTION IN NEURODEGENERATIVE DISEASES

The *in vivo* studies conducted so far, regarding the effect of metformin have generated conflicting results. Besides the large differences in study design, these outcomes are probably also due to the many biological pathways influenced by metformin. Here we will discuss some of the biological signaling pathways and biological mechanisms that are the most relevant for metformin's potential as a therapy in neurodegenerative disease (**Figure 2**).

Central Metabolism and Signaling

Central metabolism is tied to the overarching cell signaling pathways involved in proliferation, stress and survival, which are heavily implicated in human diseases including cancer and neurodegeneration. Metformin acts on central metabolism and several major signaling pathways including energy sensing (glucose metabolism and AMPK signaling), mTOR signaling, and inflammatory signaling. Mitochondrial signaling will be addressed separately.

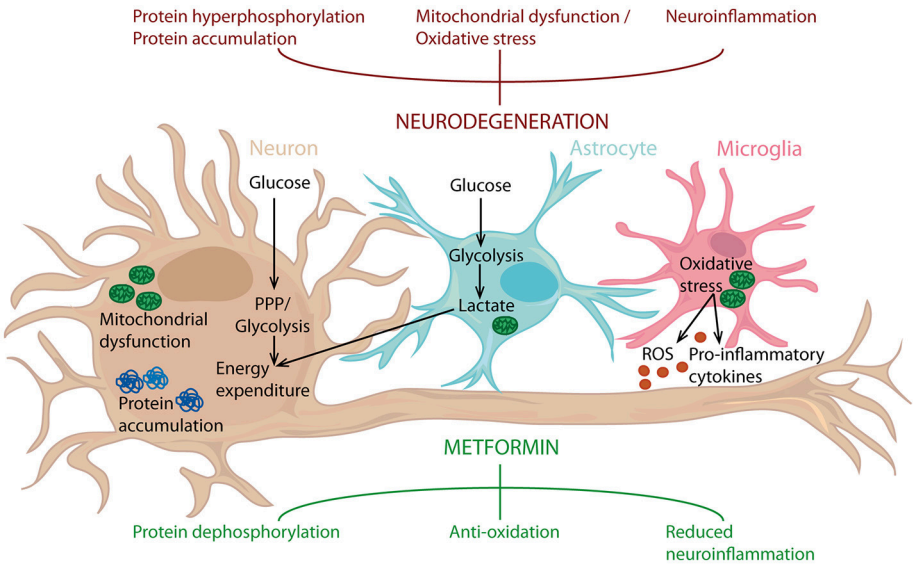

FIGURE 2 | Metformin's potential as a neuroprotective agent. Metformin can counteract protein hyperphosphorylation, oxidative stress and neuroinflammation, processes known to drive neurodegeneration. Metformin can act on neurons, but also targets astrocytes and microglia. Consequently, metformin can influence inflammatory status, along with glucose metabolism in the entire brain and thereby reduce neuroinflammation and act as an antioxidant, leading to protein dephosphorylation. PPP, Pentose phosphate pathway.

Energy Sensing and Metabolism

The brain constitutes only 2% of the total body mass, but it is one of the main energy-demanding organs in the human body utilizing around 20% of total energy expenditure. Brain cells incorporate (i) the neurons (70–80% of brain energy expenditure) and (ii) glial cells, comprising oligodendrocytes, astrocytes and microglia (accounting for the remaining 20–30% of energy expenditure). The high energy demand of neurons is one of several factors partially explaining the selective vulnerability of certain neuronal subtypes in neurodegenerative diseases. Energy metabolism has long since been thought to play a role in the etiology of neurodegenerative diseases and here we will briefly mention some of the related signaling pathways and biological mechanisms that are relevant for metformin's therapeutic potential in neurodegeneration.

AMPK signaling

AMPK is an evolutionarily conserved sensor of cellular energy status. AMPK is activated by increasing AMP levels in conditions of energy deprivation and the enzyme consequently inhibits energy consumption and stimulates catabolic pathways. Activation of AMPK has a wide range of effects, including inhibition of mTor and PI3K-Akt signaling (two important pathways discussed later).

Dysregulation of AMPK is associated with insulin resistance and T2DM (164, 165) and neuroinflammation (166–168). AMPK signaling plays a major role in AD disease progression since AMPK has been shown to regulate both Aβ generation and tau phosphorylation. Inhibition of Aβ production and tau phosphorylation in neuronal cultures is dependent on AMPK activation (169) and activation of AMPK lowers extracellular Aβ accumulation (170). Conversely, in neurons, AMPK activation has been linked to tau phosphorylation as a response to Aβ toxicity (171, 172).

Metformin inhibits complex I of the electron transport chain needed for mitochondrial respiration, thereby leading to an energy deficit and indirectly activating the AMPK pathway (173–175). Thus, stimulation of AMPK can be seen as a key consequence of metformin administration, explaining many of the known effects of the drug (**Figure 3**).

However, in the context of AD especially, more studies are needed to understand the complex role of AMPK signaling and the action of metformin. A study conducted in human neuronal stem cells proposed that activation of AMPK via metformin is neuroprotective against Aβ (176) and other *in vitro* studies showed that metformin is able to reduce tau phosphorylation via mTOR/PP2A (Protein phosphates 2A) signaling (54) and that it can reduce molecular pathologies associated with AD (177). An additional level of modulation via AMPK by metformin could come from metformin's ability to reduce BACE1 protein levels in neurons (178). Conversely, metformin was also reported to upregulate BACE1 in neurons and increase the generation of Aβ (179), suggesting detrimental effects of activating AMPK in diseased neurons.

In PD mouse models the AMPK involvement is similarly multifarious. Administration of the neurotoxin MPTP activates AMPK signaling (180). Interestingly, both AMPK overexpression and AMPK inhibition have promoted survival in neurotoxin treated PD models (180) but another study provided evidence for a protective function of AMPK activation in *in vivo* PD models (181).

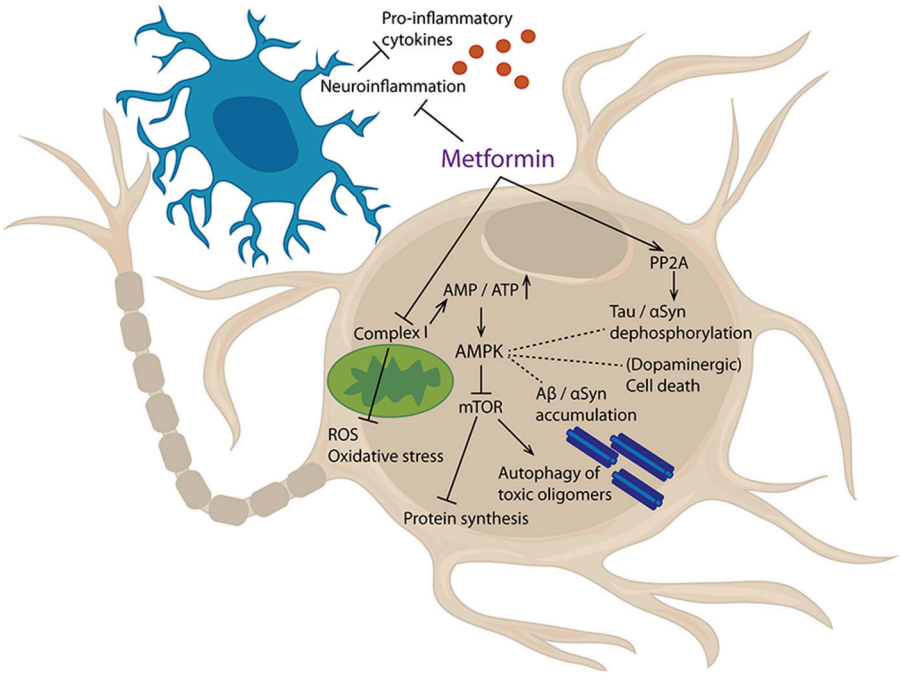

FIGURE 3 | Cellular targets of metformin. Metformin inhibits mitochondrial complex I, thereby increasing AMP/ATP ratio. This lack of energy leads to an activation of AMPK, which, amongst others, inhibits mTor signaling. Furthermore, metformin can activate PP2A and inhibit neuroinflammatory processes. Results of these events are reduced production of pro-inflammatory cytokines and reactive oxygen species (ROS), decreased oxidative stress, inhibition of protein synthesis and augmented autophagy of toxic oligomers. Additionally, protein dephosphorylation, protein aggregation, and cell death are affected.

Overexpression of aSyn in cell culture reduced AMPK activity, while inhibition of AMPK lowered resistance to aSyn toxicity (182). AMPK's subunits α1 and α2 have neuroprotective effects against aSyn toxicity with low but continuous AMPK activity almost completely preventing loss of dopaminergic neurons (183). Accordingly, in rodent PD models dietary metformin influenced neuronal function via AMPK modulated aSyn phosphorylation status (49, 51). However, several other studies point in a different direction. Over-active AMPK promotes aSyn accumulation (184) and hyperactivation of AMPK leads to aSyn binding to the GTPase PIKE-L and dopaminergic cell death (47). These studies show that lower AMPK activity may in fact be beneficial at least in aSyn models of PD.

As is the case in many neurodegenerative diseases, the underlying genetic and biological causes are heterogeneous, often causing multiple pathologies that can overlap across the disease spectrum. The action of metformin primarily via the mitochondria could have numerous and potentially opposite effects on AMPK depending on the amount of involvement and type of mitochondrial signaling in each patient or disease model at any given moment. One important aspect to consider here is that biological pathways are not necessarily fixed in a single state throughout the disease course. Neurons especially have evolved to carefully adapt to energetic needs in order to survive since they are seldom replaced. Sophisticated compensatory mechanisms are initiated for the purpose of mitochondrial rejuvenation and adaption. Such complexity has made modeling neurodegenerative diseases in human neurons challenging and has contributed to the current situation where no causative or "cure all" therapies are available.

Glucose metabolism

Glucose is an essential energy substrate needed to sustain neuronal activity and is taken up via glucose transporters expressed in the brain endothelium, astrocytes, and neurons (185). Neurons mostly rely on glucose for energy but utilize ketone bodies during fasting. In contrast to other cell types, in neurons the rate limiting glycolytic enzyme Phosphofructokinase B3 is highly turned over by the proteasome, resulting in the preferential metabolism of glucose via the pentose phosphate pathway (PPP) as opposed to glycolysis (119, 186).

A product of the PPP is the electron donor NADPH, which provides reducing power for anabolic reactions and is crucial for maintaining antioxidant potential. The PPP helps neurons to meet high energy demands, but since neurons are predominantly oxidative, maintaining a fine balance between glycolysis and PPP is essential for counteracting oxidative damage and conserving energy.

Glial cells on the other hand, predominantly metabolize glucose via glycolysis producing lactate and have only very low rates of mitochondrial oxidation. Glia metabolically support axons and lactate can be shuttled across a gradient from glia to neurons (**Figure 2**) (187, 188). Interestingly, in cell culture, neurons favor lactate over glucose (189) preferring a fast supply

of energy over metabolic efficiency. In the human brain, energy demand must be tightly regulated to offset oxidative damage and therefore cell culture and cell culture media effects should be taken into consideration when considering the conflicting data on metformin performed *in situ*.

Inhibiting the PPP and glutathione pathways causes increased levels of oxidative stress and cell death similar to that seen during neurodegeneration (119). Glucose hypometabolism has been shown in PD brains (190) and deregulation of glucose metabolism has been proposed as an early event in the pathogenesis of PD (119). Dunn et al. proposed that dysregulation of glucose metabolism occurs via dysregulation of the PPP, which causes oxidative stress because of less efficient glutathione recycling, and it is this event that underlies the increased levels of oxidative stress observed in PD (119).

Metformin can act in these pathways by slowing oxidative phosphorylation via inhibition of complex I in mitochondria and by inhibiting gluconeogenesis, having the effect of further aiding neurons to reduce their oxidative burden by minimalizing NADH utilization.

Insulin signaling

Insulin plays an important role in the brain. It is used as a hormonal signal to control body weight, food uptake, and metabolic homeostasis (191–193). Insulin has also been shown to influence expression of dopamine receptors and concentration of dopamine (194–196). Disturbances in insulin signaling have been implicated in several neurodegenerative diseases including AD, PD, and HD (197–200). Insulin is secreted in response to high blood sugar and acts in different organs including the brain. Activation of the Phosphoinositide-3-kinase (PI3K)—Akt pathway via insulin receptor activation and insulin receptor substrates plays a central role in the metabolic actions of insulin (201). Akt activation regulates proteins such as mTOR, FOXO, and BAD. Overall, Akt has over 100 known substrates and has diverse effects on cellular growth, cell proliferation, glucose uptake, protein synthesis, glycogen synthesis, and apoptosis (202). Akt is inhibited by PP2A (203), PHLPP1/2 (204), and indirectly by PTEN (205). Insulin resistance has been associated with disturbances in signaling up and downstream of Akt (206–208).

Insulin has been administered to patients to try to improve symptoms of neurodegeneration (209, 210) and has been shown to protect cells from Aβ induced death (211–213). The Insulin Degrading Enzyme (IDE), originally found to play an important role in insulin turnover (214) is involved in Aβ degradation. IDE can degrade secreted Aβ from neurons and microglia and mediate its clearance (215). Furthermore, IDE hypofunction can contribute to *in vivo* Aβ accumulation (216). In hippocampi of ApoE4 carriers reduced expression levels of IDE have been measured (217) and genetic differences in IDE expression and activity have been suggested to be involved in AD development (218–221). Reduced levels of IDE in liver and brain have been correlated with aging (222) and IDE can counteract damage from oxidative stress, suggesting a neuroprotective role (223–226).

Metformin lowers blood glucose levels through inhibition gluconeogenesis in the liver via AMPK (227, 228). AMPK inhibits PI3K/Akt signaling, the crucial pathway downstream of the insulin and IGF1 receptors (229). Metformin has also been shown to act on insulin signaling independently of AMPK. Metformin is reported to downregulate expression of insulin and IGF-1 receptors (230, 231) and reduces phosphorylation of insulin receptors (232) including IRS-1 (230, 233).

Both acute and chronic metformin administration has been found to increase levels of GLP-1, an incretin known to induce insulin secretion, in humans and mice (234–236). Very recently a randomized, double-blind, placebo-controlled trial for PD showed that a GLP-1 agonist had positive effects on motor symptoms in PD (237), generating a new potential mechanism for metformin action in neurodegeneration.

mTOR Signaling

mTOR signaling is a highly conserved and central signaling pathway integrating upstream signals such as nutrient and redox status and then controlling downstream processes such as cellular growth, motility, survival, and death (238). The mTor pathway is crucial for regulating mitochondrial biogenesis and autophagy, two processes that are defective in many neurodegenerative diseases.

mTOR is a serine/threonine protein kinase, composed of the protein complexes mTORC1 and mTORC2. mTOR signaling is targeted by the PI3K/Akt pathway, the key insulin signaling pathway (239, 240). Both PTEN (241, 242) and AMPK (243, 244) suppress mTor signaling and rapamycin is a well-studied inhibitor of mTORC1 (245–247). Although mTor signaling influences many downstream events, the most important mechanism of action is through the phosphorylation and activation of S6K1 and 4E-BP1 and subsequent control of RNA translation (238) (**Figure 4**). Interestingly, deficiency in mTor signaling has been implicated with insulin resistance and diabetes. Nutrient dependent stimulation of S6K1 can induce insulin resistance (248, 249) and S6K1 deficiency protects against high fat diet-induced insulin resistance (250).

The mTOR inhibitor rapamycin suppresses neurodegeneration phenotypes in mice (251) and protects against MPTP-induced loss of dopaminergic neurons (252). Rapamycin also prevents the development of dyskinesia without affecting the therapeutic efficacy of L-DOPA and thus, the mTORC1 signaling cascade represents a promising target for the design of anti-Parkinsonian therapies (253).

Elevated mTOR signaling has been found in AD patients and is linked to diabetes and aging (254, 255). Rapamycin abolishes cognitive deficits and reduces Aβ levels in a mouse model of AD (256). It also ameliorates AD-related phenotypes by restoring hippocampal gene expression signatures (257). Importantly, mTor regulates tau phosphorylation and degradation (258), making this pathway an interesting target for the treatment of tauopathies.

If we compare the therapeutic potential of metformin, a well-known inhibitor of mTOR signaling through activation of AMPK (259) to that of rapamycin, which is more widely accepted in the field, the obvious difference is that metformin action on mTOR is

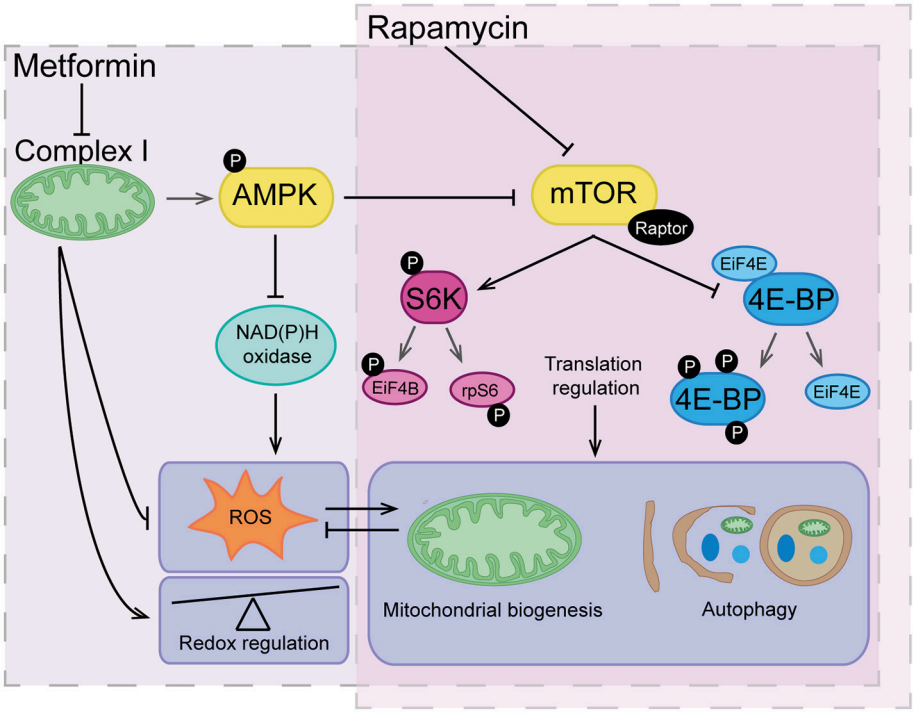

FIGURE 4 | The overlapping actions of metformin and rapamycin. Rapamycin acts by directly inhibiting mTOR and therefore translation regulation, which has a major influence of highly regulated processes such as mitochondrial biogenesis and autophagy. Metformin acts indirectly on the mTOR pathway through inhibition of complex I and activation of AMPK signaling. Metformin also reduces reactive oxygen species (ROS) via inhibitory action on complex I and NAD(P)H oxidase having an overall effect as a redox regulator. Downstream of metformin action, low level ROS can indirectly trigger signals for mitochondrial biogenesis and turnover of organelles and proteins via autophagy. Vice versa, maintenance of healthy mitochondrial networks involving autophagy and mitochondrial biogenesis further reduces build-up of damaging levels of ROS.

relatively indirect. Rapamycin forms a complex with the FKBP12 binding protein which binds and specifically alters mTORC1. Metformin acts on the mTOR pathway indirectly via multiple routes. The AMPK independent routes include inhibition of transcription factors (260), the PI3K/AKT pathway (261), and induction of REDD (262). In direct contrast to this, one study has shown that metformin can directly inhibit mTORC1 and is dependent on Rag GTPases not AMPK (263). These data support the view that metformin has more than one direct target and is likely to have many more indirect targets, thus explaining why the use of metformin and a research tool or treatment is less accepted than rapamycin.

Nevertheless, the mTOR pathway links several biological pathways underlying neurodegenerative diseases and therefore the ability of metformin to inhibit this signaling cascade endorses the argument that more mechanistic work using metformin and its inclusion in clinical trials should be positively considered.

Inflammation

Neuroinflammation is considered a major driving force in the progression of neurodegenerative diseases and the triggering of innate immune mechanisms is emerging as a crucial component in disease pathogenesis. Microglia and other cell types in the brain can be activated in response to misfolded proteins or aberrantly localized nucleic acids. This diverts microglia from their physiological and beneficial functions, and leads to their sustained release of pro-inflammatory mediators (264).

Intake of non-steroidal anti-inflammatory drugs (NSAIDs) has been reported to decrease incidence of AD later in life (265, 266) and activated microglia are found in brains of AD patients (267, 268).

In AD, an integrated network-based approach identified gene perturbations associated with innate immune pathways and microglia cells in late onset forms of the disease (269). AD patients show increased expression of inducible nitric oxide synthase (iNOS, a product of neuroinflammation) in neurons and glia, leading to augmented nitric oxide production (270, 271). Activated microglia can further induce tau phosphorylation in primary mouse neurons, activating IL1β receptor and p38 MAPK stress signaling (272).

In PD, patients show increased numbers of activated microglia and astrocytes (273, 274) and microglia activation has been associated with disease progression (273, 275, 276). aSyn has been found to activate microglia, enhancing neurotoxicity (277). Activation of microglia increases nitration of aSyn, resulting in neuronal cell death (278).

Immune signaling triggers transcriptional events, but also changes in metabolic flux, redox balance, and metabolite balance

via mitochondria (279). Mitochondrial dysfunction is associated with neuroinflammation (280) and even moderate mitochondrial DNA stress can trigger antiviral signaling (281).

Metformin reduces general inflammation parameters and inhibits NF-κB signaling as well as proinflammatory cytokines in different cell types (282–285), suggesting that metformin could protect against neuroinflammation. Interestingly, in two MPTP-induced PD mouse models, metformin reduced levels of the microglia marker Iba1 as well as the pro-inflammatory cytokines TNFα, Il-1β, IL-6, and iNOS in the *substantia nigra pars compacta* (46, 49). Here, more studies are needed but metformin seems to have a wholly positive effect against general inflammation. Neuroinflammation is a recognized event associated with neurodegenerative diseases and therefore metformin could be both a useful tool and therapy.

Mitochondria

Mitochondria are crucial organelles that produce energy and perform a plethora of other functions needed for central metabolism and cell signaling. Mitochondrial dysfunction is a phenomenon that traverses all neurodegenerative diseases and forms the basis of β-cell dysfunction in T2DM (286). One important aspect of mitochondrial dysfunction in neurological disease is that the need for tightly controlled energy metabolism in neurons can partially explain some of the vulnerabilities involved in their demise.

Parkinson's Disease

In PD, the link between mitochondrial dysfunction and disease has been proven by the identification of environmental factors and disease genes which critically affect mitochondria. The outcome has been a large body of work depicting the role of mitochondrial dysfunction in PD, yet the exact mechanisms underlying sporadic forms of PD are less defined.

Loss of function mutations in PINK1 or parkin cause PD (287–290) as a result of mitochondrial dysfunction and this has been elucidated *in vitro* (291, 292) and *in vivo* (293–296). PINK1 and parkin act in a pathway that is important for mitophagy (removal of damaged mitochondria via the lysosome) induced by mitochondrial depolarization. Here, PINK1 functions upstream of parkin (295, 297). Upon mitochondrial damage, PINK1 accumulates on the mitochondrial surface and selectively recruits parkin to mitochondria (298, 299). Mitochondrial substrates are ubiquitinylated, leading to the removal of damaged mitochondria. PINK1 is now known to be a ubiquitin kinase (300) but may have other functions yet unknown. For example, PINK1 is not required for basal mitophagy *in vivo* (301, 302) and has been proposed to regulate complex I (303), mitochondrial dynamics (304), mitochondrial proteostasis (305), and mitochondrial metabolism via TRAP1 (306, 307).

PINK1 and parkin are upregulated under metabolic stress in the vessel walls of obese and diabetic mice and have a protective action by limiting reactive oxygen species (ROS) production and mitochondrial dysfunction (308). In a diabetic mouse model, PINK1 expression in the hippocampus was in this case reduced following hydrogen peroxide treatment (309), further suggesting that PINK1 plays a role as a stress sensor and functions accordingly in diverse ways. PINK1 is generally associated with neuroprotection since loss of function causes PD, but because PINK1 is normally highly turned over at the mitochondrial outer membrane and therefore overexpression and/or altered expression might also induce unwanted downstream events. In one study, PINK1 overexpression restrained MAPK and ROS signaling and mitigated insulin resistance in cell models (310). Conversely, PINK1 loss corrupts function of islet and β-cells causing impaired glucose uptake and increased levels of plasma insulin (311). Further evidence that PD proteins play important roles in energy metabolism is a study showing that TP53INP1 deficient cells (TP53INP1 is a susceptibility locus in T2DM) causes an increase in ROS that impairs mitophagy via the PINK1-parkin pathway (312).

Parkinson's disease mutations in aSyn are associated with several cellular defects, including reduced mitochondrial integrity and function. Recent work has identified a highly neurotoxic aSyn species which induces mitochondrial damage and mitophagy in the human and animal brain (313). However, the consequences of these mitochondrial changes for bioenergetic functions remains somewhat undefined. Interestingly, aSyn toxicity is mitigated by TRAP1 (314), a mitochondrial ATPase that has been linked to metformin.

In this pathway, TRAP1 and the mitochondrial serine protease HtrA2 are both targets of the PD protein PINK1 (305, 306). HtrA2 and TRAP1 genetic variants have been found in PD patients (307, 315) but the mutations are rare and a controversial topic (316–318). Regardless of the genetic contribution to disease, TRAP1 at least appears to play an important regulatory role in mitochondria that is relevant for the fine tuning of energy metabolism. TRAP1 is well studied in cancer since TRAP1 expression is tightly regulated in tumor cells (319), TRAP1 acts as a metabolic switch (320) by targeting and inhibiting succinate dehydrogenase (321), which is important for metabolic re-purposing and inflammatory responses (322).

In ovarian cancer where TRAP1 expression was altered, metformin was effective in rendering the tumor sensitive to chemotherapy (323), suggesting that metformin might be relevant to TRAP1 mediated signaling. On this basis, metformin was then used to successfully rescue mitochondrial dysfunction in a TRAP1 cell model of PD (307). In a healthy person, fine tuning of mitochondrial energy usage via the PINK1-HtrA2-TRAP1 pathway and other regulatory mechanisms may allow cells to conserve energy and reduce oxidative burden. Metformin's ability to mimic this fine tuning role *in vitro* was beneficial in one model of sporadic PD (307). However, there are still a lot of questions that remain unanswered such as whether metformin is beneficial in non-diseased neurons, aging neurons and other forms of familial and sporadic PD. One question is whether metformin could specifically target energetic deficits in the dopaminergic neurons of the *substantia nigra pars compacta*. The question is not yet answered because selective vulnerability is still not yet fully understood. We can speculate that oxidative or metabolic burden over time could contribute to making these cells especially vulnerable. Many redox reactions happen in mitochondria as a result of mitochondrial activity. Neurons in comparison to many other cell types have a high

energy demand and because of the autonomous pacemaking in dopaminergic neurons of the *substantia nigra* (324), these cells are thought to have a higher oxidative burden. The metabolism of dopamine itself is highly oxidative and can form several toxic species. Therefore, if metformin can mildly reduce the oxidative burden at the mitochondria without interfering with normal redox signaling and stimulate autophagy and other processes which can become less effective over time, it could be seen as a very useful drug to counteract neurodegenerative diseases. Neurons have a sophisticated and unique line of quality control defenses which allow them to compensate for stress and survive against all odds because once they die, inflammation often ensues and they are seldom replaced. It just depends whether metformin treatment could be used to intervene at the right time to not interfere with necessary compensatory responses, rather enhance them.

Alzheimer's Disease

The exact mitochondrial events leading to AD are less defined than in PD, yet aging is still the greatest known risk factor. Energy metabolism and mitochondrial dysfunction have been proposed as a primary event in mechanisms underlying AD such as synaptic degeneration, Aβ deposition and formation of neurofibrillary tangles (325). There is a vast amount of evidence that mitochondrial dysfunction occurs after the early cellular events in AD and can contribute to the advancement of further degeneration, but it is often unclear whether mitochondrial dysfunction is indeed just a secondary event or whether it might be involved in primary pathogenesis. For example, in the case of tau, abnormal tau triggers oxidative stress and mitochondrial defects such as mitochondrial depolarization, impaired mitochondrial complex activities and reduced energy output (326, 327). Tau also localizes to the microtubules, the tracks on which mitochondria move along with the help of adapter proteins and defective mitochondrial movement has been shown in several models of AD (328, 329).

There is also evidence that mitochondrial metabolism is altered in AD brains (reviewed in (330)). The tricarboxylic acid (TCA) cycle enzymes pyruvate dehydrogenase, isocitrate dehydrogenase, and alpha-ketoglutarate dehydrogenase are affected in AD brain tissue and in patient-derived fibroblasts (331). Changes in these checkpoint TCA cycle enzymes are associated with metabolic re-wiring often in response to stress and redox changes. In addition to matrix enzymes, deficiencies in oxidative phosphorylation (OXPHOS) have been reported [reviewed in (332)].

In AD research, there are few mechanistic models for mitochondrial dysfunction, mainly due to the fact that there are no mitochondrial causative genes for AD. The mitochondrial mechanism of metformin action in dementia and AD is likely similar in PD, in that metformin can act on mitochondrial quality control via mitochondrial biogenesis and energy conservation.

The Complex I Paradox

Many of metformin's actions are thought to be an indirect result of complex I inhibition. The exact inhibitory mechanism of metformin on complex I is not fully understood. The inhibitory mechanisms of other complex I inhibitors such as MPTP and rotenone are better known in terms of binding site and mechanism of toxicity, especially in disease.

Complex I deficiency has long since been associated with mitochondrial dysfunction and Parkinson's disease risk [for a review see (333)]. Complex I deficiencies have also been reported in AD, HD and ALS (332). The neurotoxins MPTP and rotenone inhibit complex I and generate toxic levels of ROS, which leads to neuronal cell death. It is possible that sub-lethal concentrations of mitochondrial inhibitors that do not generate ROS (or generate less ROS) could be beneficial but little is known.

It is generally accepted that metformin does not generate dangerous levels of ROS. Pharmacologically reducing oxidative phosphorylation and thus the oxidative burden (at the right moment) without generating too much ROS is certainly a challenge. We found that sub lethal concentrations of the specific mitochondrial complex V inhibitor oligomycin, could rescue mitochondrial dysfunction in a TRAP1 deficient PD model to a similar extent as metformin (307) but since metformin is an approved compound for human consumption, we followed up the protective effects of metformin only. It might be interesting to assess the potential neuroprotective action and toxicity with a titration of several respiratory chain inhibitors that act at different sites. For example, the mitochondrial complex III inhibitor, antimycin A is known to generate large amounts of ROS (334), but oligomycin and other disrupters of the respiratory chain have been shown to generate little or no ROS (335).

Aging

The main hallmarks of aging set out by Lopez-Otin are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (336). All of these hallmarks in one way or another are associated with the pathogenesis of neurodegenerative diseases. Here we will focus attention on some specific aspects relating to these hallmarks that could be the most relevant to metformin's mechanism of action at the mitochondria.

Mitonuclear protein imbalance

Human mitochondrial DNA (mtDNA) is bound inside nucleoid bundles, has a high copy number, is inherited maternally and has a high mutation rate (337). Mitochondrial damage and/or depletion induces stress-signaling and adaptive metabolic responses. MtDNA instability is a physiologically relevant stress observed in many human diseases and aging (281). Mitonuclear protein imbalance, is a stoichiometric imbalance between nuclear and mitochondrially encoded proteins and is activated as a key longevity response across many species (338). Alterations to mtDNA are directly linked to respiratory chain dysfunction in sporadic PD patients and it has been shown that complex I is initially affected followed next by complex IV (339). It is thought that imbalance in the stoichiometry between mitochondrially translated proteins and nuclear encoded ones is both a cellular signal and marker of mitochondrial adaption. The mTOR inhibitor rapamycin is used as a tool to initiate mitonuclear

protein imbalance (338) and metformin is capable of modulating mitonuclear protein imbalance in human cells (307).

Oxidant stress and senescence

The production of reactive species is usually balanced by the cell's antioxidant defenses. An imbalance in the amount of ROS to antioxidant defense results in oxidative stress and can cause damage to proteins, lipids and nucleotides.

Mitochondria are a major source of ROS due to oxygen use in energy production through the electron transport chain. Electrons leak while they are being transferred along the complexes of the electron transport chain. Leaked electrons can react with molecular oxygen to form superoxide radicals. Superoxide can react with Mn-SOD to form hydrogen peroxide, a ROS and a signaling molecule. Hydrogen peroxide is either broken down to form water or it can react with metals to form the highly reactive hydroxyl radical. In mitochondria the main leakage sites are at the transfer of four electrons to oxygen at Complex IV, but also complex I, complex III and via certain dehydrogenases of the TCA cycle in the mitochondrial matrix. Consequences of oxidative stress include proliferation, adaption, damage, senescence, or death depending on the cell type and severity [for review see (340, 341)]. Neurons need to counteract a great deal of ROS because of high energy bursts and catecholamine neurotransmitter metabolism.

Oxidative damage is a major contributor to neurodegenerative diseases [for a review see (342)]. Both oxidative stress and oxidative damage can lead to stress adaption. One such adaptive mechanism in mitochondria might be finely-tuned inhibition of respiratory complexes or mitochondrial uncoupling via uncoupling proteins. There is mounting evidence that mitochondrial uncoupling proteins are neuroprotective [for a review see (343)]. Cellular senescence can occur when adaptive responses are unable to properly protect key molecules from damage to the extent that a cell can no longer divide.

The PD protein DJ-1 provides a link between neurodegeneration and energy metabolism. DJ-1 acts as a chaperone and protease to stabilize mitochondria and protect cells from oxidative stress (344). Several other cellular functions have been attributed to DJ-1, including; binding of Ras as a transcriptional co-activator (345), negative regulation of the phosphoinositide-3-kinase (PI3K)/AKT signaling cascade through inhibition of PTEN (309, 346, 347), chaperone function (348, 349), and RNA binding (350). Although controversial, DJ-1 has also been claimed to have glyoxalase (351) and deglycase (352) enzyme activities (353). DJ-1 also influences insulin secretion as well as β cell viability in the pancreas and DJ-1 knockout mice show increased ROS levels in islet cells, impaired glucose tolerance and decreased insulin secretion (354).

GAPS IN THE RESEARCH

Trials using metformin to treat or protect against neurodegenerative diseases in humans and animals have produced mostly conflicting results. The data shows either positive, no or even detrimental effects of metformin on neurodegenerative processes in cell cultures, animals and humans.

The outcome may depend on the species, cell type or underlying metabolic state. Two promising research areas however are neuroinflammation and aging, yet more work is needed. Very few studies have looked directly at the role of metformin in neuroinflammation, but since this is a growing research focus in the field, more metformin studies may arise. The exact role of metformin in aging is a question that needs to be at least partly understood before we can progress further in understanding its potential to treat neurodegenerative diseases. A major hurdle to this is the lack of good human aging models mainly *in vitro* but also *in vivo*.

Another gap in the knowledge is whether there are potential adverse effects of metformin use in non-diabetics. For example, it has been well documented that long term metformin use leads to vitamin B12 deficiency (355). Vitamin B12 and folate are needed for transmethylation and hydroxylation reactions from amino acids that are crucial for neurotransmitter biosynthesis. How much influence could this have in a patient with disturbed neurotransmitter metabolism and/or those receiving other medications.

THE THERAPEUTIC POTENTIAL OF METFORMIN: FEASIBILITY

There are several reasons why the use of metformin to treat neurodegeneration could bring about doubt from clinicians and scientists when considering its potential as a therapy or as a research tool. The main point being that metformin seems to be acting on a plethora of biological pathways, and therefore it is very difficult to pin down mechanisms. The second point is the controversial subject of "anti-aging" drugs in general. Since we know very little about the biological underpinnings of aging and know even less about how to efficiently model it in the laboratory, the promotion of an "anti-aging" drug often conjures up more questions than it answers. Then there are several other sticking points among researchers, one being the fact that metformin acts by inhibiting mitochondrial respiration, the exact effect that has been shown by years of research in the Parkinson's disease field to in fact contribute development of disease.

In direct contrast, there are several arguments for metformin being a feasible and useful drug. Firstly, glucose metabolism is of central importance to neuronal redox status, therefore to the long-term survival of neurons. Secondly, as a population we are increasingly insulin resistant and therefore metformin is particularly apt. Metformin is a cheap and safe drug with few side effects and therefore more work *in vitro*, *in vivo* and in trials will be welcomed.

Nir Barzilai, the director of the Institute for Aging Research at the Albert Einstein College of Medicine suggests that metformin and other related drugs can extend our years of healthy, disease-free living by decades (356). Other scientists have not specifically mentioned metformin but in his 2005 book on mitochondria, Nick Lane suggests that if we live longer to rid ourselves of diseases of old age we need more mitochondria and perhaps

a more refined free-radical detection system (357). Whether metformin is capable of modifying the detection system at the right physiological moment without deleterious effects is at least an exciting possibility.

FUTURE DEVELOPMENTS IN THE FIELD

There is potential that metformin could be beneficial in the task of counteracting aging and clinical studies imply that metformin may have positive effects on cognition in T2DM patients. A better understanding of how metformin works will help researchers in the neurodegeneration field to successfully design future research and trials. Upcoming studies such as TAME (358) will help in this respect.

The anti-aging effects of metformin could be summarized by its ability to interfere with the multistage process of energy production without producing damaging amounts of ROS. This action alone could be seen as neuroprotective and metformin may further protect by activating other biological pathways. For example, slowing mitochondrial energy production can also trigger a cascade of signaling events in the liver that result in reduced glucose and insulin. The key role of insulin in nutrient sensing which balances growth and proliferation with life-extending conservation, makes metformin an interesting drug. The field of aging research is growing and *in vivo* and *in vitro* aging models are advancing.

Probably due to the complexity of metformin action, this drug will not likely serve as a potential treatment for neurodegenerative diseases on the current stage because much more work is needed to understand the role of aging in different neurodegenerative disease forms. The greatest value of metformin today might lie in its potential to help decipher those mechanisms underlying neurodegeneration.

AUTHOR CONTRIBUTIONS

CR, GM, and JF contributed equally to the writing and the editing of the manuscript. All authors approved the final version and submission of this article.

FUNDING

CR is funded by the German Center for Neurodegenerative Diseases (DZNE). GM is supported by an Edmond J. Safra Fellowship in Movement Disorders from the Michael J. Fox Foundation. JF acknowledges funding from the German Research Council (DFG), German Federal Ministry of Education and Research (BMBF) support code 031 A 430 A, DZNE, The University of Tubingen and the EU Joint Programme—Neurodegenerative Disease Research (JPND) project.

ACKNOWLEDGMENTS

The authors would like to thank Manu Sharma, Jonas Neher, Anna Schaedler, and Laura Dunn for critically reviewing the manuscript.

REFERENCES

1. Feigin VL, Abajobir AA, Abate KH, Abd-Allah F, Abdulle AM, Abera SF, et al. Global, regional, and national burden of neurological disorders during 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Neurol*. (2017) 16, 877–97. doi: 10.1016/S1474-4422(17)30299-5
2. Goodarzi MO, Bryer-Ash M. Metformin revisited: re-evaluation of its properties and role in the pharmacopoeia of modern antidiabetic agents. *Diabetes Obes Metab*. (2005) 7:654–65. doi: 10.1111/j.1463-1326.2004.00448.x
3. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: an overview. *Clin Sci*. (2012) 122:253–70. doi: 10.1042/CS20110386
4. Lamanna C, Monami M, Marchionni N, Mannucci E. Effect of metformin on cardiovascular events and mortality: a meta-analysis of randomized clinical trials. *Diabetes Obes Metab* (2011) 13:221–8. doi: 10.1111/j.1463-1326.2010.01349.x
5. Rojas LB, Gomes MB. Metformin: an old but still the best treatment for type 2 diabetes. *Diabetol Metab Syndr*. (2013) 5:6. doi: 10.1186/1758-5996-5-6
6. Patrone C, Eriksson O, Lindholm D. Diabetes drugs and neurological disorders: new views and therapeutic possibilities. *Lancet Diabetes Endocrinol*. (2014) 2:256–62. doi: 10.1016/S2213-8587(13)70125-6
7. Mertens J, Paquola ACM, Ku M, Hatch E, Bohnke L, Ladjevardi S, et al. Directly reprogrammed human neurons retain aging-associated transcriptomic signatures and reveal age-related nucleocytoplasmic defects. *Cell Stem Cell* (2015) 17:705–18. doi: 10.1016/j.stem.2015.09.001
8. Kim Y, Zheng X, Ansari Z, Bunnell MC, Herdy JR, Traxler L, et al. Mitochondrial aging defects emerge in directly reprogrammed human neurons due to their metabolic profile. *Cell Rep*. (2018) 23:2550–8. doi: 10.1016/j.celrep.2018.04.105
9. Craft S, Stennis Watson G. Insulin and neurodegenerative disease: shared and specific mechanisms. *Lancet Neurol*. (2004) 3:169–78. doi: 10.1016/S1474-4422(04)00681-7
10. Ninomiya T. Diabetes mellitus and dementia. *Curr Diab Rep*. (2014) 14:487. doi: 10.1007/s11892-014-0487-z
11. Neumann KF, Rojo L, Navarrete LP, Farias G, Reyes P, Maccioni RB. Insulin resistance and Alzheimer's disease: molecular links & clinical implications. *Curr Alzheimer Res*. (2008) 5:438–47. doi: 10.2174/156720508785908919
12. Verdile G, Fuller SJ, Martins RN. The role of type 2 diabetes in neurodegeneration. *Neurobiol Dis*. (2015) 84:22–38. doi: 10.1016/j.nbd.2015.04.008
13. United Nations DoEaSA Population Division. World population prospects: The 2015 revision, key findings and advance tables. In: *Working Paper No ESA/P/WP*241 (2015).
14. Prince M, Comas Herrera A, Knapp M, Karagiannidou M, Guerchet M. *World Alzheimer Report 2016: Improving Healthcare for People with Dementia. Coverage, Quality and Costs Now and in the Future*. London: Alzheimer's Disease International (ADI) (2016).
15. Brion JP, Couck AM, Passareiro E, Flament-Durand J. Neurofibrillary tangles of Alzheimer's disease: an immunohistochemical study. *J Submicrosc Cytol*. (1985) 17:89–96.
16. Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI. Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proc Natl Acad Sci USA*. (1986) 83:4913–7. doi: 10.1073/pnas.83.13.4913
17. Glenner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun*. (2012) 425:534–9. doi: 10.1016/j.bbrc.2012.08.020
18. Johnson GV, Stoothoff WH. Tau phosphorylation in neuronal cell function and dysfunction. *J Cell Sci*. (2004) 117:5721–9. doi: 10.1242/jcs.01558

19. Arriagada PV, Growdon JH, Hedley-Whyte ET, Hyman BT. Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology* (1992) 42:631–9. doi: 10.1212/WNL.42.3.631
20. Sun X, Bromley-Brits K, Song W. Regulation of beta-site APP-cleaving enzyme 1 gene expression and its role in Alzheimer's disease. *J Neurochem.* (2012) 120(Suppl). 1:62–70. doi: 10.1111/j.1471-4159.2011.07515.x
21. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, et al. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* (1991) 349:704–6. doi: 10.1038/349704a0
22. Levy-Lahad E, Wasco W, Poorkaj P, Romano DM, Oshima J, Pettingell WH, et al. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science* (1995) 269:973–7. doi: 10.1126/science.7638622
23. Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, et al. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* (1995) 375:754–60. doi: 10.1038/375754a0
24. Hardy J, Bogdanovic N, Winblad B, Portelius E, Andreasen N, Cedazo-Minguez A, et al. Pathways to Alzheimer's disease. *J Intern Med*. (2014) 275:296–303. doi: 10.1111/joim.12192
25. Efthymiou AG, Goate AM. Late onset Alzheimer's disease genetics implicates microglial pathways in disease risk. *Mol Neurodegener*. (2017) 12:43. doi: 10.1186/s13024-017-0184-x
26. Huang KL, Marcora E, Pimenova AA, Di Narzo AF, Kapoor M, Jin SC, et al. A common haplotype lowers PU.1 expression in myeloid cells and delays onset of Alzheimer's disease. *Nat Neurosci*. (2017) 20:1052–1061. doi: 10.1038/nn.4587
27. Kivipelto M, Ngandu T, Fratiglioni L, Viitanen M, Kareholt I, Winblad B, et al. Obesity and vascular risk factors at midlife and the risk of dementia and Alzheimer disease. *Arch Neurol*. (2005) 62:1556–60. doi: 10.1001/archneur.62.10.1556
28. Coon KD, Myers AJ, Craig DW, Webster JA, Pearson JV, Lince DH, et al. A high-density whole-genome association study reveals that APOE is the major susceptibility gene for sporadic late-onset Alzheimer's disease. *J Clin Psychiatry* (2007) 68:613–8. doi: 10.4088/JCP.v68n0419
29. Baumgart M, Snyder HM, Carrillo MC, Fazio S, Kim H, Johns H. Summary of the evidence on modifiable risk factors for cognitive decline and dementia: a population-based perspective. *Alzheimer's Dement.* (2015) 11:718–26. doi: 10.1016/j.jalz.2015.05.016
30. Killin LO, Starr JM, Shiue IJ, Russ TC. Environmental risk factors for dementia: a systematic review. *BMC Geriatr*. (2016) 16:175. doi: 10.1186/s12877-016-0342-y
31. Li W, Risacher SL, McAllister TW, Saykin AJ. Traumatic brain injury and age at onset of cognitive impairment in older adults. *J Neurol.* (2016) 263:1280–5. doi: 10.1007/s00415-016-8093-4
32. Mody N, Agouni A, McIlroy GD, Platt B, Delibegovic M. Susceptibility to diet-induced obesity and glucose intolerance in the APP (SWE)/PSEN1 (A246E) mouse model of Alzheimer's disease is associated with increased brain levels of protein tyrosine phosphatase 1B (PTP1B) and retinol-binding protein 4 (RBP4), and basal phosphorylation of S6 ribosomal protein. *Diabetologia* (2011) 54:2143–51. doi: 10.1007/s00125-011-2160-2
33. Ruiz HH, Chi T, Shin AC, Lindtner C, Hsieh W, Ehrlich M, et al. Increased susceptibility to metabolic dysregulation in a mouse model of Alzheimer's disease is associated with impaired hypothalamic insulin signaling and elevated BCAA levels. *Alzheimer's Dement.* (2016) 12:851–61. doi: 10.1016/j.jalz.2016.01.008
34. Jimenez-Palomares M, Ramos-Rodriguez JJ, Lopez-Acosta JF, Pacheco-Herrero M, Lechuga-Sancho AM, Perdomo G, et al. Increased Abeta production prompts the onset of glucose intolerance and insulin resistance. *Am J Physiol Endocrinol Metab.* (2012) 302:E1373–80. doi: 10.1152/ajpendo.00500.2011
35. Takeda S, Sato N, Uchio-Yamada K, Sawada K, Kunieda T, Takeuchi D, et al. Diabetes-accelerated memory dysfunction via cerebrovascular inflammation and Abeta deposition in an Alzheimer mouse model with diabetes. *Proc Natl Acad Sci USA*. (2010) 107:7036–41. doi: 10.1073/pnas.1000645107
36. DiTacchio KA, Heinemann SF, Dziewczapolski G. Metformin treatment alters memory function in a mouse model of Alzheimer's disease. *J Alzheimer's Dis.* (2015) 44:43–8. doi: 10.3233/JAD-141332
37. Barini E, Antico O, Zhao Y, Asta F, Tucci V, Catelani T, et al. Metformin promotes tau aggregation and exacerbates abnormal behavior in a mouse model of tauopathy. *Mol Neurodegener.* (2016) 11:16. doi: 10.1186/s13024-016-0082-7
38. Agusti A, Garcia-Pardo MP, Lopez-Almela I, Campillo I, Maes M, Romani-Perez M, et al. Interplay between the gut-brain axis, obesity and cognitive function. *Front Neurosci*. (2018) 12:155. doi: 10.3389/fnins.2018.00155
39. Li ZG, Zhang W, Sima AA. Alzheimer-like changes in rat models of spontaneous diabetes. *Diabetes* (2007) 56:1817–24. doi: 10.2337/db07-0171
40. Pedersen WA, McMillan PJ, Kulstad JJ, Leverenz JB, Craft S, Haynatzki GR. Rosiglitazone attenuates learning and memory deficits in Tg2576 Alzheimer mice. *Exp Neurol*. (2006) 199:265–73. doi: 10.1016/j.expneurol.2006.01.018
41. De Felice FG, Vieira MN, Bomfim TR, Decker H, Velasco PT, Lambert MP, et al. Protection of synapses against Alzheimer's-linked toxins: insulin signaling prevents the pathogenic binding of Abeta oligomers. *Proc Natl Acad Sci USA*. (2009) 106:1971–6. doi: 10.1073/pnas.0809158106
42. Piriz J, Muller A, Trejo JL, Torres-Aleman I. IGF-I and the aging mammalian brain. *Exp Gerontol*. (2011) 46:96–9. doi: 10.1016/j.exger.2010.08.022
43. Salcedo I, Tweedie D, Li Y, Greig NH. Neuroprotective and neurotrophic actions of glucagon-like peptide-1: an emerging opportunity to treat neurodegenerative and cerebrovascular disorders. *Br J Pharmacol*. (2012) 166:1586–99. doi: 10.1111/j.1476-5381.2012.01971.x
44. Mattson MP, Longo VD, Harvie M. Impact of intermittent fasting on health and disease processes. *Ageing Res Rev*. (2017) 39:46–58. doi: 10.1016/j.arr.2016.10.005
45. Bayliss JA, Lemus MB, Santos VV, Deo M, Davies JS, Kemp BE, et al. Metformin prevents nigrostriatal dopamine degeneration independent of AMPK activation in dopamine neurons. *PLoS ONE* (2016) 11:e0159381. doi: 10.1371/journal.pone.0159381
46. Ismaiel AA, Espinosa-Oliva AM, Santiago M, Garcia-Quintanilla A, Oliva-Martin MJ, Herrera AJ, et al. Metformin, besides exhibiting strong in vivo anti-inflammatory properties, increases mptp-induced damage to the nigrostriatal dopaminergic system. *Toxicol Appl Pharmacol*. (2016) 298:19–30. doi: 10.1016/j.taap.2016.03.004
47. Kang H, Khang R, Ham S, Jeong GR, Kim H, Jo M, et al. Activation of the ATF2/CREB-PGC-1alpha pathway by metformin leads to dopaminergic neuroprotection. *Oncotarget* (2017) 8:48603–18. doi: 10.18632/oncotarget.18122
48. Katila N, Bhurtel S, Shadfar S, Srivastav S, Neupane S, Ojha U, et al. Metformin lowers alpha-synuclein phosphorylation and upregulates neurotrophic factor in the MPTP mouse model of Parkinson's disease. *Neuropharmacology* (2017) 125:396–407. doi: 10.1016/j.neuropharm.2017.08.015
49. Lu M, Su C, Qiao C, Bian Y, Ding J, Hu G. Metformin prevents dopaminergic neuron death in MPTP/P-induced mouse model of parkinson's disease via autophagy and mitochondrial ROS clearance. *Int J Neuropsychopharmacol.* (2016) 19:pyw047. doi: 10.1093/ijnp/pyw047
50. Patil SP, Jain PD, Ghumatkar PJ, Tambe R, Sathaye S. Neuroprotective effect of metformin in MPTP-induced Parkinson's disease in mice. *Neuroscience* (2014) 277:747–54. doi: 10.1016/j.neuroscience.2014.07.046
51. Perez-Revuelta BI, Hettich MM, Ciociaro A, Rotermund C, Kahle PJ, Krauss S, et al. Metformin lowers Ser-129 phosphorylated alpha-synuclein levels via mTOR-dependent protein phosphatase 2A activation. *Cell Death Dis*. (2014) 5:e1209. doi: 10.1038/cddis.2014.175
52. Porceddu PF, Ishola IO, Contu L, Morelli M. Metformin prevented dopaminergic neurotoxicity induced by 3,4-Methylenedioxymethamphetamine administration. *Neurotox Res*. (2016) 30:101–9. doi: 10.1007/s12640-016-9633-5
53. Chen F, Dong RR, Zhong KL, Ghosh A, Tang SS, Long Y, et al. Antidiabetic drugs restore abnormal transport of amyloid-beta across the blood-brain barrier and memory impairment in db/db mice. *Neuropharmacology* (2016) 101:123–36. doi: 10.1016/j.neuropharm.2015.07.023
54. Kickstein E, Krauss S, Thornhill P, Rutschow D, Zeller R, Sharkey J, et al. Biguanide metformin acts on tau phosphorylation via mTOR/protein phosphatase 2A (PP2A) signaling. *Proc Natl Acad Sci USA*. (2010) 107:21830–5. doi: 10.1073/pnas.0912793107

55. Li J, Deng J, Sheng W, Zuo Z. Metformin attenuates Alzheimer's disease-like neuropathology in obese, leptin-resistant mice. *Pharmacol Biochem Behav*. (2012) 101:564–74. doi: 10.1016/j.pbb.2012.03.002
56. Picone P, Nuzzo D, Caruana L, Messina E, Barera A, Vasto S, et al. Metformin increases APP expression and processing via oxidative stress, mitochondrial dysfunction and NF-kappaB activation: use of insulin to attenuate metformin's effect. *Biochim Biophys Acta* (2015) 1853:1046–59. doi: 10.1016/j.bbamcr.2015.01.017
57. Allard JS, Perez EJ, Fukui K, Carpenter P, Ingram DK, de Cabo R. Prolonged metformin treatment leads to reduced transcription of Nrf2 and neurotrophic factors without cognitive impairment in older C57BL/6J mice. *Behav Brain Res*. (2016) 301:1–9. doi: 10.1016/j.bbr.2015.12.012
58. Lennox R, Porter DW, Flatt PR, Holscher C, Irwin N, Gault VA. Comparison of the independent and combined effects of sub-chronic therapy with metformin and a stable GLP-1 receptor agonist on cognitive function, hippocampal synaptic plasticity and metabolic control in high-fat fed mice. *Neuropharmacology* (2014) 86:22–30. doi: 10.1016/j.neuropharm.2014.06.026
59. McNeilly AD, Williamson R, Balfour DJ, Stewart CA, Sutherland C. A high-fat-diet-induced cognitive deficit in rats that is not prevented by improving insulin sensitivity with metformin. *Diabetologia* (2012) 55:3061–70. doi: 10.1007/s00125-012-2686-y
60. Pintana H, Apaijai N, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. Effects of metformin on learning and memory behaviors and brain mitochondrial functions in high fat diet induced insulin resistant rats. *Life Sci*. (2012) 91:409–14. doi: 10.1016/j.lfs.2012.08.017
61. Thangthaeng N, Rutledge M, Wong JM, Vann PH, Forster MJ, Sumien N. Metformin impairs spatial memory and visual acuity in old male mice. *Aging Dis*. (2017) 8:17–30. doi: 10.14336/AD.2016.1010
62. Ma TC, Buescher JL, Oatis B, Funk JA, Nash AJ, Carrier RL, et al. Metformin therapy in a transgenic mouse model of Huntington's disease. *Neurosci Lett*. (2007) 411:98–103. doi: 10.1016/j.neulet.2006.10.039
63. Kaneb HM, Sharp PS, Rahmani-Kondori N, Wells DJ. Metformin treatment has no beneficial effect in a dose-response survival study in the SOD1(G93A) mouse model of ALS and is harmful in female mice. *PLoS ONE* (2011) 6:e24189. doi: 10.1371/journal.pone.0024189
64. Monette MC, Baird A, Jackson DL. A meta-analysis of cognitive functioning in nondemented adults with type 2 diabetes mellitus. *Can J Diab.* (2014) 38:401–8. doi: 10.1016/j.jcjd.2014.01.014
65. Palta P, Schneider AL, Biessels GJ, Touradji P, Hill-Briggs F. Magnitude of cognitive dysfunction in adults with type 2 diabetes: a meta-analysis of six cognitive domains and the most frequently reported neuropsychological tests within domains. *J Int Neuropsychol Soc.* (2014) 20:278–91. doi: 10.1017/S1355617713001483
66. Vincent C, Hall PA. Executive function in adults with type 2 diabetes: a meta-analytic review. *Psychosom Med*. (2015) 77:631–42. doi: 10.1097/PSY.0000000000000103
67. Cooper C, Sommerlad A, Lyketsos CG, Livingston G. Modifiable predictors of dementia in mild cognitive impairment: a systematic review and meta-analysis. *Am J Psychiatry* (2015) 172:323–34. doi: 10.1176/appi.ajp.2014.14070878
68. Moulton CD, Costafreda SG, Horton P, Ismail K, Fu CH. Meta-analyses of structural regional cerebral effects in type 1 and type 2 diabetes. *Brain Imaging Behav*. (2015) 9:651–62. doi: 10.1007/s11682-014-9348-2
69. Wu G, Lin L, Zhang Q, Wu J. Brain gray matter changes in type 2 diabetes mellitus: a meta-analysis of whole-brain voxel-based morphometry study. *J Diabetes Complicat.* (2017). 31:1698–703. doi: 10.1016/j.jdiacomp.2017.09.001
70. Lu FP, Lin KP, Kuo HK. Diabetes and the risk of multi-system aging phenotypes: a systematic review and meta-analysis. *PLoS ONE* (2009) 4:e4144. doi: 10.1371/journal.pone.0004144
71. Profenno LA, Porsteinsson AP, Faraone SV. Meta-analysis of Alzheimer's disease risk with obesity, diabetes, and related disorders. *Biol Psychiatry* (2010) 67:505–12. doi: 10.1016/j.biopsych.2009.02.013
72. Vagelatos NT, Eslick GD. Type 2 diabetes as a risk factor for Alzheimer's disease: the confounders, interactions, and neuropathology associated with this relationship. *Epidemiol Rev*. (2013) 35:152–60. doi: 10.1093/epirev/mxs012
73. Cheng G, Huang C, Deng H, Wang H. Diabetes as a risk factor for dementia and mild cognitive impairment: a meta-analysis of longitudinal studies. *Intern Med J*. (2012) 42:484–91. doi: 10.1111/j.1445-5994.2012.02758.x
74. Janson J, Laedtke T, Parisi JE, O'Brien P, Petersen RC, Butler PC. Increased risk of type 2 diabetes in Alzheimer disease. *Diabetes* (2004) 53:474–81. doi: 10.2337/diabetes.53.2.474
75. Matsuzaki T, Sasaki K, Tanizaki Y, Hata J, Fujimi K, Matsui Y, et al. Insulin resistance is associated with the pathology of Alzheimer disease: the Hisayama study. *Neurology* (2010) 75:764–70. doi: 10.1212/WNL.0b013e3181eee25f
76. Turner R, Craft S, Aisen P. Individuals with Alzheimer's disease exhibit a high prevalence of undiagnosed impaired glucose tolerance and type 2 diabetes mellitus. *Alzheimer's Dement.* (2013) 9:P284–5. doi: 10.1016/j.jalz.2013.05.573
77. Frolich L, Blum-Degen D, Riederer P, Hoyer S. A disturbance in the neuronal insulin receptor signal transduction in sporadic Alzheimer's disease. *Ann NY Acad Sci*. (1999) 893:290–3. doi: 10.1111/j.1749-6632.1999.tb07839.x
78. Rivera EJ, Goldin A, Fulmer N, Tavares R, Wands JR, de la Monte SM. Insulin and insulin-like growth factor expression and function deteriorate with progression of Alzheimer's disease: link to brain reductions in acetylcholine. *J Alzheimer's Dis.* (2005) 8:247–68. doi: 10.3233/JAD-2005-8304
79. Bomfim TR, Forny-Germano L, Sathler LB, Brito-Moreira J, Houzel JC, Decker H, et al. An anti-diabetes agent protects the mouse brain from defective insulin signaling caused by Alzheimer's disease- associated Abeta oligomers. *J Clin Invest*. (2012) 122:1339–53. doi: 10.1172/JCI57256
80. Talbot K, Wang HY, Kazi H, Han LY, Bakshi KP, Stucky A, et al. Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *J Clin Invest*. (2012) 122:1316–38. doi: 10.1172/JCI59903
81. Luchsinger JA, Tang MX, Shea S, Mayeux R. Hyperinsulinemia and risk of Alzheimer disease. *Neurology* (2004) 63:1187–92. doi: 10.1212/01.WNL.0000140292.04932.87
82. Baker LD, Cross DJ, Minoshima S, Belongia D, Watson GS, Craft S. Insulin resistance and Alzheimer-like reductions in regional cerebral glucose metabolism for cognitively normal adults with prediabetes or early type 2 diabetes. *Arch Neurol*. (2011) 68:51–7. doi: 10.1001/archneurol.2010.225
83. Arvanitakis Z, Schneider JA, Wilson RS, Li Y, Arnold SE, Wang Z, et al. Diabetes is related to cerebral infarction but not to AD pathology in older persons. *Neurology* (2006) 67:1960–5. doi: 10.1212/01.wnl.0000247053.45483.4e
84. Abner EL, Nelson PT, Kryscio RJ, Schmitt FA, Fardo DW, Woltjer RL, et al. Diabetes is associated with cerebrovascular but not Alzheimer's disease neuropathology. *Alzheimer's Dement.* (2016) 12:882–9. doi: 10.1016/j.jalz.2015.12.006
85. Crane PK, Walker RL, Sonnen J, Gibbons LE, Melrose R, Hassenstab J, et al. Glucose levels during life and neuropathologic findings at autopsy among people never treated for diabetes. *Neurobiol Aging* (2016) 48:72–82. doi: 10.1016/j.neurobiolaging.2016.07.021
86. Dos Santos Matioli MNP, Suemoto CK, Rodriguez RD, Farias DS, da Silva MM, Leite REP, et al. Diabetes is not associated with Alzheimer's disease neuropathology. *J Alzheimer's Dis.* (2017) 60:1035–43. doi: 10.3233/JAD-170179
87. Pruzin JJ, Schneider JA, Capuano AW, Leurgans SE, Barnes LL, Ahima RS, et al. Diabetes, hemoglobin A1C, and regional Alzheimer disease and infarct pathology. *Alzheimer Dis Assoc Disord*. (2017) 31:41–7. doi: 10.1097/WAD.0000000000000172
88. Beeri MS, Silverman JM, Davis KL, Marin D, Grossman HZ, Schmeidler J, et al. Type 2 diabetes is negatively associated with Alzheimer's disease neuropathology. *J Gerontol Ser A Biol Sci Med Sci.* (2005) 60:471–5. doi: 10.1093/gerona/60.4.471
89. Ahtiluoto S, Polvikoski T, Peltonen M, Solomon A, Tuomilehto J, Winblad B, et al. Diabetes, Alzheimer disease, and vascular dementia: a population-based neuropathologic study. *Neurology* (2010) 75:1195–202. doi: 10.1212/WNL.0b013e3181f4d7f8
90. Rahimi J, Kovacs GG. Prevalence of mixed pathologies in the aging brain. *Alzheimer's Res Ther*. (2014) 6:82. doi: 10.1186/s13195-014-0082-1

91. Norton S, Matthews FE, Barnes DE, Yaffe K, Brayne C. Potential for primary prevention of Alzheimer's disease: an analysis of population-based data. *Lancet Neurol*. (2014) 13:788–94. doi: 10.1016/S1474-4422(14)70136-X
92. Wahlqvist ML, Lee MS, Hsu CC, Chuang SY, Lee JT, Tsai HN. Metformin-inclusive sulfonylurea therapy reduces the risk of Parkinson's disease occurring with Type 2 diabetes in a Taiwanese population cohort. *Parkinsonism Relat Disord*. (2012) 18:753–8. doi: 10.1016/j.parkreldis.2012.03.010
93. Brakedal B, Flønes I, Reiter SF, Torkildsen Ø, Dölle C, Assmus J, et al. Glitazone use associated with reduced risk of Parkinson's disease. *Mov Dis* (2017) 32:1594–9. doi: 10.1002/mds.27128
94. Brakedal B, Haugarvoll K, Tzoulis C. Simvastatin is associated with decreased risk of Parkinson disease. *Ann Neurol.* (2017) 81:329–30. doi: 10.1002/ana.24857
95. Kuan YC, Huang KW, Lin CL, Hu CJ, Kao CH. Effects of metformin exposure on neurodegenerative diseases in elderly patients with type 2 diabetes mellitus. *Prog Neuropsychopharmacol Biol Psychiatry* (2017) 79:77–83. doi: 10.1016/j.pnpbp.2017.06.002
96. Hsu CC, Wahlqvist ML, Lee MS, Tsai HN. Incidence of dementia is increased in type 2 diabetes and reduced by the use of sulfonylureas and metformin. *J Alzheimer's Dis.* (2011) 24:485–93. doi: 10.3233/JAD-2011-101524
97. Cheng C, Lin CH, Tsai YW, Tsai CJ, Chou PH, Lan TH. Type 2 diabetes and antidiabetic medications in relation to dementia diagnosis. *J Gerontol Ser A Biol Sci Med Sci* (2014) 69:1299–305. doi: 10.1093/gerona/glu073
98. Heneka MT, Fink A, Doblhammer G. Effect of pioglitazone medication on the incidence of dementia. *Ann Neurol*. (2015) 78:284–94. doi: 10.1002/ana.24439
99. Ye F, Luo YJ, Xiao J, Yu NW, Yi G. Impact of insulin sensitizers on the incidence of dementia: a meta-analysis. *Dement Geriatr Cogn Disord*. (2016) 41:251–60. doi: 10.1159/000445941
100. Wang CP, Lorenzo C, Habib SL, Jo B, Espinoza SE. Differential effects of metformin on age related comorbidities in older men with type 2 diabetes. *J Diabetes Complicat*. (2017) 31:679–86. doi: 10.1016/j.jdiacomp.2017.01.013
101. Orkaby AR, Cho K, Cormack J, Gagnon DR, Driver JA. Metformin vs sulfonylurea use and risk of dementia in US veterans aged >/=65 years with diabetes. *Neurology* (2017) 89:1877–85. doi: 10.1212/WNL.0000000000004586
102. Ng TP, Feng L, Yap KB, Lee TS, Tan CH, Winblad B. Long-term metformin usage and cognitive function among older adults with diabetes. *J Alzheimer's Dis.* (2014) 41:61–8. doi: 10.3233/JAD-131901
103. Imfeld P, Bodmer M, Jick SS, Meier CR. Metformin, other antidiabetic drugs, and risk of Alzheimer's disease: a population-based case-control study. *J Am Geriatr Soc*. (2012) 60:916–21. doi: 10.1111/j.1532-5415.2012.03916.x
104. Huang CC, Chung CM, Leu HB, Lin LY, Chiu CC, Hsu CY, et al. Diabetes mellitus and the risk of Alzheimer's disease: a nationwide population-based study. *PLoS ONE* (2014) 9:e87095. doi: 10.1371/journal.pone.0087095
105. Koenig AM, Mechanic-Hamilton D, Xie SX, Combs MF, Cappola AR, Xie L, et al. Effects of the insulin sensitizer metformin in Alzheimer Disease: pilot data from a randomized placebo-controlled crossover study. *Alzheimer Dis Assoc Disord*. (2017) 31:107–13. doi: 10.1097/WAD.0000000000000202
106. Hervas D, Fornes-Ferrer V, Gomez-Escribano AP, Sequedo MD, Peiro C, Millan JM, et al. Metformin intake associates with better cognitive function in patients with Huntington's disease. *PLoS ONE* (2017) 12:e0179283. doi: 10.1371/journal.pone.0179283
107. Yokoyama H, Ogawa M, Honjo J, Okizaki S, Yamada D, Shudo R, et al. Risk factors associated with abnormal cognition in Japanese outpatients with diabetes, hypertension or dyslipidemia. *Diabetol Int.* (2015) 6:268–74. doi: 10.1007/s13340-014-0194-7
108. Luchsinger JA, Perez T, Chang H, Mehta P, Steffener J, Pradabhan G, et al. Metformin in amnestic mild cognitive impairment: results of a pilot randomized placebo controlled clinical trial. *J Alzheimer's Dis.* (2016) 51:501–14. doi: 10.3233/JAD-150493
109. de Lau LM, Breteler MM. Epidemiology of Parkinson's disease. *Lancet Neurol*. (2006) 5:525–35. doi: 10.1016/S1474-4422(06)70471-9
110. Jankovic J. Parkinson's disease: clinical features and diagnosis. *J Neurol Neurosurg Psychiatr*. (2008) 79:368–76. doi: 10.1136/jnnp.2007.131045
111. Damier P, Hirsch EC, Agid Y, Graybiel AM. The substantia nigra of the human brain. II. Patterns of loss of dopamine-containing neurons in Parkinson's disease. *Brain* (1999) 122(Pt 8), 1437–48.
112. Schneider SA, Obeso JA. Clinical and pathological features of Parkinson's disease. *Curr Top Behav Neurosci*. (2014) 22:205–20. doi: 10.1007/7854_2014_317
113. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. *Nature* (1997) 388:839–40. doi: 10.1038/42166
114. Braak H, Del Tredici K, Bratzke H, Hamm-Clement J, Sandmann-Keil D, Rub U. Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). *J Neurol.* (2002) 249(Suppl. 3):iii1–5. doi: 10.1007/s00415-002-1301-4
115. Beach TG, Adler CH, Lue L, Sue LI, Bachalakuri J, Henry-Watson J, et al. Unified staging system for Lewy body disorders: correlation with nigrostriatal degeneration, cognitive impairment and motor dysfunction. *Acta Neuropathol*. (2009) 117:613–34. doi: 10.1007/s00401-009-0538-8
116. Gasser T. Identifying PD-causing genes and genetic susceptibility factors: current approaches and future prospects. *Prog Brain Res*. (2010) 183:3–20. doi: 10.1016/S0079-6123(10)83001-8
117. Deng H, Wang P, Jankovic J. The genetics of Parkinson disease. *Ageing Res Rev*. (2017) 42:72–85. doi: 10.1016/j.arr.2017.12.007
118. Klein C, Westenberger A. Genetics of Parkinson's disease. *Cold Spring Harb Perspect Med*. (2012) 2:a008888. doi: 10.1101/cshperspect.a008888
119. Dunn L, Allen GF, Mamais A, Ling H, Li A, Duberley KE, et al. Dysregulation of glucose metabolism is an early event in sporadic Parkinson's disease. *Neurobiol Aging* (2014) 35:1111–5. doi: 10.1016/j.neurobiolaging.2013.11.001
120. Athauda D, Foltynie T. The ongoing pursuit of neuroprotective therapies in Parkinson disease. *Nat Rev Neurol.* (2015) 11:25–40. doi: 10.1038/nrneurol.2014.226
121. Choi JY, Jang EH, Park CS, Kang JH. Enhanced susceptibility to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity in high-fat diet-induced obesity. *Free Radic Biol Med*. (2005) 38:806–16. doi: 10.1016/j.freeradbiomed.2004.12.008
122. Morris JK, Bomhoff GL, Stanford JA, Geiger PC. Neurodegeneration in an animal model of Parkinson's disease is exacerbated by a high-fat diet. *Am J Physiol Regul Integr Compar Physiol.* (2010) 299:R1082–90. doi: 10.1152/ajpregu.00449.2010
123. Rotermund C, Truckenmuller FM, Schell H, Kahle PJ. Diet-induced obesity accelerates the onset of terminal phenotypes in alpha-synuclein transgenic mice. *J Neurochem*. (2014) 131:848–58. doi: 10.1111/jnc.12813
124. Morris JK, Bomhoff GL, Gorres BK, Davis VA, Kim J, Lee PP, et al. Insulin resistance impairs nigrostriatal dopamine function. *Exp Neurol*. (2011) 231:171–80. doi: 10.1016/j.expneurol.2011.06.005
125. Wang L, Zhai YQ, Xu LL, Qiao C, Sun XL, Ding JH, et al. Metabolic inflammation exacerbates dopaminergic neuronal degeneration in response to acute MPTP challenge in type 2 diabetes mice. *Exp Neurol.* (2014) 251:22–9. doi: 10.1016/j.expneurol.2013.11.001
126. Hu G, Jousilahti P, Bidel S, Antikainen R, Tuomilehto J. Type 2 diabetes and the risk of Parkinson's disease. *Diabetes Care* (2007) 30:842–7. doi: 10.2337/dc06-2011
127. Simon KC, Chen H, Schwarzschild M, Ascherio A. Hypertension, hypercholesterolemia, diabetes, and risk of Parkinson disease. *Neurology* (2007) 69:1688–95. doi: 10.1212/01.wnl.0000271883.45010.8a
128. Driver JA, Smith A, Buring JE, Gaziano JM, Kurth T, Logroscino G. Prospective cohort study of type 2 diabetes and the risk of Parkinson's disease. *Diabetes Care* (2008) 31:2003–5. doi: 10.2337/dc08-0688
129. D'Amelio M, Ragonese P, Callari G, Di Benedetto N, Palmeri B, Terruso V, et al. Diabetes preceding Parkinson's disease onset. A case-control study. *Parkinsonism Relat Disord.* (2009) 15:660–4. doi: 10.1016/j.parkreldis.2009.02.013
130. Miyake Y, Tanaka K, Fukushima W, Sasaki S, Kiyohara C, Tsuboi Y, et al. Case-control study of risk of Parkinson's disease in relation to hypertension, hypercholesterolemia, and diabetes in Japan. *J Neurol Sci*. (2010) 293:82–6. doi: 10.1016/j.jns.2010.03.002

131. Schernhammer E, Hansen J, Rugbjerg K, Wermuth L, Ritz B. Diabetes and the risk of developing Parkinson's disease in Denmark. *Diabetes Care* (2011) 34:1102–8. doi: 10.2337/dc10-1333
132. Xu Q, Park Y, Huang X, Hollenbeck A, Blair A, Schatzkin A, et al. Diabetes and risk of Parkinson's disease. *Diabetes Care* (2011) 34:910–5. doi: 10.2337/dc10-1922
133. Lu L, Fu DL, Li HQ, Liu AJ, Li JH, Zheng GQ. Diabetes and risk of Parkinson's disease: an updated meta-analysis of case-control studies. *PLoS ONE* (2014) 9:e85781. doi: 10.1371/journal.pone.0085781
134. Cereda E, Barichella M, Pedrolli C, Klersy C, Cassani E, Caccialanza R, et al. Diabetes and risk of Parkinson's disease: a systematic review and meta-analysis. *Diabetes Care* (2011) 34:2614–23. doi: 10.2337/dc11-1584
135. Yue X, Li H, Yan H, Zhang P, Chang L, Li T. Risk of Parkinson disease in diabetes mellitus: an updated meta-analysis of population-based cohort studies. *Medicine* (2016) 95:e3549. doi: 10.1097/MD.0000000000003549
136. Crane PK, Walker R, Hubbard RA, Li G, Nathan DM, Zheng H, et al. Glucose levels and risk of dementia. *N Engl J Med*. (2013) 369:540–8. doi: 10.1056/NEJMoa1215740
137. Walker Z, Possin KL, Boeve BF, Aarsland D. Lewy body dementias. *Lancet* (2015) 386:1683–97. doi: 10.1016/S0140-6736(15)00462-6
138. Ong M, Foo H, Chander RJ, Wen MC, Au WL, Sitoh YY, et al. Influence of diabetes mellitus on longitudinal atrophy and cognition in Parkinson's disease. *J Neurol Sci*. (2017) 377:122–6. doi: 10.1016/j.jns.2017.04.010
139. Bosco D, Plastino M, Cristiano D, Colica C, Ermio C, De Bartolo M, et al. Dementia is associated with insulin resistance in patients with Parkinson's disease. *J Neurol Sci*. (2012) 315:39–43. doi: 10.1016/j.jns.2011.12.008
140. Secnik J, Cermakova P, Fereshtehnejad SM, Dannberg P, Johnell K, Fastbom J, et al. Diabetes in a large dementia cohort: clinical characteristics and treatment from the Swedish dementia registry. *Diabetes Care* (2017) 40:1159–66. doi: 10.2337/dc16-2516
141. Levy G, Tang MX, Cote LJ, Louis ED, Alfaro B, Mejia H, et al. Do risk factors for Alzheimer's disease predict dementia in Parkinson's disease? An exploratory study. *Mov Disord.* (2002) 17:250–7. doi: 10.1002/mds.10086
142. Malek N, Lawton MA, Swallow DM, Grosset KA, Marrinan SL, Bajaj N, et al. Vascular disease and vascular risk factors in relation to motor features and cognition in early Parkinson's disease. *Mov Disord.* (2016) 31:1518–26. doi: 10.1002/mds.26698
143. Pilotto A, Turrone R, Liepelt-Scarfone I, Bianchi M, Poli L, Borroni B, et al. Vascular risk factors and cognition in Parkinson's disease. *J Alzheimer's Dis.* (2016) 51:563–70. doi: 10.3233/JAD-150610
144. Xu Y, Yang J, Shang H. Meta-analysis of risk factors for Parkinson's disease dementia. *Transl Neurodegener*. (2016) 5:11. doi: 10.1186/s40035-016-0058-0
145. Malfitano AM, Marasco G, Proto MC, Laezza C, Gazzerro P, Bifulco M. Statins in neurological disorders: an overview and update. *Pharmacol Res.* (2014) 88:74–83. doi: 10.1016/j.phrs.2014.06.007
146. Abe K, Aoki M, Tsuji S, Itoyama Y, Sobue G, Togo M, et al. Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. *Lancet Neurol.* (2017) 16:505–12. doi: 10.1016/S1474-4422(17)30115-1
147. Mullard A. 2017 FDA drug approvals. *Nat Rev Drug Discov.* (2018) 17:81. doi: 10.1038/nrd.2018.4
148. Kioumourtzoglou MA, Rotem RS, Seals RM, Gredal O, Hansen J, Weisskopf MG. Diabetes mellitus, obesity, and diagnosis of amyotrophic lateral sclerosis: a population-based study. *JAMA Neurol*. (2015) 72:905–11. doi: 10.1001/jamaneurol.2015.0910
149. Mariosa D, Kamel F, Bellocco R, Ye W, Fang F. Association between diabetes and amyotrophic lateral sclerosis in Sweden. *Eur J Neurol.* (2015) 22:1436–42. doi: 10.1111/ene.12632
150. Jawaid A, Salamone AR, Strutt AM, Murthy SB, Wheaton M, McDowell EJ, et al. ALS disease onset may occur later in patients with pre-morbid diabetes mellitus. *Eur J Neurol.* (2010) 17:733–9. doi: 10.1111/j.1468-1331.2009.02923.x
151. D'Ovidio F, d'Errico A, Carna P, Calvo A, Costa G, Chio A. The role of pre-morbid diabetes on developing amyotrophic lateral sclerosis. *Eur J Neurol.* (2018) 25:164–70. doi: 10.1111/ene.13465
152. Paganoni S, Hyman T, Shui A, Allred P, Harms M, Liu J, et al. Pre-morbid type 2 diabetes mellitus is not a prognostic factor in amyotrophic lateral sclerosis. *Muscle Nerve* (2015) 52:339–43. doi: 10.1002/mus.24688
153. Sun Y, Lu CJ, Chen RC, Hou WH, Li CY. Risk of amyotrophic lateral sclerosis in patients with diabetes: a nationwide population-based cohort study. *J Epidemiol.* (2015) 25:445–51. doi: 10.2188/jea.JE20140176
154. Hollinger SK, Okosun IS, Mitchell CS. Antecedent disease and amyotrophic lateral sclerosis: what is protecting whom? *Front Neurol.* (2016) 7:47. doi: 10.3389/fneur.2016.00047
155. Park Y, Park J, Kim Y, Baek H, Kim SH. Association between nutritional status and disease severity using the amyotrophic lateral sclerosis (ALS) functional rating scale in ALS patients. *Nutrition* (2015) 31:1362–7. doi: 10.1016/j.nut.2015.05.025
156. Dupuis L, Dengler R, Heneka MT, Meyer T, Zierz S, Kassubek J, et al. A randomized, double blind, placebo-controlled trial of pioglitazone in combination with riluzole in amyotrophic lateral sclerosis. *PLoS ONE* (2012) 7:e37885. doi: 10.1371/journal.pone.0037885
157. Wills AM, Hubbard J, Macklin EA, Glass J, Tandan R, Simpson EP, et al. Hypercaloric enteral nutrition in patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled phase 2 trial. *Lancet* (2014) 383:2065–72. doi: 10.1016/S0140-6736(14)60222-1
158. Levine TD, Bowser R, Hank NC, Gately S, Stephan D, Saperstein DS, et al. A pilot trial of pioglitazone HCl and tretinoin in ALS: cerebrospinal fluid biomarkers to monitor drug efficacy and predict rate of disease progression. *Neurol Res Int*. (2012) 2012:582075. doi: 10.1155/2012/582075
159. Lalic NM, Maric J, Svetel M, Jotic A, Stefanova E, Lalic K, et al. Glucose homeostasis in Huntington disease: abnormalities in insulin sensitivity and early-phase insulin secretion. *Arch Neurol.* (2008) 65:476–80. doi: 10.1001/archneur.65.4.476
160. Montojo MT, Aganzo M, Gonzalez N. Huntington's disease and diabetes: chronological sequence of its association. *J Huntington's Dis.* (2017) 6:179–88. doi: 10.3233/JHD-170253
161. Hu Y, Liang J, Yu S. High prevalence of diabetes mellitus in a five-generation Chinese family with Huntington's disease. *J Alzheimer's Dis.* (2014) 40:863–8. doi: 10.3233/JAD-131847
162. Boesgaard TW, Nielsen TT, Josefsen K, Hansen T, Jorgensen T, Pedersen O, et al. Huntington's disease does not appear to increase the risk of diabetes mellitus. *J Neuroendocrinol*. (2009) 21:770–6. doi: 10.1111/j.1365-2826.2009.01898.x
163. Russo CV, Salvatore E, Sacca F, Tucci T, Rinaldi C, Sorrentino P, et al. Insulin sensitivity and early-phase insulin secretion in normoglycemic Huntington's disease patients. *J Huntington's Dis.* (2013) 2:501–7. doi: 10.3233/JHD-130078
164. Sriwijitkamol A, Coletta DK, Wajcberg E, Balbontin GB, Reyna SM, Barrientes J, et al. Effect of acute exercise on AMPK signaling in skeletal muscle of subjects with type 2 diabetes: a time-course and dose-response study. *Diabetes* (2007) 56:836–48. doi: 10.2337/db06-1119
165. Xu XJ, Gauthier MS, Hess DT, Apovian CM, Cacicedo JM, Gokce N, et al. Insulin sensitive and resistant obesity in humans: AMPK activity, oxidative stress, and depot-specific changes in gene expression in adipose tissue. *J Lipid Res*. (2012) 53:792–801. doi: 10.1194/jlr.P022905
166. Culmsee C, Monnig J, Kemp BE, Mattson MP. AMP-activated protein kinase is highly expressed in neurons in the developing rat brain and promotes neuronal survival following glucose deprivation. *J Mol Neurosci.* (2001) 17:45–58. doi: 10.1385/JMN:17:1:45
167. Garcia-Gil M, Pesi R, Perna S, Allegrini S, Giannecchini M, Camici M, et al. 5'-aminoimidazole-4-carboxamide riboside induces apoptosis in human neuroblastoma cells. *Neuroscience* (2003) 117:811–20. doi: 10.1016/S0306-4522(02)00836-9
168. Meares GP, Qin H, Liu Y, Holdbrooks AT, Benveniste EN. AMP-activated protein kinase restricts IFN-gamma signaling. *J Immunol.* (2013) 190:372–80. doi: 10.4049/jimmunol.1202390
169. Greco SJ, Sarkar S, Johnston JM, Tezapsidis N. Leptin regulates tau phosphorylation and amyloid through AMPK in neuronal cells. *Biochem Biophys Res Commun*. (2009) 380:98–104. doi: 10.1016/j.bbrc.2009.01.041
170. Vingtdeux V, Giliberto L, Zhao H, Chandakkar P, Wu Q, Simon JE, et al. AMP-activated protein kinase signaling activation by resveratrol modulates amyloid-beta peptide metabolism. *J Biol Chem*. (2010) 285:9100–13. doi: 10.1074/jbc.M109.060061

171. Thornton C, Bright NJ, Sastre M, Muckett PJ, Carling D. AMP-activated protein kinase (AMPK) is a tau kinase, activated in response to amyloid beta-peptide exposure. *Biochem J*. (2011) 434:503–12. doi: 10.1042/BJ20101485
172. Mairet-Coello G, Courchet J, Pieraut S, Courchet V, Maximov A, Polleux F. The CAMKK2-AMPK kinase pathway mediates the synaptotoxic effects of Abeta oligomers through Tau phosphorylation. *Neuron* (2013) 78:94–108. doi: 10.1016/j.neuron.2013.02.003
173. Maziere JC, Maziere C, Mora L, Gardette J, Salmon S, Auclair M, et al. The antidiabetic drug metformin decreases cholesterol metabolism in cultured human fibroblasts. *Atherosclerosis* (1988) 71:27–33. doi: 10.1016/0021-9150(88)90299-7
174. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, DePinho RA, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* (2005) 310:1642–6. doi: 10.1126/science.1120781
175. Stephenne X, Foretz M, Taleux N, van der Zon GC, Sokal E, Hue L, et al. Metformin activates AMP-activated protein kinase in primary human hepatocytes by decreasing cellular energy status. *Diabetologia* (2011) 54:3101–10. doi: 10.1007/s00125-011-2311-5
176. Chiang MC, Cheng YC, Chen SJ, Yen CH, Huang RN. Metformin activation of AMPK-dependent pathways is neuroprotective in human neural stem cells against Amyloid-beta-induced mitochondrial dysfunction. *Exp Cell Res*. (2016) 347:322–31. doi: 10.1016/j.yexcr.2016.08.013
177. Gupta A, Bisht B, Dey CS. Peripheral insulin-sensitizer drug metformin ameliorates neuronal insulin resistance and Alzheimer's-like changes. *Neuropharmacology* (2011) 60:910–20. doi: 10.1016/j.neuropharm.2011.01.033
178. Hettich MM, Matthes F, Ryan DP, Griesche N, Schroder S, Dorn S, et al. The anti-diabetic drug metformin reduces BACE1 protein level by interfering with the MID1 complex. *PLoS ONE* (2014) 9:e102420. doi: 10.1371/journal.pone.0102420
179. Chen Y, Zhou K, Wang R, Liu Y, Kwak YD, Ma T, et al. Antidiabetic drug metformin (GlucophageR) increases biogenesis of Alzheimer's amyloid peptides via up-regulating BACE1 transcription. *Proc Natl Acad Sci USA*. (2009) 106:3907–12. doi: 10.1073/pnas.0807991106
180. Choi JS, Park C, Jeong JW. AMP-activated protein kinase is activated in Parkinson's disease models mediated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Biochem Biophys Res Commun*. (2010) 391:147–51. doi: 10.1016/j.bbrc.2009.11.022
181. Ng CH, Guan MS, Koh C, Ouyang X, Yu F, Tan EK, et al. AMP kinase activation mitigates dopaminergic dysfunction and mitochondrial abnormalities in Drosophila models of Parkinson's disease. *J Neurosci*. (2012) 32:14311–7. doi: 10.1523/JNEUROSCI.0499-12.2012
182. Dulovic M, Jovanovic M, Xilouri M, Stefanis L, Harhaji-Trajkovic L, Kravic-Stevovic T, et al. The protective role of AMP-activated protein kinase in alpha-synuclein neurotoxicity in vitro. *Neurobiol Dis*. (2014) 63:1–11. doi: 10.1016/j.nbd.2013.11.002
183. Bobela W, Nazeeruddin S, Knott G, Aebischer P, Schneider BL. Modulating the catalytic activity of AMPK has neuroprotective effects against alpha-synuclein toxicity. *Mol Neurodegener*. (2017) 12:80. doi: 10.1186/s13024-017-0220-x
184. Jiang P, Gan M, Ebrahim AS, Castanedes-Casey M, Dickson DW, Yen SH. Adenosine monophosphate-activated protein kinase overactivation leads to accumulation of alpha-synuclein oligomers and decrease of neurites. *Neurobiol Aging* (2013) 34, 1504–15. doi: 10.1016/j.neurobiolaging.2012.11.001
185. Duran-Aniotz C, Hetz C. Glucose metabolism: a sweet relief of Alzheimer's disease. *Curr Biol*. (2016) 26:R806–9. doi: 10.1016/j.cub.2016.07.060
186. Herrero-Mendez A, Almeida A, Fernandez E, Maestre C, Moncada S, Bolanos JP. The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nat Cell Biol*. (2009) 11:747–52. doi: 10.1038/ncb1881
187. Lee Y, Morrison BM, Li Y, Lengacher S, Farah MH, Hoffman PN, et al. Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* (2012) 487:443–8. doi: 10.1038/nature11314
188. Funfschilling U, Supplie LM, Mahad D, Boretius S, Saab AS, Edgar J, et al. Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature* (2012) 485:517–21. doi: 10.1038/nature11007
189. Bouzier-Sore AK, Voisin P, Canioni P, Magistretti PJ, Pellerin L. Lactate is a preferential oxidative energy substrate over glucose for neurons in culture. *J Cereb Blood Flow Metab*. (2003) 23:1298–306. doi: 10.1097/01.WCB.0000091761.61714.25
190. Borghammer P. Perfusion and metabolism imaging studies in Parkinson's disease. *Dan Med J*. (2012) 59:B4466.
191. Woods SC, Lotter EC, McKay LD, Porte D Jr. Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature* (1979) 282:503–5. doi: 10.1038/282503a0
192. Obici S, Zhang BB, Karkanias G, Rossetti L. Hypothalamic insulin signaling is required for inhibition of glucose production. *Nat Med*. (2002) 8:1376–82. doi: 10.1038/nm1202-798
193. Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. *Nature* (2006) 443:289–95. doi: 10.1038/nature05026
194. Lozovsky D, Saller CF, Kopin IJ. Dopamine receptor binding is increased in diabetic rats. *Science* (1981) 214:1031–3. doi: 10.1126/science.6458088
195. Lozovsky DB, Kopin IJ, Saller CF. Modulation of dopamine receptor supersensitivity by chronic insulin: implication in schizophrenia. *Brain Res*. (1985) 343:190–3. doi: 10.1016/0006-8993(85)91178-3
196. Danguir J, Elghozi JL, Laude D. Increased dopamine and serotonin metabolites in CSF during severe insulin-induced hypoglycemia in freely moving rats. *Neurochem Int* (1984) 6:71–5. doi: 10.1016/0197-0186(84)90028-7
197. Colin E, Regulier E, Perrin V, Durr A, Brice A, Aebischer P, et al. Akt is altered in an animal model of Huntington's disease and in patients. *Eur J Neurosci*. (2005) 21:1478–88. doi: 10.1111/j.1460-9568.2005.03985.x
198. Griffin RJ, Moloney A, Kelliher M, Johnston JA, Ravid R, Dockery P, et al. Activation of Akt/PKB, increased phosphorylation of Akt substrates and loss and altered distribution of Akt and PTEN are features of Alzheimer's disease pathology. *J Neurochem*. (2005) 93:105–17. doi: 10.1111/j.1471-4159.2004.02949.x
199. Malagelada C, Jin ZH, Greene LA. RTP801 is induced in Parkinson's disease and mediates neuron death by inhibiting Akt phosphorylation/activation. *J Neurosci*. (2008) 28:14363–71. doi: 10.1523/JNEUROSCI.3928-08.2008
200. Timmons S, Coakley MF, Moloney AM, O' Neill C. Akt signal transduction dysfunction in Parkinson's disease. *Neurosci Lett*. (2009) 467:30–5. doi: 10.1016/j.neulet.2009.09.055
201. Shepherd PR, Withers DJ, Siddle K. Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *Biochem J*. (1998) 333(Pt 3):471–90.
202. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* (2007) 129:1261–74. doi: 10.1016/j.cell.2007.06.009
203. Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, et al. Role of translocation in the activation and function of protein kinase B. *J Biol Chem*. (1997) 272:31515–24. doi: 10.1074/jbc.272.50.31515
204. Brognard J, Sierecki E, Gao T, Newton AC. PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. *Mol Cell* (2007) 25:917–31. doi: 10.1016/j.molcel.2007.02.017
205. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, et al. Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* (1998) 95:29–39. doi: 10.1016/S0092-8674(00)81780-8
206. Yamauchi T, Tobe K, Tamemoto H, Ueki K, Kaburagi Y, Yamamoto-Honda R, et al. Insulin signalling and insulin actions in the muscles and livers of insulin-resistant, insulin receptor substrate 1-deficient mice. *Mol Cell Biol*. (1996) 16:3074–84. doi: 10.1128/MCB.16.6.3074
207. Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, et al. A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell* (1998) 2:559–69. doi: 10.1016/S1097-2765(00)80155-0
208. Previs SF, Withers DJ, Ren JM, White MF, Shulman GI. Contrasting effects of IRS-1 versus IRS-2 gene disruption on carbohydrate and lipid metabolism in vivo. *J Biol Chem*. (2000) 275:38990–4. doi: 10.1074/jbc.M006490200
209. Reger MA, Watson GS, Frey WH II, Baker LD, Cholerton B, Keeling ML, et al. Effects of intranasal insulin on cognition in memory-impaired older adults: modulation by APOE genotype. *Neurobiol Aging* (2006) 27:451–8. doi: 10.1016/j.neurobiolaging.2005.03.016

210. McNay EC, Ong CT, McCrimmon RJ, Cresswell J, Bogan JS, Sherwin RS. Hippocampal memory processes are modulated by insulin and high-fat-induced insulin resistance. *Neurobiol Learn Mem*. (2010) 93:546–53. doi: 10.1016/j.nlm.2010.02.002
211. Takadera T, Sakura N, Mohri T, Hashimoto T. Toxic effect of a beta-amyloid peptide (beta 22-35) on the hippocampal neuron and its prevention. *Neurosci Lett*. (1993) 161:41–4. doi: 10.1016/0304-3940(93)90135-8
212. Rensink AA, Otte-Holler I, de Boer R, Bosch RR, ten Donkelaar HJ, de Waal RM, et al. Insulin inhibits amyloid beta-induced cell death in cultured human brain pericytes. *Neurobiol Aging* (2004) 25:93–103. doi: 10.1016/S0197-4580(03)00039-3
213. Di Carlo M, Picone P, Carrotta R, Giacomazza D, San Biagio PL. Insulin promotes survival of amyloid-beta oligomers neuroblastoma damaged cells via caspase 9 inhibition and Hsp70 upregulation. *J Biomed Biotechnol*. (2010) 2010:147835. doi: 10.1155/2010/147835
214. Mirsky IA, Broh-Kahn RH. The inactivation of insulin by tissue extracts; the distribution and properties of insulin inactivating extracts. *Arch Biochem*. (1949) 20:1–9.
215. Vekrellis K, Ye Z, Qiu WQ, Walsh D, Hartley D, Chesneau V, et al. Neurons regulate extracellular levels of amyloid beta-protein via proteolysis by insulin-degrading enzyme. *J Neurosci*. (2000) 20:1657–65. doi: 10.1523/JNEUROSCI.20-05-01657.2000
216. Farris W, Mansourian S, Chang Y, Lindsley L, Eckman EA, Frosch MP, et al. Insulin-degrading enzyme regulates the levels of insulin, amyloid β-protein, and the β-amyloid precursor protein intracellular domain *in vivo*. *Proc Natl Acad Sci USA*. (2003) 100:4162–7. doi: 10.1073/pnas.0230450100
217. Cook DG, Leverenz JB, McMillan PJ, Kulstad JJ, Ericksen S, Roth RA, et al. Reduced hippocampal insulin-degrading enzyme in late-onset Alzheimer's disease is associated with the apolipoprotein E-epsilon4 allele. *Am J Pathol*. (2003) 162:313–9. doi: 10.1016/S0002-9440(10)63822-9
218. Ertekin-Taner N, Allen M, Fadale D, Scanlin L, Younkin L, Petersen RC, et al. Genetic variants in a haplotype block spanning IDE are significantly associated with plasma Abeta42 levels and risk for Alzheimer disease. *Hum Mutat*. (2004) 23:334–42. doi: 10.1002/humu.20016
219. Zuo X, Jia J. Promoter polymorphisms which modulate insulin degrading enzyme expression may increase susceptibility to Alzheimer's disease. *Brain Res*. (2009) 1249:1–8. doi: 10.1016/j.brainres.2008.10.034
220. Reitz C, Cheng R, Schupf N, Lee JH, Mehta PD, Rogaeva E, et al. Association between variants in IDE-KIF11-HHEX and plasma amyloid beta levels. *Neurobiol Aging* (2012) 33:199.e113–197. doi: 10.1016/j.neurobiolaging.2010.07.005
221. Wang S, He F, Wang Y. Association between polymorphisms of the insulin-degrading enzyme gene and late-onset Alzheimer disease. *J Geriatr Psychiatry Neurol*. (2015) 28:94–8. doi: 10.1177/0891988714554707
222. Kochkina EG, Plesneva SA, Vasilev DS, Zhuravin IA, Turner AJ, Nalivaeva NN. Effects of ageing and experimental diabetes on insulin-degrading enzyme expression in male rat tissues. *Biogerontology* (2015) 16:473–84. doi: 10.1007/s10522-015-9569-9
223. Barber AJ, Nakamura M, Wolpert EB, Reiter CE, Seigel GM, Antonetti DA, et al. Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. *J Biol Chem*. (2001) 276:32814–21. doi: 10.1074/jbc.M104738200
224. Duarte AI, Santos MS, Seica R, de Oliveira CR. Insulin affects synaptosomal GABA and glutamate transport under oxidative stress conditions. *Brain Res*. (2003) 977:23–30. doi: 10.1016/S0006-8993(03)02679-9
225. Yu XR, Jia GR, Gao GD, Wang SH, Han Y, Cao W. Neuroprotection of insulin against oxidative stress-induced apoptosis in cultured retinal neurons: involvement of phosphoinositide 3-kinase/Akt signal pathway. *Acta Biochim Biophys Sin*. (2006) 38:241–8. doi: 10.1111/j.1745-7270.2006.00152.x
226. Moosavi M, Maghsoudi N, Zahedi-Asl S, Naghdi N, Yousefpour M, Trounce IA. The role of PI3/Akt pathway in the protective effect of insulin against corticosterone cell death induction in hippocampal cell culture. *Neuroendocrinology* (2008) 88:293–8. doi: 10.1159/000150441
227. Cusi K, Consoli A, DeFronzo RA. Metabolic effects of metformin on glucose and lactate metabolism in noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab*. (1996) 81:4059–67.
228. Hawley SA, Gadalla AE, Olsen GS, Hardie DG. The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* (2002) 51:2420–5. doi: 10.2337/diabetes.51.8.2420
229. Adak T, Samadi A, Unal AZ, Sabuncuoglu S. A reappraisal on metformin. *Regul Toxicol Pharmacol*. (2017) 92:324–32. doi: 10.1016/j.yrtph.2017.12.023
230. Zakikhani M, Blouin MJ, Piura E, Pollak MN. Metformin and rapamycin have distinct effects on the AKT pathway and proliferation in breast cancer cells. *Breast Cancer Res Treat*. (2010) 123:271–9. doi: 10.1007/s10549-010-0763-9
231. Sarfstein R, Friedman Y, Attias-Geva Z, Fishman A, Bruchim I, Werner H. Metformin downregulates the insulin/IGF-I signaling pathway and inhibits different uterine serous carcinoma (USC) cells proliferation and migration in p53-dependent or -independent manners. *PLoS ONE* (2013) 8:e61537. doi: 10.1371/journal.pone.0061537
232. Memmott RM, Mercado JR, Maier CR, Kawabata S, Fox SD, Dennis PA. Metformin prevents tobacco carcinogen–induced lung tumorigenesis. *Cancer Prevent Res*. (2010) 3:1066–76. doi: 10.1158/1940-6207.CAPR-10-0055
233. Karnevi E, Said K, Andersson R, Rosendahl AH. Metformin-mediated growth inhibition involves suppression of the IGF-I receptor signalling pathway in human pancreatic cancer cells. *BMC Cancer* (2013) 13:235. doi: 10.1186/1471-2407-13-235
234. Mannucci E, Ognibene A, Cremasco F, Bardini G, Mencucci A, Pierazzuoli E, et al. Effect of metformin on glucagon-like peptide 1 (GLP-1) and leptin levels in obese nondiabetic subjects. *Diabetes Care* (2001) 24:489–94. doi: 10.2337/diacare.24.3.489
235. Mannucci E, Tesi F, Bardini G, Ognibene A, Petracca MG, Ciani S, et al. Effects of metformin on glucagon-like peptide-1 levels in obese patients with and without Type 2 diabetes. *Diabetes Nutr Metab*. (2004) 17:336–42.
236. Maida A, Lamont BJ, Cao X, Drucker DJ. Metformin regulates the incretin receptor axis via a pathway dependent on peroxisome proliferator-activated receptor-alpha in mice. *Diabetologia* (2011) 54:339–49. doi: 10.1007/s00125-010-1937-z
237. Athauda D, Maclagan K, Skene SS, Bajwa-Joseph M, Letchford D, Chowdhury K, et al. Exenatide once weekly versus placebo in Parkinson's disease: a randomised, double-blind, placebo-controlled trial. *Lancet* (2017) 390:1664–75. doi: 10.1016/S0140-6736(17)31585-4
238. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell* (2012) 149:274–93. doi: 10.1016/j.cell.2012.03.017
239. Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, et al. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol Cell* (2003) 11:1457–66. doi: 10.1016/S1097-2765(03)00220-X
240. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* (2005) 307:1098–101. doi: 10.1126/science.1106148
241. Walsh PT, Buckler JL, Zhang J, Gelman AE, Dalton NM, Taylor DK, et al. PTEN inhibits IL-2 receptor-mediated expansion of CD4+ CD25+ Tregs. *J Clin Invest*. (2006) 116:2521–31. doi: 10.1172/JCI28057
242. Garcia-Cao I, Song MS, Hobbs RM, Laurent G, Giorgi C, de Boer VC, et al. Systemic elevation of PTEN induces a tumor-suppressive metabolic state. *Cell* (2012) 149:49–62. doi: 10.1016/j.cell.2012.02.030
243. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* (2003) 115:577–90. doi: 10.1016/S0092-8674(03)00929-2
244. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell* (2008) 30:214–26. doi: 10.1016/j.molcel.2008.03.003
245. Brown EJ, Beal PA, Keith CT, Chen J, Shin TB, Schreiber SL. Control of p70 s6 kinase by kinase activity of FRAP in vivo. *Nature* (1995) 377:441–6. doi: 10.1038/377441a0
246. Brunn GJ, Hudson CC, Sekulic A, Williams JM, Hosoi H, Houghton PJ, et al. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science* (1997) 277:99–101. doi: 10.1126/science.277.5322.99

247. Yip CK, Murata K, Walz T, Sabatini DM, Kang SA. Structure of the human mTOR complex I and its implications for rapamycin inhibition. *Mol Cell* (2010) 38:768–74. doi: 10.1016/j.molcel.2010.05.017
248. Khamzina L, Veilleux A, Bergeron S, Marette A. Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. *Endocrinology* (2005) 146:1473–81. doi: 10.1210/en.2004-0921
249. Tremblay F, Krebs M, Dombrowski L, Brehm A, Bernroider E, Roth E, et al. Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. *Diabetes* (2005) 54:2674–84. doi: 10.2337/diabetes.54.9.2674
250. Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* (2004) 431:200–5. doi: 10.1038/nature02866
251. Siddiqui A, Bhaumik D, Chinta SJ, Rane A, Rajagopalan S, Lieu CA, et al. Mitochondrial quality control via the PGC1α-TFEB signaling pathway is compromised by parkin Q311X mutation but independently restored by rapamycin. *J Neurosci.* (2015) 35:12833–44. doi: 10.1523/JNEUROSCI.0109-15.2015
252. Zhang Y, He X, Wu X, Lei M, Wei Z, Zhang X, et al. Rapamycin upregulates glutamate transporter and IL-6 expression in astrocytes in a mouse model of Parkinson's disease. *Cell Death Dis.* (2017) 8:e2611. doi: 10.1038/cddis.2016.491
253. Santini E, Heiman M, Greengard P, Valjent E, Fisone G. Inhibition of mTOR signaling in Parkinson's disease prevents L-DOPA–induced dyskinesia. *Sci Signal.* (2009) 2:ra36. doi: 10.1126/scisignal.2000308
254. Dazert E, Hall MN. mTOR signaling in disease. *Curr Opin Cell Biol.* (2011) 23:744–55. doi: 10.1016/j.ceb.2011.09.003
255. Wang C, Yu JT, Miao D, Wu ZC, Tan MS, Tan, L (2014). Targeting the mTOR signaling network for Alzheimer's disease therapy. *Mol Neurobiol.* 49:120–35. doi: 10.1007/s12035-013-8505-8
256. Spilman P, Podlutskaya N, Hart MJ, Debnath J, Gorostiza O, Bredesen D, et al. Inhibition of mTOR by rapamycin abolishes cognitive deficits and reduces amyloid-beta levels in a mouse model of Alzheimer's disease. *PLoS ONE* (2010) 5:e9979. doi: 10.1371/journal.pone.0009979
257. Caccamo A, De Pinto V, Messina A, Branca C, and Oddo S. Genetic reduction of mammalian target of rapamycin ameliorates Alzheimer's disease-like cognitive and pathological deficits by restoring hippocampal gene expression signature. *J Neurosc.* (2014) 34:7988–98. doi: 10.1523/JNEUROSCI.0777-14.2014
258. Caccamo A, Magri A, Medina DX, Wisely EV, Lopez-Aranda MF, Silva AJ, et al. mTOR regulates tau phosphorylation and degradation: implications for Alzheimer's disease and other tauopathies. *Aging Cell* (2013) 12:370–80. doi: 10.1111/acel.12057
259. Shi WY, Xiao D, Wang L, Dong LH, Yan ZX, Shen ZX, et al. Therapeutic metformin/AMPK activation blocked lymphoma cell growth via inhibition of mTOR pathway and induction of autophagy. *Cell Death Dis.* (2012) 3:e275. doi: 10.1038/cddis.2012.13
260. Nair V, Pathi S, Jutooru I, Sreevalsan S, Basha R, Abdelrahim M, et al. Metformin inhibits pancreatic cancer cell and tumor growth and downregulates Sp transcription factors. *Carcinogenesis* (2013) 34:2870–9. doi: 10.1093/carcin/bgt231
261. Slomovitz BM, Coleman RL. The PI3K/AKT/mTOR pathway as a therapeutic target in endometrial cancer. *Clin Cancer Res.* (2012) 18:5856–64. doi: 10.1158/1078-0432.CCR-12-0662
262. Ben Sahra I, Regazzetti C, Robert G, Laurent K, Le Marchand-Brustel Y, Auberger P, et al. Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1. *Cancer Res*. (2011) 71:4366–72. doi: 10.1158/0008-5472.CAN-10-1769
263. Kalender A, Selvaraj A, Kim SY, Gulati P, Brule S, Viollet B, et al. Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell Metab*. (2010) 11:390–401. doi: 10.1016/j.cmet.2010.03.014
264. Heneka MT, Kummer MP, Latz E. Innate immune activation in neurodegenerative disease. *Nat Rev Immunol.* (2014) 14:463–77. doi: 10.1038/nri3705
265. Weggen S, Eriksen JL, Das P, Sagi SA, Wang R, Pietrzik CU, et al. A subset of NSAIDs lower amyloidogenic Abeta42 independently of cyclooxygenase activity. *Nature* (2001) 414:212–6. doi: 10.1038/35102591
266. Sastre M, Dewachter I, Landreth GE, Willson TM, Klockgether T, van Leuven F, et al. Nonsteroidal anti-inflammatory drugs and peroxisome proliferator-activated receptor-gamma agonists modulate immunostimulated processing of amyloid precursor protein through regulation of beta-secretase. *J Neurosci.* (2003) 23:9796–804. doi: 10.1523/JNEUROSCI.23-30-09796.2003
267. Perlmutter LS, Barron E, Chui HC. Morphologic association between microglia and senile plaque amyloid in Alzheimer's disease. *Neurosci Lett*. (1990) 119:32–6. doi: 10.1016/0304-3940(90)90748-X
268. Wisniewski HM, Wegiel J, Wang KC, Lach B. Ultrastructural studies of the cells forming amyloid in the cortical vessel wall in Alzheimer's disease. *Acta Neuropathol.* (1992) 84:117–27. doi: 10.1007/BF00311383
269. Zhang Y, Zhou B, Deng B, Zhang F, Wu J, Wang Y, et al. Amyloid-beta induces hepatic insulin resistance *in vivo* via JAK2. *Diabetes* (2013) 62:1159–66. doi: 10.2337/db12-0670
270. Vodovotz Y, Lucia MS, Flanders KC, Chesler L, Xie QW, Smith TW, et al. Inducible nitric oxide synthase in tangle-bearing neurons of patients with Alzheimer's disease. *J Exp Med*. (1996) 184:1425–33. doi: 10.1084/jem.184.4.1425
271. Heneka MT, Wiesinger H, Dumitrescu-Ozimek L, Riederer P, Feinstein DL, Klockgether T. Neuronal and glial coexpression of argininosuccinate synthetase and inducible nitric oxide synthase in Alzheimer disease. *J Neuropathol Exp Neurol.* (2001) 60:906–16. doi: 10.1093/jnen/60.9.906
272. Bhaskar K, Konerth M, Kokiko-Cochran ON, Cardona A, Ransohoff RM, Lamb BT. Regulation of tau pathology by the microglial fractalkine receptor. *Neuron* (2010) 68:19–31. doi: 10.1016/j.neuron.2010.08.023
273. McGeer PL, Itagaki S, Boyes BE, McGeer EG. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* (1988) 38:1285–91. doi: 10.1212/WNL.38.8.1285
274. Damier P, Hirsch EC, Zhang P, Agid Y, Javoy-Agid F. Glutathione peroxidase, glial cells and Parkinson's disease. *Neuroscience* (1993) 52:1–6. doi: 10.1016/0306-4522(93)90175-F
275. Czlonkowska A, Kohutnicka M, Kurkowska-Jastrzebska I, Czlonkowski A. Microglial reaction in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced Parkinson's disease mice model. *Neurodegeneration* (1996) 5:137–43.
276. Sugama S, Yang L, Cho BP, DeGiorgio LA, Lorenzl S, Albers DS, et al. Age-related microglial activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration in C57BL/6 mice. *Brain Res.* (2003) 964:288–94. doi: 10.1016/S0006-8993(02)04085-4
277. Zhang W, Wang T, Pei Z, Miller DS, Wu X, Block ML, et al. Aggregated alpha-synuclein activates microglia: a process leading to disease progression in Parkinson's disease. *FASEB J.* (2005) 19:533–42. doi: 10.1096/fj.04-2751com
278. Giasson BI, Duda JE, Murray IV, Chen Q, Souza JM, Hurtig HI, et al. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* (2000) 290:985–9. doi: 10.1126/science.290.5493.985
279. Sancho D, Enamorado M, Garaude J. Innate immune function of mitochondrial metabolism. *Front Immunol.* (2017) 8:527. doi: 10.3389/fimmu.2017.00527
280. Torres-Odio S, Key J, Hoepken H.-H., Canet-Pons J, Valek L, Roller B, et al. Progression of pathology in PINK1-deficient mouse brain from splicing via ubiquitination, ER stress, and mitophagy changes to neuroinflammation. *J Neuroinflammation* (2017) 14:154. doi: 10.1186/s12974-017-0928-0
281. West AP, Khoury-Hanold W, Staron M, Tal MC, Pineda CM, Lang SM, et al. Mitochondrial DNA stress primes the antiviral innate immune response. *Nature* (2015) 520:553–7. doi: 10.1038/nature14156
282. Isoda K, Young JL, Zirlik A, MacFarlane LA, Tsuboi N, Gerdes N, et al. Metformin inhibits proinflammatory responses and nuclear factor-kappaB in human vascular wall cells. *Arterioscler Thromb Vasc Biol*. (2006) 26:611–7. doi: 10.1161/01.ATV.0000201938.78044.75

283. Gu J, Ye S, Wang S, Sun W, Hu Y. Metformin inhibits nuclear factor-κB activation and inflammatory cytokines expression induced by high glucose via adenosine monophosphate-activated protein kinase activation in rat glomerular mesangial cells *in vitro*. *Chin Med J (Engl)* (2014) 127:1755–60. doi: 10.3760/cma.j.issn.0366-6999.20132781
284. Woo SL, Xu H, Li H, Zhao Y, Hu X, Zhao J, et al. Metformin ameliorates hepatic steatosis and inflammation without altering adipose phenotype in diet-induced obesity. *PLoS ONE* (2014) 9:e91111. doi: 10.1371/journal.pone.0091111
285. Cameron AR, Morrison VL, Levin D, Mohan M, Forteath C, Beall C, et al. Anti-inflammatory effects of metformin irrespective of diabetes status. *Circ Res*. (2016) 119:652–65. doi: 10.1161/CIRCRESAHA.116.308445
286. Kaufman BA, Li C, Soleimanpour SA. Mitochondrial regulation of beta-cell function: maintaining the momentum for insulin release. *Mol Aspects Med*. (2015) 42:91–104. doi: 10.1016/j.mam.2015.01.004
287. Lucking CB, Durr A, Bonifati V, Vaughan J, De Michele G, Gasser T, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene. *N Engl J Med*. (2000) 342:1560–7. doi: 10.1056/NEJM200005253422103
288. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* (2003) 299:256–9. doi: 10.1126/science.1077209
289. Valente EM, Abou-Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* (2004) 304:1158–60. doi: 10.1126/science.1096284
290. Winklhofer KF, Haass C. Mitochondrial dysfunction in Parkinson's disease. *Biochim Biophys Acta* (2010) 1802:29–44. doi: 10.1016/j.bbadis.2009.08.013
291. Exner N, Treske B, Paquet D, Holmstrom K, Schiesling C, Gispert S, et al. Loss-of-function of human PINK1 results in mitochondrial pathology and can be rescued by parkin. *J Neurosci.* (2007) 27:12413–8. doi: 10.1523/JNEUROSCI.0719-07.2007
292. Mortiboys H, Aasly J, Bandmann O. Ursocholanic acid rescues mitochondrial function in common forms of familial Parkinson's disease. *Brain* (2013) 136:3038–50. doi: 10.1093/brain/awt224
293. Greene JC, Whitworth AJ, Kuo I, Andrews LA, Feany MB, Pallanck LJ. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. *Proc Natl Acad Sci USA*. (2003) 100:4078–83. doi: 10.1073/pnas.0737556100
294. Palacino JJ, Sagi D, Goldberg MS, Krauss S, Motz C, Wacker M, et al. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. *J Biol Chem*. (2004) 279:18614–22. doi: 10.1074/jbc.M401135200
295. Park J, Lee SB, Lee S, Kim Y, Song S, Kim S, et al. Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. *Nature* (2006) 441:1157–61. doi: 10.1038/nature04788
296. Gispert S, Ricciardi F, Kurz A, Azizov M, Hoepken HH, Becker D, et al. Parkinson phenotype in aged PINK1-deficient mice is accompanied by progressive mitochondrial dysfunction in absence of neurodegeneration. *PLoS ONE* (2009) 4:e5777. doi: 10.1371/journal.pone.0005777
297. Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR, Seol JH, et al. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* (2006) 441:1162–6. doi: 10.1038/nature04779
298. Geisler S, Holmstrom KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, et al. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol*. (2010) 12:119–31. doi: 10.1038/ncb2012
299. Narendra DP, Jin SM, Tanaka A, Suen DF, Gautier CA, Shen J, et al. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol*. (2010) 8:e1000298. doi: 10.1371/journal.pbio.1000298
300. Koyano F, Okatsu K, Kosako H, Tamura Y, Go E, Kimura M, et al. Ubiquitin is phosphorylated by PINK1 to activate parkin. *Nature* (2014) 510:162–6. doi: 10.1038/nature13392
301. Lee JJ, Sanchez-Martinez A, Zarate AM, Benincá C, Mayor U, Clague MJ, et al. (2018). Basal mitophagy is widespread in *Drosophila* but minimally affected by loss of Pink1 or parkin. *J Cell Biol.* 217:1613–22. doi: 10.1083/jcb.201801044
302. McWilliams TG, Prescott AR, Montava-Garriga L, Ball G, Singh F, Barini E, et al. Basal mitophagy occurs independently of PINK1 in mouse tissues of high metabolic demand. *Cell Metab*. (2018) 27:439-449.e435. doi: 10.1016/j.cmet.2017.12.008
303. Morais VA, Haddad D, Craessaerts K, De Bock PJ, Swerts J, Vilain S, et al. PINK1 loss-of-function mutations affect mitochondrial complex I activity via NdufA10 ubiquinone uncoupling. *Science* (2014) 344:203–7. doi: 10.1126/science.1249161
304. Wang X, Winter D, Ashrafi G, Schlehe J, Wong YL, Selkoe D, et al. PINK1 and parkin target miro for phosphorylation and degradation to arrest mitochondrial motility. *Cell* (2011) 147:893–906. doi: 10.1016/j.cell.2011.10.018
305. Plun-Favreau H, Klupsch K, Moisoi N, Gandhi S, Kjaer S, Frith D, et al. The mitochondrial protease HtrA2 is regulated by Parkinson's disease-associated kinase PINK1. *Nat Cell Biol*. (2007) 9:1243–52. doi: 10.1038/ncb1644
306. Pridgeon JW, Olzmann JA, Chin LS, Li L. PINK1 protects against oxidative stress by phosphorylating mitochondrial chaperone TRAP1. *PLoS Biol*. (2007) 5:e172. doi: 10.1371/journal.pbio.0050172
307. Fitzgerald JC, Zimprich A, Carvajal Berrio DA, Schindler KM, Maurer B, Schulte C, et al. Metformin reverses TRAP1 mutation-associated alterations in mitochondrial function in Parkinson's disease. *Brain* (2017) 140:2444–59. doi: 10.1093/brain/awx202
308. Wu W, Xu H, Wang Z, Mao Y, Yuan L, Luo W, et al. PINK1-parkin-mediated mitophagy protects mitochondrial integrity and prevents metabolic stress-induced endothelial injury. *PLoS ONE* (2015) 10:e0132499. doi: 10.1371/journal.pone.0132499
309. Choi J, Ravipati A, Nimmagadda V, Schubert M, Castellani RJ, Russell JW. Potential roles of PINK1 for increased PGC-1α-mediated mitochondrial fatty acid oxidation and their associations with Alzheimer disease and diabetes. *Mitochondrion* (2014) 18:41–8. doi: 10.1016/j.mito.2014.09.005
310. Cang X, Wang X, Liu P, Wu X, Yan J, Chen J, et al. PINK1 alleviates palmitate induced insulin resistance in HepG2 cells by suppressing ROS mediated MAPK pathways. *Biochem Biophys Res Commun*. (2016) 478:431–8. doi: 10.1016/j.bbrc.2016.07.004
311. Deas E, Piipari K, Machhada A, Li A, Gutierrez-del-Arroyo A, Withers DJ, et al. (2014). PINK1 deficiency in β-cells increases basal insulin secretion and improves glucose tolerance in mice. *Open Biol.* 4:140051. doi: 10.1098/rsob.140051
312. Seillier M, Pouyet L, N'Guessan P, Nollet M, Capo F, Guillaumond F, et al. Defects in mitophagy promote redox-driven metabolic syndrome in the absence of TP53INP1. *EMBO Mol Med*. (2015) 7:802–18. doi: 10.15252/emmm.201404318
313. Grassi D, Howard S, Zhou M, Diaz-Perez N, Urban NT, Guerrero-Given D, et al. Identification of a highly neurotoxic alpha-synuclein species inducing mitochondrial damage and mitophagy in Parkinson's disease. *Proc Natl Acad Sci USA*. (2018) 115:E2634–43. doi: 10.1073/pnas.1713849115
314. Butler EK, Voigt A, Lutz AK, Toegel JP, Gerhardt E, Karsten P, et al. The mitochondrial chaperone protein TRAP1 mitigates alpha-Synuclein toxicity. *PLoS Genet*. (2012) 8:e1002488. doi: 10.1371/journal.pgen.1002488
315. Strauss KM, Martins LM, Plun-Favreau H, Marx FP, Kautzmann S, Berg D, et al. Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. *Hum Mol Genet*. (2005) 14:2099–111. doi: 10.1093/hmg/ddi215
316. Simon-Sanchez J, Singleton AB. Sequencing analysis of OMI/HTRA2 shows previously reported pathogenic mutations in neurologically normal controls. *Hum Mol Genet*. (2008) 17:1988–93. doi: 10.1093/hmg/ddn096
317. Fitzgerald JC, Zimprich A, Reddy Bobbili D, Sharma M, May P, Kruger R. Reply: No evidence for rare TRAP1 mutations influencing the risk of idiopathic Parkinson's disease. *Brain* (2018) 141:e17. doi: 10.1093/brain/awx380
318. Gaare JJ, Nido GS, Sztromwasser P, Knappskog PM, Dahl O, Lund-Johansen M, et al. No evidence for rare TRAP1 mutations influencing the risk of idiopathic Parkinson's disease. *Brain* (2018) 141:e16. doi: 10.1093/brain/awx378
319. Rasola A, Neckers L, Picard D. Mitochondrial oxidative phosphorylation TRAP(1)ped in tumor cells. *Trends Cell Biol*. (2014) 24:455–63. doi: 10.1016/j.tcb.2014.03.005
320. Yoshida S, Tsutsumi S, Muhlebach G, Sourbier C, Lee MJ, Lee S, et al. Molecular chaperone TRAP1 regulates a metabolic switch between mitochondrial respiration and aerobic glycolysis. *Proc Natl Acad Sci USA*. (2013) 110:E1604–12. doi: 10.1073/pnas.1220659110

321. Sciacovelli M, Guzzo G, Morello V, Frezza C, Zheng L, Nannini N, et al. The mitochondrial chaperone TRAP1 promotes neoplastic growth by inhibiting succinate dehydrogenase. *Cell Metab*. (2013) 17:988–99. doi: 10.1016/j.cmet.2013.04.019
322. Mills EL, Kelly B, Logan A, Costa ASH, Varma M, Bryant CE, et al. Succinate dehydrogenase supports metabolic repurposing of mitochondria to drive inflammatory macrophages. *Cell* (2016) 167:457–470.e413. doi: 10.1016/j.cell.2016.08.064
323. Matassa DS, Amoroso MR, Lu H, Avolio R, Arzeni D, Procaccini C, et al. Oxidative metabolism drives inflammation-induced platinum resistance in human ovarian cancer. *Cell Death Differ*. (2016) 23:1542–54. doi: 10.1038/cdd.2016.39
324. Guzman JN, Sanchez-Padilla J, Chan CS, Surmeier DJ. Robust pacemaking in substantia nigra dopaminergic neurons. *J Neurosci*. (2009) 29:11011–9. doi: 10.1523/JNEUROSCI.2519-09.2009
325. Swerdlow RH, Burns JM, Khan SM. The Alzheimer's disease mitochondrial cascade hypothesis. *J Alzheimer's Dis*. (2010) 20(Suppl. 2):S265–79. doi: 10.3233/JAD-2010-100339
326. David DC, Hauptmann S, Scherping I, Schuessel K, Keil U, Rizzu P, et al. Proteomic and functional analyses reveal a mitochondrial dysfunction in P301L tau transgenic mice. *J Biol Chem*. (2005) 280:23802–14. doi: 10.1074/jbc.M500356200
327. Rhein V, Song X, Wiesner A, Ittner LM, Baysang G, Meier F, et al. Amyloid-β and tau synergistically impair the oxidative phosphorylation system in triple transgenic Alzheimer's disease mice. *Proc Natl Acad Sci USA*. (2009) 106:20057–62. doi: 10.1073/pnas.0905529106
328. Vossel KA, Zhang K, Brodbeck J, Daub AC, Sharma P, Finkbeiner S, et al. Tau reduction prevents Abeta-induced defects in axonal transport. *Science* (2010) 330:198. doi: 10.1126/science.1194653
329. Calkins MJ, Reddy PH. Amyloid beta impairs mitochondrial anterograde transport and degenerates synapses in Alzheimer's disease neurons. *Biochim Biophys Acta* (2011) 1812:507–13. doi: 10.1016/j.bbadis.2011.01.007
330. Beal MF. Mitochondria take center stage in aging and neurodegeneration. *Ann Neurol*. (2005) 58:495–505. doi: 10.1002/ana.20624
331. Bubber P, Haroutunian V, Fisch G, Blass JP, Gibson GE. Mitochondrial abnormalities in Alzheimer brain: mechanistic implications. *Ann Neurol*. (2005) 57:695–703. doi: 10.1002/ana.20474
332. Johri A, Beal MF. Mitochondrial dysfunction in neurodegenerative diseases. *J Pharmacol Exp Ther*. (2012) 342:619–30. doi: 10.1124/jpet.112.192138
333. Schapira AH, Jenner P. Etiology and pathogenesis of Parkinson's disease. *Mov Disord*. (2011) 26:1049–55. doi: 10.1002/mds.23732
334. Cadenas E, Davies KJ. Mitochondrial free radical generation, oxidative stress, and aging. *Free Radic Biol Med*. (2000) 29:222–30. doi: 10.1016/S0891-5849(00)00317-8
335. Li N, Oquendo E, Capaldi RA, Robinson JP, He YD, Hamadeh HK, et al. A systematic assessment of mitochondrial function identified novel signatures for drug-induced mitochondrial disruption in cells. *Toxicol Sci* (2014) 142:261–73. doi: 10.1093/toxsci/kfu176
336. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell* (2013) 153:1194–217. doi: 10.1016/j.cell.2013.05.039
337. Pakendorf B, Stoneking M. MITOCHONDRIAL DNA AND HUMAN EVOLUTION. *Annu Rev Genomics Hum Genet*. (2005) 6:165–83. doi: 10.1146/annurev.genom.6.080604.162249
338. Houtkooper RH, Mouchiroud L, Ryu D, Moullan N, Katsyuba E, Knott G, et al. Mitonuclear protein imbalance as a conserved longevity mechanism. *Nature* (2013) 497:451–7. doi: 10.1038/nature12188
339. Dolle C, Flones I, Nido GS, Miletic H, Osuagwu N, Kristoffersen S, et al. Defective mitochondrial DNA homeostasis in the substantia nigra in Parkinson disease. *Nat Commun*. (2016) 7:13548. doi: 10.1038/ncomms13548
340. Finkel T. Signal transduction by reactive oxygen species. *J Cell Biol*. (2011) 194:7–15. doi: 10.1083/jcb.201102095
341. Giorgio M, Trinei M, Migliaccio E, Pelicci PG. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nat Rev Mol Cell Biol*. (2007) 8:722–8. doi: 10.1038/nrm2240
342. Andersen JK. Oxidative stress in neurodegeneration: cause or consequence? *Nat Med*. (2004) 10(Suppl.) S18–25. doi: 10.1038/nrn1434
343. Andrews ZB, Diano S, Horvath TL. Mitochondrial uncoupling proteins in the cns: in support of function and survival. *Nat Rev Neurosci*. (2005) 6:829. doi: 10.1038/nrn1767
344. Kahle PJ, Waak J, Gasser T. DJ-1 and prevention of oxidative stress in Parkinson's disease and other age-related disorders. *Free Radic Biol Med*. (2009) 47:1354–61. doi: 10.1016/j.freeradbiomed.2009.08.003
345. Xu J, Zhong N, Wang H, Elias JE, Kim CY, Woldman I, et al. The Parkinson's disease-associated DJ-1 protein is a transcriptional co-activator that protects against neuronal apoptosis. *Hum Mol Genet*. (2005) 14:1231–41. doi: 10.1093/hmg/ddi134
346. Kim RH, Peters M, Jang Y, Shi W, Pintilie M, Fletcher GC, et al. DJ-1, a novel regulator of the tumor suppressor PTEN. *Cancer Cell* (2005) 7:263–73. doi: 10.1016/j.ccr.2005.02.010
347. Wu L, Xu H, Cao L, Li T, Li R, Feng Y, et al. Salidroside protects against MPP+-induced neuronal injury through DJ-1-Nrf2 antioxidant pathway. *Evid Based Complement Altern Med*. (2017) 2017:5398542. doi: 10.1155/2017/5398542
348. Zhou W, Zhu M, Wilson MA, Petsko GA, Fink AL. The oxidation state of DJ-1 regulates its chaperone activity toward alpha-synuclein. *J Mol Biol*. (2006) 356:1036–48. doi: 10.1016/j.jmb.2005.12.030
349. Bjorkblom B, Adilbayeva A, Maple-Grodem J, Piston D, Okvist M, Xu XM, et al. Parkinson disease protein DJ-1 binds metals and protects against metal-induced cytotoxicity. *J Biol Chem*. (2013) 288:22809–20. doi: 10.1074/jbc.M113.482091
350. van der Brug MP, Blackinton J, Chandran J, Hao LY, Lal A, Mazan-Mamczarz K, et al. RNA binding activity of the recessive parkinsonism protein DJ-1 supports involvement in multiple cellular pathways. *Proc Natl Acad Sci USA*. (2008) 105:10244–9. doi: 10.1073/pnas.0708518105
351. Lee JY, Song J, Kwon K, Jang S, Kim C, Baek K, et al. Human DJ-1 and its homologs are novel glyoxalases. *Hum Mol Genet*. (2012) 21:3215–25. doi: 10.1093/hmg/dds155
352. Richarme G, Mihoub M, Dairou J, Bui LC, Leger T, Lamouri A. Parkinsonism-associated protein DJ-1/Park7 is a major protein deglycase that repairs methylglyoxal- and glyoxal-glycated cysteine, arginine, and lysine residues. *J Biol Chem*. (2015) 290:1885–97. doi: 10.1074/jbc.M114.597815
353. Richarme G, Dairou J. Parkinsonism-associated protein DJ-1 is a bona fide deglycase. *Biochem Biophys Res Commun*. (2017) 483:387–91. doi: 10.1016/j.bbrc.2016.12.134
354. Eberhard D, Lammert E. The role of the antioxidant protein DJ-1 in type 2 diabetes mellitus. *Adv Exp Med Biol*. (2017) 1037:173–86. doi: 10.1007/978-981-10-6583-5_11
355. De Jager J, Kooy A, Lehert P, Wulffelé MG, Van Der Kolk J, Bets D, et al. Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. (2010) *BMJ* 340:c2181. doi: 10.1136/bmj.c2181
356. Warasila W. *Forget the Blood of Teens*. This Pill Promises to Extend Life for a Nickel a Pop. Wired Magazine (2017).
357. Lane N. *Power, Sex, Suicide. Mitochondria and the Meaning of Life*. Oxford: Oxford University Press (2005).
358. Barzilai N, Crandall JP, Kritchevsky SB, Espeland MA. Metformin as a tool to target aging. *Cell Metab*. (2016) 23:1060–5. doi: 10.1016/j.cmet.2016.05.011

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 10 August 2018
doi: 10.3389/fendo.2018.00446

Metformin, an Anti-diabetic Drug to Target Leukemia

*Giulia Biondani and Jean-François Peyron**

Team #4: Leukemia: Molecular Addictions, Resistances & Leukemic Stem Cells, Université Nice Côte d'Azur, C3M-Inserm U1065, Paris, France

Metformin, a widely used anti-diabetic molecule, has attracted a strong interest in the last 10 years as a possible new anti-cancer molecule. Metformin acts by interfering with mitochondrial respiration, leading to an activation of the AMPK tumor-suppressive pathway to promote catabolic-energy saving reactions and block anabolic ones that are associated with abnormal cell proliferation. Metformin also acts at the organism level. In type 2 diabetes patients, metformin reduces hyperglycemia and increases insulin sensitivity by enhancing insulin-stimulated glucose uptake in muscles, liver, and adipose tissue and by reducing glucose output by the liver. Lowering insulin and insulin-like growth factor 1 (IGF-1) levels that stimulate cancer growth could be important features of metformin's mode of action. Despite continuous progress in treatments with the use of targeted therapies and now immunotherapies, acute leukemias are still of very poor prognosis for relapse patients, demonstrating an important need for new treatments deriving from the identification of their pathological supportive mechanisms. In the last decade, it has been realized that if cancer cells modify and reprogram their metabolism to feed their intense biochemical needs associated with their runaway proliferation, they develop metabolic addictions that could represent attractive targets for new therapeutic strategies that intend to starve and kill cancer cells. This Mini Review explores the anti-leukemic potential of metformin and its mode of action on leukemia metabolism.

Keywords: metformin, leukemia, chemotherapy, adjuvant, AMPK pathway, metabolism and bioenergetics

OPEN ACCESS

Edited by:
Benoit Viollet,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Giulia Cantini,
Università degli Studi di Firenze, Italy
Marta Letizia Hribal,
Università degli Studi Magna Græcia di Catanzaro, Italy
Jerome Tamburini,
Université Paris Descartes, France

****Correspondence:***
Jean-François Peyron
peyron@unice.fr

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *02 May 2018*
Accepted: *19 July 2018*
Published: *10 August 2018*

Citation:
Biondani G and Peyron J-F (2018) Metformin, an Anti-diabetic Drug to Target Leukemia. Front. Endocrinol. 9:446. doi: 10.3389/fendo.2018.00446

METFORMIN: A TALE OF DRUG REPOSITIONING IN CANCER

Metformin is an active biguanide derivative extracted from the French Lilac (Galega officinalis), a plant discovered during the Middle Age for its healing effects on the diabetic condition. Metformin/Glucophage® was first prescribed in Europe in 1979, then in the United States by 1994 and is now the first-line treatment for type 2 diabetes (T2D) as more than 120 million patients are treated worldwide (1).

In 2001 metformin appeared on the cancer scene when it was observed that in hepatocytes it stimulated the AMP-activated serine threonine protein kinase (AMPK) (2), a sensor of the energetic cellular status and an important tumor suppressor pathway (3, 4).

This discovery prompted clinicians and researchers to measure cancer frequency in T2D patients under metformin. It was first shown in 2005 that metformin significantly reduced cancer incidence in a cohort of 983 T2D patients (5). Other studies confirmed that metformin was associated with a lower risk of cancer in treated diabetic patients (6–8).

These striking results led the renowned cancer researcher Lewis Cantley to consider that "Metformin may have saved more people from cancer deaths than any drug in history" (9).

Numerous investigations worldwide rapidly demonstrated direct anti-cancer effects of metformin on various models (10–12). *In vitro*, metformin exhibits a strong anti-proliferative action on cancer cell lines derived from breast, colon, ovaries, pancreas, lung, and prostate (13–15). These results were strengthened by pre-clinical *in vivo* experiments using xenografts or transgenic mice and chemically-induced cancers. As an example, in a tobacco-induced lung carcinogenesis mouse model, metformin decreases tumor burden by 72% (16). Evidences show that metformin can act through an AMPK dependent (17, 18) or independent (19) way. However, despite metformin is widely used in clinic, its molecular mechanism of action is still under debate.

METFORMIN: MODE(S) OF ACTION

From the different reports it appears that metformin exerts a double action at both organism and molecular levels.

Metformin's Systemic Effects

Within the organism, metformin has an anti-hyperglycemic action but as it does not decrease insulin secretion there is no risk of hypoglycemia in normal subjects (20). In muscles, metformin reduces hyperglycemia through different mechanisms: by enhancing insulin-stimulated glucose uptake and reducing hepatic glucose output (21). It lowers the production of glucose by the liver, and increases glucose utilization by muscles and adipocytes. This results in a decreased insulinemia and an amelioration of insulin sensitivity, likely counteracting the increased glucose uptake by insulin, which facilitates tumor initiation and progression (22). It was thus envisioned that the anti-cancer effects of metformin could be due to its ability to reduce circulating levels of glucose and consequently of insulin and insulin-like growth factor 1(IGF-1) that are suspected to feed different cancers expressing the receptors for these growth factors on their surface (23–26).

Diabetes, in particular T2D, and obesity are clearly associated with an increased risk to develop various cancers (27). However, no increased incidence was observed for hematologic malignancies (28) suggesting at first that the systemic effects of metformin may not apply to leukemia. Nevertheless, a metabolic syndrome with insulin resistance has been reported in leukemic patients exposed to high dose glucocorticoids (29). This could favor a therapy-induced obesity with hyperinsulinemia that supports leukemic cell survival and worsens patient's outcome. Insulin and IGF-1 receptors were found expressed on acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) (30, 31) and insulin stimulates *in vitro* the proliferation of ALL cell lines and primary cells that were sensitive to metformin (32). At the molecular level, an IGF1-IGF-1R autocrine loop is responsible for activation of a leukemia-supportive PI3K/Akt/mTOR pathway (33). Pharmacological interference with the insulin receptor and/or IGF1R autocrine loops affects leukemic proliferation (34) and potentiates the apoptotic action of etoposide (31). Similarly, targeting IGF-1R interferes with the growth of chronic lymphocytic leukemia (CLL) (35).

If insulin/IGF-1 do not appear to be strong oncogenic drivers for acute leukemias, they are likely trophic factors, supporting the rational use of metformin to decrease hyperinsulinemia and to indirectly affect leukemic cells.

Metformin's Molecular Effects

As shown in **Figure 1**, metformine inhibits oxidative respiration by acting on the complex I of the mitochondrial respiratory chain (17, 18), leading to a drop in ATP synthesis, tilting the AMP/ATP balance toward AMP, with the consequent stimulation of AMPK. It is well known that the LKB1/AMPK pathway also regulates the protein synthesis rate through the control of mTOR. Activated AMPK stimulates tuberous sclerosis complex 1/2 (TSC1/2) through phosphorylation and its GTPase-activating protein (GAP) function toward the small G-protein Rheb (Ras homolog enriched in brain), thus determining the switching off of Rheb and resulting in the inhibition of mTOR activity (36–38). AMPK activation requires binding and phosphorylation by the tumor suppressor liver kinase B1 (LKB1) (39, 40). Therefore, the absence of LKB1 impedes an AMPK-negative regulation of cancerous cell metabolism.

The AMPK pathway is a major repressor of the mTOR pathway that uses energy and nutrients to stimulate ATP-consuming anabolic reactions, favoring growth and proliferation (41). Activation of the PI3K/Akt pathway, a major upstream activator of mTOR, is restrained by the lipid phosphatase and tumor suppressor PTEN (phosphatase and tensin homolog), frequently inactivated in cancer (42, 43). Defects in control by PTEN lead to a constitutive activation of the Akt pathway that is involved in the etiology of various pathological conditions such as diabetes, aging, and cancer (44).

The mTOR serine/threonine kinase is the active central component of the mTORC1 and mTORC2 cellular complexes that function to coordinately stimulate cell growth (44). mTORC1 is crucial for the synthesis of proteins, lipids, and nucleic acids while mTORC2 phosphorylates Akt to stimulate proliferation and survival (45, 46). Furthermore, AMPK promotes phosphorylation of TORC2 (transducer of regulated CREB activity 2) to block its nuclear translocation and association with phospho-CREB (CRE binding protein), thus impairing the transcription of genes involved in gluconeogenesis such as peroxisome-proliferator-activated receptor-γ co-activator-1α (PGC-1α), glucose-6-phosphatase (G6Pase), and phosphoenolpyruvate carboxykinase (PEPCK) (38, 47).

Through AMPK stimulation, metformin interferes with mTORC1 activation. In addition, AMPK inhibits ATP formation through fatty acid oxidation (FAO) (48) and stimulates glycolysis by phosphorylation-induced activation of phosphofructo-2-kinase (PFK2) (49). Also, AMPK modulates gene expression for important metabolic enzymes (50) and induces a metabolic cell cycle checkpoint through p53 activation (51). Therefore, AMPK agonists as well as indirect activators such as metformin can be envisioned as promising anti-cancer compounds.

AMPK-Independent Effects of Metformin

Not all actions of metformin are mediated by AMPK (19). Metformin together with hexokinase 2 (HK2) depletion

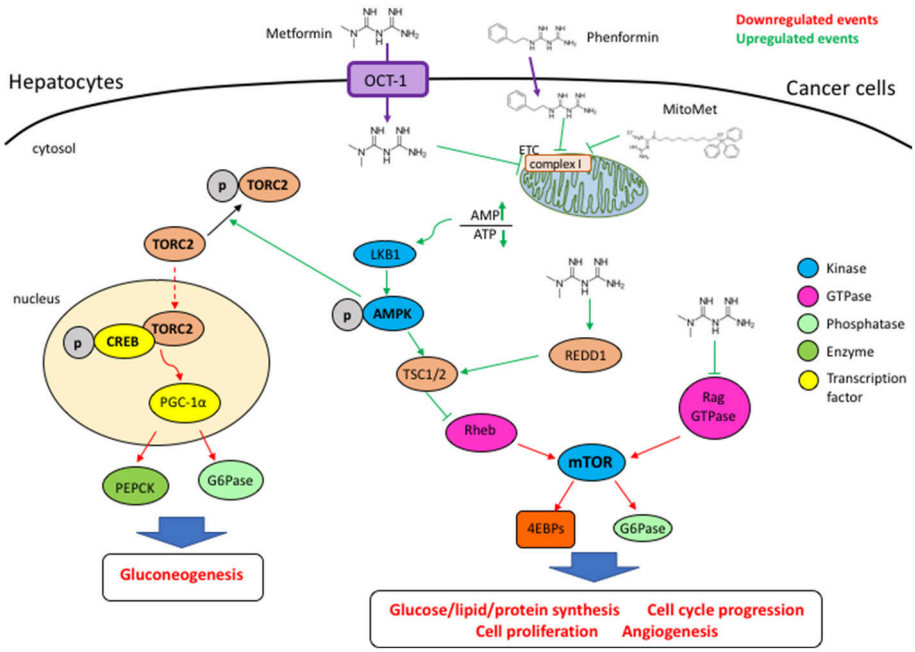

FIGURE 1 | The entrance of metformin is mediated by the OCT1 transporter. By blocking the mitochondrial respiratory chain complex I, metformin and phenformin determine an increase of the AMP/ATP ratio, a condition that activates AMPK through phosphorylation by LKB1. Metformin-activated AMPK counteracts the activation of the mTORC1 complex, impairing cell cycle progression and proliferation, angiogenesis, as well as lipid and protein syntheses. Metformin can induce REDD1 and inhibit Rag GTPases, thus leading to the blocking of mTORC1 through an AMPK-independent way. In an AMPK-dependent way metformin promotes TORC2 phosphorylation and blocks its nuclear translocation, its association with phospho-CREB, impairing the transcription of genes such as PGC-1α, G6Pase, and PEPCK whose products promotes gluconeogenesis.

synergistically interferes with mTORC1 activation through the induction of the mTORC1 inhibitor REDD1 (regulated in development and DNA damage) in hepatocellular carcinoma cells, even upon depletion of AMPKα1 and AMPKα2 (52). Repression of G6Pase and of hepatic glucose production by metformin still occurs in both AMPK and LKB1-deficient hepatocytes (53).

In AML cells metformin can block proliferation at either G0/G1 or S-G2/M, depending on the cell line analyzed. Furthermore, by using a siRNA of AMPKα1/2, Scotland and colleagues showed that metformin-induced cell death is not dependent by AMPK activation in AML cells (54). In prostate cancer cell lines, metformin has AMPK-independent anti-proliferative effects through induction of REDD1 (55). In breast cancer cells metformin interferes with purine/pyrimidine and glutathione synthesis upstream of AMPK (56).

Metformin and resveratrol synergistically block pancreatic cancer cell proliferation *in vitro* and *in vivo* by inhibiting vascular endothelial growth factor B (VEGF-B) signaling pathway (57).

LEUKEMIA

Leukemia represent 2.8% of all cancers and 3.4% of deaths from cancer worldwide, with 351,000 new cases/year. Leukemia results from the transformation of hematopoietic stem-progenitor cells (HSPCs). Acute lymphoid or myeloid leukemia (ALL/AML) show an intense proliferation of immature leukemic blasts arrested at various stages of differentiation (58, 59). Despite important progress in treatments, the 5-year survival for T-ALL is 70–75% for children and only 35–40% for adults (59). New therapeutic strategies should therefore be identified to eradicate leukemia.

Finding New Therapeutic Options for Leukemia

Targeting the energetic metabolism of cancer cells is emerging as an attractive option (60) as cancer/leukemic cells reprogram their metabolism to fulfill their intense metabolic needs. Consequently, they develop metabolic addictions that can be used as new targeting options to starve and kill them (60).

The PI3K/Akt/mTOR Axis Supports Leukemic Growth

The control of the PI3K/Akt/mTOR axis by PTEN is fundamental for the self-renewal of HSCs and PTEN knock-out generates leukemia in mice (61). A common biochemical feature among acute leukemia is the abnormal and constitutive activation of the PI3K/Akt/mTOR pathway (62, 63). Separated or combined pharmacological targeting of PI3K, Akt, or mTOR triggers leukemic cell death in AML and ALL (64, 65). PI-103, a dual inhibitor of PI3K and mTOR displays anti-leukemic properties (66). Unfortunately, the immunosuppressant rapalogs

(Temsirolimus, Everolimus) that target mTORC1 activation, showed a limited anti-cancer activity as they failed to inhibit mTORC2 activity and reactivated the tumor supportive Akt pathway (41, 67, 68). Torkinibs, ATP-competitive inhibitors of the mTOR kinase activity, target both mTOR complexes (69, 70) and have already displayed promising anti-cancer properties on leukemia models (71–73).

Metformin: A New Treatment for Leukemia?

Metformin represents an interesting opportunity to target leukemia through inhibition of constitutive mTOR, a pathological hallmark in leukemogenesis. In 2010 metformin was shown to interfere with AML proliferation and clonogenic activity and to induce apoptosis in human immortalized cell lines and primary samples while it did not affect normal CD34+ HSCs (37). Metformin, after blocking mTORC1 activation, prevents initiation of translation, in particular of c-myc, cyclin D1, and Bcl-xL that are crucial for cancer proliferation (37). Metformin induces apoptosis of leukemic megakaryoblasts from acute megakaryoblastic leukemia (AMKL) which is a rare type of leukemia with poor prognosis (74). Metformin could be an option for the DNA repair defective Fanconi Anemia pre-leukemic disorder as it is toxic after inhibiting the respiratory chain (75).

In T-ALL cells, metformin stimulates AMPK to inhibit mTOR and trigger an autophagic response that precedes apoptosis. By affecting protein synthesis, metformin strongly decreases c-myc and Bcl-xL levels (76). This apoptotic action of metformin in T-ALL also involves an AMPK-dependent activation of the ER stress/unfolded protein response (UPR) (77). In this model, metformin induces a compensatory, anti-apoptotic activation of Akt and of PIM-2, that could be reversed by inhibitors, synergizing with metformin for cell death induction.

Genetic defects in the PTEN tumor suppressor gene are leading to the constitutive activation of the PI3K/Akt/mTOR pathway in T-ALL (78) and are associated with a poor outcome in pediatric T-ALL (79). Tumor cells from a mouse T-ALL model generated by the T-cell specific deletion of PTEN display a constitutive activation of PI3K/Akt/mTOR that could be inhibited by metformin through AMPK activation and by torkinibs (80). Deletion of LKB1 in mice with a PTEN+/- background increases lymphoma incidence that appeared with a shorter latency and were sensitive to metformin (81).

Metformin counteracted the activation of the PI3K/Akt/mTOR pathway triggered by several oncogenes such as the Bcr-abl fusion tyrosine kinase in CML and Phi+ T-ALL and B-ALL and the Tax oncoprotein in HTLV-1-induced ATL (human T-lymphotropic virus type 1-induced adult-T-cell leukemia). Through AMPK activation, metformin suppresses proliferation and clonogenic activity of various CML lines, including those expressing the imatinib-resistant T315I Bcr-abl mutant (82). In ATL, LKB1/AMPK activation by metformin inhibits leukemic proliferation by reducing Tax expression (83). In CLL, metformin prevents cell cycle entry of leukemic cells *in vitro* after engagement of a CD40-CD40L proliferative stimulus (84). CLL cells that are sensitive to the tyrosine kinase inhibitor dasatinib appears to be selectively killed by metformin (85).

Leukemic stem cells (LSCs) are the rare cells at the origin of leukemia and also of relapse because of their intrinsic mechanisms of resistance to chemotherapies (86). Interestingly, in T-ALL metformin targets the Hoescht 33342^{low} side population and the CD34+CD7-CD4- subset that are known to be enriched in LSCs (76). Similarly, cancer stem cells (CSCs) in different solid tumors appears to be highly sensitive to low doses of metformin (87, 88).

Metformin in Combination Therapies

The eradication of cancer will require the combination of multiple therapeutic strategies in a personalized manner. Anti-cancer clinical protocols and drug cocktails would need to be adapted to the specific genetic defects of each patient. Nevertheless, targeting a common dysregulated cellular function such as the reprogrammed cancer metabolism with a metabolic disruptor such as metformin is likely to be an interesting adjuvant approach.

Metformin has already been associated to several classical chemotherapeutic drugs with promising results. Metformin shows additive effects with anthracyclines (doxorubicin, daunorubicin) to reduce growth and survival of lymphoma cells (80), T-ALL cells (89), and ALL (32). The use of metformin could help to reduce the dose of doxorubicin necessary to prolong remission (88) and consequently to reduce cardiac toxicity of anthracyclines.

In T-ALLs metformin synergizes with dexamethasone, the glucocorticoid used as first line treatment for acute leukemias (80), and also potentiates the effect of the microtubule-disrupting agent vincristine (90) and of the topoisomerase II inhibitor etoposide (32).

All-trans retinoic acid (ATRA) is used in acute promyeloid leukemia (APL) to overcome the differentiation block induced by the PML-RAR fusion oncoprotein. By inducing PML-RAR degradation, metformin synergized with ATRA to induce APL cell death (91).

Triggering leukemia apoptosis at the mitochondrial level with the bcl-2 inhibitor ABT-737 is a promising therapy which was shown to be enhanced by metformin-induced mitochondrial membrane depolarization (92). The anti-leukemic activity of the Flt3 inhibitor sorafenib, that was developed to target poor prognosis-ITD Flt3 AML cells, could be enhanced by metformin, thus inducing a strong decrease in the expression of several components of the mTOR pathway (93).

Metformin and Other Metabolic Disruptors

In several studies metformin displays strong potentiating effects when combined with molecules affecting metabolism, in particular glycolysis, such as ritonavir in multiple myeloma (MM) (94) and CLL (95). *In silico*, metformin was predicted to combine with an inhibitor of the Glut4 glucose transporter to affect MM (96).

Disruption of the mitochondrial respiratory complex I by metformin is followed by a compensatory upregulation of glucose

uptake and glycolysis (97, 98). As a consequence metformin was shown to synergize with the non-metabolizable glucose analog and hexokinase inhibitor 2-deoxy-glucose (2-DG) in T-ALL (80) and in CML (99) and with the glycolysis inhibitor sodium dichloroacetate (DCA) in B-CLL (100). Similar effects have been observed in Flt3-positive AML when metformin was associated with the metabolic inhibitor 6-BT (101). Cell death induction of MM cells after disrupting protein homeostasis with the proteasome inhibitor bortezomib can be enhanced by metformin, preventing a protective autophagic response (102). ALL cells display a metabolic dependency on asparagine that can be targeted with L-asparaginase, an effect further amplified by metformin (80).

What Is Better: Targeting the Warburg's Effect or Mitochondria in Leukemia?

In the 1920s, Otto Warburg and colleagues observed for the first time that cancer tissues were taking up enormous amounts of glucose compared to the surrounding tissue. Later, in 1956 Otto Warburg proposed that cancer cells have defective mitochondria because they utilize glucose through aerobic glycolysis, unlike normal cells which use glucose to produce ATP through oxidative phosphorylation (OXPHOS) in mitochondria (103). It was realized a couple of years ago that despite a far less efficient ATP production, this metabolic reprogramming represents an adaptation to optimize the utilization of nutrients to produce the biomass necessary for the generation of new proliferating cancer cells (104–106). Nevertheless, cancer and leukemic cells need active mitochondria for their fitness. Targeting the mitochondria respiratory function by inhibiting the electron transport chain (80), mitochondrial translation (107), or the FAO (108), are all new efficient approaches to kill leukemic cells. Recently, a mitochondrial transfer from stromal cells toward leukemic AML cells provided them with a survival advantage toward chemotherapy (109, 110). An important metabolic plasticity appears to take place as the environment of leukemic cells is changing (111). AML cells can become more sensitive to metformin when cultured in low-glucose medium or after downregulating glycolysis with 2-DG or an Akt inhibitor (54). Similarly, pharmacological approaches to inhibit OXPHOS markedly enhanced the anti-leukemic effects of cytarabine (112).

Metformin for Cancer Patients: Dose and Effects

There are at least two important questions pending about the use of metformin in cancer.

First, will the ability of metformin to control hyperinsulinemia and glycemia in T2D patients stand for non-diabetic people? The 306 registered clinical trials on metformin and cancer will provide important answers. In relation to this review, metformin is tested (NCT01324180) in relapsed childhood ALL in association with vincristine, dexamethasone, doxorubicin, and PEG-asparaginase that are classical drugs for these leukemias. Metformin will be evaluated as a monotherapy for untreated or relapsed CLL patients in a phase 2 pilot study (NCT01750567).

Second, the doses of metformin that are efficient *in vitro* on cancer models are in the mM range, far above those obtained in treated T2D patients (6–30 μM) (113, 114). The cellular entry of the highly hydrophilic metformin is limited by expression of the organic cation transporter (OCT) (115). Interesting areas of research aim at facilitating metformin uptake through specific encapsulation, use of nanocarriers, or after chemical modifications. Coupling a mitochondrial vector to metformin (MitoMet) increases its ability to interfere with OXPHOS and consequently its efficiency to affect proliferation and to trigger ROS-dependent apoptosis in pancreatic cancer *in vitro* and *in vivo*, without affecting normal fibroblasts (116).

Phenformin, a hydrophobic metformin derivative is more active than metformin (81, 117) but was rapidly withdrawn from the market in the late 1970s because of numerous deadly cases of lactic acidosis. We now believe that phenformin could be worth testing as an adjuvant molecule for cancer patients with a monitoring of lactic acidosis. A clinical trial (NCT03026517) will evaluate phenformin in combination with dabrafenib and trametinib for patients with BRAF-mutated melanoma.

Recently Higurashi et al demonstrated the important role of metformin in chemoprevention of colorectal cancer (118). Other clinical trials are ongoing for coloncancer and other tumor types (e.g., NCT03047837; NCT02581137; NCT01312467; NCT01579812; NCT02581137).

CONCLUSIONS AND PERSPECTIVES

Many studies support to use metformin and derivatives like phenformin as global adjuvants for classical anti-leukemic drugs. Improving metformin entry and access to its cellular target(s) through chemical modifications or the use of nanocarriers could be important means to increase the potential of this interesting anti-metabolic molecule.

AUTHOR CONTRIBUTIONS

GB and J-FP equally wrote the article. GB did the illustration work.

FUNDING

This article is supported by an institutional grant from Inserm. GB is supported by a postdoctoral fellowship from INCa.

REFERENCES

1. Bailey CJ, Turner RC. Metformin. *N Engl J Med.* (1996) 334:574–9. doi: 10.1056/NEJM199602293340906

2. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest.* (2001) 108:1167–74. doi: 10.1172/JCI 13505

3. Shackelford DB, Shaw RJ. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. *Nat Rev Cancer* (2009) 9:563–75. doi: 10.1038/nrc2676
4. Hardie DG. AMPK: positive and negative regulation, and its role in whole-body energy homeostasis. *Curr Opin Cell Biol.* (2015) 33:1–7. doi: 10.1016/j.ceb.2014.09.004
5. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ* (2005) 330:1304–5. doi: 10.1136/bmj.38415.708634.F7
6. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, et al. Diabetes and cancer: a consensus report. *CA Cancer J Clin.* (2010) 60:207–21. doi: 10.3322/caac.20078
7. Zhang P, Li H, Tan X, Chen L, Wang S. Association of metformin use with cancer incidence and mortality: a meta-analysis. *Cancer Epidemiol.* (2013) 37:207–18. doi: 10.1016/j.canep.2012.12.009
8. Singh S, Singh H, Singh PP, Murad MH, Limburg PJ. Antidiabetic medications and the risk of colorectal cancer in patients with diabetes mellitus: a systematic review and meta-analysis. *Cancer Epidemiol Biomarkers Prev.* (2013) 22:2258–68. doi: 10.1158/1055-9965.EPI-13-0429
9. Taubes G. Cancer research. Cancer prevention with a diabetes pill. *Science* (2012) 335: 29. doi: 10.1126/science.335.6064.29
10. Ben Sahra I, Le Marchand-Brustel Y, Tanti JF, Bost F. Metformin in cancer therapy: a new perspective for an old antidiabetic drug *Mol Cancer Ther.* (2010) 9:1092–9. doi: 10.1158/1535-7163.MCT-09-1186
11. Dowling RJ, Niraula S, Stambolic V, Goodwin PJ. Metformin in cancer: translational challenges. *J Mol Endocrinol.* (2012) 48:R31–43. doi: 10.1530/JME-12-0007
12. Pollak M. Potential applications for biguanides in oncology. *J Clin Invest.* (2013) 123:3693–700. doi: 10.1172/JCI67232
13. Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F, et al. Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. *Cancer Res.* (2007) 67:6745–52. doi: 10.1158/0008-5472.CAN-06-4447
14. Wang LW, Li ZS, Zou DW, Jin ZD, Gao J, Xu GM. Metformin induces apoptosis of pancreatic cancer cells. *World J Gastroenterol.* (2008) 14:7192–8. doi: 10.3748/wjg.v14.i47.7192
15. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, et al. The antidiabetic drug metformin exerts an antitumoral effect *in vitro* and *in vivo* through a decrease of cyclin Dl level. *Oncogene* (2008) 27:3576–86. doi: 10.1038/sj.onc.1211024
16. Memmott RM, Mercado JR, Maier CR, Kawabata S, Fox SD, Dennis PA. Metformin prevents tobacco carcinogen–induced lung tumorigenesis. *Cancer Prev Res.* (2010) 3:1066–76. doi: 10.1158/1940-6207.CAPR-10-0055
17. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex of the mitochondrial respiratory chain. *Biochem J.* (2000) 348(Pt 3):607–14. doi: 10.1042/bj3480607
18. El-Mir MY, Nogueira V, Fontaine E, Avéret N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem.* (2000) 275:223–8. doi: 10.1074/jbc.275.1.223
19. Miller RA, Birnbaum MJ. An energetic tale of AMPK-independent effects of metformin. *J Clin Invest.* (2010) 120:2267–70. doi: 10.1172/JCI43661
20. Barnett AH. Avoiding hypoglycaemia while achieving good glycaemic control in type diabetes through optimal use of oral agent therapy. *Curr Med Res Opin.* (2010) 26:1333–42. doi: 10.1185/03007991003738063
21. Klip A, Leiter LA. Cellular mechanism of action of metformin. *Diabetes Care* (1990) 13:696–704.
22. Yu X, Mao W, Zhai Y, Tong C, Liu M, Ma L, et al. Anti-tumor activity of metformin: from metabolic and epigenetic perspectives. *Oncotarget* (2017) 8:5619–28. doi: 10.18632/oncotarget.13639
23. Taubes G. Cancer research. Unraveling the obesity-cancer connection. *Science* (2012) 335:28, 30–2. doi: 10.1126/science.335.6064.28
24. Cohen DH, LeRoith D. Obesity, type diabetes, and cancer: the insulin and IGF connection. *Endocr Relat Cancer* (2012) 19:F27–45. doi: 10.1530/ERC-11-0374
25. Gallagher EJ, LeRoith D. The proliferating role of insulin and insulin-like growth factors in cancer. *Trends Endocrinol Metab.* (2010) 21:610–8. doi: 10.1016/j.tem.20
26. Frasca F, Pandini G, Sciacca L, Pezzino V, Squatrito S, Belfiore A, et al. The role of insulin receptors and IGF-I receptors in cancer and other diseases. *Arch Physiol Biochem.* (2008) 114:23–37. doi: 10.1080/13813450801969715
27. Chowdhury TA. Diabetes and cancer. *QJM* (2010) 103:905–15. doi: 10.1093/qjmed/hcq149
28. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med.* (2003) 348:1625–38. doi: 10.1056/NEJMoa021423
29. Chow EJ, Pihoker C, Friedman DL, Lee SJ, McCune JS, Wharton C, et al. Glucocorticoids and insulin resistance in children with acute lymphoblastic leukemia. *Pediatr Blood Cancer* (2013) 60:621–6. doi: 10.1002/pbc.24364
30. Esber EC, Buell DN, Leikin SL. Insulin binding of acute lymphocytic leukemia cells. *Blood* (1976) 48:33–9.
31. Doepfner KT, Spertini O, Arcaro A. Autocrine insulin-like growth factor-I signaling promotes growth and survival of human acute myeloid leukemia cells via the phosphoinositide 3-kinase/Akt pathway. *Leukemia* (2007) 21:1921–30. doi: 10.1038/sj.leu.2404813
32. Pan J, Chen C, Jin Y, Fuentes-Mattei E, Velazquez-Tores G, Benito JM, et al. Differential impact of structurally different anti-diabetic drugs on proliferation and chemosensitivity of acute lymphoblastic leukemia cells. *Cell Cycle* (2012) 11:2314–26. doi: 10.4161/cc.20770
33. Chapuis N, Tamburini J, Cornillet-Lefebvre P, Gillot L, Bardet V, Willems L, et al. Autocrine IGF-1/IGF-1R signaling is responsible for constitutive PI3K/Akt activation in acute myeloid leukemia: therapeutic value of neutralizing anti-IGF-1R antibody. *Haematologica* (2010) 95:415–23. doi: 10.3324/haematol.2009.010785
34. Tazzari PL, Tabellini G, Bortul R, Papa V, Evangelisti C, Grafone T, et al. The insulin-like growth factor-I receptor kinase inhibitor NVP-AEW5 induces apoptosis in acute myeloid leukemia cells exhibiting autocrine insulin-like growth factor-I secretion. *Leukemia* (2007) 21:886–96. doi: 10.1038/sj.leu.2404643
35. Yaktapour N, Übelhart R, Schüler J, Aumann K, Dierks C, Burger M, et al. Insulin-like growth factor-receptor (IGF1R) as a novel target in chronic lymphocytic leukemia. *Blood* (2013) 122:1621–33. doi: 10.1182/blood-2013-02-484386
36. Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. *Cell* (2003) 115:577–90. doi: 10.1016/S0092-8674(03)00929-2
37. Green AS, Chapuis N, Maciel TT, Willems L, Lambert M, Arnoult C, et al. The LKB1/AMPK signaling pathway has tumor suppressor activity in acute myeloid leukemia through the repression of mTOR-dependent oncogenic mRNA translation. *Blood* (2010) 116:4262–73. doi: 10.1182/blood-2010-02-269837
38. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F, Cellular and molecular mechanisms of metformin: an overview. *Clin Sci.* (2012) 122:253–70. doi: 10.1042/CS20110386
39. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, DePinho RA, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci USA.* (2004) 101:3329–35. doi: 10.1073/pnas.0308061100
40. Woods A, Johnstone SR, Dickerson K, Leiper FC, Fryer LG, Neumann D, et al. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol.* (2003) 13:2004–8. doi: 10.1016/j.cub.2003.10.031
41. Foster KG, Fingar DC. Mammalian target of rapamycin (mTOR): conducting the cellular signaling symphony. *J Biol Chem.* (2010) 285:14071–7. doi: 10.1074/jbc.R109.094003
42. Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, et al. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.that is mutated in multiple advanced cancers. *Nat Genet.* (1997) 15: 356-62. doi: 10.1038/ng0497-356
43. Salmena L, Carracedo A, Pandolfi PP. Tenets of PTEN tumor suppression *Cell* (2008) 133:403–14. doi: 10.1016/j.cell.2008.04.013
44. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol.* (2011) 12:21–35. doi: 10.1038/nrm3025
45. Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Mol Cell* (2010) 40:310–22. doi: 10.1016/j.molcel.2010.09.026

46. Ben-Sahra I, Manning BD. mTORCsignaling and the metabolic control of cell growth. *Curr Opin Cell Biol.* (2017) 45:72–82. doi: 10.1016/j.ceb.2017.02.012
47. Koo SH, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, et al. The CREB coactivator TORCis a key regulator of fasting glucose metabolism. *Nature* (2005) 437:1109–11. doi: 10.1038/nature03967
48. Carling D, Zammit VA, Hardie DG. A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. *FEBS Lett.* (1987) 223:217–22.
49. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, et al. Phosphorylation and activation of heart PFK-by AMPK has a role in the stimulation of glycolysis during ischaemia. *Curr Biol.* (2000) 10:1247–55. doi: 10.1016/S0960-9822(00)00742-9
50. Mihaylova MM, Shaw RJ. The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol.* (2011) 13:1016–23. doi: 10.1038/ncb2329
51. Jones RG, Plas DR, Kubek S, Buzzai M, Mu, J. Xu Y, et al. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell* (2005) 18:283–93. doi: 10.1016/j.molcel.2005.03.027
52. DeWaal D, Nogueira V, Terry AR, Patra KC, Jeon SM, Guzman G, et al. Hexokinase-depletion inhibits glycolysis and induces oxidative phosphorylation in hepatocellular carcinoma and sensitizes to metformin. *Nat Commun.* (2018) 9:446. doi: 10.1038/s41467-017-02733-4
53. Foretz M, Hébrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, et al. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest.* (2010) 120:2355–69. doi: 10.1172/JCI40671
54. Scotland S, Saland E, Skuli N, de Toni F, Boutzen H, Micklow E, et al. Mitochondrial energetic and AKT status mediate metabolic effects and apoptosis of metformin in human leukemic cells. *Leukemia* (2013) 27:2129–38. doi: 10.1038/leu.2013.107
55. Ben Sahra I, Regazzetti C, Robert G, Laurent K, Le Marchand-Brustel Y, Auberger P, et al. Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1. *Cancer Res.* (2011) 71:4366–72. doi: 10.1158/0008-5472.CAN-10-1769
56. Corominas-Faja B, Quirantes-Piné R, Oliveras-Ferraros C, Vazquez-Martin A, Cufí S, et al. Metabolomic fingerprint reveals that metformin impairs one-carbon metabolism in a manner similar to the antifolate class of chemotherapy drugs. *Aging* (2012) 4:480–98. doi: 10.18632/aging.100472
57. Zhu M, Zhang Q, Wang X, Kang L, Yang Y, Liu Y, et al. Metformin potentiates anti-tumor effect of resveratrol on pancreatic cancer by down-regulation of VEGF-B signaling pathway. *Oncotarget* (2016) 7:84190–200. doi: 10.18632/oncotarget.12391
58. Löwenberg B, Downing JR, Burnett A. Acute myeloid leukemia. *N Engl J Med.* (1999) 341:1051–62. doi: 10.1056/NEJM199909303411407
59. Pui CH, Evans WE. Treatment of acute lymphoblastic leukemia. *N Engl J Med.* (2006) 354:166–78. doi: 10.1056/NEJMra052603
60. Vander Heiden MG. Exploiting tumor metabolism: challenges for clinical translation *J Clin Invest.* (2013) 123:3648–51. doi: 10.1172/JCI72391
61. Yilmaz OH, Valdez R, Theisen BK, Guo W, Ferguson DO, Wu H, et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* (2006) 441: 475-82. doi: 10.1038/nature04703
62. Barrett D, Brown VI, Grupp SA, Teachey DT. Targeting the PI3K/AKT/mTOR signaling axis in children with hematologic malignancies. *Paediatr Drugs* (2012) 14:299–316. doi: 10.2165/11594740-000000000-00000
63. Polak R, Buitenhuis, M. The PI3K/PKB signaling module as key regulator of hematopoiesis: implications for therapeutic strategies in leukemia. *Blood* (2012) 119:911–23. doi: 10.1182/blood-2011-07-366203
64. Martelli AM, Evangelisti C, Chiarini F, Grimaldi C, Manzoli L, McCubrey JA. Targeting the PI3K/AKT/mTOR signaling network in acute myelogenous leukemia. *Expert Opin Investig Drugs* (2009) 18:1333–49. doi: 10.1517/14728220903136775
65. Simioni C, Neri LM, Tabellini G, Ricci F, Bressanin D, Chiarini F, et al. Cytotoxic activity of the novel Akt inhibitor, MK-2206, in T-cell acute lymphoblastic leukemia. *Leukemia* (2012) 26:2336–42. doi: 10.1038/leu.2012.136
66. Park S, Chapuis N, Bardet V, Tamburini J, Gallay N, Willems L, et al. PI-103, a dual inhibitor of Class IA phosphatidylinositide 3-kinase and mTOR, has antileukemic activity in AML. *Leukemia* (2008) 22:1698–706. doi: 10.1038/leu.2008.144
67. Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, Alimonti A, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. *J Clin Invest.* (2008) 118:3065–74. doi: 10.1172/JCI34739
68. Efeyan A, Sabatini DM. mTOR and cancer: many loops in one pathway. *Curr Opin Cell Biol.* (2010) 22:169–76. doi: 10.1016/j.ceb.2009.10.007
69. García-Martínez JM, Moran J, Clarke RG, Gray A, Cosulich SC, Chresta CM et al. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). *Biochem J* (2009) 421:29–42. doi: 10.1042/BJ20090489
70. Sun SY. mTOR kinase inhibitors as potential cancer therapeutic drugs. *Cancer Lett.* (2013) 340:1–8. doi: 10.1016/j.canlet.2013.06.017
71. Janes MR, Limon JJ, So L, Chen J, Lim RJ, Chavez MA, et al. Effective and selective targeting of leukemia cells using a TORC1/kinase inhibitor. *Nat Med.* (2010) 16:205–13. doi: 10.1038/nm.2091
72. Evangelisti C, Ricci F, Tazzari P, Tabellini G, Battistelli M, Falcieri E, et al. Targeted inhibition of mTORC1 and mTORC2 by active-site mTOR inhibitors has cytotoxic effects in T-cell acute lymphoblastic leukemia. *Leukemia* (2011) 25:781–91. doi: 10.1038/leu.2011.20
73. Willems L, Chapuis N, Puissant A, Maciel TT, Green AS, Jacque N, et al. The dual mTORC1 and mTOR2 Cinhibitor AZD8055 has anti-tumor activity in acute myeloid leukemia. *Leukemia* (2012) 26:1195–202. doi: 10.1038/leu.2011.339
74. Liang X, Kong P, Wang J, Xu Y, Gao C, Guo G. Effects of metformin on proliferation and apoptosis of human megakaryoblastic Dami and MEG-cells. *J Pharmacol Sci.* (2017) 135:14–21. doi: 10.1016/j.jphs.2017.08.003
75. Ravera S, Cossu V, Tappino B, Nicchia E, Dufour C, Cavani S, et al. Concentration-dependent metabolic effects of metformin in healthy and Fanconi anemia lymphoblast cells. *J Cell Physiol.* (2018) 233:1736–51. doi: 10.1002/jcp.26085
76. Grimaldi C, Chiarini F, Tabellini G, Ricci F, Tazzari PL, Battistelli M, et al. AMP-dependent kinase/mammalian target of rapamycin complex signaling in T-cell acute lymphoblastic leukemia: therapeutic implications. *Leukemia* (2012) 26:91–100. doi: 10.1038/leu.2011.269
77. Leclerc GM, Leclerc GJ, Kuznetsov JN, DeSalvo J, Barredo JC. Metformin induces apoptosis through AMPK-dependent inhibition of UPR signaling in ALL lymphoblasts. *PLoS ONE* (2013) 8:e74420. doi: 10.1371/journal.pone.0074420
78. Silva A, Yunes JA, Cardoso BA, Martins LR, Jotta PY, Abecasis M, et al. PTEN posttranslational inactivation and hyperactivation of the PI3K/Akt pathway sustain primary T cell leukemia viability. *J Clin Invest.* (2008) 118:3762–74. doi: 10.1172/JCI34616
79. Jotta PY, Ganazza MA, Silva A, Viana MB, da Silva MJ, Zambaldi LJ, et al. Negative prognostic impact of PTEN mutation in pediatric T-cell acute lymphoblastic leukemia. *Leukemia* (2010) 24:239–42. doi: 10.1038/leu.2009.209
80. Rosilio C, Lounnas N, Nebout M, Imbert V, Hagenbeek T, Spits H, et al. The metabolic perturbators metformin, phenformin and AICAR interfere with the growth and survival of murine PTEN-deficient T cell lymphomas and human T-ALL/T-LL cancer cells. *Cancer Lett.* (2013) 336:114–26. doi: 10.1016/j.canlet.2013.04.015
81. Huang X, Wullschleger S, Shpiro N, McGuire VA, Sakamoto K, Woods YL, et al. Important role of the LKB1-AMPK pathway in suppressing tumorigenesis in PTEN-deficient mice. *Biochem J.* (2008) 412:211–21. doi: 10.1042/BJ20080557
82. Vakana E, Altman JK, Glaser H, Donato NJ, Platanias LC. Antileukemic effects of AMPK activators on BCR-ABL-expressing cells. *Blood* (2011) 118:6399–402. doi: 10.1182/blood-2011-01-332783
83. Tang HM, Gao WW, Chan CP, Siu YT, Wong CM, Kok KH, et al. LKB1 tumor suppressor and salt-inducible kinases negatively regulate human T-cell leukemia virus type transcription. *Retrovirology* (2013) 10:40. doi: 10.1186/1742-4690-10-40
84. Bruno S, Ledda B, Tenca C, Ravera S, Orengo AM, Mazzarello AN, et al. Metformin inhibits cell cycle progression of B-cell chronic lymphocytic leukemia cells. *Oncotarget* (2015) 6:22624–40. doi: 10.18632/oncotarget.4168
85. Martinez Marignac VL, Smith S, Toban N, Bazile M, Aloyz, R. Resistance to Dasatinib in primary chronic lymphocytic leukemia lymphocytes involves AMPK-mediated energetic re-programming. *Oncotarget* (2013) 4:2550–66. doi: 10.18632/oncotarget.1508

86. Huntly BJ, Gilliland DG. Leukaemia stem cells and the evolution of cancer-stem-cell research. *Nat Rev Cancer* (2005) 5:311–21. doi: 10.1038/nrc1592
87. Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res.* (2009) 69:7507–11. doi: 10.1158/0008-5472.CAN-09-2994
88. Iliopoulos D, Hirsch HA, Struhl K. Metformin decreases the dose of chemotherapy for prolonging tumor remission in mouse xenografts involving multiple cancer cell types. *Cancer Res.* (2011) 71:3196–201. doi: 10.1158/0008-5472.CAN-10-3471
89. Shi WY, Xiao D, Wang L, Dong LH, Yan ZX, Shen ZX, et al. Therapeutic metformin/AMPK activation blocked lymphoma cell growth via inhibition of mTOR pathway and induction of autophagy. *Cell Death Dis.* (2012) 3:e275. doi: 10.1038/cddis.2012.13
90. Yi Y, Gao L, Wu M, Ao J, Zhang C, Wang X, et al. Metformin Sensitizes Leukemia Cells to Vincristine via Activation of AMP-activated Protein Kinase. *J Cancer* (2017) 8:2636–42. doi: 10.7150/jca.19873
91. Huai L, Wang C, Zhang C, Li Q, Chen Y, Jia Y, et al. Metformin induces differentiation in acute promyelocytic leukemia by activating the MEK/ERK signaling pathway. *Biochem Biophys Res Commun.* (2012) 422:398–404. doi: 10.1016/j.bbrc.2012.05.001
92. Velez J, Pan R, Lee JT, Enciso L, Suarez M, Duque JE, et al. Biguanides sensitize leukemia cells to ABT-737-induced apoptosis by inhibiting mitochondrial electron transport. *Oncotarget* (2016) 7:51435–49. doi: 10.18632/oncotarget.9843
93. Wang F, Liu Z, Zeng J, Zhu H, Li J, Cheng X, et al. Metformin synergistically sensitizes FLT3-ITD-positive acute myeloid leukemia to sorafenib by promoting mTOR-mediated apoptosis and autophagy. *Leuk Res.* (2015) 39:1421–7. doi: 10.1016/j.leukres.2015.09.016
94. Dalva-Aydemir S, Bajpai R, Martinez M, Adekola KU, Kandela I, Wei C, et al. Targeting the metabolic plasticity of multiple myeloma with FDA-approved ritonavir and metformin. *Clin Cancer Res.* (2015) 21:1161–71. doi: 10.1158/1078-0432.CCR-14-1088
95. Adekola KU, Dalva Aydemir S, Ma S, Zhou Z, Rosen ST, Shanmugam M. Investigating and targeting chronic lymphocytic leukemia metabolism with the human immunodeficiency virus protease inhibitor ritonavir and metformin. *Leuk Lymphoma* (2015) 56:450–9. doi: 10.3109/10428194.2014.922180
96. Mishra RK, Wei C, Hresko RC, Bajpai R, Heitmeier M, Matulis SM, et al. *In silico* modeling-based identification of Glucose Transporter (GLUT4)-selective Inhibitors for Cancer Therapy. *J Biol Chem.* (2015) 290:14441–53. doi: 10.1074/jbc.M114.628826
97. Pryor PR, Liu SC, Clark AE, Yang J, Holman GD, Tosh D. Chronic insulin effects on insulin signalling and GLUT4 endocytosis are reversed by metformin. *Biochem J.* (2000) 3(Pt 1):83–91. doi: 10.1042/0264-6021:3480083
98. Ben Sahra I, Laurent K, Giuliano S, Larbret F, Ponzio G, Gounon P, et al. Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells. *Cancer Res.* (2010) 70:2465–75. doi: 10.1158/0008-5472.CAN-09-2782
99. Xue C, Wang C, Liu Q, Meng Q, Sun H, Huo X, et al. Targeting P-glycoprotein expression and cancer cell energy metabolism: combination of metformin and 2-deoxyglucose reverses the multidrug resistance of K562/Dox cells to doxorubicin. *Tumour Biol.* (2016) 37:8587–97. doi: 10.1007/s13277-015-4478-8
100. Voltan R, Rimondi E, Melloni E, Gilli P, Bertolasi V, Casciano F, et al. Metformin combined with sodium dichloroacetate promotes B leukemic cell death by suppressing anti-apoptotic protein Mcl-1. *Oncotarget* (2016) 7:18965–77. doi: 10.18632/oncotarget.7879
101. Sabnis HS, Bradley HL, Tripathi S, Yu WM, Tse W, Qu CK, et al. Synergistic cell death in FLT3-ITD positive acute myeloid leukemia by combined treatment with metformin and 6-benzylthioinosine. *Leuk Res.* (2016) 50:132–40. doi: 10.1016/j.leukres.2016
102. Jagannathan S, Abdel-Malek MA, Malek E, Vad N, Latif T, Anderson KC, et al. Pharmacologic screens reveal metformin that suppresses GRP78-dependent autophagy to enhance the anti-myeloma effect of bortezomib. *Leukemia* (2015) 29:2184–91. doi: 10.1038/leu.2015.157
103. Warburg O. On the origin of cancer cells. *Science* (1956) 123:309–14.
104. Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. *Cell* (2008) 134:703–7. doi: 10.1016/j.cell.2008.08.021
105. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* (2009) 324:1029–33. doi: 10.1126/science.1160809
106. Ward PS, Thompson CB. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer Cell* (2012) 21:297–308. doi: 10.1016/j.ccr.2012.02.014
107. Skrtić M, Sriskanthadevan S, Jhas B, Gebbia M, Wang X, Wang Z, et al. Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. *Cancer Cell* (2011) 20:674–88. doi: 10.1016/j.ccr.2011.10.015
108. Samudio I, Harmancey R, Fiegl M, Kantarjian H, Konopleva M, Korchin B, et al. Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction. *J Clin Invest.* (2010) 120:142–56. doi: 10.1172/JCI38942
109. Moschoi R, Imbert V, Nebout M, Chiche J, Mary D, et al. Protective mitochondrial transfer from bone marrow stromal cells to acute myeloid leukemic cells during chemotherapy. *Blood* (2016) 128:253–64. doi: 10.1182/blood-2015-07-655860
110. Griessinger E, Moschoi R, Biondani G, Peyron JF. Mitochondrial transfer in the leukemia microenvironment. *Trends Cancer* (2017) 3:828–39. doi: 10.1016/j.trecan.2017.10.003
111. Suganuma K, Miwa H, Imai N, Shikami M, Gotou M, Goto M, et al. Energy metabolism of leukemia cells: glycolysis versus oxidative phosphorylation. *Leuk Lymphoma* (2010) 51:2112–9. doi: 10.3109/10428194.20
112. Farge T, Saland E, de Toni F, Aroua N, Hosseini M, Perry R, et al. Chemotherapy-resistant human acute myeloid leukemia cells are not enriched for leukemic stem cells but require oxidative metabolism. *Cancer Discov.* (2017) 7:716–35. doi: 10.1158/2159-8290.CD-16-0441
113. Scheen AJ. Clinical pharmacokinetics of metformin. *Clin Pharmacokinet* (1996) 30:359–71.
114. Hundal RS, Inzucchi SE. Metformin: new understandings, new uses. *Drugs* (2003) 63:1879–94. doi: 10.2165/00003495-200363180-00001
115. Segal ED, Yasmeen A, Beauchamp MC, Rosenblatt J, Pollak M, Gotlieb WH. Relevance of the OCT1 transporter to the antineoplastic effect of biguanides. *Biochem Biophys Res Commun.* (2011) 414:694–9. doi: 10.1016/j.bbrc.2011.09.134
116. Boukalova S, Stursa J, Werner L, Ezrova Z, Cerny J, Bezawork-Geleta A, et al. Mitochondrial targeting of metformin enhances its activity against pancreatic cancer. *Mol Cancer Ther.* (2016) 15:2875–86. doi: 10.1158/1535-7163.MCT-15-1021
117. Caraci F, Chisari M, Frasca G, Chiechio S, Salomone S, Pinto A, et al. Effects of phenformin on the proliferation of human tumor cell lines. *Life Sci.* (2003) 74:643–50. doi: 10.1016/j.lfs.2003.07.015
118. Higurashi T, Hosono K, Takahashi H, Komiya Y, Umezawa S, Sakai E, et al. Metformin for chemoprevention of metachronous colorectal adenoma or polyps in post-polypectomy patients without diabetes: a multicentre double-blind, placebo-controlled, randomised phase trial. *Lancet Oncol.* (2016) 17:475–83. doi: 10.1016/S1470-2045(15)00565-3

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a shared affiliation, though no other collaboration, with the authors at time of review.

frontiers in Endocrinology

REVIEW
published: 21 August 2018
doi: 10.3389/fendo.2018.00472

Metformin: Focus on Melanoma

Emilie Jaune [1,2] *and Stéphane Rocchi* [1,2]*

[1] INSERM U1065, Centre Méditerranéen de Médecine Moléculaire (C3M), Nice, France, [2] Université de Nice Sophia Antipolis, UFR de Médecine, Nice, France

Metformin is the most common biguanide used in the treatment of diabetes, with 120 million treated patients worldwide. Metformin decreases hyperglycemia without inducing hypoglycemia in diabetic patients and is very well tolerated. The principal effects of metformin are to decrease hepatic gluconeogenesis and increase glucose absorption by skeletal muscles. These effects are primarily due to metformin's action on mitochondria, which requires the activation of metabolic checkpoint AMP-activated protein kinase (AMPK). AMPK is implicated in several pathways, and following metformin activation, it decreases protein synthesis and cell proliferation. Many studies have examined the role of metformin in the regulation of cancer cells, particularly its effects on cancer cell proliferation and cell death. Encouraging results have been obtained in different types of cancers, including prostate, breast, lung, and skin cancers (melanoma). Furthermore, many retrospective epidemiological studies in diabetes patients have shown that metformin treatment decreased the risk of cancers compared with other antidiabetic treatments. In this review, we will discuss the effects of metformin on melanoma cells. Together, our novel data demonstrate the importance of developing metformin and new biguanide-derived compounds as potential treatments against a number of different cancers, particularly melanoma.

Keywords: biguanides, metformin, melanoma skin cancer, cancer treatment, AMPK pathway

OPEN ACCESS

Edited by:
Graham Rena,
University of Dundee, United Kingdom

Reviewed by:
Silvio Naviglio,
Università degli Studi della Campania "Luigi Vanvitelli" Caserta, Italy
Susanna Morano,
Sapienza Università di Roma, Italy

***Correspondence:**
Stéphane Rocchi
srocchi@unice.fr

Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *09 April 2018*
Accepted: *31 July 2018*
Published: *21 August 2018*

Citation:
Jaune E and Rocchi S (2018) Metformin: Focus on Melanoma. Front. Endocrinol. 9:472. doi: 10.3389/fendo.2018.00472

INTRODUCTION

Biguanides are molecules derived from guanidine and are used in diabetes treatment. Guanidine is extracted from *Galega officinalis*, a plant used in medicine for many years. Indeed, this plant was used for its antidiabetic properties before its effects on glycaemia were discovered in the 1920s (1). Since this time, many guanidine-derived compounds have been used in type 2 diabetes, such as buformin, phenformin, and metformin. At first, metformin was not truly compared with other guanidine-derived compounds because of its less important effects on insulin sensitivity. Other biguanides, phenformin, and buformin were widely used in diabetic treatments starting in 1920 until their high toxicity in patients was discovered in 1930 (2). Afterwards, biguanides were no longer used in type 2 diabetes treatment until a study by French chemist Jean Sterne in 1957, where he showed metformin's effects on type 2 diabetes without apparent toxicity (3). Thanks to this study, metformin received marketing authorization in Europe in 1958 and in the USA in 1995 (2). Currently, metformin is the most prescribed antidiabetic medication in the world, and it is used to treat more than 120 million people (4).

After years of treatment with metformin, retrospectives studies showed that diabetes patients had decreased cancer incidence with metformin compared to treatment with another antidiabetic drug (5). Afterwards, studies confirmed these results (6, 7), and many groups focused their research on metformin's effects on cancer cells. In this study, we will particularly focus on metformin's effects on melanoma.

Cutaneous melanoma is a malignant cancer that rises from the transformation of melanocytes. These cells are normally responsible for the synthesis of melanin, which is a photoprotector pigment. Melanoma is widespread with 200,000 new cases every year and 65,000 melanoma-associated deaths. Its incidence doubles every 10 years, and although it represents only 10% of all skin cancers, melanoma is responsible for 80% of skin cancer deaths, which constitutes a real public health problem (8). Melanoma is the most aggressive skin cancer and possesses a strong invasive capability that enables the development of metastasis principally in the lymph node, liver, lung but also in the central nervous system. Metastatic melanoma is one of the deadliest cancers because of the inefficacy of current therapies.

For 15 years, targeted therapy against BRAF(mutated in 50–60% of primary melanoma) or MEK protein has been developed, and some of these treatments have been commercialized, including BRAF inhibitors, such as vemurafenib (or PLX4032) and dabrafenib, and MEK inhibitors, such as cobimetinib, or trametinib (9). The first results with these therapies seem promising with an increase in overall survival and shrinkage of the primary tumor. However, after few weeks of treatment, patients develop a strong resistance to these therapies, enabling metastatic growth and relapse (10, 11). Furthermore, melanoma cells have the ability to escape the immune response. Due to this observation, current therapeutic approaches try to allow immune system activation to kill melanoma cells (12, 13). Currently, two different antibodies are commercialized: ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1). ipilimumab targets CD4+ T cells, whereby its inhibition allows T-cell activation. This treatment increases patient survival rate, but only 15–20% of patients respond to this treatment (14). PD-1 is also expressed on T cells, and its expression inhibits T-cell activation. Its target, PDL-1, is widely found in melanoma cells. PD-1 treatment shows a response in ∼30–40% of patients (15). Even if these responses result in an objective and long-lasting response, ∼55–60% of patients do not respond to these treatments. The identification of new antimelanoma compounds is essential for developing new therapies.

PRINCIPAL EFFECTS OF METFORMIN IN TYPE 2 DIABETES TREATMENTS

In type 2 diabetes patients, metformin (N,N-dimethylbiguanide) exerts its antidiabetic function by decreasing the insulin resistance of glucose-intolerant patients and hepatic gluconeogenesis. Indeed, the liver is considered to be the principal site of action of metformin, where it can act on gluconeogenesis, glycolysis, and glycogen synthesis. In type 2 diabetes patients, hepatic gluconeogenesis is increased relative to healthy patients. However, under metformin treatment, glucose absorption and general levels of glucose can decrease to 75% (16). Furthermore, this molecule also increases the high absorption of glucose by skeletal muscles, which improves its effects on glucose homeostasis. In general, metformin increases glucose absorption by increasing the plasma membrane translocation of glucose receptors, such as glucose transporter 1 (GLUT-1), in both hepatic cells (17) and skeletal muscle cells (18). In addition, this compound highly increases the expression of insulin receptor substrate 1 and 2 (IRS-1 and 2), which enhances glucose absorption.

Interestingly, metformin also blocks the effects of glucagon, which normally enhances gluconeogenesis, by inhibiting essential enzymes in this process and stimulating glycolysis via the alteration of numerous enzymes in this signaling pathway (19).

However, we currently do not understand all the mechanisms of actions of metformin in these patients. Interestingly, a recent study showed the effect of metformin on the intestinal microbiota and its impact on metabolism in obese mice (20). Indeed, type 2 diabetes seems to be impacted by the intestinal microbiota (21), and therefore, the effects on the microbiota could be partly responsible for metformin's effects in type 2 diabetes patients.

METFORMIN ACTS AS AN ANTICANCER AGENT

Retrospective Studies

Diabetic patients possess a higher risk of developing cancers than healthy patients, which is partly due to increasing levels of circulating growth factors, such as insulin or insulin growth factor 1 and 2 (IGF-1 and 2) (22). In this context, many retrospective analyses in type 2 diabetes patients have shown that metformin possesses antitumoral proprieties (5–7). In *Evans* et al. diabetic patients treated with metformin presented less cancer than patients treated with other antidiabetics. Following this study, many investigations have shown the antineoplastic effects of metformin in numerous cancer types (6, 23–25). For example, a study compared the effects of three different treatments, metformin, insulin, or sulfonylureas, over 5 years in ∼10,300 diabetes patients. The results showed that patients treated with metformin have a lower cancer-related mortality rate than patients treated with other treatments (23). Inversely, the study by Currie et al. showed that patients treated with insulin developed more solid cancer than those treated with metformin (25). Another study observed that 7.3% of type 2 diabetes patients treated with metformin developed cancers compared with 11.6% of patients treated with other antidiabetics (6). In a more specific retrospective study, it was shown that the use of metformin for long-term treatment in men decreased prostate cancer development by 34% compared to patients treated with other antidiabetic drugs (26). In women, metformin treatment induced a 56% decrease in the breast cancer risk of diabetic patients (24). Recently, a study in a Korean population with type 2 diabetes showed a decrease in cancer development for patients treated with long-term metformin (5.8 years) with an incidence of 13.2 per 1000 compared with an incidence of 21.8 per 1000 in patients with another treatments (27).

In 2010, a short-term clinical study (1 month) performed in non-diabetic patients showed the significant effect of metformin on the development of rectal aberrant crypt foci (precancerous lesions) and the proliferation of colonic epithelial cells (28). Currently, 304 clinical trials have been registered on metformin

treatment in different cancer types (ClinicalTrial.gov; March 2018).

Mechanisms of Action of Metformin on Cancer Cells

Consequently, many laboratories have tried to understand the mechanisms of action of metformin in different types of cancers, such as lung, prostate, and ovarian cancers or melanoma. The *in vitro* effects of metformin, alone or in combination with other drugs, have been studied in many different cancers (29–32). Moreover, numerous *in vivo* studies have demonstrated the efficacy of metformin in decreasing tumoral growth (33, 34).

Indirect Effects of Metformin

In these studies, different mechanisms have been identified to explain metformin's effects on cancer cells. The first mechanism is an indirect effect of metformin. Indeed, in different cancers, such as breast, colon, or prostate cancer, hyperinsulinemia and obesity induced by insulin and IGF1/2 are associated with poor prognosis (35). Interestingly, metformin decreases circulating insulin levels in patients. Indeed, the transcription of key genes inhibits gluconeogenesis by metformin in the liver, and increased glucose absorption in skeletal muscle cells involves a decrease in blood glucose levels, decreasing circulating insulin levels (36). Therefore, metformin decreases tumoral growth by its inhibition of circulating insulin levels (**Figure 1**). Furthermore, in a mouse model, metformin inhibited lung cancer cell growth induced by hyperinsulinemia and obesity by decreasing the circulating level of insulin and by activating the AMPK pathway (37). Finally, in non-diabetic woman with breast cancer, a study showed that metformin decreased circulating insulin levels by 22% and increased insulin sensitivity by 25% (38). These results confirm that a decrease in insulin induced by metformin can be considered a new potential mechanism in metformin inhibition of tumorigenesis. As we described previously, metformin seems to impact the microbiota in type 2 diabetes patients (20). Therefore, it will be interesting to study the impact of metformin on the microbiota in different cancer types.

Direct Effects of Metformin

However, the principal effects of metformin on cancer cells are direct effects, which predominantly induce mammalian target of rapamycin complex 1 (mTORC1) inhibition (**Figure 1**). mTORC1 is a protein complex composed of five different proteins: DEP domain-containing mTOR interacting protein (DEPTOR), mammalian LST8/G-protein β-subunit like protein (mLST8), regulatory-associated protein of mTOR (RAPTOR), proline-rich AKT substrate of 40 kDa (PRAS40), and mammalian target of rapamycin (mTOR). This complex is implicated in many cellular processes but principally in protein synthesis regulation, which is essential for cellular growth. This complex is often activated in cancer cells and can be associated with cancer therapy resistance. Furthermore, mTORC1 plays a critical role in the proliferation and growth of normal stem cells and cancer stem cells. mTOR's implication in cancer stem cell proliferation has been demonstrated in various cancer types, such as colon, pancreas, or breast cancer (39–41).

Depending on the cancer type, many different mechanisms have been discovered to explain the inhibition of mTORC1. The principal one induces AMPK pathway activation after mitochondria dysregulation by metformin. Indeed, at the cellular level, metformin principally acts on mitochondria by inhibiting complex I of the mitochondrial respiratory chain, which disrupts ATP production in the cell (42) and induces AMPK activation (43). A recent study also showed that metformin dysregulates mitochondrial functions *via* calcium flux release (44). Indeed, metformin induces endoplasmic reticulum (ER) stress, which releases calcium into the cytoplasm of the cell. This calcium release induces higher calcium absorption by the mitochondria, which results in mitochondrial swelling. AMPK is an energy sensor that plays an important role in many metabolic pathways involved in restoring energetic balance within the cell (45). In addition, when AMPK activation is sustained, it can play an important role in different cellular processes, such as cell growth and proliferation, cell cycle regulation, cell polarity, apoptosis, and autophagy (46). After metformin induction, AMPK seems to be activated in cancer cells on threonine 172 by liver kinase B1 (LKB1) (47). LKB1 is deleted in many different tumors, such as tumors in cervical or lung cancer, showing the link between LKB1 expression and cancer predisposition. The LKB1/AMPK pathway inhibits mTOR expression *via* the activation of tuberculous sclerosis complex 1 and 2 (TSC1 and TSC2), which induces the dysregulation of protein synthesis, thereby inhibiting tumoral cell proliferation. Interestingly, AMPK can also directly inhibit RAPTOR, a positive regulator of mTOR (48).

Furthermore, metformin can inhibit mTORC1 by AMPK-independent effects (49). Some of these effects are due to mTORC1 inhibition *via* recombinant activating gene (RAG) GTPase family protein inhibition (50). Indeed, RAG GTPases recruit mTORC1 *via* RAPTOR interactions at lysosomal surfaces, where they are activated by Ras homolog enriched in brain (RHEB). Metformin can also directly inhibit Ragulator (51). In prostate cancer, it has been shown that metformin can induce cancer cell death by p53/regulation in development and DNA damage responses 1 (REDD1) pathway activation, which induces the inhibition of mTOR, thereby inhibiting tumor growth (52).

Metformin inhibits cancer cell proliferation by mTORC1-independent mechanisms. Indeed, AMPK can directly phosphorylate p53 on serine 15, which increases p21 expression and enhances cell cycle arrest (53). It has also been described that metformin-induced cell cycle arrest is mediated by cyclin D1 inhibition and Rb dephosphorylation in prostate cancer cells (29) or by an AMPK-dependent mechanism requiring the downregulation of cyclin D1 and implication of p21 and p27 in breast cancer cells (54). A new mechanism implicating the upregulation of micro-RNA34a in renal cancer cells has been described to induce G0-G1 cell cycle arrest under metformin treatment (55). Furthermore, metformin-induced G1-cell cycle arrest has also been observed in pancreatic, glioma, endometrial, and ovarian cancer cells (56). Recent studies in glioblastoma and ovarian cancer cells have also shown cell cycle arrest in G2/M induced by metformin (57, 58). In addition, metformin can induce cell cycle arrest in the S phase in triple-negative breast cancer (54). Metformin inhibits different genes implicated in

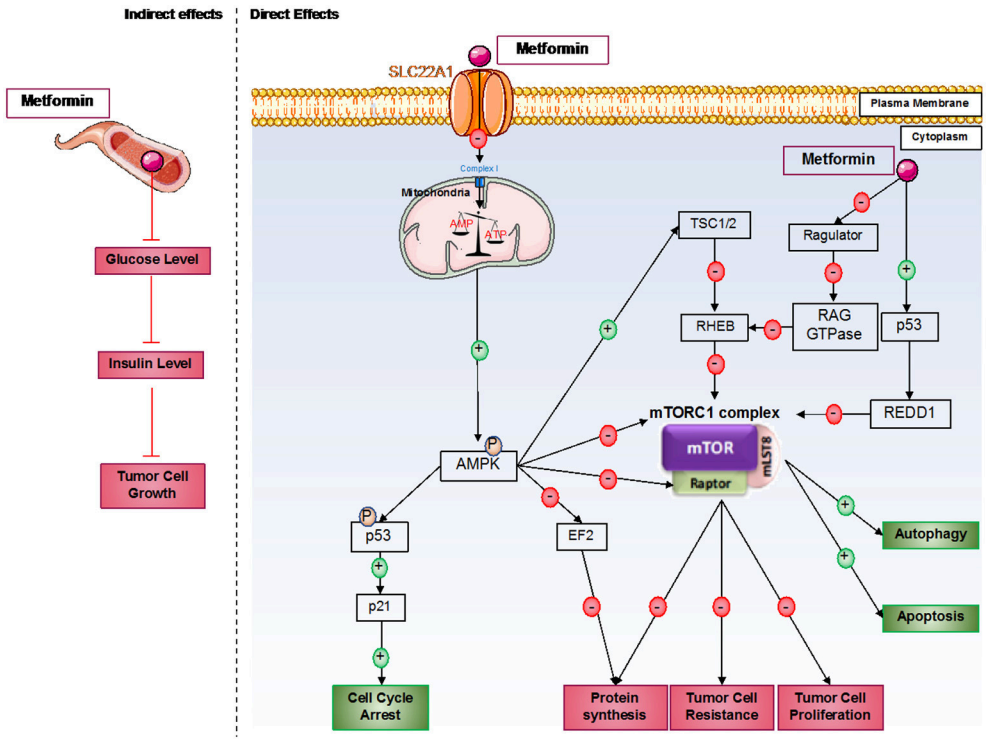

FIGURE 1 | Direct and indirect mechanisms of action of metformin in cancer cells. Metformin induces antitumorigenic effects by both indirect and direct mechanisms. In the blood circulation, metformin decreases glucose levels and therefore insulin levels; insulin can act as a growth factor in tumor cells. For direct effects, metformin acts by AMPK-dependent and independent effects. Generally, metformin induces inhibition of the mTORC1 pathway, which involves an essential protein complex in cellular processes, including protein synthesis and cell proliferation; this complex also promotes tumor cell resistance to therapies. Furthermore, metformin induces cell cycle arrest by p53 activation.

cell division, such as genes encoding tubulin or histones, which enhances cell cycle arrest (56). AMPK also inhibits protein synthesis *via the* inhibition of elongation factor 2 (EF2) protein (59). Furthermore, under AICAR stimulation, active AMPK can decrease fatty acid synthase (FAS) expression in prostate cancer cells (60). This enzyme is essential for fatty acid synthesis, which is also essential for cell proliferation.

Finally, the inhibition of mTORC1 also induces cell death mechanism activation, which induces cancer cell death. For the autophagy process, mTORC1 inhibits the initial step of phagophore formation. This complex also inhibits unc-51 like autophagy activating kinase 1 (ULK1), an essential kinase for autophagy induction (61). Activated AMPK induced by metformin enhances autophagy initiation *via* inhibition of the mTORC1 complex by phosphorylation of TSC2 on serine 1345 (62, 63). AMPK directly phosphorylates ULK1 and induces mTOR-independent autophagy (61). For the apoptosis process in cancer cells, it has been shown that autophagy induction enhances caspase-dependent apoptosis (64). In adipocytes and under AICAR stimulation, AMPK activates apoptosis processes *via* eukaryotic initiation factor 2 α (eIF2α) regulation (65). Moreover, activation of AMPK stimulates the phosphorylation of p53 on serine 46, which is essential in apoptotic type I programmed cell death induction (66). Different studies in the pancreas and in endometrial cancers showed that the antitumor effects of metformin involve the induction of AMPK-dependent apoptosis (67). Finally, the LKB1/AMPK pathway activated by nutrient deprivation increases cyclin-dependent kinase inhibitor 1B (p27), which enhances autophagy and apoptosis processes in cancer cells (68).

In addition, AMPK activation by metformin induces many different antitumor effects *via* the inhibition of c-MYC or hypoxia-inducible factor-1 α (HIF-1α) (69). Metformin activates the DNA damage reparation pathway *via* ataxia telangiectasia mutated (ATM) activation, which inhibits tumor growth (70).

In each case, metformin acts as a major metabolism disruptor in cancer cells, induces dysregulation in energetic metabolism and protein synthesis, and activates autophagy and apoptosis processes.

METFORMIN AND MELANOMA

Metformin can induce cancer cell death by different mechanisms. However, what is known about metformin in melanoma cells?

As previously described, melanoma is the most aggressive form of skin cancer, and currently, efficient treatments have not been developed for most patients. The discovery of new treatments for this cancer appears to be essential. In this context, different laboratories, including ours, have shown that

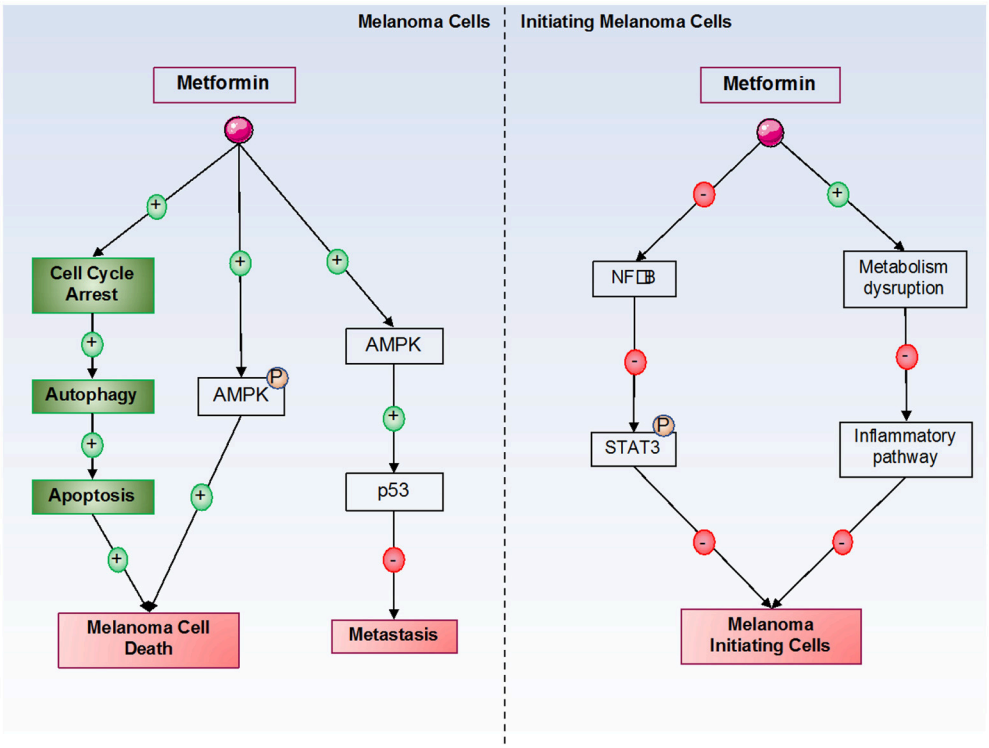

FIGURE 2 | Metformin's effects on melanoma cells. Metformin induces melanoma cell death by both AMPK-dependent and -independent pathways. By an unknown mechanism, metformin induces cell cycle arrest in melanoma cells, which is responsible for the activation of autophagy, and in turn, for the activation of apoptosis, leading to melanoma cell death. In initiating melanoma cells, metformin decreased cell transformation and proliferation by inhibiting the NF-κB pathway and the inflammatory pathway.

metformin or phenformin (another biguanide compound) can inhibit melanoma cell proliferation (33, 71–73).

As previously discussed, metformin can inhibit cancer cell proliferation and induce cancer cell death by many different mechanisms. In melanoma cells, it has been shown that metformin induces cell cycle arrest in melanoma cells in the G0-G1 phase after 24 h of treatment at 10 mM. However, the molecular mechanism of this cell cycle arrest has not been identified in melanoma cells. Furthermore, our laboratory has also shown that this cell cycle arrest is responsible for autophagy (at 72 h) and apoptosis (at 96 h) induction by metformin in melanoma cells (71, 74). In this model, we also showed that inhibition of AMPK (by siRNA) induces a partial restoration of melanoma cell viability under metformin treatment, suggesting that AMPK plays a partial role in metformin-induced melanoma cell death. This finding also suggests that another AMPK-independent mechanism is implicated in metformin-induced melanoma cell death (**Figure 2**). Interestingly, in xenograft mouse models, metformin decreases the tumoral volume of melanoma cells. In addition, no cellular death has been observed in normal human cells, such as melanocytes, even if endogenous AMPK is expressed. Similar results have also been observed by other laboratories (75, 76). In another study, metformin induced autophagy activation in melanoma cells by inhibiting a new potential therapeutic target, tribbles pseudokinase 3 (TRIB3) (33). In this study, the authors showed that metformin attenuated melanoma growth and metastasis by reducing TRIB3 expression in non-diabetic and diabetic mouse models.

Interestingly, a recent study showed that metformin can directly act not only on melanoma cells to induce cell death but also on the tumor microenvironment, particularly in the context of an immune response (77). This study showed that metformin activated both autophagy and apoptosis in melanoma cancer cells *in vitro* and confirmed the results *in vivo* in mouse models challenged with B16 murine melanoma cells. The results showed that metformin activity on melanoma cells was partly due to the immune system and that the antitumor activity of metformin was lost on immunodeficient (NSG) mice. This group also showed that metformin interaction with the immune system was principally associated with T cells (77). As described in the introduction, the immune system is very important in melanoma therapies, and current immunotherapies show very interesting objective responses, but they occur in very few patients. This study showed the interactions between the immune system and metformin; thus, it will be interesting to test a combination of metformin treatment and immunotherapies, such as anti-PD1, to increase the effects of immunotherapies in melanoma cells.

Another study from our laboratory showed that metformin inhibited the proliferation of melanoma cells (78). Indeed, in this study, we showed *in vitro* that metformin modulates

the expression of different proteins, such as Slug, Snail, and matrix metalloproteinases 2 and 9 (MMP2 and 9), the latter two of which are implicated in the epithelial-mesenchymal transition *via* an AMPK- and p53-dependent mechanism. However, even if metformin can inhibit cell invasion, it has no effect on the migration ability of melanoma cells. Furthermore, *in vivo*, we showed that metformin inhibits melanoma metastasis development in the lung. In general, a study on UV-induced skin cancers showed that metformin, by an AMPK-dependent pathway, inhibited tumorigenesis in skin cells (79). Indeed, the authors showed that AMPK was inhibited by UVB irradiation, demonstrating its important role as a tumor suppressor in skin cancers. This inhibition enhanced decrease of the DNA damage response pathway *via* reduction of xeroderma pigmentosum C (XPC). However, under stimulation with AICAR and metformin, the DNA damage response pathway was reactivated, decreasing in cancer cell development. Therefore, the authors showed the importance of AMPK activation by different treatments, such as metformin, in decreasing cancer cell development and proliferation induced by UVB, such as in melanoma development.

Interestingly, melanoma is one of cancer that is the most dependent on and impacted by metabolism (80). Melanoma is a cancer that requires glycolytic metabolism, which is mediated by mitochondrial activity (81). Moreover, resistance to BRAF inhibitor cells have shown increased oxidative metabolism and mitochondrial dependence for cell survival (82). Therefore, in both sensitive and resistant to BRAF inhibitor melanoma cells, mitochondria, and metabolism appear to be essential, and a drug, such as metformin or another biguanide, that alters this metabolism could be an interesting prospect for new melanoma treatments. This information suggests that testing a drug such as metformin, which disrupts metabolism, in combination with other therapies, such as targeted therapies (BRAF inhibitors) or immunotherapies (anti-PD1) in melanoma cells, could increase objective responses and inhibit primary or acquired resistance to these treatments.

Studies have examined the effects of metformin in combination with BRAF inhibitors, such as vemurafenib (Zelboraf). Indeed, many groups have used combination treatments with BRAF inhibitors to inhibit resistance in melanoma cells. The combination of vemurafenib (BRAF inhibitor) and metformin showed encouraging results with synergistic effects for inducing melanoma cell death (76). Indeed, *in vitro* experiments show synergistic antiproliferative effects, particularly in BRAFV600E mutant cell lines. In other studies, metformin increased the toxicity of cisplatin, a chemotherapeutic drug, in melanoma cells (83, 84).

These results seem interesting, but further study is needed. Indeed, in certain studies, the combination of metformin and a BRAF inhibitor stimulated the proliferation of melanoma cells mutated by NRAS (76). It will be interesting to observe the metabolic characteristics of melanoma cells after a treatment combination of metformin and BRAF inhibitors to better understand active mechanisms.

Furthermore, metformin effects were analyzed in combination with immunotherapies (anti-CTLA4, anti-PD-1 or anti-PD-L1). As previously stated, immunotherapies have been developed for melanoma treatment for a few years. These therapies, which tend to reactivate the patient's immune system, show very efficient and durable responses, but they are effective in only 15–30% of patients. Therefore, we can imagine that combinations with another molecule, such as metformin or another biguanide can increase the objective responses obtained with immunotherapies and decrease resistance to these treatments. Interestingly, a recent study showed that phenformin, another biguanide, potentiated the effect of immunotherapy (85). In this study, the authors showed that phenformin induced the production of reactive oxygen species in granulocytic myeloid-derived suppressor cells, which increased the effect of immunotherapies on melanoma cells. Indeed, in combination with anti-PD-1, phenformin enhanced melanoma inhibition in a BRAF V600E/PTEN-null melanoma mouse model. In these mice, CD8+ lymphocytes were activated, which increased melanoma cell death. In addition, results from the study by Scharping et al. suggested that tumor hypoxia plays a role as a barrier against immunotherapy and that metformin, which can reduce intratumoral hypoxia, can improve immunotherapy efficacy against melanoma cells (86). Taken together, these results suggest that biguanides, such as metformin, could be used in combination with targeted therapies against BRAF or with immunotherapies to synergize treatment effects on melanoma cells.

Finally, certain studies have examined metformin's effects on melanoma initiating cells (MIC). Indeed, melanoma is a heterogenic tumor, and some studies including ours have suggested that MIC could be responsible for the metastatic potential of melanoma, which could be implicated in resistance to BRAF inhibitor therapies (87, 88). These MIC constitute a chemoresistant cell population that expresses specific markers. However, independently of MIC numbers, characteristics, or mechanisms that regulate the transition between MIC and proliferative cells, it is clear that melanoma cell populations with different tumorigenic potentials exist (89). A study has shown that STAT3 (Signal Transducer and Activator of Transcription 3) pathway activation is necessary to acquire "MIC properties" (90). Furthermore, a recent study showed that a combination of stattic and metformin decreased brain tumor initiating cells by STAT3-dependent mechanisms (91). Interestingly, metformin blocks the inflammatory pathway responsible for stem cell transformation and growth due to cellular metabolism disruption (84). Finally, our laboratory has shown that metformin can decrease MIC populations (unpublished results) (**Figure 2**).

CLINICAL TRIALS OF METFORMIN ON MELANOMA TREATMENT

In this context, 304 clinical trials have been or are currently being performed to test the effects of metformin on different cancer types (ClinicalTrials.gov).

In our laboratory, thanks to the *in vitro* and *in vivo* results obtained from metformin treatment against melanoma,

we developed a phase II clinical trial that was performed in the dermatology department at the University Hospital Centre in Nice. This clinical trial allowed us to determine the efficacy of metformin treatment on metastatic melanoma patients (92). In this study, patients were experiencing therapeutic failure to chemotherapies and BRAF inhibitors and were not eligible for or not responsive to immunotherapy treatment. The study was performed on 17 patients with ages ranging from 49 to 88 years (mean of 74 years). Metformin was prescribed at 1,000 mg three times daily. After 4 months, 11 patients showed melanoma progression, 3 patients were deceased due to the disease and 2 patients had to stop treatment. After 6 months, the only patient still being treated with metformin did not show a significant response. These results were not very encouraging; it could have been concluded that metformin treatment at similar doses to those in type 2 diabetes did not induce significant efficacy in this population of metastatic melanoma patients, independently of mutational status. However, the poor efficacy of metformin treatment could be linked to different barriers in this study. Indeed, treated patients were in total therapeutic failure and had notable progression of melanoma disease. Therefore, it will be interesting to test metformin or new biguanide-derived compounds with better efficacy at an early stage of disease progression.

Currently, other clinical trials are still in progress, and their results will be truly important for understanding metformin treatments against melanoma and continuing their use. Another phase I/II clinical trial is currently being performed in the United States in Louisville, and metformin's treatment effects in combination with vemurafenib, a BRAF inhibitor, is being evaluated in 55 patients with BRAF-mutated melanoma (ClinicalTrials.gov, NCT01638676). At the University of Louisville, another clinical trial evaluating the combination of dabrafenib/or trametinib and metformin is being evaluated. This phase I/II clinical trial started in 2014 on 53 participants, and no results have been published (ClinicalTrials.gov, NCT02143050). Other clinical trials are currently in progress to evaluate the effects of metformin in combination with different treatments; a study of metformin combined with pembrolizumab (immunotherapy) is being conducted in Pittsburgh (ClinicalTrials.gov, NCT03311308), and a study on metformin combined with dacarbazine (chemotherapy) is being conducted in Petersburg (ClinicalTrials.gov, NCT02190838).

In conclusion, metformin, and more generally biguanides, seem to be good candidates for the development of new therapies against melanoma. Their impact on metabolism and the activation of cell death mechanisms in melanoma cells could be promising in melanoma treatment. Furthermore, studies in which metformin, or other biguanides, is combined with current therapies show a synergistic response in melanoma cells, and therefore, their results could be interesting for the development of new therapy combinations against melanoma and even other cancer cell types.

AUTHOR CONTRIBUTIONS

EJ wrote the first draft and made the figures of the review. SR oversaw the work and made the corrections and the final modifications.

REFERENCES

1. Watanabe C. Studies in the metabolic changes induced by administration of guanidine bases. Influence of injected guanidine hydrochloride upon blood sugar content. *J Biol Chem.* (1918).
2. Pasik C, editor. Diabetes and the biguanides: the mystery of each. In: Glucophage: *Serving Diabetology for 40 Years*. Lyon: Groupe Lipha (1997). p. 79.
3. Sterne J. Du nouveau dans les antidiabétiques. La NN dimethylamine guanyl guanidine (NNDG). *Maroc Med.* (1957).
4. Bridgeman SC, Ellison GC, Melton PE, Newsholme P, Mamotte C. Epigenetic effects of metformin: from molecular mechanisms to clinical implications. *Diabetes Obes Metab.* (2018) 20:1553–62. doi: 10.1111/dom.13262
5. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ* (2005) 330:1304–5. doi: 10.1136/bmj.38415.708634.F7
6. Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD, Evans JM. New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes. *Diabetes Care* (2009) 32:1620–5. doi: 10.2337/dc08-2175
7. Monami M, Marchionni N, Mannucci E. Glucagon-like peptide-1 receptor agonists in type 2 diabetes: a meta-analysis of randomized clinical trials. *Eur J Endocrinol.* (2009) 160:909–17. doi: 10.1530/EJE-09-0101
8. Demierre M-F. Epidemiology and prevention of cutaneous melanoma. *Curr Treat Options Oncol.* (2006) 7:181–6. doi: 10.1007/s11864-006-0011-z
9. Smalley, K. S. (2010). PLX-4032, a small-molecule B-Raf inhibitor for the potential treatment of malignant melanoma. *Curr Opin Invest Drugs* (2000) 11:699–706.
10. Aplin AE, Kaplan FM, Shao Y. Mechanisms of resistance to RAF inhibitors in melanoma. *J Invest Dermatol.* (2011) 131:1817–20. doi: 10.1038/jid.2011.147
11. Flaherty KT, Infante JR, Daud A, Gonzalez R, Kefford RF, Sosman J, et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med.* (2012) 367:1694–703. doi: 10.1056/NEJMoa1210093
12. Zeiser R, Schnitzler M, Andrlova H, Hellige T, Meiss F. Immunotherapy for malignant melanoma. *Curr Stem Cell Res Ther.* (2012) 7:217–28. doi: 10.2174/157488812799859883
13. Eggermont AM, Spatz A, Robert C. Cutaneous melanoma. *Lancet* (2014) 383:816–27. doi: 10.1016/S0140-6736(13)60802-8
14. Weber JS, O'day S, Urba W, Powderly J, Nichol G, Yellin M, et al. Phase I/II study of ipilimumab for patients with metastatic melanoma. *J Clin Oncol.* (2008) 26:5950–6. doi: 10.1200/JCO.2008.16.1927
15. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol.* (2010) 28:3167–75. doi: 10.1200/JCO.2009.26.7609
16. Stumvoll M, Nurjhan N, Perriello G, Dailey G, Gerich JE. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. *N Engl J Med.* (1995) 333:550–4. doi: 10.1056/NEJM199508313330903
17. Gunton JE, Delhanty PJ, Takahashi SI, Baxter RC. Metformin rapidly increases insulin receptor activation in human liver and signals preferentially through insulin-receptor substrate-2. *J Clin Endocrinol Metab.* (2003) 88:1323–32. doi: 10.1210/jc.2002-021394
18. Fischer Y, Thomas J, Rösen P, Kammermeier H. Action of metformin on glucose transport and glucose transporter GLUT1 and GLUT4 in heart muscle cells from healthy and diabetic rats. *Endocrinology* (1995) 136:412–20. doi: 10.1210/endo.136.2.7835271

19. Fulgencio JP, Kohl C, Girard J, Pégorier JP. Effect of metformin on fatty acid and glucose metabolism in freshly isolated hepatocytes and on specific gene expression in cultured hepatocytes. *Biochem Pharmacol.* (2001) 62:439–46. doi: 10.1016/S0006-2952(01)00679-7
20. Lee H, Lee Y, Kim J, An J, Lee S, Kong H, et al. Modulation of the gut microbiota by metformin improves metabolic profiles in aged obese mice. *Gut Microbes* (2017) 9:155–65. doi: 10.1080/19490976.2017.1405209
21. Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature* (2015) 528:262–6. doi: 10.1038/nature 15766
22. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, et al. Diabetes and cancer: a consensus report. *CA Cancer J Clin.* (2010) 60:207–21. doi: 10.3322/caac.20078
23. Bowker SL, Majumdar SR, Veugelers P, Johnson JA. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes Care* (2006) 29:254–8. doi: 10.2337/diacare.29.02.06.dc05-1558
24. Bodmer M, Meier C, Krähenbühl S, Jick SS, Meier CR. Long-term metformin use is associated with decreased risk of breast cancer. *Diabetes Care* (2010) 33:1304–8. doi: 10.2337/dc09-1791
25. Currie CJ, Poole CD, Jenkins-Jones S, Gale EA, Johnson JA, Morgan CL. Mortality after incident cancer in people with and without type 2 diabetes: impact of metformin on survival. *Diabetes Care* (2012) 35, 299–304. doi: 10.2337/dc11-1313
26. Murtola TJ, Tammela TLJ, Lahtela J, Auvinen A. Antidiabetic medication and prostate cancer risk: a population-based case-control study. *Am J Epidemiol.* (2008) 168:925–31. doi: 10.1093/aje/kwn190
27. Kim HJ, Lee S, Chun KH, Jeon JY, Han SJ, Kim DJ, et al. Metformin reduces the risk of cancer in patients with type 2 diabetes: an analysis based on the Korean National Diabetes Program Cohort. *Medicine* (2018) 97:e0036. doi: 10.1097/MD.0000000000010036
28. Hosono K, Endo H, Takahashi H, Sugiyama M, Sakai E, Uchiyama T, et al. Metformin suppresses colorectal aberrant crypt foci in a short-term clinical trial. *Cancer Prev Res.* (2010) 3:1077–83. doi: 10.1158/1940-6207.CAPR-10-0186
29. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, et al. The antidiabetic drug metformin exerts an antitumoral effect *in vitro* and *in vivo* through a decrease of cyclin D1 level. *Oncogene* (2008) 27:3576–86. doi: 10.1038/sj.onc.1211024
30. Aljada A, Mousa SA. Metformin and neoplasia: implications and indications. *Pharmacol Ther.* (2012) 133:108–15. doi: 10.1016/j.pharmthera.2011.09.004
31. Valtorta S, Dico AL, Raccagni I, Gaglio D, Belloli S, Politi LS, et al. Metformin and temozolomide, a synergic option to overcome resistance in glioblastoma multiforme models. *Oncotarget* (2017) 8:113090–104. doi: 10.18632/oncotarget.23028
32. Roos JF, Qudsi M, Samara A, Rahim MM, Al-Bayedh SA, and Ahmed H. Metformin for lung cancer prevention and improved survival: a novel approach. *Eur. J. Cancer Prev.* (2018) doi: 10.1097/CEJ.0000000000000442
33. Li K, Zhang TT, Wang F, Cui B, Zhao CX, Yu JJ, et al. Metformin suppresses melanoma progression by inhibiting KAT5-mediated SMAD3 acetylation, transcriptional activity and TRIB3 expression. *Oncogene* (2018) 37:2967–81. doi: 10.1038/s41388-018-0172-9
34. Zhang JW, Zhao F, Sun Q. Metformin synergizes with rapamycin to inhibit the growth of pancreatic cancer *in vitro* and *in vivo*. *Oncol Lett.* (2018) 15:1811–6. doi: 10.3892/ol.2017.7444
35. Pollak M. The insulin and insulin-like growth factor receptor family in neoplasia: an update. *Nat Rev Cancer* (2012) 12:159–69. doi: 10.1038/ nrc3215
36. Cusi K, Consoli A, Defronzo RA. Metabolic effects of metformin on glucose and lactate metabolism in noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab.* (1996) 81:4059–67.
37. Algire C, Zakikhani M, Blouin M-J, Shuai JH, Pollak M. Metformin attenuates the stimulatory effect of a high-energy diet on *in vivo* LLC1 carcinoma growth. *Endocr Relat Cancer* (2008) 15:833–9. doi: 10.1677/ERC-08-0038
38. Goodwin PJ, Pritchard KI, Ennis M, Clemons M, Graham M, Fantus IG. Insulin-lowering effects of metformin in women with early breast cancer. *Clin Breast Cancer* (2008) 8:501–5. doi: 10.3816/CBC.2008.n.060
39. Cai Z, Ke J, He X, Yuan R, Chen Y, Wu X, et al. Significance of mTOR signaling and its inhibitor against cancer stem-like cells in colorectal cancer. *Ann Surg Oncol.* (2014) 21:179–88. doi: 10.1245/s10434-013-3146-8
40. Zeng J-Y, Sharma S, Zhou Y-Q, Yao H-P, Hu X, Zhang R, et al. Synergistic activities of MET/RON inhibitor BMS-777607 and mTOR inhibitor AZD8055 to polyploid cells derived from pancreatic cancer and cancer stem cells. *Mol Cancer Ther.* (2014) 13:37–48. doi: 10.1158/1535-7163.MCT-13-0242
41. Karthik G-M, Ma R, Lövrot J, Kis LL, Lindh C, Blomquist L, et al. mTOR inhibitors counteract tamoxifen-induced activation of breast cancer stem cells. *Cancer Lett.* (2015) 367:76–87. doi: 10.1016/j.canlet.2015.07.017
42. Miller RA, Birnbaum MJ. An energetic tale of AMPK-independent effects of metformin. *J Clin Invest.* (2010) 120:2267–70. doi: 10.1172/JCI43661
43. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest.* (2001) 108:1167–74. doi: 10.1172/JCI13505
44. Loubiere C, Clavel S, Gilleron J, Harisseh R, Fauconnier J, Ben-Sahra I, et al. The energy disruptor metformin targets mitochondrial integrity via modification of calcium flux in cancer cells. *Sci Rep.* (2017) 7:5040. doi: 10.1038/s41598-017-05052-2
45. Shaw RJ, Kosmatka M, Bardeesy N, Hurley RL, Witters LA, Depinho RA, et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci USA.* (2004) 101:3329–35. doi: 10.1073/pnas.0308061100
46. Luo Z, Zang M, Guo W. AMPK as a metabolic tumor suppressor: control of metabolism and cell growth. *Future Oncol.* (2010) 6:457–70. doi: 10.2217/fon.09.174
47. Hawley SA, Boudeau J. Reid JL, Mustard KJ, Udd L, Mäkelä TP, et al. (2003). Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *J. Biol.* 2:28. doi: 10.1186/1475-4924-2-28
48. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol Cell* (2008) 30:214–26. doi: 10.1016/j.molcel.2008.03.003
49. Ikhlas S, Ahmad M. Metformin: Insights into its anticancer potential with special reference to AMPK dependent and independent pathways. *Life Sci.* (2017) 185:53–62. doi: 10.1016/j.lfs.2017.07.029
50. Kalender A, Selvaraj A, Kim SY, Gulati P, Brûlé S, Viollet B, et al. (2010). Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell Metab.* 11, 390–401. doi: 10.1016/j.cmet.2010.03.014
51. Pierotti MA, Berrino F, Gariboldi M, Melani C, Mogavero A, Negri T, et al. Targeting metabolism for cancer treatment and prevention: metformin, an old drug with multi-faceted effects. *Oncogene* (2013) 32:1475–87. doi: 10.1038/onc.2012.181
52. Ben Sahra I, Regazzetti C, Robert G, Laurent K, Le Marchand-Brustel Y, Auberger P, et al. Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1. *Cancer Res.* (2011) 71:4366–72. doi: 10.1158/0008-5472.CAN-10-1769
53. Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, et al. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell* (2005) 18:283–93. doi: 10.1016/j.molcel.2005.03.027
54. Alimova IN, Liu B, Fan Z, Edgerton SM, Dillon T, Lind SE, et al. Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest *in vitro*. *Cell Cycle* (2009) 8:909–15. doi: 10.4161/cc.8.6.7933
55. Xie W, Wang L, Sheng H, Qiu J, Zhang D, Zhang L, et al. Metformin induces growth inhibition and cell cycle arrest by upregulating microRNA34a in renal cancer cells. *Med Sci Monit.* (2017) 23:29–37. doi: 10.12659/MSM. 898710
56. Oliveras-Ferraros C, Vazquez-Martin A, Menendez JA. Genome-wide inhibitory impact of the AMPK activator metformin on [kinesins, tubulins, histones, auroras and polo-like kinases] M-phase cell cycle genes in human breast cancer cells. *Cell Cycle* (2009) 8:1633–6. doi: 10.4161/cc.8.10.8406
57. Adeberg S, Bernhardt D, Harrabi SB, Nicolay NH, Hörner-Rieber J, König L, et al. Metformin enhancedin vitroradiosensitivity associates with g2/m cell cycle arrest and elevated adenosine-5'-monophosphate-activated protein kinase levels in Glioblastoma. *Radiol Oncol.* (2017) 51:431–7. doi: 10.1515/raon-2017-0042
58. Fu YL, Zhang QH, Wang XW, He H. Antidiabetic drug metformin mitigates ovarian cancer SKOV3 cell growth by triggering G2/M cell cycle arrest and

inhibition of m-TOR/PI3K/Akt signaling pathway. *Eur Rev Med Pharmacol Sci.* (2017) 21:1169–75.
59. Horman S, Browne G, Krause U, Patel J, Vertommen D, Bertrand L, et al. Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. *Curr Biol.* (2002) 12:1419–23. doi: 10.1016/S0960-9822(02)01077-1
60. Xiang X, Saha AK, Wen R, Ruderman NB, Luo Z. AMP-activated protein kinase activators can inhibit the growth of prostate cancer cells by multiple mechanisms. *Biochem Biophys Res Commun.* (2004) 321:161–7. doi: 10.1016/j.bbrc.2004.06.133
61. Egan D, Kim J, Shaw RJ, Guan K-L. The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR. *Autophagy* (2011) 7:643–4. doi: 10.4161/auto.7.6.15123
62. Inoki K, Li Y, Zhu T, Wu J, Guan K-L. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol.* (2002) 4:648–57. doi: 10.1038/ncb839
63. Meley D, Bauvy C, Houben-Weerts JH, Dubbelhuis PF, Helmond MT, Codogno P, et al. AMP-activated protein kinase and the regulation of autophagic proteolysis. *J Biol Chem.* (2006) 281:34870–9. doi: 10.1074/jbc.M605488200
64. Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, et al. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev.* (2007) 21:1621–35. doi: 10.1101/gad.1565707
65. Dagon Y, Avraham Y, Berry EM. AMPK activation regulates apoptosis, adipogenesis, and lipolysis by eIF2alpha in adipocytes. *Biochem Biophys Res Commun.* (2006) 340:43–7. doi: 10.1016/j.bbrc.2005.11.159
66. Okoshi R, Ozaki T, Yamamoto H, Ando K, Koida N, Ono S, et al. Activation of AMP-activated protein kinase induces p53-dependent apoptotic cell death in response to energetic stress. *J Biol Chem.* (2008) 283:3979–87. doi: 10.1074/jbc.M705232200
67. Wang LW, Li ZS, Zou DW, Jin ZD, Gao J, Xu GM. Metformin induces apoptosis of pancreatic cancer cells. *World J Gastroenterol.* (2008) 14:7192–8. doi: 10.3748/wjg.14.7192
68. Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, et al. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. *Nat Cell Biol.* (2007) 9:218–24. doi: 10.1038/ncb1537
69. Blandino G, Valerio M, Cioce M, Mori F, Casadei L, Pulito C, et al. Metformin elicits anticancer effects through the sequential modulation of DICER and c-MYC. *Nat Commun.* (2012) 3:865. doi: 10.1038/ncomms1859
70. Vazquez-Martin A, Oliveras-Ferraros C, Cufí S, Martin-Castillo B, and Menendez JA. Metformin activates an ataxia telangiectasia mutated (ATM)/Chk2-regulated DNA damage-like response. *Cell Cycle* (2011) 10:1499–501. doi: 10.4161/cc.10.9.15423
71. Tomic T, Botton T, Cerezo M, Robert G, Luciano F, Puissant A, et al. Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. *Cell Death Dis.* (2011) 2:e199. doi: 10.1038/cddis.2011.86
72. Petti C, Vegetti C, Molla A, Bersani I, Cleris L, Mustard KJ, et al. AMPK activators inhibit the proliferation of human melanomas bearing the activated MAPK pathway. *Melanoma Res.* (2012) 22:341–50. doi: 10.1097/CMR.0b013e3283544929
73. Yuan P, Ito K, Perez-Lorenzo R, Del Guzzo C, Lee JH, Shen CH, et al. Phenformin enhances the therapeutic benefit of BRAF(V600E) inhibition in melanoma. *Proc Natl Acad Sci USA.* (2013) 110:18226–31. doi: 10.1073/pnas.1317577110
74. Cerezo M, Tomic T, Ballotti R, Rocchi S. Is it time to test biguanide metformin in the treatment of melanoma? *Pigment Cell Melanoma Res.* (2015) 28:8–20. doi: 10.1111/pcmr.12267
75. Janjetovic K, Harhaji-Trajkovic L, Misirkic-Marjanovic M, Vucicevic L, Stevanovic D, Zogovic N, et al. *In vitro* and *in vivo* anti-melanoma action of metformin. *Eur J Pharmacol.* (2011) 668:373–82. doi: 10.1016/j.ejphar.2011.07.004
76. Niehr F, Von Euw E, Attar N, Guo D, Matsunaga D, Sazegar H, et al. Combination therapy with vemurafenib (PLX4032/RG7204) and metformin in melanoma cell lines with distinct driver mutations. *J Transl Med.* (2011) 9:76. doi: 10.1186/1479-5876-9-76
77. Pereira FV, Melo ACL, Low JS, De Castro ÍA, Braga TT, Almeida DC, et al. Metformin exerts antitumor activity via induction of multiple death pathways in tumor cells and activation of a protective immune response. *Oncotarget* (2018) 9:25808–25. doi: 10.18632/oncotarget.25380
78. Cerezo M, Tichet M, Abbe P, Ohanna M, Lehraiki A, Rouaud F, et al. Metformin blocks melanoma invasion and metastasis development in AMPK/p53-dependent manner. *Mol Cancer Ther.* (2013) 12:1605–15. doi: 10.1158/1535-7163.MCT-12-1226-T
79. Wu CL, Qiang L, Han W, Ming M, Viollet B, He YY. Role of AMPK in UVB-induced DNA damage repair and growth control. *Oncogene* (2013) 32:2682–9. doi: 10.1038/onc.2012.279
80. Haq R. Metabolic dysregulation in melanoma: cause or consequence? *Cancer Discov.* (2014) 4:390–1. doi: 10.1158/2159-8290.CD-14-0173
81. Scott DA, Richardson AD, Filipp FV, Knutzen CA, Chiang GG, Ronai ZA, et al. Comparative metabolic flux profiling of melanoma cell lines: beyond the Warburg effect. *J Biol Chem.* (2011) 286:42626–34. doi: 10.1074/jbc.M111.282046
82. Baenke F, Chaneton B, Smith M, Van Den Broek N, Hogan K, Tang H, et al. Resistance to BRAF inhibitors induces glutamine dependency in melanoma cells. *Mol Oncol.* (2016) 10:73–84. doi: 10.1016/j.molonc.2015.08.003
83. Janjetovic K, Vucicevic L, Misirkic M, Vilimanovich U, Tovilovic G, Zogovic N, et al. Metformin reduces cisplatin-mediated apoptotic death of cancer cells through AMPK-independent activation of Akt. *Eur. J. Pharmacol.* (2011) 651:41–50. doi: 10.1016/j.ejphar.2010.11.005
84. Hirsch HA, Iliopoulos D, Struhl K. Metformin inhibits the inflammatory response associated with cellular transformation and cancer stem cell growth. *Proc Natl Acad Sci USA.* (2013) 110:972–7. doi: 10.1073/pnas.1221055110
85. Kim SH, Li M, Trousil S, Zhang Y, Pasca Di Magliano M, Swanson KD, et al. Phenformin inhibits myeloid-derived suppressor cells and enhances the anti-tumor activity of PD-1 blockade in melanoma. *J Invest Dermatol.* (2017) 137:1740–8. doi: 10.1016/j.jid.2017.03.033
86. Scharping NE, Menk AV, Whetstone RD, Zeng X, Delgoffe GM. Efficacy of PD-1 blockade is potentiated by metformin-induced reduction of tumor hypoxia. *Cancer Immunol Res.* (2017) 5:9–16. doi: 10.1158/2326-6066.CIR-16-0103
87. Zabierowski SE, Herlyn M. Learning the ABCs of melanoma-initiating cells. *Cancer Cell* (2008) 13:185–7. doi: 10.1016/j.ccr.2008.02.015
88. Lehraiki A, Cerezo M, Rouaud F, Abbe P, Allegra M, Kluza J, et al. Increased CD271 expression by the NF-kB pathway promotes melanoma cell survival and drives acquired resistance to BRAF inhibitor vemurafenib. *Cell Discov.* (2015) 1:15030. doi: 10.1038/celldisc.2015.30
89. Hoek KS, Goding CR. Cancer stem cells versus phenotype-switching in melanoma. *Pigment Cell Melanoma Res.* (2010) 23:746–59. doi: 10.1111/j.1755-148X.2010.00757.x
90. Ohanna M, Giuliano S, Bonet C, Imbert V, Hofman V, Zangari J, et al. Senescent cells develop a PARP-1 and nuclear factor-{kappa}B-associated secretome (PNAS). *Genes Dev.* (2011) 25:1245–61. doi: 10.1101/gad.625811
91. Leidgens V, Proske J, Rauer L, Moeckel S, Renner K, Bogdahn U, et al. Stattic and metformin inhibit brain tumor initiating cells by reducing STAT3-phosphorylation. *Oncotarget* (2017) 8:8250–63. doi: 10.18632/oncotarget.14159
92. Montaudié H, Cerezo M, Bahadoran P, Roger C, Passeron T, Machet L, et al. Metformin monotherapy in melanoma: a pilot, open-label, prospective, and multicentric study indicates no benefit. *Pigment Cell Melanoma Res.* (2017) 30:378–80. doi: 10.1111/pcmr.12576

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 21 August 2018
doi: 10.3389/fendo.2018.00372

Metabolic Profiles Associated With Metformin Efficacy in Cancer

Sylvia Andrzejewski[1,2], *Peter M. Siegel*[1,2] *and Julie St-Pierre*[3]*

[1] Department of Biochemistry, McGill University, Montreal, QC, Canada, [2] Goodman Cancer Research Centre, McGill University, Montreal, QC, Canada, [3] Department of Biochemistry, Microbiology and Immunology, and Ottawa Institute of Systems Biology, University of Ottawa, Ottawa, ON, Canada

Metformin is one of the most commonly prescribed medications for the treatment of type 2 diabetes. Numerous reports have suggested potential anti-cancerous and cancer preventive properties of metformin, although these findings vary depending on the intrinsic properties of the tumor, as well as the systemic physiology of patients. These intriguing studies have led to a renewed interest in metformin use in the oncology setting, and fueled research to unveil its elusive mode of action. It is now appreciated that metformin inhibits complex I of the electron transport chain in mitochondria, causing bioenergetic stress in cancer cells, and rendering them dependent on glycolysis for ATP production. Understanding the mode of action of metformin and the consequences of its use on cancer cell bioenergetics permits the identification of cancer types most susceptible to metformin action. Such knowledge may also shed light on the varying results to metformin usage that have been observed in clinical trials. In this review, we discuss metabolic profiles of cancer cells that are associated with metformin sensitivity, and rationalize combinatorial treatment options. We use the concept of bioenergetic flexibility, which has recently emerged in the field of cancer cell metabolism, to further understand metabolic rearrangements that occur upon metformin treatment. Finally, we advance the notion that metabolic fitness of cancer cells increases during progression to metastatic disease and the emergence of therapeutic resistance. As a result, sophisticated combinatorial approaches that prevent metabolic compensatory mechanisms will be required to effectively manage metastatic disease.

Keywords: metformin, phenformin, mitochondria, diabetes, cancer, breast cancer, metabolism, mitochondrial drugs

OPEN ACCESS

Edited by:
Frederic Bost,
Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Bin Zheng,
Harvard Medical School, United States
Daniel Rotroff,
Cleveland Clinic, United States
Federica Barbieri,
Università di Genova, Italy

***Correspondence:**
Julie St-Pierre
julie.st-pierre@uottawa.ca

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *04 May 2018*
Accepted: *21 June 2018*
Published: *21 August 2018*

Citation:
Andrzejewski S, Siegel PM and St-Pierre J (2018) Metabolic Profiles Associated With Metformin Efficacy in Cancer. Front. Endocrinol. 9:372. doi: 10.3389/fendo.2018.00372

METFORMIN

A Drug With a Long History

Metformin was first discovered in the 1920s by a French physician from a plant called Goat's Rue (1). It was found that animals grazing on this plant had low blood glucose levels [reviewed in Witters (2)]. Subsequently, it was determined that the active compound responsible for lowering blood glucose was a guanidine moiety. Early synthetic homologues of guanidine were created for the treatment of diabetes, although they proved to be hepatotoxic and were rapidly discontinued. Renewed interest in guanidine in the 1960s led to the creation of a family of biguanide compounds [reviewed in White (3)]. Phenformin was the first biguanide family member prescribed to diabetic patients (4); however, its use was associated with the development of lactic acidosis (5). The biguanide metformin was better tolerated relative to phenformin by diabetic patients and was

approved by the Food and Drug Administration (FDA) in the 1990s for the treatment of type 2 diabetes (6). Metformin is an extremely safe medication; rarely associated with the development of lactic acidosis (7). Additionally, metformin has global appeal as it is a low cost medication with generic versions also available.

It has been reported that patients with diabetes are more likely to develop cancer in their lifetime compared to non-diabetic individuals (8). A retrospective report published in 2005 suggested that metformin users have lower incidences of cancer relative to patients prescribed other type 2 diabetic medications (9). Moreover, users of diabetic medications other than metformin displayed increased cancer-related mortality (10). The study by Evans et al. (9) sparked great interest in the academic community, and metformin has been, or currently is being investigated in 310 individual clinical trials for its role in the prevention or treatment of various types of cancer. However, there is currently no consensus regarding which cancers are most likely to benefit from metformin treatment. Completed clinical trials have varied in outcome depending on trial design, cancer type, stage of cancer, timing of metformin treatment, and combinatorial therapies or treatments given in addition to metformin. Individual clinical studies have shown that metformin is associated with increased survival of diabetic patients with lung (11), colorectal (12), and prostate (13, 14) cancers. Moreover, metformin is associated with reduced risk of developing pancreatic (15), breast (16), colorectal (17) or liver (18) cancers. Recently, studies have been developed to investigate potential anti-cancer roles of metformin in non-diabetic patients given the increasing literature supporting its action in cancers, as well as the fact that metformin is associated with less hypoglycemic episodes than other diabetic medications (19). One randomized control trial on metformin monotherapy in advanced melanoma showed no benefit; however, the authors propose a more effective strategy would involve combining metformin with BRAF inhibitors and screening for patients with p53 polymorphisms (20). Such a trial in advanced melanoma has been completed (NCT02143050)[1], and another combining metformin with cancer immunotherapy is ongoing (NCT03311308)[2]. One randomized trial of metformin combinatorial treatment with standard of care chemotherapy showed no benefit in advanced pancreatic cancer (21), despite large meta-analysis showing significant survival in metformin treated pancreatic patients (22). These studies highlight a need for more rigorous planning of clinical trials that focus more on potential predictive biomarkers (23). Additionally, a randomized trial with metformin monotherapy in early stage breast cancer is ongoing (NCT01101438)[3], as well as a trial combining metformin with neo-adjuvant chemotherapy in HER2+ breast cancer (NCT03238495)[4]. These studies will reveal whether metformin's mode of action in cancer extends beyond its ability to reduce blood glucose levels, as glucose levels in healthy patients will not be affected by metformin treatment. Overall, the current available data support continued efforts toward examining the potential therapeutic role of metformin in various cancers, both in diabetic and non-diabetic patients.

Molecular Targets of Metformin

Metformin is known to act on the liver, gut and skeletal muscle to globally lower blood glucose levels in diabetic patients with hyperglycemia (24) (**Figure 1**). The first report of a direct molecular target of metformin was in 2000 (25) showing that metformin acts on complex I of the electron transport chain (ETC) of mitochondria. However, the experiments in this study were performed under harsh experimental conditions that included incubation of mitochondria at low temperature (8°C) for extended periods of time (400 min) in the presence of high dose (10 mM) of metformin. The conclusions were rapidly challenged when a study showed that metformin had no direct effect on mitochondrial complex I (26). As a result, this controversy remained, and for over a decade following these initial observations the molecular mechanism of metformin was characterized as unknown or incompletely described. Various targets have been proposed by several groups, including complex II and IV of the ETC (27), LKB1/AMPK (28–30), adenylate cyclase (31), AMP deaminase (32), NADPH oxidase (33) and mitochondrial glycerophosphate dehydrogenase (34). Elucidation of a key molecular target of metformin came in 2014 when three groups, using differential approaches and experimental conditions published novel and conclusive evidence on the inhibitory properties of metformin on complex I (35–37). This included work on permeabilized cells and cancer cells that do not express complex I (37), isolated mitochondria (35, 36) and purified complex I (36). It is now generally accepted that a direct molecular target of metformin is complex I (24, 38, 39). Many of the other proposed effects and targets of metformin may be explained by a shift in NAD/NADH caused by complex I inhibition, leading to decreased activity of enzymes that depend on the fine balance of cellular NAD/NADH. Inhibition of mitochondrial glycerophosphate dehydrogenase could also perturb NAD/NADH (40). The controversies surrounding the action of metformin on cells may be partly explained by the varying concentrations used in experimental systems (28).

Bioenergetic Stress: Metabolic Disruption

Complex I is the entry point for reduced NADH in the ETC. Direct inhibition of complex I by metformin in cells decreases the proton gradient and mitochondrial oxygen consumption rate (35), diminishes tricarboxylic acid cycle (TCA) activity and metabolites (35, 41–45) and leads to decreased cellular ATP

[1]NCT02143050 Study of Dabrafenib, Trametinib and Metformin for Melanoma Patients., (https://clinicaltrials.gov/show/NCT02143050).

[2]NCT03311308 A Trial of Pembrolizumab and Metformin Versus Pembrolizumab Alone in Advanced Melanoma., (https://clinicaltrials.gov/show/NCT03311308).

[3]NCT01101438 A Phase III Randomized Trial of Metformin vs Placebo in Early Stage Breast Cancer., (https://clinicaltrials.gov/show/NCT01101438).

[4]NCT03238495 Randomized Trial of Neo-adjuvant Chemotherapy With or Without Metformin for HER2 Positive Operable Breast Cancer., (https://clinicaltrials.gov/show/NCT03238495).

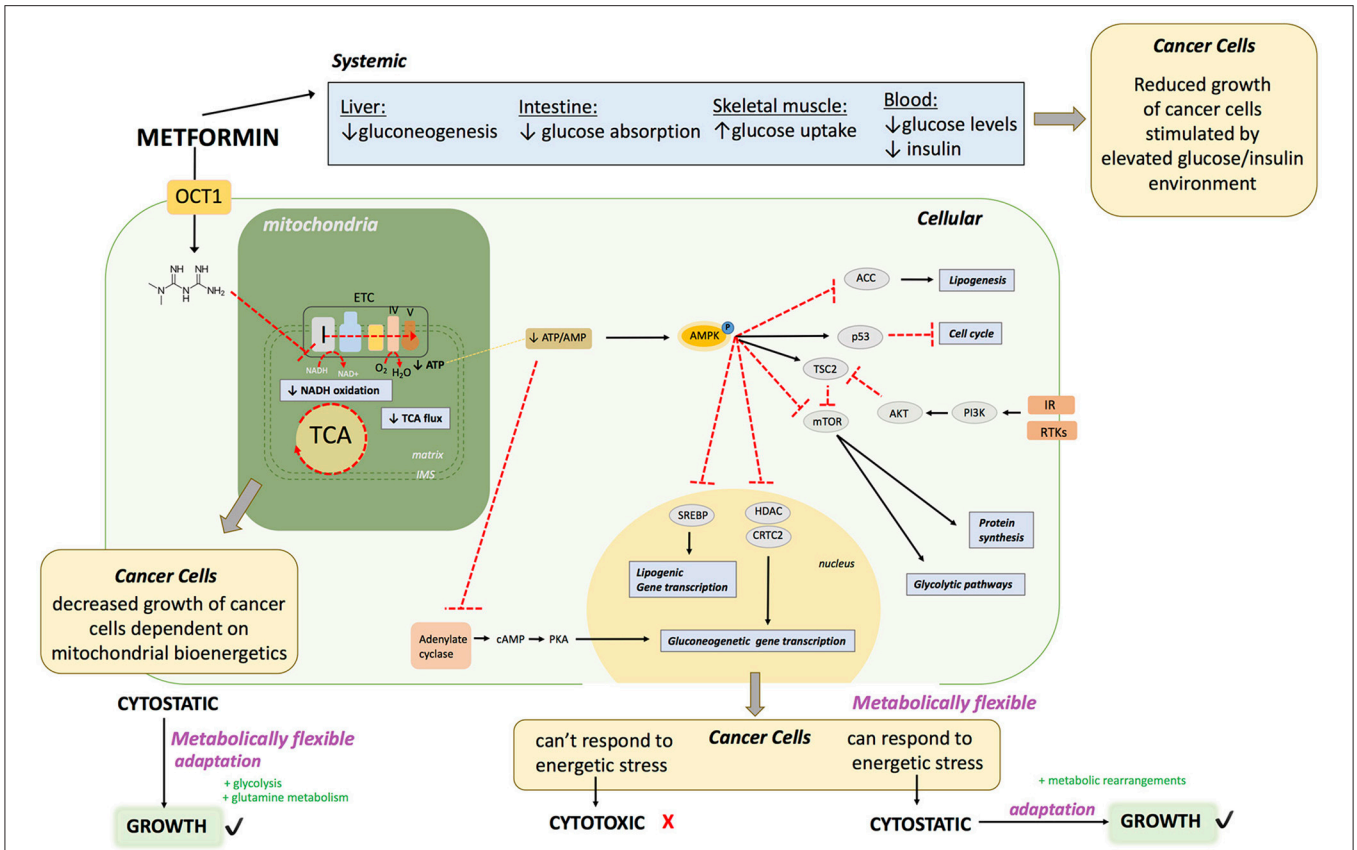

FIGURE 1 | Proposed Molecular Mechanisms of Metformin Action.

levels (25, 26, 43) (**Figure 1**). The inhibition of mitochondrial respiration and ATP production by metformin results in a compensatory increase in glycolysis (35, 43) as well as increased activity of glycolytic enzymes. These metabolic adaptations are engaged in an attempt to restore cellular ATP levels. However, if the compensatory activation of glycolysis cannot meet the cellular ATP requirements, AMPK becomes activated in order to potentiate catabolic metabolism, and inhibit anabolic reactions (30, 43, 46, 47). Phosphorylation and activation of AMPK leads to phosphorylation and inactivation of ACC, one of the most characterized targets of AMPK, causing a reduction in lipogenesis (48, 49). Metformin increases the levels of AMP causing inhibition of adenylate cyclase (31). Metformin also inhibits mTORC1 signaling (50, 51). Overall, metformin treatment causes at least a transient decrease in cellular energy status, leading to a global decrease in ATP consuming processes. In proliferating cells, this can elicit a cytostatic state that is associated with reduced proliferation, explaining some clinical observations of decreased progression of cancer cell growth. Cancer cells that cannot eventually compensate for this reduced energy status may undergo apoptosis (52, 53).

Metformin enters the cell via an OCT transporter; commonly OCT1 expressed on the surface of hepatocytes. Metformin acts directly on mitochondria to inhibit complex I of the ETC. This causes 1) diminished NADH oxidation at complex I, resulting in a buildup of NADH, 2) diminished TCA cycle activity due to allosteric inhibition of enzymes in the TCA cycle from increased NADH/NAD, 3) diminished flow of electrons throughout the ETC, and ultimately diminished oxygen consumption and ATP production at complex V (ATP synthase). This can lead to decreased growth of a subset of cancers that heavily rely on mitochondrial bioenergetics. Failure to rearrange metabolic programs leads to decreased ATP levels. Diminished ATP levels in the cell leads to AMPK activation. In hepatocytes, this drop in ATP leads to a decrease in gluconeogenesis due to allosteric inhibition of several leads to a decreased absorption of glucose. In the muscles, this leads to increased glucose uptake and eventually a decrease in hyperglycemia in the blood; with reduced glucose and insulin levels. The reduction in blood glucose and insulin levels may impair the growth of a subset of cancers that proliferate in an environment dictated by type 2 diabetes. At a cellular level, activation of AMPK leads (1) to inactivation of ACC, leading to a decrease in lipogenesis, (2) activation of p53 leading to a decrease in cell cycle progression, (3) inactivation of mTOR leading to decreased protein synthesis and glycolytic pathways. Inactivation of mTOR may be useful in a subset of cancers that have RTKs or IR activation. AMPK activation also leads to (4) decreased transcription of gluconeogenic genes by inhibition of HDAC and CRTC2, which is also achieved by (5) adenylate cyclase inhibition. Furthermore, AMPK activation causes (6) a decrease of lipogenic gene expression by inhibition of SREBP. The end

result of metformin exposure is cellular energetic stress. If the cancer cells are metabolically flexible, allowing them to successfully respond to this stress by rearranging metabolic programs, metformin has a cytostatic effect, however if cells fail to cope, metformin has a cytotoxic effect.

OCT1: organic transporter 1, **TCA:** tricarboxylic acid cycle, **AMPK**: 5′ adenosine monophosphate- activated protein kinase, **IR:** insulin receptor **ACC** acetyl-CoA carboxylase: **mTOR:** mammalian target of rapamycin, **RTKs**: receptor tyrosine kinases. **cAMP:** Cyclic adenosine monophosphate, **AKT**: protein kinase B, **SREBP:** Sterol regulatory element binding protein, **HDAC:** histone deacetylase, **CRTC2:** CREB-regulated transcription coactivator 2.

METFORMIN: BIOENERGETIC MEDICINE

Bioenergetic Medicine

Metformin is now classified as a bioenergetic disruptor and such drugs represent an exciting strategy to treat metabolic disorders, including cancer. Bioenergetic drugs affect ATP generating pathways, namely glycolysis, and oxidative phosphorylation. Bioenergetics is undeniably coupled to the proliferative potential of cancer cells. The focus of bioenergetic medicine (54) is not solely to impact ATP production, but also to disrupt biosynthetic pathways that rely on precursor metabolites found in ATP generating pathways for cancer cell proliferation. For example, glucose metabolism has been targeted using glycolysis inhibitors, such as 2-deoxyglucose (2-DG), a non-metabolizable glucose analog, which has been employed in clinical trials for various cancer types. Although many *in vitro* or murine studies demonstrate profound effects of 2-DG treatment on the growth of various cancer cell models (55–57), many clinical trials with 2-DG have been terminated early due to lack of early clinical efficacy as well as side effects, notably extreme exhaustion and cardiac arrhythmias in patients (NCT00633087)[5]. A completed study investigating an optimal dosage of 2-DG for solid tumors in combination with docetaxel treatment noted only moderate effects on stabilizing disease (58). However, significant side effects, including fatigue and nausea, were noted in many of patients (58).

In addition to glucose, many cancers are dependent on glutamine for their growth and are said to suffer from glutamine "addiction" (59). The expression of glutaminase is also up regulated in various cancer types (60–62). Murine tumor xenografts show promising anti-growth responses to inhibition of glutamine (glutaminase) metabolism (63, 64), and clinical trials are currently ongoing to test the efficacy of inhibiting glutaminase using a small molecule inhibitor (CB-839, Calithera Biosciences) in multiple types and stages of cancer (NCT02071862[6]; NCT02071888[7]; NCT03163667)[8]. It has also been suggested that metastatic progression is accompanied by increased glutamine utilization, and thus more aggressive prostate cancer cells were more sensitive to the glutaminase inhibitor CB-839 (65). However, to date, there are no glutaminase inhibitors approved for usage in cancer treatment.

Sensitivity to Metformin: A Metabolic Profile

Performing clinical trials in patients to determine which cancer type will benefit most from metformin treatment is undeniably important to understand the potential of this drug in oncology. With recent advancements, especially the identification of a molecular target of metformin, an alternative strategy to elucidate metformin's potential in oncology is to establish a "metformin sensitivity" profile at the cellular level to identify those cancer cell types most sensitive to its effects (**Figure 2**)**.** This entails (1) understanding the metabolic changes that occur upon metformin treatment, (2) determining the cancer cell types most susceptible to these changes, (3) identifying those patients that would best benefit from metformin treatment and lastly, 4) defining combinatorial therapies that work best with metformin treatment in order to prevent compensatory mechanisms. This approach represents a rational and streamlined method to identify patients who would be most responsive to metformin treatment. However, it is difficult to predict whether the effects observed at the cellular level will translate *in vivo*. Therefore, the comparisons of the results obtained *in vitro*, *in vivo* and in clinical trials are necessary to reveal the full potential of metformin in the oncology setting.

To identify cancer cells most susceptible to metformin, we first need to recognize its mechanism of action and identify the internal cellular changes that occur upon treatment. Metformin inhibits complex I of the ETC in mitochondria, leading to perturbation in NAD/NADH and decreased oxygen consumption. This leads to diminished TCA activity and metabolite levels, as well as potential energetic stress leading to AMPK activation. Cells compensate for these metformin-mediated effects by increasing glucose uptake and glycolysis, and switching to glutamine utilization, as a way of refueling the TCA and providing biosynthetic intermediates for lipid production required to synthesize membranes (35, 42, 43, 66, 67). Hence, cancer cells exposed to metformin need to rearrange and reroute metabolic flux. It is increasingly evident that metformin alters substrate utilization in the mitochondria (45). As a result, cancer cells that would be most susceptible to metformin's action would have a high reliance on OXPHOS as a source of ATP and lack metabolic flexibility to efficiently engage glycolysis. For example, cancer cells with defective mitochondria may not be able to successfully switch mitochondrial substrate utilization due to mutations or defects in these metabolic pathways. As a result, cells with defective mitochondria could be more sensitive to metformin treatment due to their inability to alter mitochondrial substrate utilization. In support of this point, complex I mutations have been shown to predict sensitivity to phenformin (68). It is possible that cancer cells with oxidative phosphorylation deficits may thus be more sensitive to biguanides than normal tissues.

[5]NCT00633087 A Phase I/II Trial of 2-Deoxyglucose (2DG) for the Treatment of Advanced Cancer and Hormone Refractory Prostate Cancer. (https://clinicaltrials.gov/show/NCT00633087).

[6]NCT02071862 Study of the Glutaminase Inhibitor CB-839 in Solid Tumors.

[7]NCT02071888 Study of the Glutaminase Inhibitor CB-839 in Hematological Tumors.

[8]NCT03163667 CB-839 With Everolimus vs. Placebo With Everolimus in Patients With RCC.

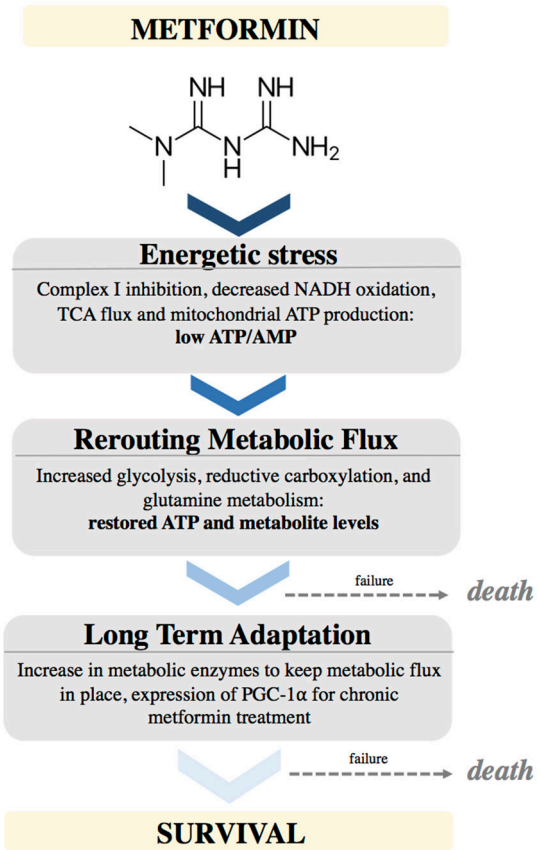

FIGURE 2 | Schematic depicting the effects of Metformin on cellular metabolism and adaptations needed to support cancer cell survival.

It is becoming more apparent that numerous metabolic programs and adaptations in cancer cells are mediated by the metabolic regulator PGC-1α (69). We predict that cancer cells expressing low PGC-1α levels, or that fail to upregulate PGC-1α in the presence of metformin, would be more sensitive as they may not efficiently engage adaptive programs to promote survival. Additionally, cancer cells with inactive or impaired AMPK signaling may be more sensitive to metformin treatment, as AMPK is the main energy sensor in the cell, and AMPK activation upon exposure to metformin contributes significantly to the upregulation of PGC-1α and its adaptive programs (70). Although previously controversial, AMPK is not required for metformin action; however, AMPK signaling is advantageous as an adaptive response to cope with energetic stress (71).

Metformin causes energetic stress in cells by inhibiting complex I of the electron transport chain in mitochondria. This causes a decrease in NADH oxidation, decreased TCA flux, leading to low levels of TCA metabolites. This causes a temporarily low ATP/AMP ratio. Cells react by rewiring metabolic flux. This includes up regulating pathways to support increased glycolysis, increased glutamine utilization to provide alternative sources of ATP as well as metabolites. Cells that fail to metabolically adapt to this stress will undergo cell death. After longer exposure to metformin, cells will adapt by stably increasing enzymes needed to maintain these metabolic pathways, partially by upregulating PGC-1α expression (**Figure 2**).

Metabolic Flexibility: Targeting Metformin Resistance

It has recently been shown that chronic exposure to metformin in cancer cells ultimately leads to drug resistance and that this is linked to increased PGC-1α levels (41). Metformin resistant cells are metabolically flexible and able to switch fuel sources from oxidative metabolism to glycolysis and glutamine metabolism in the context of metformin-mediated inhibition of oxidative phosphorylation. Although at first it may seem counterintuitive to increase the level of PGC-1α, a key regulator of OXPHOS and mitochondrial biogenesis, upon inhibition of OXPHOS by metformin, it is now appreciated that PGC-1α clearly has functions outside of its classic role in mitochondrial metabolism. We argue that PGC-1α supports metabolic flexibility upon bioenergetic stresses. Elevated PGC-1α levels in the presence of metformin reprograms cellular metabolism and creates a new metabolic state that promotes an alternate source of ATP production through stimulation of glycolysis as well as facilitating anabolic metabolism by diverting mitochondrial metabolites that would normally be used for ATP production for use in anabolic reactions. In support of this point, PGC-1α controls numerous metabolic programs in cancer, notably glucose (41, 72), glutamine (73), fat (74), and one carbon metabolism (70). This ability of PGC-1α to support numerous metabolic programs in breast cancer cells allows for an enhanced fuel flexibility to cope with bioenergetic stressors such as metformin (41).

After developing a greater understanding of the metabolic rearrangements that occur upon metformin treatment (**Figure 2**), rational combinatorial treatments can be devised to combat adaptive mechanisms (**Figure 3**). The most immediate strategy would be to combine metformin with glycolysis inhibitors to prevent the adaptive glycolytic activity seen with metformin treatment alone. Blocking oxidative phosphorylation and glycolysis would stop the two main sources of ATP production, ultimately leading to cell death. Indeed, when breast cancer cells treated with metformin are deprived of glucose, this results in almost 100% cell death in just 72 h, even in the presence of glutamine (35). Additionally, it has been shown that cells with mutations leading to either impaired glucose utilization or mitochondrial DNA mutations are more sensitive to the effects of biguanides (68). Other reports have shown similar results by combining metformin with inhibitors of glycolysis and thus preventing ATP production (75, 76). One concern is that all cells are capable of engaging glycolysis and OXPHOS for ATP production, although their degree of dependence on either pathway can vary. Rapidly proliferating cells require much more ATP than differentiated cells, thus targeting ATP producing pathways could prove beneficial, as this rationale has been the basis of chemotherapy for decades. Another potential metabolic combination therapy could be the targeting of regulators of metabolic flexibility, notably PGC-1α. A small molecule compound was recently found to

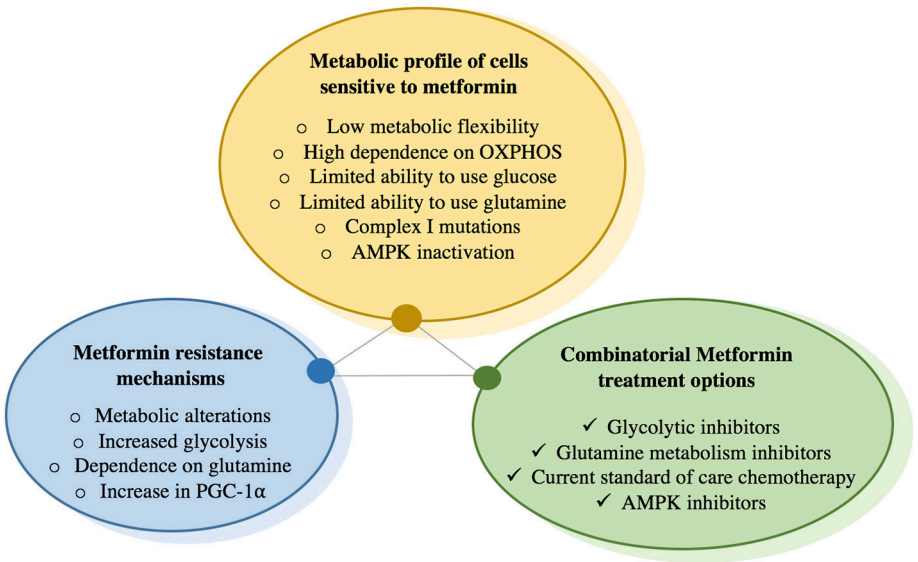

FIGURE 3 | Concepts for metformin sensitivity, resistance, and combinatorial treatment options.

reduce PGC-1α-dependent gluconeogenic activity in the liver by increasing PGC-1α acetylation, leading to an amelioration of glucose homeostasis in a murine model of diabetes (77). However, the role of PGC-1α in gluconeogenesis is far more developed than in cancer, and cancer specific post-translational modifications on PGC-1α are far less understood.

In addition, metformin is currently used in clinical trials as a combinational therapy with already established treatment options, such as chemotherapy. It has been suggested that combinatorial treatment of metformin with chemotherapy may sensitize cancer cells to chemotherapy treatment, leading to improved treatment efficacy and lower doses of administered chemotherapy (78–82). In murine models, combinatorial metformin treatment with the chemotherapeutic agent doxorubicin led to reduced mammary tumor mass and relapse compared to either drug alone when performing xenograft experiments (83, 84). There is also data showing that metformin has synergistic effects with various chemotherapy agents, including Pemetrexed in cell lines of non-small cell lung cancer (NSCLC), (85) EGFR-TKI in patients with NSCLC (86), Trichostatin in osteosarcoma cell lines (87), Simvastatin in animal models of metastatic prostate cancer (88) and Nelfinavir in cervical cancer xenografts (89). It has also been suggested that metformin may lead to a re-sensitization of cancer cells that have become resistance to chemotherapy, the predominant cause of treatment failure in patients undergoing treatment (45, 84, 90, 91). One study showed that metformin reduces the differences in metabolism between chemotherapy resistant and sensitive cells (92). Furthermore, metformin was shown to target metabolic programs that chemoresistant cancer cells become reliant on, including OXPHOS and glutamine metabolism (92).

Overall, metabolic flexibility is required to adapt to bioenergetic stress, such as metformin exposure. Additionally, cancer cells treated with chemotherapeutic agents display vast metabolic arrangements enabling them to become resistant. Targeting this flexibility by inhibiting compensatory metabolic shifts, such as using inhibitors of glycolysis or glutamine metabolism, may prove useful. It is becoming clear that attacking only one aspect of cellular growth or one metabolic pathway will ultimately lead to metabolic rearrangements and the emergence of resistance. Targeting both cellular proliferation and metabolism could prove to be a more efficacious strategy. Another approach could be to overload the compensatory metabolic pathways by drastically increasing ATP demand through the use of chemotherapeutic agents.

The features of cancer cells that would make them most sensitive to metformin treatment are described. Cells become resistant to chronic exposure to metformin by increasing glucose uptake as well as glycolysis, increasing glutamine utilization as a vital metabolite precursor for biosynthetic needs, as well as increase in PGC-1α expression, which has been shown to increase metabolic flexibility that is needed to overcome metformin-mediated bioenergetic stress. To prevent compensatory mechanisms by cells exposed to metformin, this drug can be combined with glycolysis inhibitors that prevent metabolism of glucose to lactate, or glutamine metabolism inhibitors, which prevent glutamine utilization. There is also data suggesting that metformin has synergetic effects with certain chemotherapies and may re-sensitize cancer cells that have become resistant to chemotherapy.

FUTURE OF METFORMIN IN ONCOLOGY

Development of Novel Complex I Inhibitors in Oncology

In addition to metformin, various mitochondrial drugs are being developed for potential uses in oncology, and have been

shown to alter mitochondrial metabolism. These include: (1) small molecule BAY 87-2242 that was developed as a complex I inhibitor, leading to a reduction of melanoma tumor growth in murine models (93), (2) Xanthohumol that leads to the overproduction of ROS and eventual apoptosis in cancer cells (94), (3) Canagliflozin, a proposed inhibitor of complex I and mitochondrial glutamate dehydrogenase (95), which reduces the proliferation of prostate and lung cancer cells (96), 4] Fenofibrate, another proposed complex I inhibitor, that depletes cellular ATP and induces cytotoxicity in glioblastoma (97) and 5) small molecule inhibitor JC1-20679 developed to inhibit complex I, slowing the growth of a panel of cancer cell lines (98). These results highlight the importance of mitochondrial metabolism in cancer and support the notion of targeting mitochondria for cancer therapeutic purposes. At this stage, it is unknown whether some of these molecules will be approved for usage in clinical trials, as toxicity in humans has not yet been demonstrated for all these drugs. However, Canaglifozin is already used for the treatment of type 2 diabetes; but the FDA has recently added additional *Warning and Precautions* stating that this drug causes increased ketoacidosis, decreased bone density, and increased risk of leg and foot amputations (99, 100). Developing an effective drug for oncology is clearly not as simple as just synthesizing potent mitochondrial inhibitors. It is important to appreciate that complex I inhibitors, like rotenone or MPTP, can induce neurodegeneration in murine models (101, 102). However, metformin intake has been associated with better cognitive function in patients with Huntington's Disease (103). Indeed, it has been shown that metformin confers protection against mutant Huntingtin by modulating mitochondrial dynamics and activating AMPK (104).

In addition to metformin, phenformin is being revisited for usage in cancer therapy. Phenformin, like metformin, is a complex I inhibitor (36); however, it is transported with a greater affinity and kinetics into cells (105). For this reason, phenformin rapidly accumulates in cancer cells. Additionally, phenformin uptake will not depend on the genetic variation of transporters (OCT family), which have been shown to influence metformin uptake and efficacy due to individual polymorphisms (106). Phenformin is currently in a few clinical trials including a phase I clinical trial to determine optimal dosage for combined treatment with small molecule targeted therapies (Dabrafenib and Trametinib) for patients with BRAF mutated melanoma (NCT03026517)[9]. It is being examined whether phenformin can reduce melanoma resistance to traditional targeted therapies. It is possible that phenformin will become more rapidly used in future clinical trials; however, accurate dosage, which is effective yet minimizes side effects, has always been an issue, and is a key reason for its rapid discontinued use in diabetes (107). Therefore, there is still a need to determine optimal doses of phenformin for oncology application, while minimizing side effects such as lactic acidosis and gastrointestinal distress. With optimal dosage of phenformin, it may even be possible to decrease the dosage of chemotherapeutic agents.

Lastly, an emerging field in cancer metabolism is the development of organelle targeted therapeutics (108), which could be utilized to specifically localize and compartmentalize therapies to potentially minimize adverse effects. This notion could be used to reduce administered doses of therapy, while maximizing dose in the compartmentalized region, although this research area needs to be developed further.

AUTHOR CONTRIBUTIONS

SA wrote most of the text for this manuscript. SA, PS and JS-P contributed to the concepts, writing and editing of the manuscript.

FUNDING

Research conducted in the authors' laboratories were supported from grants from the Terry Fox Research Institute (TFF-PPG-242122) and the CIHR (PJT-148650).

[9]NCT03026517 Clinical Trial of Phenformin in Combination With Dabrafenib and Trametinib for Patients With BRAF-mutated Melanoma.

REFERENCES

1. Bailey CJ, Day C. Traditional plant medicines as treatments for diabetes. *Diabetes Care* (1989) 12:553–64. doi: 10.2337/diacare.12.8.553
2. Witters LA. The blooming of the French lilac, *J Clin Invest.* (2001) 108:1105–7. doi: 10.1172/JCI14178
3. White JR Jr. a brief history of the development of diabetes medications. *Diabetes Spectr.* (2014) 27:82–6. doi: 10.2337/diaspect.27.2.82
4. Nattrass M, Alberti KG. Biguanides. *Diabetologia* (1978) 14:71–4. doi: 10.1007/BF01263443
5. McGuinness ME, Talbert RL. Phenformin-induced lactic acidosis: a forgotten adverse drug reaction. *Ann Pharmacother* (1993) 27:1183–7. doi: 10.1177/106002809302701004
6. Bailey CJ, Turner RC. Metformin. *N Engl J Med.* (1996) 334:574–9. doi: 10.1056/NEJM199602293340906
7. Lalau JD, Race JM. Lactic acidosis in metformin therapy: searching for a link with metformin in reports of "metformin-associated lactic acidosis". *Diabetes Obes Metab.* (2001) 3:195–201. doi: 10.1046/j.1463-1326.2001.00128.x
8. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, et al. Diabetes and cancer: a consensus report. *Diabetes Care* (2010) 33:1674–85. doi: 10.2337/dc10-0666
9. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ* (2005) 330:1304–5. doi: 10.1136/bmj.38415.708634.F7
10. Bowker SL, Majumdar SR, Veugelers P, Johnson JA. Increased cancer-related mortality for patients with type 2 diabetes who use sulfonylureas or insulin. *Diabetes Care* (2006) 29:254–8. doi: 10.2337/diacare.29.02.06.dc05-1558
11. Tian RH, Zhang YG, Wu Z, Liu X, Yang JW, Ji HL. Effects of metformin on survival outcomes of lung cancer patients with type 2 diabetes mellitus: a meta-analysis. *Clin Transl Oncol.* (2016) 18:641–9. doi: 10.1007/s12094-015-1412-x
12. Meng F, Song L, Wang W. Metformin improves overall survival of colorectal cancer patients with diabetes: a meta-analysis. *J Diabetes Res.* (2017) 2017:5063239. doi: 10.1155/2017/5063239

13. He XX, Tu SM, Lee MH, Yeung SC. Thiazolidinediones and metformin associated with improved survival of diabetic prostate cancer patients. *Ann Oncol.* (2011) 22:2640–5. doi: 10.1093/annonc/mdr020
14. Spratt DE, Zhang C, Zumsteg ZS, Pei X, Zhang Z, Zelefsky MJ. Metformin and prostate cancer: reduced development of castration-resistant disease and prostate cancer mortality. *Eur Urol.* (2013) 63:709–16. doi: 10.1016/j.eururo.2012.12.004
15. Wang Z, Lai ST, Xie L, Zhao JD, Ma NY, Zhu J, et al. Metformin is associated with reduced risk of pancreatic cancer in patients with type 2 diabetes mellitus: a systematic review and meta-analysis. *Diabetes Res Clin Pract.* (2014) 106:19–26. doi: 10.1016/j.diabres.2014.04.007
16. Col NF, Ochs L, Springmann V, Aragaki AK, Chlebowski RT. Metformin and breast cancer risk: a meta-analysis and critical literature review. *Breast Cancer Res Treat.* (2012) 135:639–46. doi: 10.1007/s10549-012-2170-x
17. Nie Z, Zhu H, Gu M. Reduced colorectal cancer incidence in type 2 diabetic patients treated with metformin: a meta-analysis. *Pharm Biol.* (2016) 54:2636–42. doi: 10.1080/13880209.2016.1176057
18. Zhang H, Gao C, Fang L, Zhao HC, Yao SK. Metformin and reduced risk of hepatocellular carcinoma in diabetic patients: a meta-analysis. *Scand J Gastroenterol.* (2013) 48:78–87. doi: 10.3109/00365521.2012.719926
19. Bodmer M, Meier C, Krahenbuhl S, Jick SS, Meier CR. Metformin, sulfonylureas, or other antidiabetes drugs and the risk of lactic acidosis or hypoglycemia: a nested case-control analysis. *Diabetes Care* (2008) 31:2086–91. doi: 10.2337/dc08-1171
20. Montaudie H, Cerezo M, Bahadoran P, Roger C, Passeron T, Machet L, Arnault JP, Verneuil L, Maubec E, Aubin F, et al. Metformin monotherapy in melanoma: a pilot, open-label, prospective, and multicentric study indicates no benefit. *Pigment Cell Melanoma Res.* (2017) 30:378–80. doi: 10.1111/pcmr.12576
21. Kordes S, Pollak MN, Zwinderman AH, Mathot RA, Weterman MJ, Beeker A, et al. Metformin in patients with advanced pancreatic cancer: a double-blind, randomised, placebo-controlled phase 2 trial. *Lancet Oncol.* (2015) 16:839–47. doi: 10.1016/S1470-2045(15)00027-3
22. Li X, Li T, Liu Z, Gou S, Wang C. The effect of metformin on survival of patients with pancreatic cancer: a meta-analysis. *Sci Rep.* (2017) 7:5825. doi: 10.1038/s41598-017-06207-x
23. Bhaw-Luximon A, Jhurry D. Metformin in pancreatic cancer treatment: from clinical trials through basic research to biomarker quantification. *J Cancer Res Clin Oncol.* (2016) 142:2159–2171. doi: 10.1007/s00432-016-2178-4
24. Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B. Metformin: from mechanisms of action to therapies. *Cell Metab.* (2014) 20:953–66. doi: 10.1016/j.cmet.2014.09.018
25. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J.* (2000) 348 (Pt 3):607–14. doi: 10.1042/bj3480607
26. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem.* (2000) 275:223–8. doi: 10.1074/jbc.275.1.223
27. Drahota Z, Palenickova E, Endlicher R, Milerova M, Brejchova J, Vosahlikova M, et al. Biguanides inhibit complex I, II and IV of rat liver mitochondria and modify their functional properties. *Physiol Res.* (2014) 63:1–11. Available online at: http://www.biomed.cas.cz/physiolres/pdf/63/63_1.pdf
28. He L, Wondisford FE. Metformin action: concentrations matter. *Cell Metab.* (2015) 21:159–62. doi: 10.1016/j.cmet.2015.01.003
29. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* (2005) 310:1642–6. doi: 10.1126/science.1120781
30. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest.* (2001) 108:1167–74. doi: 10.1172/JCI13505
31. Miller RA, Chu Q, Xie J, Foretz M, Viollet B, Birnbaum MJ. Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature* (2013) 494:256–60. doi: 10.1038/nature11808
32. Ouyang J, Parakhia RA, Ochs RS. Metformin activates AMP kinase through inhibition of AMP deaminase. *J Biol Chem.* (2011) 286:1–11. doi: 10.1074/jbc.M110.121806
33. Piwkowska A, Rogacka D, Jankowski M, Dominiczak MH, Stepinski JK, Angielski S. Metformin induces suppression of NAD(P)H oxidase activity in podocytes. *Biochem Biophys Res Commun.* (2010) 393:268–73. doi: 10.1016/j.bbrc.2010.01.119
34. Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, Prigaro BJ, Wood JL, Bhanot S, MacDonald MJ, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature* (2014) 510:542–6. doi: 10.1038/nature13270
35. Andrzejewski S, Gravel SP, Pollak M, St-Pierre J. Metformin directly acts on mitochondria to alter cellular bioenergetics. *Cancer Metab.* (2014) 2:12. doi: 10.1186/2049-3002-2-12
36. Bridges HR, Jones AJ, Pollak MN, Hirst J. Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria. *Biochem J.* (2014) 462:475–87. doi: 10.1042/BJ20140620
37. Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, Glasauer A, Dufour E, Mutlu GM, Budigner GS, et al. Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *Elife* (2014) 3:e02242. doi: 10.7554/eLife.02242
38. Luengo A, Sullivan LB, Heiden MG. Understanding the complex-I-ty of metformin action: limiting mitochondrial respiration to improve cancer therapy. *BMC Biol.* (2014) 12:82. doi: 10.1186/s12915-014-0082-4
39. Pernicova I, Korbonits M. Metformin–mode of action and clinical implications for diabetes and cancer. *Nat Rev Endocrinol.* (2014) 10:143–56. doi: 10.1038/nrendo.2013.256
40. Baur JA, Birnbaum MJ. Control of gluconeogenesis by metformin: does redox trump energy charge? *Cell Metab.* (2014) 20:197–99. doi: 10.1016/j.cmet.2014.07.013
41. Andrzejewski S, Klimcakova E, Johnson RM, Tabaries S, Annis MG, McGuirk S, et al. PGC-1α promotes breast cancer metastasis and confers bioenergetic flexibility against metabolic drugs. *Cell Metab.* (2017) 26:778–87.e5. doi: 10.1016/j.cmet.2017.09.006
42. Fendt SM, Bell EL, Keibler MA, Davidson SM, Wirth GJ, Fiske B, et al. (2013). Metformin decreases glucose oxidation and increases the dependency of prostate cancer cells on reductive glutamine metabolism. *Cancer Res.* (2017) 73:4429–38. doi: 10.1158/0008-5472.CAN-13-0080
43. Griss T, Vincent EE, Egnatchik R, Chen J, Ma EH, Faubert B, et al. Metformin antagonizes cancer cell proliferation by suppressing mitochondrial-dependent biosynthesis. *PLoS Biol.* (2015) 13:e1002309. doi: 10.1371/journal.pbio.1002309
44. Janzer A, German NJ, Gonzalez-Herrera KN, Asara JM, Haigis MC, Struhl K. Metformin and phenformin deplete tricarboxylic acid cycle and glycolytic intermediates during cell transformation and NTPs in cancer stem cells. *Proc Natl Acad Sci USA.* (2014) 111:105749. doi: 10.1073/pnas.1409844111
45. Liu X, Romero IL, Litchfield LM, Lengyel E, Locasale JW. Metformin targets central carbon metabolism and reveals mitochondrial requirements in human cancers. *Cell Metab.* (2016) 24:728–39. doi: 10.1016/j.cmet.2016.09.005
46. Bogachus LD, Turcotte LP. Genetic downregulation of AMPK-alpha isoforms uncovers the mechanism by which metformin decreases FA uptake and oxidation in skeletal muscle cells. *Am J Physiol Cell Physiol.* (2010) 299:C1549–61. doi: 10.1152/ajpcell.00279.2010
47. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. *Diabetologia* (2017) 60:1577–85. doi: 10.1007/s00125-017-4342-z
48. Hardie DG, Ross FA, Hawley SA. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol.* (2017) 13:251–62. doi: 10.1038/nrm3311
49. Loubiere C, Goiran T, Laurent K, Djabari Z, Tanti JF, Bost F. Metformin-induced energy deficiency leads to the inhibition of lipogenesis in prostate cancer cells. *Oncotarget* (2015) 6:15652–61. doi: 10.18632/oncotarget.3404
50. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. *Cancer Res.* (2007) 67:10804–12. doi: 10.1158/0008-5472.CAN-07-2310
51. Liu X, Chhipa RR, Pooya S, Wortman M, Yachyshin S, Chow LM, Kumar A, Zhou X, Sun Y, Quinn B, et al. Discrete mechanisms of mTOR and cell

cycle regulation by AMPK agonists independent of AMPK. *Proc Natl Acad Sci USA*. (2014) 111:E435–44. doi: 10.1073/pnas.1311121111
52. Bhat M, Yanagiya A, Graber T, Razumilava N, Bronk S, Zammit D, Zhao Y, Zakaria C, Metrakos P, Pollak M, et al. Metformin requires 4E-BPs to induce apoptosis and repress translation of Mcl-1 in hepatocellular carcinoma cells. *Oncotarget* (2017) 8:50542–56. doi: 10.18632/oncotarget.10671
53. Takahashi A, Kimura F, Yamanaka A, Takebayashi A, Kita N, Takahashi K, et al. Metformin impairs growth of endometrial cancer cells via cell cycle arrest and concomitant autophagy and apoptosis. *Cancer Cell Int.* (2014) 14:53. doi: 10.1186/1475-2867-14-53
54. Swerdlow RH. Bioenergetic medicine. *Br J Pharmacol.* (2014) 171:1854–69. doi: 10.1111/bph.12394
55. Ben Sahra I, Laurent K, Giuliano S, Larbret F, Ponzio G, Gounon P, Le Marchand-Brustel Y, Giorgetti-Peraldi S, Cormont M, Bertolotto C, et al. Targeting cancer cell metabolism: the combination of metformin and 2-deoxyglucose induces p53-dependent apoptosis in prostate cancer cells. *Cancer Res.* (2010) 70:2465–75. doi: 10.1158/0008-5472.CAN-09-2782
56. Huang CC, Wang SY, Lin LL, Wang PW, Chen TY, Hsu WM, et al. Glycolytic inhibitor 2-deoxyglucose simultaneously targets cancer and endothelial cells to suppress neuroblastoma growth in mice. *Dis Model Mech.* (2015) 8:1247–54. doi: 10.1242/dmm.021667
57. Kurtoglu M, Gao N, Shang J, Maher JC, Lehrman MA, Wangpaichitr M, et al. Under normoxia, 2-deoxy-D-glucose elicits cell death in select tumor types not by inhibition of glycolysis but by interfering with N-linked glycosylation. *Mol Cancer Ther.* (2007) 6:3049–58. doi: 10.1158/1535-7163.MCT-07-0310
58. Raez LE, Papadopoulos K, Ricart AD, Chiorean EG, Dipaola RS, Stein MN, Rocha Lima CM, Schlesselman JJ, Tolba K, Langmuir VK, et al. A phase I dose-escalation trial of 2-deoxy-D-glucose alone or combined with docetaxel in patients with advanced solid tumors. *Cancer Chemother Pharmacol.* (2013) 71:523–30. doi: 10.1007/s00280-012-2045-1
59. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. *Trends Biochem Sci.* (2010) 35:427–33. doi: 10.1016/j.tibs.2010.05.003
60. Craze ML, Cheung H, Jewa N, Coimbra NDM, Soria D, El-Ansari R, Aleskandarany MA, Wai Cheng K, Diez-Rodriguez M, Nolan CC, et al. MYC regulation of glutamine-proline regulatory axis is key in luminal B breast cancer. *Br J Cancer* (2018) 118:258–65. doi: 10.1038/bjc.2017.387
61. Huang F, Zhang Q, Ma H, Lv Q, Zhang T. Expression of glutaminase is upregulated in colorectal cancer and of clinical significance. *Int J Clin Exp Pathol.* (2014) 7:1093–100. Available online at: http://www.ijcep.com/files/ijcep1401009.pdf
62. Pan T, Gao L, Wu G, Shen G, Xie S, Wen H, et al. Elevated expression of glutaminase confers glucose utilization via glutaminolysis in prostate cancer. *Biochem Biophys Res Commun.* (2015) 456:452–8. doi: 10.1016/j.bbrc.2014.11.105
63. Wang JB, Erickson JW, Fuji R, Ramachandran S, Gao P, Dinavahi R, Wilson KF, Ambrosio AL, Dias SM, Dang CV, et al. Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. *Cancer Cell* (2010) 18:207–19. doi: 10.1016/j.ccr.2010.08.009
64. Xiang Y, Stine ZE, Xia J, Lu Y, O'Connor RS, Altman BJ, Hsieh AL, Gouw AM, Thomas AG, Gao P, et al. Targeted inhibition of tumor-specific glutaminase diminishes cell-autonomous tumorigenesis. *J Clin Invest.* (2015) 125:2293–306. doi: 10.1172/JCI75836
65. Zacharias NM, McCullough C, Shanmugavelandy S, Lee J, Lee Y, Dutta P, McHenry J, Nguyen L, Norton W, Jones LW, et al. Metabolic differences in glutamine utilization lead to metabolic vulnerabilities in prostate cancer. *Sci Rep.* (2017) 7:16159. doi: 10.1038/s41598-017-16327-z
66. Javeshghani S, Zakikhani M, Austin S, Bazile M, Blouin MJ, Topisirovic I, et al. Carbon source and myc expression influence the antiproliferative actions of metformin. *Cancer Res.* (2012) 72:6257–67. doi: 10.1158/0008-5472.CAN-12-2907
67. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: an overview. *Clin Sci.* (2012) 122:253–70. doi: 10.1042/CS20110386
68. Birsoy K, Possemato R, Lorbeer FK, Bayraktar EC, Thiru P, Yucel B, et al. Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. *Nature* (2014) 508:108–12. doi: 10.1038/nature13110
69. Deblois G, St-Pierre J, Giguere V. The PGC-1/ERR signaling axis in cancer. *Oncogene* (2013) 32:3483–90. doi: 10.1038/onc.2012.529
70. Audet-Walsh E, Papadopoli DJ, Gravel SP, Yee T, Bridon G, Caron M, et al. The PGC-1alpha/ERRalpha axis represses one-carbon metabolism and promotes sensitivity to anti-folate therapy in breast cancer. *Cell Rep.* (2016) 14:920–31. doi: 10.1016/j.celrep.2015.12.086
71. Chaube B, Malvi P, Singh SV, Mohammad N, Viollet B, Bhat MK. AMPK maintains energy homeostasis and survival in cancer cells via regulating p38/PGC-1alpha-mediated mitochondrial biogenesis. *Cell Death Discov.* (2015) 1:15063. doi: 10.1038/cddiscovery.2015.63
72. Klimcakova E, Chenard V, McGuirk S, Germain D, Avizonis D, Muller WJ, et al. PGC-1alpha promotes the growth of ErbB2/Neu-induced mammary tumors by regulating nutrient supply. *Cancer Res.* (2012) 72:1538–46. doi: 10.1158/0008-5472.CAN-11-2967
73. McGuirk S, Gravel SP, Deblois G, Papadopoli DJ, Faubert B, Wegner A, Hiller K, Avizonis D, Akavia UD, Jones RG, et al. PGC-1alpha supports glutamine metabolism in breast cancer. *Cancer Metab.* (2013) 1:22. doi: 10.1186/2049-3002-1-22
74. Bhalla K, Hwang BJ, Dewi RE, Ou L, Twaddel W, Fang HB, Vafai SB, Vazquez F, Puigserver P, Boros L, et al. PGC1alpha promotes tumor growth by inducing gene expression programs supporting lipogenesis. *Cancer Res.* (2011) 71:6888–98. doi: 10.1158/0008-5472.CAN-11-1011
75. Bost F. "Double hit" makes the difference. *Cell Cycle* (2012) 11:2979. doi: 10.4161/cc.21532
76. Chaube B, Malvi P, Singh SV, Mohammad N, Meena AS, Bhat MK. Targeting metabolic flexibility by simultaneously inhibiting respiratory complex I and lactate generation retards melanoma progression. *Oncotarget* (2015) 6:37281–99. doi: 10.18632/oncotarget.6134
77. Sharabi K, Lin H, Tavares CDJ, Dominy JE, Camporez JP, Perry RJ, Schilling R, Rines AK, Lee J, Hickey M, et al. Selective chemical inhibition of PGC-1alpha gluconeogenic activity ameliorates type 2 diabetes. *Cell* (2017) 169:148–60 e115. doi: 10.1016/j.cell.2017.03.001
78. Hanna RK, Zhou C, Malloy KM, Sun L, Zhong Y, Gehrig PA, et al. Metformin potentiates the effects of paclitaxel in endometrial cancer cells through inhibition of cell proliferation and modulation of the mTOR pathway. *Gynecol Oncol.* (2012) 125:458–69. doi: 10.1016/j.ygyno.2012.01.009
79. Iliopoulos D, Hirsch HA, Struhl K. Metformin decreases the dose of chemotherapy for prolonging tumor remission in mouse xenografts involving multiple cancer cell types. *Cancer Res.* (2011) 71:3196–201. doi: 10.1158/0008-5472.CAN-10-3471
80. Lin CC, Yeh HH, Huang WL, Yan JJ, Lai WW, Su WP, et al. Metformin enhances cisplatin cytotoxicity by suppressing signal transducer and activator of transcription-3 activity independently of the liver kinase B1-AMP-activated protein kinase pathway. *Am J Respir Cell Mol Biol.* (2013) 49:241–50. doi: 10.1165/rcmb.2012-0244OC
81. Rocha GZ, Dias MM, Ropelle ER, Osorio-Costa F, Rossato FA, Vercesi AE, et al. Metformin amplifies chemotherapy-induced AMPK activation and antitumoral growth. *Clin Cancer Res.* (2011) 17:3993–4005. doi: 10.1158/1078-0432.CCR-10-2243
82. Zhang HH, Guo XL. Combinational strategies of metformin and chemotherapy in cancers. *Cancer Chemother Pharmacol.* (2016) 78:13–26. doi: 10.1007/s00280-016-3037-3
83. Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res.* (2009) 69:7507–11. doi: 10.1158/0008-5472.CAN-09-2994
84. Shafiei-Irannejad V, Samadi N, Yousefi B, Salehi R, Velaei K, Zarghami N. Metformin enhances doxorubicin sensitivity via inhibition of doxorubicin efflux in P-gp-overexpressing MCF-7 cells. *Chem Biol Drug Des.* (2017) 91:269–76. doi: 10.1111/cbdd.13078
85. Zhang Y, Feng X, Li T, Yi E, Li Y. Metformin synergistic pemetrexed suppresses non-small-cell lung cancer cell proliferation and invasion *in vitro*. *Cancer Med.* (2017) 6:1965–75. doi: 10.1002/cam4.1133
86. Chen H, Yao W, Chu Q, Han R, Wang Y, Sun J, et al. Synergistic effects of metformin in combination with EGFR-TKI in the treatment of patients with advanced non-small cell lung cancer and type 2 diabetes. *Cancer Lett.* (2015) 369:97–102. doi: 10.1016/j.canlet.2015.08.024
87. Duo J, Ma Y, Wang G, Han X, Zhang C. Metformin synergistically enhances antitumor activity of histone deacetylase inhibitor trichostatin

a against osteosarcoma cell line. *DNA Cell Biol.* (2013) 32:156–64. doi: 10.1089/dna.2012.1926
88. Babcook MA, Shukla S, Fu P, Vazquez EJ, Puchowicz MA, Molter JP, Oak CZ, MacLennan GT, Flask CA, Lindner DJ, et al. Synergistic simvastatin and metformin combination chemotherapy for osseous metastatic castration-resistant prostate cancer. *Mol Cancer Ther.* (2014) 13:2288–302. doi: 10.1158/1535-7163.MCT-14-0451
89. Xia C, Chen R, Chen J, Qi Q, Pan Y, Du L, et al. Combining metformin and nelfinavir exhibits synergistic effects against the growth of human cervical cancer cells and xenograft in nude mice. *Sci Rep.* (2017) 7:43373. doi: 10.1038/srep43373
90. Qu C, Zhang W, Zheng G, Zhang Z, Yin J, He Z. Metformin reverses multidrug resistance and epithelial-mesenchymal transition (EMT) via activating AMP-activated protein kinase (AMPK) in human breast cancer cells. *Mol Cell Biochem.* (2014) 386:63–71. doi: 10.1007/s11010-013-1845-x
91. Yang SH, Li S, Lu G, Xue H, Kim DH, Zhu JJ, et al. Metformin treatment reduces temozolomide resistance of glioblastoma cells. *Oncotarget* (2016) 7:78787–803. doi: 10.18632/oncotarget.12859
92. Cioce M, Valerio M, Casadei L, Pulito C, Sacconi A, Mori F, Biagioni F, Manetti C, Muti P, Strano S, et al. Metformin-induced metabolic reprogramming of chemoresistant ALDHbright breast cancer cells. *Oncotarget* (2014) 5:4129–43. doi: 10.18632/oncotarget.1864
93. Schockel L, Glasauer A, Basit F, Bitschar K, Truong H, Erdmann G, Algire C, Hagebarth A, Willems PH, Kopitz C, et al. Targeting mitochondrial complex I using BAY 87-2243 reduces melanoma tumor growth. *Cancer Metab.* (2015) 3:11. doi: 10.1186/s40170-015-0138-0
94. Zhang B, Chu W, Wei P, Liu Y, Wei T. Xanthohumol induces generation of reactive oxygen species and triggers apoptosis through inhibition of mitochondrial electron transfer chain complex I. *Free Radic Biol Med.* (2015) 89:486–97. doi: 10.1016/j.freeradbiomed.2015.09.021
95. Secker PF, Beneke S, Schlichenmaier N, Delp J, Gutbier S, Leist M, et al. Canagliflozin mediated dual inhibition of mitochondrial glutamate dehydrogenase and complex I: an off-target adverse effect. *Cell Death Dis.* (2018) 9:226. doi: 10.1038/s41419-018-0273-y
96. Villani LA, Smith BK, Marcinko K, Ford RJ, Broadfield LA, Green AE, et al. The diabetes medication Canagliflozin reduces cancer cell proliferation by inhibiting mitochondrial complex-I supported respiration. *Mol Metab.* (2016) 5:1048–56. doi: 10.1016/j.molmet.2016.08.014
97. Wilk A, Wyczechowska D, Zapata A, Dean M, Mullinax J, Marrero L, Parsons C, Peruzzi F, Culicchia F, Ochoa A, et al. Molecular mechanisms of fenofibrate-induced metabolic catastrophe and glioblastoma cell death. *Mol Cell Biol.* (2015) 35:182–98. doi: 10.1128/MCB.00562-14
98. Akatsuka A, Kojima N, Okamura M, Dan S, Yamori T. A novel thiophene-3-carboxamide analog of annonaceous acetogenin exhibits antitumor activity via inhibition of mitochondrial complex I. *Pharmacol Res Perspect.* (2016) 4:e00246. doi: 10.1002/prp2.246
99. FDA (2015). *FDA Drug Safety Communication: FDA Revises Label of Diabetes Drug Canagliflozin* (*Invokana, Invokamet) to Include Updates on Bone Fracture Risk and New Information on Decreased Bone Mineral Density*. Drug Safety and Availability (FDA Drug Safety Communication).
100. FDA (2017). *FDA Drug Safety Communication: Interim Clinical Trial Results Find Increased Risk of Leg and Foot Amputations, Mostly Affecting the Toes, with the Diabetes Medicine Canagliflozin.* Drug Safety and Availability (FDA Drug Safety Communication).
101. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci.* (2000) 3:1301–6. doi: 10.1038/81834
102. Sherer TB, Kim JH, Betarbet R, Greenamyre JT. Subcutaneous rotenone exposure causes highly selective dopaminergic degeneration and alpha-synuclein aggregation. *Exp Neurol.* (2003) 179:9–16. doi: 10.1006/exnr.2002.8072
103. Hervas D, Fornes-Ferrer V, Gomez-Escribano AP, Sequedo MD, Peiro C, Millan JM, et al. Metformin intake associates with better cognitive function in patients with Huntington's disease. *PLoS ONE* (2017) 12:e0179283. doi: 10.1371/journal.pone.0179283
104. Jin J, Gu H, Anders NM, Ren T, Jiang M, Tao M, et al. Metformin protects cells from mutant huntingtin toxicity through activation of AMPK and modulation of mitochondrial dynamics. *Neuromolecular Med.* (2016) 18:581–92. doi: 10.1007/s12017-016-8412-z
105. Sogame Y, Kitamura A, Yabuki M, Komuro S. A comparison of uptake of metformin and phenformin mediated by hOCT1 in human hepatocytes. *Biopharm Drug Dispos.* (2009) 30:476–84. doi: 10.1002/bdd.684
106. Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, Ianculescu AG, Yue L, Lo JC, Burchard EG, et al. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest.* (2007) 117:1422–31. doi: 10.1172/JCI30558
107. Assan R, Heuclin C, Girard JR, LeMaire F, Attali JR. Phenformin-induced lactic acidosis in diabetic patients. *Diabetes* (1975) 24:791–800. doi: 10.2337/diab.24.9.791
108. Kalyanaraman B, Cheng G, Hardy M, Ouari O, Lopez M, Joseph J, et al. A review of the basics of mitochondrial bioenergetics, metabolism, and related signaling pathways in cancer cells: Therapeutic targeting of tumor mitochondria with lipophilic cationic compounds. *Redox Biol.* (2018) 14:316–27. doi: 10.1016/j.redox.2017.09.020

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 23 October 2018
doi: 10.3389/fendo.2018.00622

Metformin and Colorectal Cancer

*Takuma Higurashi and Atsushi Nakajima**

Department of Gastroenterology and Hepatology, Yokohama City University School of Medicine, Yokohama, Japan

Colorectal Cancer (CRC) is one of the most frequently encountered neoplasms in humans. The incidence of CRC has been increasing and new strategies for prevention, including chemoprevention, are required to lower its incidence and associated mortality. Metformin is a biguanide compound commonly used for the treatment of diabetes mellitus. Many recent basic research, epidemiological and clinical trial studies have indicated that metformin has benefits not only in diabetes treatment, but also in lowering the risk of developing cancer (including CRC). These studies indicate that metformin may be a candidate chemoprevention agent for CRC. This review article shall discuss the present evidence of metformin treatment and CRC, as well as outline our challenge in the investigation of metformin use in chemoprevention therapy for colorectal tumors.

Keywords: metfromin, colorectal cancer, chemoprevention, epidemiology, basic research, review, clinical trials

OPEN ACCESS

Edited by:
Frederic Bost, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Vladimir Nikolaevich Anisimov, N.N. Petrov National Medical Research Center of Oncology, Russia
Susanna Morano, Università degli Studi di Roma La Sapienza, Italy

****Correspondence:***
Atsushi Nakajima
nakajima-tky@umin.ac.jp

Specialty section:
This article was submitted to Cancer Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *04 June 2018*
Accepted: *28 September 2018*
Published: *23 October 2018*

Citation:
Higurashi T and Nakajima A (2018) Metformin and Colorectal Cancer. Front. Endocrinol. 9:622. doi: 10.3389/fendo.2018.00622

INTRODUCTION

Colorectal Cancer (CRC) is one of the most frequently encountered neoplasms across the world. The incidence of CRC has rising in many low- and middle-income countries, and some highly-developed countries (1). Despite great advances in cancer treatment over the last two decades, such as the development of more effective drugs with improved safety and more precise molecular targeting, unwanted adverse effects remain a major problem. New cancer treatments are also extremely expensive. The prevention or reduced incidence of cancer would help lower rising medical costs (2), providing a cheaper and more effective strategy of decreasing cancer mortality. The resection of colorectal polyps lowers the risk of future development of advanced adenoma and CRC (3). Yet patients with polyps (adenomas and/or hyperplastic polyps) remain at high risk for the development of future colorectal polyps and CRC (4). This ongoing risk highlights the need for a conceptual change, from surveillance and detection of adenomas and cancer (the former often being treated by endoscopic resection) to new strategies for prevention, including chemoprevention, to lower the incidence and associated mortality of CRC.

A number of agents have been reported to have a chemopreventive effect against colorectal carcinogenesis. In regard to epidemiology, the 2011 World Cancer Research Fund and American Institute for Cancer Research reported beneficial food and nutrition for decreasing the incidence of CRC (5) (**Table 1**). However, effective clinical trials have been limited. Nonsteroidal anti-inflammatory drugs (NSAIDs), notably cyclooxygenase-2 (COX-2) inhibitors, used either alone or in combination with other agents, have offered the most potential for lowering the risk of CRC. Unfortunately, there is an elevated risk of serious cardiovascular events associated with the administration of COX-2 inhibitors (6, 7). Considering these cardiovascular side effects and the lack of demonstrable efficacy of other drugs that initially showed potential in this setting, novel agents are required that are clinically effective and safe for CRC prevention. An increased incidence of CRC in adults is also associated with obesity and diabetes mellitus (8, 9). Therefore, we predicted that these conditions may provide novel targets for the chemoprevention of CRC.

TABLE 1 | Foods and nutrients with supporting findings in epidemiological studies.

Convincing	None
Probable	Food containing dietary fiber
	Garlic
	Milk
	Calcium
Limited-suggestive	Non-starch vegetables
	Fruits
	Food containing folate
	Fish
	Food containing selenium
	Food containing vitamin D
	Selenium

Many recent reports, including basic research, epidemiological and clinical trial studies, suggested that metformin also lowered the risk of developing malignant disease, such as CRC. Accumulating data indicates that metformin may be a candidate chemoprevention agent for CRC. We shall discuss the current evidence of metformin administration and CRC risk and outline our challenge of using metformin for the chemoprevention of colorectal tumors.

METFORMIN AND COLORECTAL CANCER

Epidemiological Research

The first report of a relationship between metformin administration and the risk of CRC was published in 2004 (10). Subsequently, many population-based and case-control cohort studies, and associated meta-analysis, have evaluated metformin use and the risk of CRC. Different studies reported a decreased risk (11–17), no association (18–21), or an increased risk of CRC (22, 23). The reason for different conclusions between certain studies may be related to time-related biases, which were proposed to account for some of the inverse associations observed between metformin administration and cancer risk reported in epidemiologic studies (24, 25). These include immortal time bias when unexposed time is misclassified, as in cohort studies, time-window bias when the time window for capturing exposure differs between cases and controls in case/control studies, or time-lag bias when treatment differs across stages of the disease (with disease stage also associated with the outcome risk). A recent cohort study that minimized these biases concluded there was an inverse association between long-term administration of metformin and CRC risk (26). Further studies and detailed analyses are needed to clarify the potential clinical benefits of metformin upon the incidence and associated mortality of CRC.

Basic Research

In preclinical research, metformin suppressed cell proliferation, increased apoptosis, caused cell cycle arrest, and suppressed the incidence and growth of experimental tumors *in vitro* and *in vivo* (27–29). The underlying molecular mechanism of metformin action was shown to involve liver kinase B1 (LKB-1)-dependent activation of AMP-activated protein kinase (AMPK) (30, 31). Molecular mechanisms of metformin actions were mostly studied in adipose and liver tissue in relation to glucose homeostasis and insulin actions. Recent studies reported involvement of the AMPK/mammalian target of rapamycin (mTOR) pathway in the induction of various cancers (32, 33). Downstream targets of mTOR signaling include proteins that control translational machinery, including the ribosomal protein S6 kinases (S6K) that regulate the initiation and elongation phases of translation (34). The upstream regulation of mTOR involves signaling pathways of several oncoproteins or tumor suppressors, including AMPK, phosphatidyl inositol 3-kinase and phosphatase and tensin homolog (35). In particular, upregulation of AMPK directly suppresses mTOR, resulting in the inhibition of cell proliferation (36). In addition, *in vitro* analysis demonstrated that the metformin-induced suppression of the growth of breast cancer cells was associated with decreased activation of mTOR and S6 kinase (37).

The above findings indicated that metformin was effective at reducing carcinogenesis *in vitro*. We will now focus on reported *in vivo* experiments and our study of colon carcinogenesis using several animals models. The first report of phenformin that inhibit metabolic immunodepression in rats 1977 (38). From then, several reports showed that biguanide prevent colon carcinogenesis. Experimental rodent models of CRC can be broadly separated into genetic (such as $Apc^{Min/+}$ mice, a murine model of familial adenomatous polyposis coli (APC)) and chemical carcinogen-induced (such as azoxymethane (AOM)-induced) sporadic models. Many studies of chemoprevention have used both rodent models of CRC, however, some studies reported that candidate agents had consistent preventive effects in both models, whereas other studies reported inconsistent and contradictory results (39). Therefore, it is important to investigate the ability of candidate chemoprevention agents to suppress tumorigenesis in both the genetic and sporadic cancer models. First, we examined the effect of metformin on intestinal polyp growth in $Apc^{Min/+}$ mice. Nine-week-old $Apc^{Min/+}$ mice were split into two groups: one received metformin (250 mg/kg per day in the diet) treatment, the other received a normal diet without metformin, and the number and size of polyps were analyzed in both groups after 10 weeks. Administration of metformin significantly suppressed the number of intestinal large polyps formed in $Apc^{Min/+}$ mice (40). Second, we investigated a carcinogen-induced sporadic colorectal cancer model. Seven-week-old mice were administered AOM by intraperitoneal injection and then treated with or without metformin for 6 weeks (to investigate aberrant crypt foci (ACF) formation) or 32 weeks (for tumor formation). Metformin treatment significantly inhibited ACF and polyp formation. Furthermore, western blot analysis showed that metformin treatment stimulated AMPK phosphorylation, and significantly inhibited the phosphorylation of mTOR, S6K and S6 proteins. It was proposed that metformin suppressed colonic mucosal proliferation via activation of AMPK and then the downstream suppression of the mTOR pathway (41). In other animal model, it has been shown that metformin dosedependently inhibits the

development of colon tumors induced by 1,2dimethylhydrazine (DMH) in rats (42, 43). In this way, many reports showed that metformin is effective for colorectal carcinogenesis both *in vivo* and *in vitro*.

Clinical Trials

Previous basic research and epidemiological studies indicated that metformin had a chemopreventive effect upon CRC. However, confirmation of metformin efficacy required a prospective interventional trial. In chemoprevention trials targeting CRC, the incidence of adenomas or the cancer itself was generally used as the main endpoint. While the occurrence of CRC is a clear endpoint, its low incidence in the general population, and the required long-term observational period make this endpoint unsuitable for chemoprevention trials (44). The use of surrogate biomarkers for cancer detection may allow evaluation of drug efficacy in a shorter timeframe. Aberrant crypt foci are very small lesions that develop in the earliest stage of colorectal carcinogenesis, and consist of large, thick crypts that can be detected by dense methylene blue staining (45–47), as shown in **Figure 1**. The ACF were reported to be precursor lesions for human colorectal carcinogenesis (48), and were proposed as a surrogate endpoint in chemoprevention trials for CRC. Several studies have examined the correlation between the presence and number of ACF and use of candidate chemopreventive agents for CRC in humans. The presence and number of ACF were found to be suppressed by certain chemopreventive agents (49, 50). There are several advantages to using colorectal ACF as the primary endpoint in CRC chemoprevention trials. First, a long-term observational period is not needed to evaluate agent effects; thus avoiding long-term trials, which require considerable effort and may expose trial participants to an increased risk of carcinoma occurrence. Second, ACF can be estimated quantitatively. In 2010, there were no reported prospective metformin chemoprevention trials, so we implemented a pilot prospective clinical trial to examine the efficacy and safety of metformin use and its effects upon ACF formation. We prospectively randomized 26 participants with colorectal ACF to receive treatment with metformin (250 mg/d) or no treatment, followed by evaluation of the number of ACF. Magnifying colonoscopy was used to determine the number of rectal ACF and other laboratory endpoints (using blind analysis) in each patient at baseline and after 1 month of treatment. Prior to treatment, there were no significant differences in the number of rectal ACF and other baseline clinical characteristics between the two groups. At 1 month, the mean number of ACF per patient was significantly reduced in the metformin group (8.78 ± 6.45 before treatment vs. 5.11 ± 4.99 at 1 month, $P = 0.007$), whereas the mean ACF number was unchanged in the control group (7.23 ± 6.65 vs. 7.56 ± 6.75, $P = 0.609$). This initial trial provided preliminary data suggesting that metformin inhibited human rectal ACF formation (51). However, this prospective trial had some limitations. First, the trial duration was only 1 month. Second, although ACF were considered a convenient surrogate biomarker of colorectal carcinogenesis (48), their biological significance remains controversial. Generally, the occurrence of CRC would be the most reliable endpoint in chemoprevention trials for CRC. However, there would be serious ethical issues in withholding endoscopic removal when resectable lesions (that develop into cancer) were detected in annual colonoscopies. In previous CRC chemoprevention trials, such as those investigating NSAIDs and aspirin, detection of the metachronous adenoma was set as the primary endpoint. Therefore, we used metachronous colorectal adenomas/polyps as the endpoint in our subsequent metformin chemoprevention trial. Previous CRC chemoprevention trials also involved initial short-term trials to establish safety and efficacy, followed by expanded trials of longer duration. Long-term trials require a large amount of resources and may expose the study participants to the risk of cancer. There have been no reported randomized control trials for CRC chemoprevention using metformin, and the safety of subjects would need careful attention in the design and execution of such a trial. Considering these issues, we designed a 1-year clinical trial to evaluate the safety and chemopreventive effect of metformin on sporadic CRC in patients at high risk of adenoma recurrence, as a preliminary study before considering long-term CRC chemoprevention trials. The trial protocol was previously published (52).

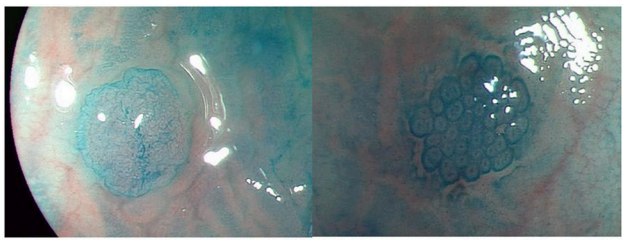

FIGURE 1 | Endoscopic features of Aberrant Crypt Foci (ACF).

In all, 498 subjects were screened for eligibility, and 347 subjects were excluded for the reasons shown in **Figure 1**. Of these participants, 183 cases were excluded owing to inadequate colon cleaning, such as an incompletely cleaned polypectomy, poor bowel preparation, short observation time or lack of insertion to caecum (the major reason was incomplete polypectomy). The 151 eligible patients were randomly allocated into two groups; 79 and 72 in the metformin and placebo groups, respectively (**Figure 2**). Of these 151 patients, five were lost to follow-up (three in the metformin group, two in placebo the group) and 13 withdrew their informed consent during the follow-up period. The remaining 133 patients (71 and 62 in the metformin and placebo groups, respectively) received a 1-year follow-up colonoscopy. **Table 2** shows the baseline characteristics of the subjects. There were no diabetes mellitus patients in either group (exclusion criteria). In both groups, the proportion of subjects with advanced adenoma (including early carcinoma) and multiple adenomas was approximately 70%. The incidence of total polyps (adenomas plus hyperplastic

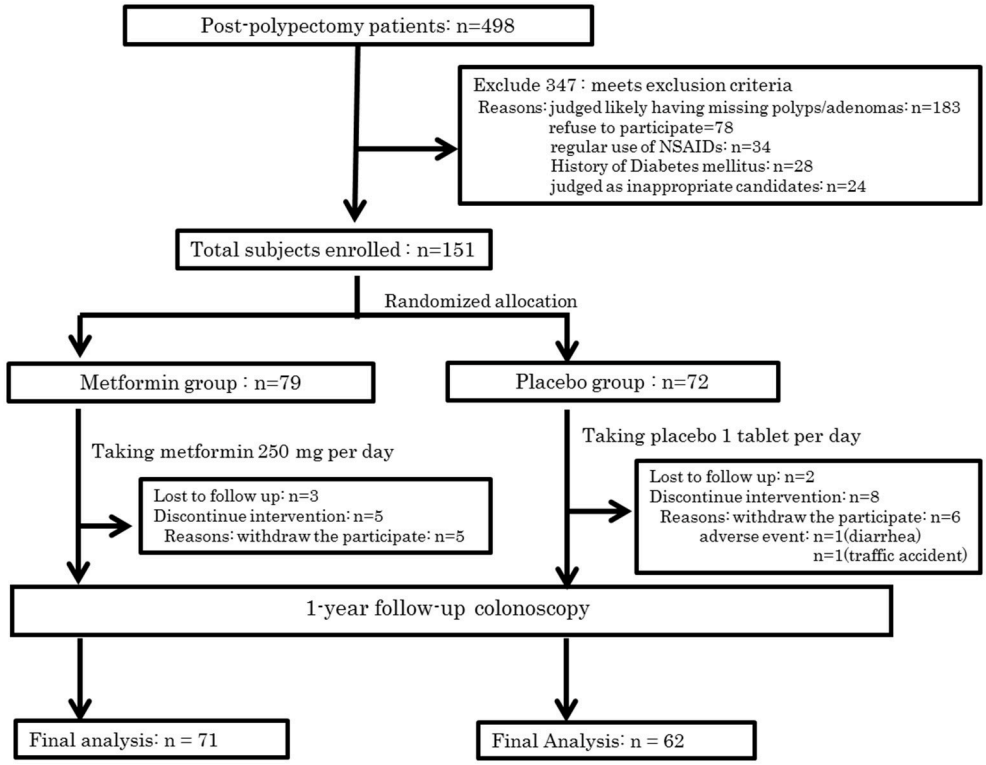

FIGURE 2 | Trial profile. This figure is reproduced from (53) with permission.

polyps) in the metformin group was significantly lower than that in the placebo group [metformin group had 27/71, 38.0% (95% confidence interval (CI), 26.7–49.3) vs. the placebo group with 35/62, 56.5% (95% CI, 44.1–68.8); $p = 0.034$]. The risk ratio (RR) was 0.674: 95% CI, 0.466–0.974. The incidence of adenomas in the metformin group was also significantly lower than that in the placebo group [metformin group had 22/71, 30.6% (95% CI, 19.9–41.2) vs. the placebo group with 32/62, 51.6% (95% CI, 39.2–64.1), $p = 0.016$]. The RR was 0.600 (95% CI, 0.393–0.916; **Table 3**). The incidence of adverse events was approximately 10% and equivalent between the two groups (**Table 4**). All adverse events were considered very mild, such as abdominal pain, diarrhea, and exanthema.

This study was the first clinical trial to examine the chemoprevention effect of low-dose metformin on metachronous colorectal adenoma/polyp formation. Metformin was shown to suppress metachronous colorectal adenoma/polyp formation (53). This clinical trial had possible limitations. First, the follow-up colonoscopy at 1 year may be too soon, because many chemoprevention trials for metachronous adenoma formation had used study durations of 3 years to 5 years. However, no previous metformin chemoprevention trials were reported, and a trial longer than 1 year may present ethical concerns. In an attempt to overcome these issues, we choose participants who were at high risk of adenoma and cancer occurrence. Patients who have had multiple and advanced adenomas (high-grade dysplasia, large adenomas >10 mm, and villous features) are known to be at high risk of CRC (3), and surveillance after endoscopic resection is recommended for up to 3 years (54). In the current trial, almost 70% of subjects in each group had previously exhibited advanced adenoma (including early carcinoma) or multiple adenomas. However, long-term observation of post-polypectomy patients, a high-risk group for CRC, may entail ethical problems. Placebo

TABLE 2 | Baseline characteristics of the subjects.

	Metformin	Placebo
No of subject	71	62
Age, (mean ± SD), y	63.1 ± 8.5	63.5 ± 10.2
Sex (M/F)	54/17	49/13
BMI	23.1 ± 2.6	23.9 ± 3.5
Family history of CRC	8 (11%)	10 (16%)
Current smoker	23 (32%)	25 (40%)
History of Diabetes	0	0
History of Hyperlipidemia	15 (21%)	7 (11%)
History of Hypertension	20 (28%)	20 (32%)
Finding of baseline CS		
Multiple & Advanced adenoma + early carcinoma	51 (72%)	43 (69%)

This table is reproduced from (53) with permission.
CS, colonoscopy; Multiple, more than 3 adenomas; Advanced adenomas, high-grade dysplasia, large size (>10 mm), or villous features.

TABLE 3 | Incidence of total polyps and adenomas 1 year after the start of treatment.

	Metformin	Placebo	*p*-value
Incidence of total polyp (95%CI)	27/71 (38.0%) (26.7–49.3)	35/62 (56.5%) (44.1–68.8)	0.034
Risk ratio (95%CI)	0.674 (0.466–0.974)	1(reference)	
Incidence of total adenomas (95%CI)	22/71 (30.6%) (19.9–41.2)	32/62 (51.6%) (39.2–64.1)	0.016
Risk ratio (95%CI)	0.600 (0.393–0.916)	1 (reference)	

This table is reproduced from (53) with permission.
CI, confidence interval; IQR, interquartile range.

TABLE 4 | Adverse events in the metformin and placebo groups.

Adverse events	Metformin	Placebo
Abdominal pain	0	1
Diarrhea	1	4
Rash	2	0
Constipation	3	3
Alopecia	0	1
Total	6	9

This table is reproduced from (53) with permission.
All adverse events were NCI-CTCAE grade 1.

group subjects who received resection of advanced or multiple adenomas showed a high rate (30/43, 70%) of recurrence, and this rate was a little higher than that found in previous chemoprevention trials for adenoma recurrence. However, there was no CRC detected in any subjects in the 1 year follow-up colonoscopy. To validate the efficacy of metformin for the prevention of CRC, further long-term studies are needed. The second limitation is that the trial did not study dose–response effects of metformin on metachronous colorectal adenomas/polyps. Previous trials of metformin for cancer prevention and adjuvant treatment have been conducted using high-doses of metformin (500–2,000 mg/day). Unfortunately, high-dose metformin is associated with an increased risk of developing lactic acidosis and adverse gastrointestinal effects, such as diarrhea. Gontier et al. reported a PET/CT trial in which subjects received medication with anti-diabetic drugs, including metformin, and exhibited high and diffuse intestinal uptake of ^{18}F-fluorodeoxyglucose (55). This finding indicates that AMPK is abundant in intestinal mucosa and that activation of AMPK by metformin up-regulates the expression of glucose transporters. Therefore, metformin-induced chemoprevention in the colorectum appears to be a reasonable strategy targeting key molecular pathways. In a previous study, we found that oral low-dose metformin (250 mg/day) was safe and inhibited human colorectal ACF and metachronous adenoma formation (51, 53). We predict that oral low-dose metformin also has clinical efficacy for CRC chemoprevention. The third limitation of this study was that many participants in this trial were at high risk of adenoma and cancer recurrence. Around 70% of participants had advanced and multiple adenomas (or early carcinoma). This proportion was high compared with other chemoprevention trials. However, because of the randomization process, there was no internal bias in the groups. Nevertheless, our trial did not directly determine the efficacy of metformin for patients with an average risk of CRC (external validity). Finally, this trial was conducted in a small region of Japan and the sample size was small. Many previous adenoma prevention trials, including that of celecoxib, were carried out in Western countries. Future well-designed clinical chemoprevention trails are required that include larger sample sizes and involve many multinational institutions and more ethnic groups.

CONCLUSION AND FUTURE PERSPECTIVE

A practical chemoprevention agent generally requires the following attributes: safety, good compliance, cost effectiveness, and a clear mechanism. Metformin meets these criteria. To date, NSAIDs, especially COX-2 inhibitors, have provided the most reliable risk reduction for CRC, but they also confer an increased risk of severe cardiovascular events (6, 7). Metformin, first synthesized in the 1920s, has been used worldwide for treating diabetes mellitus, metabolic syndrome and polycystic ovary syndrome (56). In the present clinical study, the use of low-dose metformin for 1 year caused few adverse events, which were all very mild. These findings indicate that low-dose metformin is safe. In addition, metformin is an inexpensive medicine suitable for daily use. Generally, patients need chemopreventive agents as a long term therapy. Metformin is suitable in these conditions. Finally, the mechanism of action has been well elucidated for metformin. Metformin is known to activate AMPK, which inhibits the mTOR pathway that plays an important role in cellular translational processes and progression (30). Although more than 100 randomized controlled trials of metformin and cancer are currently registered at ClinicalTrials.gov, the vast majority are testing the effect of metformin in cancer treatment rather than prevention. This situation perhaps underscores the inherent challenge of doing chemoprevention trials with cancer endpoints, which mandate follow-up of many individuals over many years. As an efficient and feasible alternative, trials designed to examine the effect of metformin on cancer biomarkers or surrogate endpoints over a shorter time horizon are an important next step before embarking on expensive larger scale trials (57). For colorectal cancer prevention specifically, a large-scale randomized controlled trial of metformin (perhaps in combination with aspirin, an established chemopreventive agent) for adenoma recurrence in a population with a broader risk profile appears warranted (58).

In conclusion, metformin has the potential to provide a novel chemoprevention therapy for CRC. However, to fully clarify the chemopreventive effect of metformin on CRC, further large-sample size and long-term clinical trials are required.

AUTHOR CONTRIBUTIONS

TH and AN conceived the study. All the authors have read the final manuscript and approved its submission for publication.

ACKNOWLEDGMENTS

We thank Charles Allan, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. *CA Cancer J Clin.* (2018) 68:7–30. doi: 10.3322/caac.21442
2. Penny LK, Wallace HM. The challenges for cancer chemoprevention. *Chem Soc Rev.* (2015) 44:8836–47. doi: 10.1039/C5CS00705D
3. Winawer SJ, Zauber AG, Ho MN, O'Brien MJ, Gottlieb LS, Sternberg SS, et al. Prevention of colorectal cancer by colonoscopic polypectomy. the national polyp study workgroup. *N Engl J Med.* (1993) 329:1977–81. doi: 10.1056/NEJM199312303292701
4. Imperiale TF, Glowinski EA, Lin-Cooper C, Larkin GN, Rogge JD, Ransohoff DF. Five-year risk of colorectal neoplasia after negative screening colonoscopy. *N Engl J Med.* (2008) 359:1218–24. doi: 10.1056/NEJMoa0803597
5. Research WCRFAIfC. *Colorectal Cancer 2011 Report.* (2011).
6. Drazen JM. COX-2 inhibitors–a lesson in unexpected problems. *N Engl J Med.* (2005) 352:1131–2. doi: 10.1056/NEJMe058038
7. Zell JA, Pelot D, Chen WP, McLaren CE, Gerner EW, Meyskens FL. Risk of cardiovascular events in a randomized placebo-controlled, double-blind trial of difluoromethylornithine plus sulindac for the prevention of sporadic colorectal adenomas. *Cancer Prev Res.* (2009) 2:209–12. doi: 10.1158/1940-6207.CAPR-08-0203
8. Limburg PJ, Vierkant RA, Fredericksen ZS, Leibson CL, Rizza RA, Gupta AK, et al. Clinically confirmed type 2 diabetes mellitus and colorectal cancer risk: a population-based, retrospective cohort study. *Am J Gastroenterol.* (2006) 101:1872–9. doi: 10.1111/j.1572-0241.2006.00725.x
9. Frezza EE, Wachtel MS, Chiriva-Internati M. Influence of obesity on the risk of developing colon cancer. *Gut* (2006) 55:285–91. doi: 10.1136/gut.2005.073163
10. Yang YX, Hennessy S, Lewis JD. Insulin therapy and colorectal cancer risk among type 2 diabetes mellitus patients. *Gastroenterology* (2004) 127:1044–50. doi: 10.1053/j.gastro.2004.07.011
11. Cardel M, Jensen SM, Pottegard A, Jorgensen TL, Hallas J. Long-term use of metformin and colorectal cancer risk in type II diabetics: a population-based case-control study. *Cancer Med.* (2014) 3:1458–66. doi: 10.1002/cam4.306
12. Currie CJ, Poole CD, Gale EA. The influence of glucose-lowering therapies on cancer risk in type 2 diabetes. *Diabetologia* (2009) 52:1766–77. doi: 10.1007/s00125-009-1440-6
13. Lee MS, Hsu CC, Wahlqvist ML, Tsai HN, Chang YH, Huang YC. Type 2 diabetes increases and metformin reduces total, colorectal, liver and pancreatic cancer incidences in Taiwanese: a representative population prospective cohort study of 800,000 individuals. *BMC Cancer* (2011) 11:20. doi: 10.1186/1471-2407-11-20
14. Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD, Evans JM. New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes. *Diabetes Care* (2009) 32:1620–5. doi: 10.2337/dc08-2175
15. Sehdev A, Shih YC, Vekhter B, Bissonnette MB, Olopade OI, Polite BN. Metformin for primary colorectal cancer prevention in patients with diabetes: a case-control study in a US population. *Cancer* (2015) 121:1071–8. doi: 10.1002/cncr.29165
16. Tseng CH. Diabetes, metformin use, and colon cancer: a population-based cohort study in Taiwan. *Eur J Endocrinol.* (2012) 167:409–16. doi: 10.1530/EJE-12-0369
17. Zhang ZJ, Zheng ZJ, Kan H, Song Y, Cui W, Zhao G, et al. Reduced risk of colorectal cancer with metformin therapy in patients with type 2 diabetes: a meta-analysis. *Diabetes Care* (2011) 34:2323–8. doi: 10.2337/dc11-0512
18. Ferrara A, Lewis JD, Quesenberry CP Jr, Peng T, Strom BL, Van Den Eeden SK, et al. Cohort study of pioglitazone and cancer incidence in patients with diabetes. *Diabetes Care* (2011) 34:923–9. doi: 10.2337/dc10-1067
19. Kowall B, Stang A, Rathmann W, Kostev K. No reduced risk of overall, colorectal, lung, breast, and prostate cancer with metformin therapy in diabetic patients: database analyses from Germany and the UK. *Pharmacoepidemiol Drug Saf.* (2015) 24:865–74. doi: 10.1002/pds.3823
20. Lin CM, Huang HL, Chu FY, Fan HC, Chen HA, Chu DM, et al. Association between gastroenterological malignancy and diabetes mellitus and anti-diabetic therapy: a nationwide, population–based cohort study. *PLoS ONE* (2015) 10:e0125421. doi: 10.1371/journal.pone.0125421
21. Smiechowski B, Azoulay L, Yin H, Pollak MN, Suissa S. The use of metformin and colorectal cancer incidence in patients with type II diabetes mellitus. *Cancer Epidemiol Biomarkers Prev.* (2013) 22:1877–83. doi: 10.1158/1055-9965.EPI-13-0196
22. Bodmer M, Becker C, Meier C, Jick SS, Meier CR. Use of metformin is not associated with a decreased risk of colorectal cancer: a case-control analysis. *Cancer Epidemiol Biomarkers Prev.* (2012) 21:280–6. doi: 10.1158/1055-9965.EPI-11-0992-T
23. Knapen LM, Dittrich ST, de Vries F, Starup-Linde J, Vestergaard P, Henry RM, et al. Use of biguanides and the risk of colorectal cancer: a register-based cohort study. *Curr Drug Saf.* (2013) 8:349–56. doi: 10.2174/15680266113136660068
24. Suissa S, Azoulay L. Metformin and the risk of cancer: time-related biases in observational studies. *Diabetes Care* (2012) 35:2665–73. doi: 10.2337/dc12-0788
25. Gandini S, Puntoni M, Heckman-Stoddard BM, Dunn BK, Ford L, DeCensi A, et al. Metformin and cancer risk and mortality: a systematic review and meta-analysis taking into account biases and confounders. *Cancer Prev Res.* (2014) 7:867–85. doi: 10.1158/1940-6207.CAPR-13-0424
26. Bradley MC, Ferrara A, Achacoso N, Ehrlich SF, Quesenberry CP, Habel LA. A cohort study of metformin and colorectal cancer risk among patients with diabetes mellitus. *Cancer Epidemiol Biomarkers Prev.* (2018) 27:525–30. doi: 10.1158/1055-9965.EPI-17-0424
27. Alimova IN, Liu B, Fan Z, Edgerton SM, Dillon T, Lind SE, et al. Metformin inhibits breast cancer cell growth, colony formation and induces cell cycle arrest *in vitro*. *Cell Cycle* (2009) 8:909–15. doi: 10.4161/cc.8.6.7933
28. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, et al. The antidiabetic drug metformin exerts an antitumoral effect *in vitro* and *in vivo* through a decrease of cyclin D1 level. *Oncogene* (2008) 27:3576–86. doi: 10.1038/sj.onc.1211024
29. Vladimir NA. Metformin for prevention and treatment of colon cancer: a reappraisal of experimental and clinical data. *Curr Drug Targets* (2016) 17:439–46. doi: 10.2174/1389450116666150309113305
30. Shaw RJ, Lamia KA, Vasquez D, Koo SH, Bardeesy N, Depinho RA, et al. The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. *Science* (2005) 310:1642–6. doi: 10.1126/science.1120781
31. Hawley SA, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, et al. Use of cells expressing γ subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab.* (2010) 11:554–65. doi: 10.1016/j.cmet.2010.04.001
32. Carretero J, Medina PP, Blanco R, Smit L, Tang M, Roncador G, et al. Dysfunctional AMPK activity, signalling through mTOR and survival in response to energetic stress in LKB1-deficient lung cancer. *Oncogene* (2007) 26:1616–25. doi: 10.1038/sj.onc.1209951
33. Motoshima H, Goldstein BJ, Igata M, Araki E. AMPK and cell proliferation–AMPK as a therapeutic target for atherosclerosis and cancer. *J Physiol.* (2006) 574(Pt 1):63–71. doi: 10.1113/jphysiol.2006.108324

34. Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N. mTOR, translation initiation and cancer. *Oncogene* (2006) 25:6416–22. doi: 10.1038/sj.onc.1209888
35. Yang Q, Guan KL. Expanding mTOR signaling. *Cell Res.* (2007) 17:666–81. doi: 10.1038/cr.2007.64
36. Hay N. The Akt-mTOR tango and its relevance to cancer. *Cancer Cell* (2005) 8:179–83. doi: 10.1016/j.ccr.2005.08.008
37. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. *Cancer Res.* (2006) 66:10269–73. doi: 10.1158/0008-5472.CAN-06-1500
38. Dilman VM. METABOLIC IMMUNODEPRESSION WHICH INCREASES THE RISK OF CANCER. *Lancet* (1977) 310:1207–9. doi: 10.1016/S0140-6736(77)90442-1
39. Corpet DE, Pierre F. Point: from animal models to prevention of colon cancer. Systematic review of chemoprevention in min mice and choice of the model system. *Cancer Epidemiol Biomarkers Prev*. (2003) 12:391–400.
40. Tomimoto A, Endo H, Sugiyama M, Fujisawa T, Hosono K, Takahashi H, et al. Metformin suppresses intestinal polyp growth in ApcMin/+ mice. *Cancer Sci.* (2008) 99:2136–41. doi: 10.1111/j.1349-7006.2008.00933.x
41. Hosono K, Endo H, Takahashi H, Sugiyama M, Uchiyama T, Suzuki K, et al. Metformin suppresses azoxymethane-induced colorectal aberrant crypt foci by activating AMP-activated protein kinase. *Mol Carcinog.* (2010) 49:662–71. doi: 10.1002/mc.20637
42. Bekusova VV, Patsanovskii VM, Nozdrachev AD, Anisimov VN. Metformin inhibits development of colon malignant tumors induced by 1,2-dimethylhydrazine in rats. *Dokl Biol Sci.* (2016) 468:97–100. doi: 10.1134/S0012496616030017
43. Bekusova VV, Patsanovskii VM, Nozdrachev AD, Trashkov AP, Artemenko MR, Anisimov VN. Metformin prevents hormonal and metabolic disturbances and 1,2-dimethylhydrazine-induced colon carcinogenesis in non-diabetic rats. *Cancer Biol Med*. (2017) 14:100–7. doi: 10.20892/j.issn.2095-3941.2016.0088
44. Rougier P, Mitry E. Epidemiology, treatment and chemoprevention in colorectal cancer. *Ann Oncol*. (2003) 14(Suppl 2):ii3–5. doi: 10.1093/annonc/mdg722
45. Bird RP. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. *Cancer Lett.* (1995) 93:55–71. doi: 10.1016/0304-3835(95)03788-X
46. Pretlow TP, Barrow BJ, Ashton WS, O'Riordan MA, Pretlow TG, Jurcisek JA, et al. Aberrant crypts: putative preneoplastic foci in human colonic mucosa. *Cancer Res.* (1991) 51:1564–7.
47. Roncucci L, Stamp D, Medline A, Cullen JB, Bruce WR. Identification and quantification of aberrant crypt foci and microadenomas in the human colon. *Hum Pathol.* (1991) 22:287–94. doi: 10.1016/0046-8177(91)90163-J
48. Takayama T, Katsuki S, Takahashi Y, Ohi M, Nojiri S, Sakamaki S, et al. Aberrant crypt foci of the colon as precursors of adenoma and cancer. *N Engl J Med*. (1998) 339:1277–84. doi: 10.1056/NEJM199810293391803
49. Takayama T, Nagashima H, Maeda M, Nojiri S, Hirayama M, Nakano Y, et al. Randomized double-blind trial of sulindac and etodolac to eradicate aberrant crypt foci and to prevent sporadic colorectal polyps. *Clin Cancer Res.* (2011) 17:3803–11. doi: 10.1158/1078-0432.CCR-10-2395
50. Takahashi H, Hosono K, Uchiyama T, Sugiyama M, Sakai E, Endo H, et al. PPARgamma ligand as a promising candidate for colorectal cancer chemoprevention: a pilot study. *PPAR Res.* (2010) 2010:257835. doi: 10.1155/2010/257835
51. Hosono K, Endo H, Takahashi H, Sugiyama M, Sakai E, Uchiyama T, et al. Metformin suppresses colorectal aberrant crypt foci in a short-term clinical trial. *Cancer Prev Res.* (2010) 3:1077–83. doi: 10.1158/1940-6207.CAPR-10-0186
52. Higurashi T, Takahashi H, Endo H, Hosono K, Yamada E, Ohkubo H, et al. Metformin efficacy and safety for colorectal polyps: a double-blind randomized controlled trial. *BMC Cancer* (2012) 12:118. doi: 10.1186/1471-2407-12-118
53. Higurashi T, Hosono K, Takahashi H, Komiya Y, Umezawa S, Sakai E, et al. Metformin for chemoprevention of metachronous colorectal adenoma or polyps in post-polypectomy patients without diabetes: a multicentre double-blind, placebo-controlled, randomised phase 3 trial. *Lancet Oncol.* (2016) 17:475–83. doi: 10.1016/S1470-2045(15)00565-3
54. Lieberman DA, Rex DK, Winawer SJ, Giardiello FM, Johnson DA, Levin TR. Guidelines for colonoscopy surveillance after screening and polypectomy: a consensus update by the US multi-society task force on colorectal cancer. *Gastroenterology* (2012) 143:844–57. doi: 10.1053/j.gastro.2012.06.001
55. Gontier E, Fourme E, Wartski M, Blondet C, Bonardel G, Le Stanc E, et al. High and typical 18F-FDG bowel uptake in patients treated with metformin. *Eur J Nucl Med Mol Imaging* (2008) 35:95–9. doi: 10.1007/s00259-007-0563-6
56. Pierotti MA, Berrino F, Gariboldi M, Melani C, Mogavero A, Negri T, et al. Targeting metabolism for cancer treatment and prevention: metformin, an old drug with multi-faceted effects. *Oncogene* (2013) 32:1475–87. doi: 10.1038/onc.2012.181
57. Chan AT. Metformin for cancer prevention: a reason for optimism. *Lancet Oncol.* (2016) 17:407–9. doi: 10.1016/S1470-2045(16)00006-1
58. Chan AT, Ladabaum U. Where do we stand with aspirin for the prevention of colorectal cancer? The USPSTF recommendations. *Gastroenterology* (2016) 150:14–8. doi: 10.1053/j.gastro.2015.11.018

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 24 October 2018
doi: 10.3389/fendo.2018.00620

Pharmaceutical Impact of *Houttuynia Cordata* and Metformin Combination on High-Fat-Diet-Induced Metabolic Disorders: Link to Intestinal Microbiota and Metabolic Endotoxemia

Jing-Hua Wang[1], *Shambhunath Bose*[2], *Na Rae Shin*[1], *Young-Won Chin*[3], *Young Hee Choi*[4] *and Hojun Kim*[1]*

[1] Department of Oriental Rehabilitation Medicine, Dongguk University, Goyang, South Korea, [2] NosQuest, Seongnam-si, South Korea, [3] College of Pharmacy, Dongguk University-Seoul, Goyang, South Korea, [4] College of Pharmacy and Integrated Research Institute for Drug Development, Dongguk University-Seoul, Seoul, South Korea

OPEN ACCESS

Edited by:
Frederic Bost,
Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Venu Lagishetty,
University of California, Los Angeles, United States
Leslie S. Satin,
University of Michigan, United States

***Correspondence:**
Hojun Kim
kimklar@dongguk.ac.kr

Specialty section:
This article was submitted to Diabetes, a section of the journal Frontiers in Endocrinology

Received: *12 April 2018*
Accepted: *27 September 2018*
Published: *24 October 2018*

Citation:
Wang J-H, Bose S, Shin NR, Chin Y-W, Choi YH and Kim H (2018) Pharmaceutical Impact of Houttuynia Cordata and Metformin Combination on High-Fat-Diet-Induced Metabolic Disorders: Link to Intestinal Microbiota and Metabolic Endotoxemia. Front. Endocrinol. 9:620. doi: 10.3389/fendo.2018.00620

Purpose: Metformin and *Houttuynia cordata* are representative anti-diabetic therapeutic agents in western and oriental medicinal fields, respectively. The present study examined the therapeutic effects of *houttuynia cordata* extract (HCE) and metformin in combination in a dysmetabolic mouse model.

Methods: Metabolic disorders were induced in C57BL/6J mice by high fat diet (HFD) for 14 weeks.

Results: Combination of metformin and HCE significantly lowered body weight, abdominal fat, perirenal fat, liver and kidney weights, but did not change epididymal fat in HFD-fed animals. Metformin + HCE treatment markedly attenuated the elevated serum levels of TG, TC, AST, ALT, and endotoxin and restored the depleted HDL level. Both HCE and metformin + HCE treatment ameliorated glucose tolerance and high level of fasting blood glucose in association with AMPK activation. Moreover, treatment with HCE + metformin dramatically suppressed inflammation in HFD-fed animals via inhibition of proinflammatory cytokines (MCP-1 and IL-6) and LPS receptor (TLR4). Histopathological findings showed that exposure of HFD-treated animals to metformin + HCE ameliorated fatty liver, shrinkage of intestinal villi and adipocytes enlargement. Furthermore, HCE and metformin + HCE treatments markedly modulated the abundance of gut Gram-negative bacteria, including *Escherichia coli* and *Bacteriodetes fragilis*, but not universal Gram-positive bacteria.

Conclusions: Overall, HCE and metformin cooperatively exert their therapeutic effects via modulation of gut microbiota, especially reduction of Gram-negative bacteria, resulting in alleviation of endotoxemia.

Keywords: ***Houttuynia cordata*, type 2 diabetes, high fat diet, gut microbiota, endotoxin**

INTRODUCTION

Metabolism is an essential biochemical event in the body that keeps one alive and healthy. However, morbidity due to metabolic diseases such as obesity and diabetes have been continuously increasing and epidemics of these conditions are occurring in both developed and developing countries (1). Many factors, main including genetic and environmental conditions, can disrupt the normal physiological homeostasis, resulting in metabolic disorders (2). Excessive consumption of high-fat-diet (HFD) is one of the main factors that leads to metabolic disorders (3); however, energy imbalance and hereditary reasons do not completely account for the current epidemic status. Recently, increasing studies have reported that the genetic background determines the predisposition of metabolic disorders (4). Metabolic disorders are widely viewed as chronic systemic diseases because they sustain low-grade inflammation due to gut microbial dysbiosis (5). Therefore, intestinal commensal microbiota become another vital factor during the development of metabolic disorders, especially obesity and type 2 diabetes. HFD-altered gut microbiota obviously improve obesity and inflammation via the toll-like receptor 4 signaling pathway (6). In addition, HFD increases intestinal permeability, which leads to elevated serum lipopolysaccharide (LPS) levels because of gut microbiota dysbiosis (5).

Houttuynia cordata (HC) is a medicinal and edible herb with an aromatic smell that has long been used in Asia to treat pneumonia, hypertension, constipation, and hyperglycemia via detoxification, reduction of heat and diuretic action. There is accumulating evidence of multiple pharmaceutical effects of HC, such as anti-cancer (7), anaphylactic inhibitory (8), anti-mutagenic (9), anti-inflammatory (10), anti-allergic (11), anti-oxidative (12), anti-viral (13), anti-bacterial (14), anti-obesity (15), and anti-diabetic (16) activities. Moreover, metformin, a well-known biguanide antidiabetic agent that has been used for more than 60 years, exerts multiple-properties such as inhibition of hepatic gluconeogenesis, enhancement of insulin sensitivity and augmentation of peripheral glucose uptake (17, 18). Despite its beneficial impacts, metformin produces a large number of side effects, such as diarrhea, nausea, cramps, vomiting, bloating, lactic acidosis, and abdominal pain, which usually occur in clinics (19). The best-known mechanism of action of metformin is regulation of AMP-activated protein kinase (AMPK) and its downstream signaling pathway (20). Metformin has also been found to reduce hepatic gluconeogenesis and hyperglycemia independently of the AMPK pathway (21). Moreover, metformin induced augmentation of *Akkermansia muciniphila* was shown to improve glucose homeostasis in a HFD induced obese model (22). Although both HC and metformin have beneficial impacts on metabolic disorders, their combination has not been evaluated to date. Therefore, we examined an innovative agent that was formulated by combining HC with metformin to synergistically enhance the therapeutic efficacy and/or decrease side effects relative to HC or metformin alone. Specifically, the therapeutic effects of *Houttuynia cordata* extract (HCE) and metformin in combination were investigated using high-fat-diet (HFD) induced metabolic dysfunction of mice model. We also explored the corresponding potential mechanisms, especially regarding alteration of gut microbiota and systemic endotoxemia.

MATERIALS AND METHODS

Houttuynia Cordata Extract (HCE) and Metformin

Houttuynia cordata was obtained from the pharmacy of Dongguk University Ilsan International Hospital (Goyang, South Korea). After grinding, powder of *Houttuynia cordata* was extracted by 5 L ethanol recycling reflux for 4 h. The extract was then filtered and vacuum lyophilized at −70°C, which gave a 5.82% yield. The HCE contained 3.63% quercitrin, 0.45% quercetin and 0.99% of isoquercitrin (23). Metformin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals and Experimental Schedule

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC-2015-037) of Dongguk University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, USA; National Academy Press: Washington D.C., 1996). Specific-pathogen-free (SPF) C57BL/6j male mice were obtained from Koatech (Gyeonggi-do, South Korea). After 1 week of acclimatization, 40 mice were equally divided into five groups by average body weight. The normal group was fed a control diet (**Table S1**) (AIN-93G diet) for 14 weeks, while the other four groups were continuously fed 60% calorie high fat diet (HFD) (**Table S1**) for 14 weeks (**Figure 1A**). From week five to 14, among the HFD-fed mice, eight were treated with metformin (100 mg/kg/day; metformin group), eight with HCE (400 mg/kg/day), eight were treated with a combination of metformin (50 mg/kg/day) and HCE (200 mg/kg/day) and the remaining eight were administrated distilled water as a negative control group. The experimental doses of metformin and HCE were determined based on their clinical dosages and the Guidance for Industry (2005). On the last experimental day, fresh stool samples were collected, and after 12 h of fasting all the animals were weighed and anesthetized using Zoletil (tiletamine-zolazepam, Virbac, Carros, France) and Rompun (xylazine-hydrochloride, Bayer, Leverkusen, Germany) in a 1:1 v/v combination. Blood was then collected from the ventral aorta and rapidly transferred into a BD Vacutainer (Franklin Lakes, NJ, USA) for serum separation. Liver, intestine and fat tissues were removed, weighed and rapidly stored in liquid nitrogen for future analysis.

Oral Glucose Tolerance Test (OGTT)

In the last week of the animal experiment, rats were fasted for 12 h, and then orally dosed with glucose solution (2 g/kg, Sigma-Aldrich, St. Louis, MO, USA). The blood glucose levels were then measured by ACCU-CHEK Active (Mannheim, Germany) using blood collected from the tail vain at 0, 30, 60, 90, 120 min post-glucose dosing. The OGTT results were also expressed as areas under the curves (AUC) to evaluate the degree of glucose tolerance impairment.

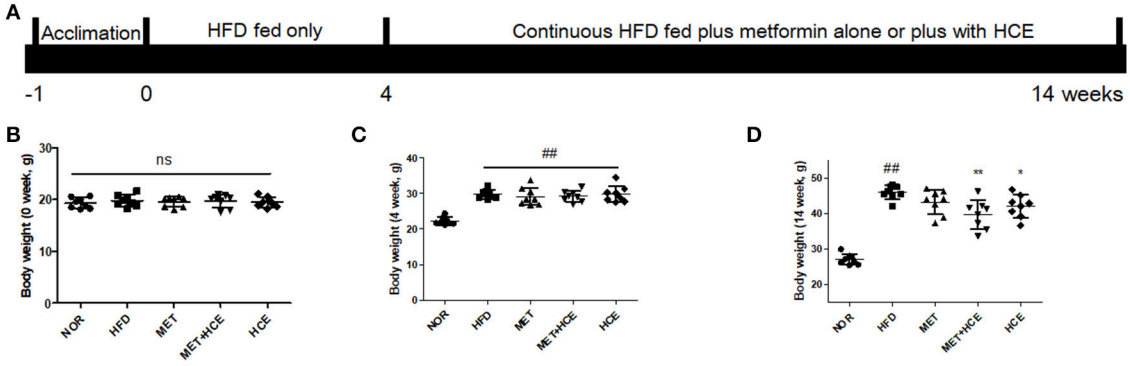

FIGURE 1 | Animal experimental schedule and body mass**.** The experimental design is illustrated intuitively **(A)** and body weight of mice were recorded at the start **(B)**, week 4 **(C)** and week 14 of the experiment **(D)**. Data were expressed as the means ± SD and evaluated using one-way ANOVA followed by the LSD *post-hoc* test. $^{\#\#}P < 0.01$ compared to the normal group; $^{*}P < 0.05$ compared to the HFD group; $^{**}P < 0.01$ compared to the HFD group ($n = 7$). "ns" means none statistic significant.

Serum Biochemical Analysis

Blood collected from the ventral aorta was centrifuged at 3,000 × g for 15 min to separate the serum. The serum levels of triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), aspartate transaminase (AST), and alanine transaminase (ALT) were subsequently determined using commercial enzymatic assay kits (Asan Pharmaceutical Co., Seoul, Korea) according to the manufacturer's instructions.

Serum Endotoxin Analysis

Serum endotoxin levels was measured using a Limulus Amebocyte Lysate (LAL) kit (ENDOSAFE, SC, USA) according to the kit manufacturer's instructions. Briefly, 10× dilutions of mice serum samples were added to the kit supplied plate and wells were spiked with 5 EU/mL standard. Following the addition of 100 μL of LAL reagent, the kinetic absorbance of the mixture was measured at 405 nm and the reaction onset times of the samples were compared to the standard curve.

Oil Red O and H&E Staining

Liver, jejunum and adipose tissues were embedded in FSC 22 frozen section compound (Leica Biosystems, Richmond, IL, USA), then frozen and sectioned at 5 mm using a Leica CM1860 Cryostat (Leica Microsystems, Nussloch, Germany). Sections were then stained with oil red O solution or hematoxylin and eosin (Cayman chemical, USA), after which they were mounted on silicone-coated slides (Leica, USA) and examined using an Olympus BX61 microscope (Tokyo, Japan) and photographed using an Olympus DP70 digital camera (Tokyo, Japan).

Real-Time PCR for Analyzing Gene Expression in Liver Tissue

Total RNA was isolated from liver tissues using TRIsure™ (BIOLINE, MA, USA). cDNA was synthesized using an AccuPower RT premix kit (Bioneer, Daejeon, Korea) and real-time PCR amplification reactions were conducted with the corresponding primers (**Table S2**) using a LightCycler® FastStart DNA Master SYBR Green kit and a LightCycler instrument (Roche Applied Science, Indianapolis, ID, USA). The reaction was conducted in a total reaction volume of 20 μl consisting of PCR mix, 1 μl of cDNA, and gene-specific primers (10 pmol each). The relative gene expression was represented by $2^{-\Delta Ct}$ using β-actin as a housekeeping gene for normalization, where Ct is the crossing threshold value and ΔCt = Ct (target gene) - Ct (β-actin).

Western Blot Analysis

Mice liver tissues were homogenized in RIPA buffer (Abcam, USA) containing protease and phosphatase inhibitors (Abcam, USA). The supernatant was isolated, and total protein concentrations was measured using a BCA kit (Thermo Scientific, USA). Denatured proteins were separated in 10% SDS-PAGE gel, then transferred to polyvinylidene fluoride (PVDF) membrane (GE Healthcare Life Science, Germany) using the Mini-PROTEAN Tetra Cell System (BioRad Laboratories Inc., CA, USA). The membranes were blocked by 5% skim milk with TBST and Tris-buffered saline, then washed with Tween 20 for 1 h and treated with primary antibody (1:10,000) overnight at 4°C. Samples were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000, beta actin manufactured by Santa Cruz, USA; AMPK, phosphorylated-AMPK and GLUT2 manufactured by Cell Signaling, USA) for 1 h. Detailed information regarding the antibodies is shown in **Table S3**. Finally, the band on membranes were detected using SUPEX ECL solution and photographed using a FUJIFILM LAS3000 Image Analyzer (FUJI, Japan).

Fecal Microbial Analysis Using RFLP (Restriction Fragment Length Polymorphism) and Real-Time PCR

Fecal genomic DNA was isolated using a QIAamp DNA Stool Mini Kit (Qiagen, CA, USA) for RFLP and real-time PCR analyses. The 16S rRNA genes were PCR amplified using the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), which were 5′ end-labeled with 5-FAM and 1492R (5′-GGTTACCTTGTTACGACTT-3′).

PCR amplification was conducted using an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 1 min at 94°C, 45 s at 53°C and 2 min at 72°C. The reaction was completed with a final primer elongation step at 72°C for 10 min. Following confirmation by agarose gel electrophoresis, PCR products were digested with the MspI restriction enzyme (TaKaRa, Shiga, Japan). The DNA samples containing the extension products were then added to Hi-Di formamide (Applied Biosystems) and GeneScan™ 1200 LIZ® Size Standard (Applied Biosystems, Foster City, CA, USA). The mixture was subsequently incubated at 95°C for 5 min, placed on ice for 5 min, then analyzed using a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). Next, T-RFLP electropherograms were imaged using GeneMapper® v5.0 and the Peak Scanner 2 software (Applied Biosystems). The relative peak areas of each terminal restriction fragment (TRF) were determined by dividing the area of the peak of interest by the total area of peaks within the following threshold values: lower threshold = 50 bp; upper threshold = 500 bp. Data were normalized by applying a threshold value for relative abundance at 0.5% and only TRFs with higher relative abundances were included in the remaining analyses.

Roche LightCycler FastStart DNA Master SYBR Green was used to conduct real-time PCR using the LightCycler 480 system (Roche Applied Science, Indianapolis, IN, USA). The primer sequences targeting the 16S rRNA gene of the bacteria are listed in **Table S2**. The standard conditions for the PCR amplification reactions were applied as previously described (23). The relative quantification of bacterial abundance is shown by 2^{-Ct} calculations (Ct, threshold cycle). The final results are expressed as normalized fold values relative to the normal group.

Cells Culture and Viability Assay

All cell lines were cultured in an incubator at 37°C in presence of humidified air of 5% CO_2. Mouse myoblasts (C2C12; Korea Cell Line Bank, Seoul, Korea) were cultured in DMEM or RPMI-1640 (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, CA, USA), 2 mM L-glutamine (GIBCO, Carlsbad, CA, USA), 100 U/ml penicillin (GIBCO, Carlsbad, CA, USA), and 100 μg/ml streptomycin (GIBCO, Carlsbad, CA, USA). The cell viability was determined using an EZ-cytox enhanced cell viability assay kit (DOGEN, Seoul, Korea). Briefly, after achieving approximately 80% confluency, the cells were treated for 24 h with quercitrin or quercetin (Sigma, USA) at 1, 5, 10, 20, 50, or 100 μM concentrations. EZ-Cytox was added to the cells 2 h prior to the end of the treatment schedule. Following completion of the reaction, the culture media were transferred to a fresh 96-well microplate. The absorbance of the wells was then read at 450 nm (650 nm as a reference wavelength) (Spectramax Plus, Molecular Devices, CA, USA). The viability of the control cells, in terms of their absorbance, was set to 100%.

Determination of Glucose Uptake *in vitro*

The C2C12 cells were seeded at 1×10^4 cells per well in 96-well black, clear bottom culture plates (Greiner Bio-One, Frickenhausen, Germany) together with 10% FBS/DMEM (GIBCO, Carlsbad, CA, USA) plus antibiotics (GIBCO, Carlsbad, CA, USA) for 24 h at 37°C in presence of humidified air of 5% CO_2. The cells were then incubated in glucose-free DMEM supplemented with 2% horse serum for 96 h until more than 90% differentiation was achieved (approximately 96 h). Next, cells were treated with 10 mM glucosamine (Sigma-Aldrich, MO, USA) and/or 200 nM insulin (Sigma, USA) for 4 h. Finally, cells were treated with metformin (750 μM) alone or in combination with HCE (100 μg/mL), quercitrin (8 μM) or quercetin (2 μM) for 12 h and subsequently treated with 75 μg/mL of 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG, Life Technologies, CA, USA) for 2 h. Eventually, the uptake of 2-NBDG by the cells was measured by fluorescence microscopy (Olympus BX-61, Tokyo, Japan) and determined using a SpectraMax M3 fluorescence reader (Molecular Devices, CA, USA) with excitation and emission wavelengths of 475 and 515 nm, respectively.

Statistical Analysis

All experimental data were analyzed by one-way ANOVA followed by the LSD (least significant difference) *post-hoc* test using SPSS 17.0 (Chicago, IL, USA). The results were expressed as the means ± standard deviations (SD) and a $P < 0.05$ was considered statistically significant.

RESULTS

Reduction of Body, Organ, and Fat Weights

Following termination of the experimental schedule at week 14, the body, fat, liver, and kidney weights of HFD-fed mice were significantly higher compared to animals fed normal diet treatment, as expected. Treatment of HFD-fed animals with both HCE and metformin + HCE markedly reduced the body, fat, liver and kidney weights. Moreover, exposure of HFD-fed mice to metformin reduced the abdominal fat weight, but less significantly than the HCE and metformin + HCE treatments (**Figures 1B–D**, **Table 1**). Furthermore, metformin treatment did not produce any significant effect on body, perirenal, epididymal or total fat of HFD-fed animals. Although not statistically significant, combination of metformin and HCE showed greater anti-obesity effects than either compound alone (**Table 1**).

Amelioration of Serum Lipid Parameters and Hepatic Transaminases

As expected, treatment with HFD significantly increased the levels of serum TG, TC, AST and ALT, and markedly decreased the levels of serum HDL. Combined metformin and HCE treatment significantly attenuated the levels of TG, TC, AST and ALT, and significantly increased the levels of serum HDL in the HFD group. Metformin treatment alone only significantly lowered the level of serum ALT, while HCE treatment alone markedly lowered the levels of serum TC and ALT relative to the HFD group. Overall, combination of metformin and HCE group ameliorated the serum lipid profile and liver transaminases to a greater extent than metformin or HCE alone (**Table 2**).

TABLE 1 | Comparison of body, fat and organ weights.

Groups	normal	HFD	metformin	metformin + HCE	HCE
Body weight gain (g/week)	0.48 ± 0.07	1.62 ± 0.22$^{\#\#}$	1.40 ± 0.23	1.04 ± 0.46**	1.22 ± 0.37*
Food intake (g/week)	16.9 ± 0.8	17.4 ± 0.8	15.6 ± 0.8**	16.5 ± 0.8*	16.4 ± 0.7*
Food efficiency ratio	0.028	0.094	0.085	0.059	0.072
Abdominal fat (g)	0.29 ± 0.05	1.51 ± 0.09$^{\#\#}$	1.23 ± 0.31*	0.74 ± 0.36**	1.12 ± 0.23**
Perirenal fat (g)	0.29 ± 0.11	0.99 ± 0.09$^{\#\#}$	0.98 ± 0.14	0.80 ± 0.15*	0.83 ± 0.14*
Epididymal fat (g)	0.86 ± 0.21	1.93 ± 0.29	2.31 ± 0.46	2.20 ± 0.38	2.20 ± 0.43
Total fat (g)	1.49 ± 0.37	4.40 ± 0.38$^{\#}$	4.52 ± 0.47	3.76 ± 0.80*	4.14 ± 0.53
Liver weight (g)	0.93 ± 0.08	1.86 ± 0.15$^{\#\#}$	1.40 ± 0.28**	1.16 ± 0.33**	1.24 ± 0.21**
Kidney weight (g)	0.32 ± 0.02	0.39 ± 0.03$^{\#\#}$	0.34 ± 0.03**	0.36 ± 0.02*	0.34 ± 0.03**

TC, total cholesterol; HDL, high density lipoprotein; TG, triglyceride. Data were expressed as Mean ± SD, different letters indicate significantly different at measured by One-Way ANOVA followed by LSD ($P < 0.05$, $n = 8$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to the normal group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the HFD group.

TABLE 2 | Comparison of serum biochemistry parameters.

Groups	normal	HFD	metformin	metformin + HCE	HCE
TG (mg/dL)	159 ± 38	182 ± 28	195 ± 26	154 ± 18*	176 ± 32
TC (mg/dL)	128 ± 15	187 ± 24$^{\#\#}$	177 ± 11	155 ± 21**	173 ± 33*
HDL (mg/dL)	27.19 ± 4.72	18.67 ± 2.29$^{\#\#}$	20.42 ± 1.45	22.95 ± 2.04**	17.66 ± 1.99
AST	24.88 ± 8.62	35.87 ± 6.29$^{\#}$	32.24 ± 7.96	22.87 ± 4.81**	28.49 ± 7.42
ALT	6.43 ± 3.15	23.73 ± 4.90$^{\#\#}$	14.56 ± 9.19*	6.39 ± 3.97**	9.82 ± 5.78**

TC, total cholesterol; HDL, high density lipoprotein; TG, triglyceride. Data were expressed as Mean ± SD, different letters indicate significantly different at measured by One-Way ANOVA followed by LSD ($P < 0.05$, $n = 7$). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to the normal group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the HFD group

Improvement of Hyperglycemia and Glucose Tolerance *in vivo* and Glucose Uptake *in vitro*

As anticipated, HFD treatment significantly increased the fasting glucose relative to the normal group. Both metformin and HCE alone and in combination notably lowered the high fasting blood glucose (FBG) relative to HFD treatment. Combined treatment with metformin and HCE showed more efficient reduction of hyperglycemia than treatment with metformin and HCE alone. In addition, OGTT (AUC) was markedly increased by HFD treatment relative to the normal group, while HCE and HCE + metformin treatment significantly reduced the OGTT (AUC) relative to HFD treatment. Finally, HCE + metformin treatment showed more effective amelioration of glucose tolerance than HCE alone (**Figures 2A–C**, **Table S4**).

The *in vitro* results showed that treatment of C2C12 cells with either metformin alone or metformin + HCE remarkably elevated the glucose uptake. Interestingly, metformin in combination with quercitrin plus quercetin treatment, but not metformin + quercitrin or quercetin, exhibited a similar ability for glucose uptake in HepG2 cells as metformin + HCE treatment (**Figure 2D**).

Alleviation of Systemic Endotoxin

Serum endotoxin level was significantly elevated in the HFD group relative to the normal group. However, the metformin + HCE group more significantly reduced the serum endotoxin concentration than HCE or metformin alone relative to the HFD group.

Histopathological Alteration

Staining of hepatic tissue with oil red o revealed that HFD treatment induced lipid droplet deposition in the liver (**Figure 3A**). Additionally, HFD treatment markedly decreased the length and volume of intestinal villi and obviously reduced the size of adipocytes relative to the normal group (**Figures 3B–E**). However, these alterations were recovered in all of the medicine-treated groups. Indeed, the hepatic lipid accumulation, intestinal villi atrophy, and adipocytes enlargement in the HFD-fed animals were more prominently ameliorated by metformin + HCE treatment than metformin or HCE treatment alone.

Activation of AMPK and GLUT2

Treatment of HFD-fed animals with metformin + HCE, but not metformin or HCE alone, resulted in a significant increase in hepatic gene expression of AMPK. Moreover, treatment of HFD-fed animals with metformin + HCE enhanced the pAMPK/AMPK ratio. However, treatment of HFD-fed animals with metformin or HCE led to less enhancement of the pAMPK/AMPK ratio than their combination. Moreover, exposure of HFD-fed animals to all treatments significantly

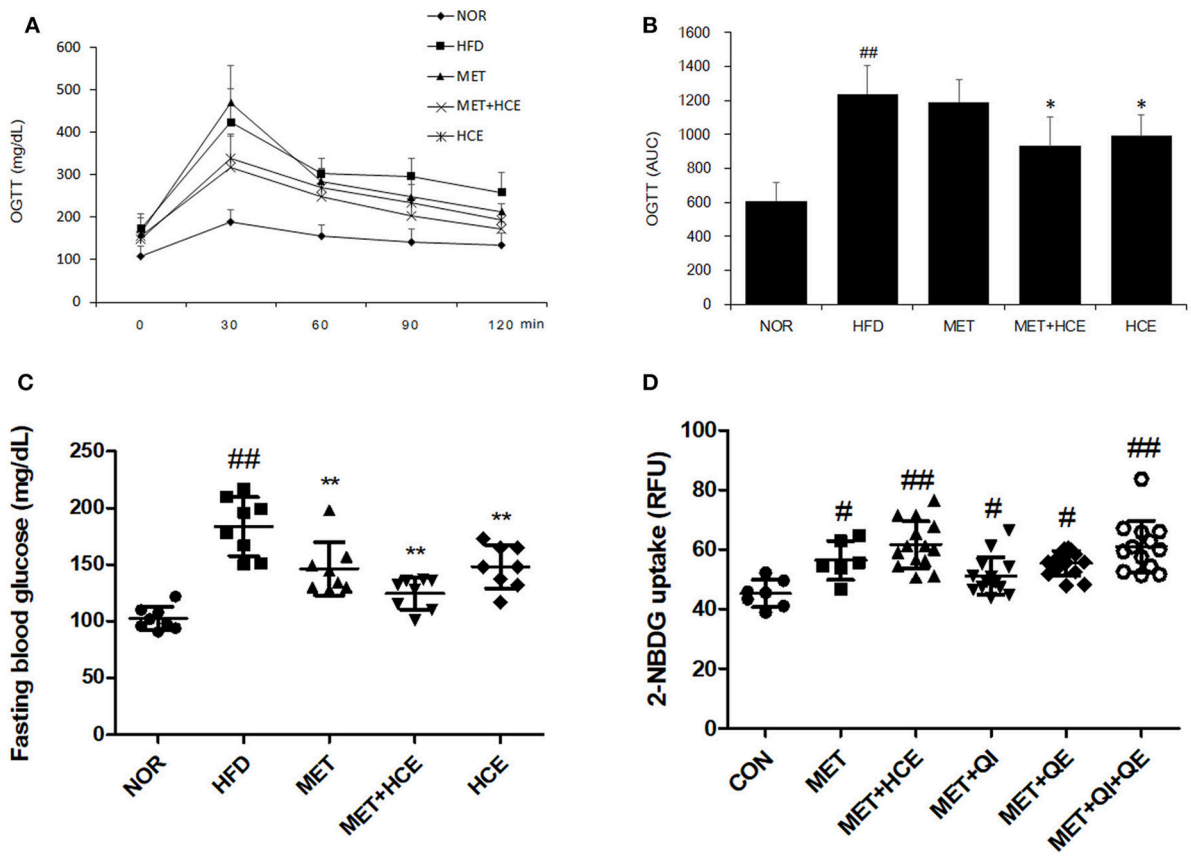

FIGURE 2 | Glucose related parameters *in vivo* and *in vitro.* Impact of metformin either alone or in combination with HCE on insulin sensitivity and glucose tolerance in OLETF rats. Oral glucose tolerance tests (OGTTs) **(A)** of the animals were conducted in the last week and the areas under the curves (AUCs) **(B)** were constructed as described in the Materials and Methods section. Fasting blood glucose **(C)** was also recorded on the last day of the *in vivo* experiment. The glucose uptake ability **(D)** of metformin (750 μM), HCE (100 μg/mL) and its main compounds (8 μM of quercitrin, 2 μM of quercetin) were assessed in C2C12 cells. Data were expressed as the means ± SD and evaluated using one-way ANOVA followed by the LSD *post-hoc* test. **(B)** $^{\#\#}P < 0.01$ compared to the normal group; $^{*}P < 0.05$ compared to the HFD group ($n = 5$). **(C)** $^{\#\#}P < 0.01$ compared to the normal group; $^{**}P < 0.01$ compared to the HFD group ($n = 7$). **(D)** $^{\#}P < 0.05$ compared to the control group; $^{\#\#}P < 0.01$ compared to the control group ($n = 14$).

elevated hepatic gene expression of GLUT2 and markedly increased the hepatic GLUT2 protein level (**Figure 4**).

Attenuation of Inflammation

AS expected, HFD treatment significantly up-regulated gene expression of the TLR4 and downstream signaling proteins, such as IL-6 and MCP-1, relative to the normal group. Nevertheless, HCE + metformin treatment showed greater inhibition of the TLR4 and MCP-1 expression than HFD treatment rather relative to metformin or HCE alone (**Figure 5**).

Modification of Gut Microbial Distribution

PCoA analysis of RFLP data revealed unique characteristics of the gut microbial community in normal, HFD, metformin and HCE groups. More specifically, the distribution pattern of the gut microbial community in the metformin + HCE group had more similarity with the metformin alone group than with other groups (**Figure 6**). Exposure to HFD resulted in a significant increase in the abundance of Gram-negative bacteria in the animals. Additionally, treatment with HFD-fed animals with metformin + HCE, but neither metformin nor HCE alone, significantly decreased the population of universal Gram-negative bacteria. Conversely, exposure of HFD-fed animals to all three medicines significantly reduced the population of *Escherichia coli*. No significant differences in the abundance of universal Gram-positive bacteria were observed among groups. However, exposure of HFD-fed animals to metformin + HCE, but neither metformin nor HCE alone, significantly decreased the population of *Clostridium leptum*. In contrast, treatment of HFD-fed animals with metformin or HCE alone resulted in a greater increase in *Bacteriodetes fragilis* abundance when compared to HFD-fed animals treated with metformin + HCE (**Figure 7**).

DISCUSSION

Although substantial studies have shown that HC and metformin individually could improve metabolic activities (24, 25), to the best of our knowledge, this is the first report to evaluate the

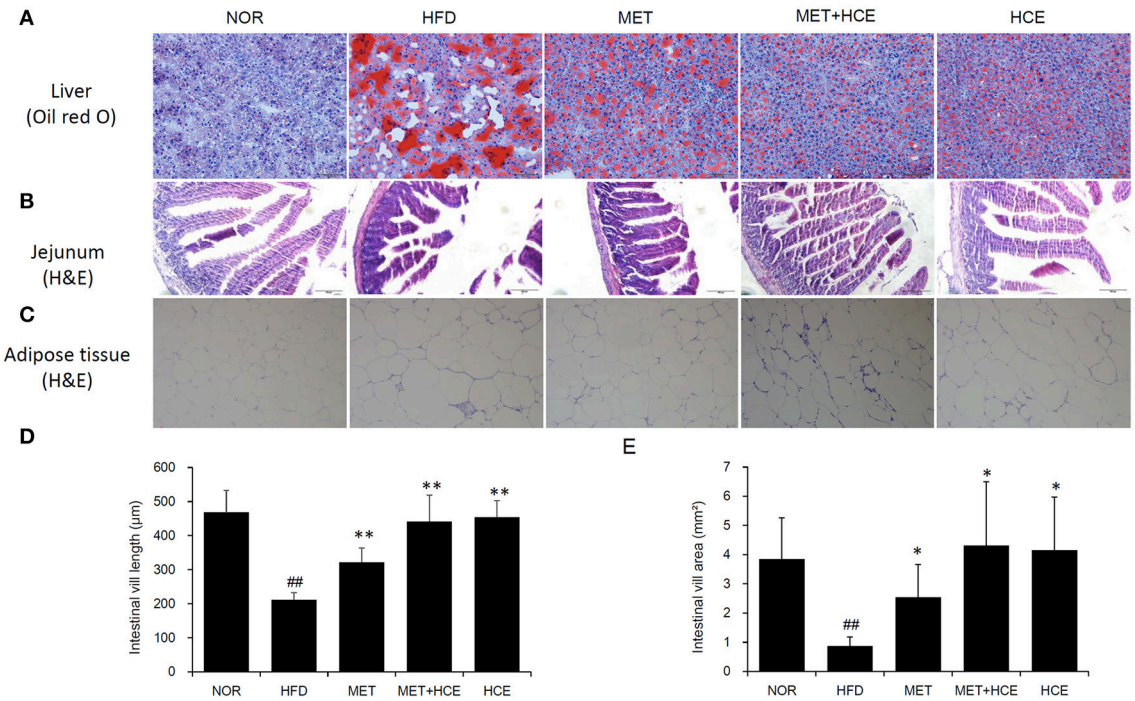

FIGURE 3 | Histopathological analysis. On the final experimental day, the liver **(A)**, jejunum **(B)** and adipose tissue **(C)** were removed rapidly, after which tissue sections were prepared and stained with oil red O or hematoxylin and eosin. Histological examination of the tissue sections was conducted under a light microscope (200× magnification). Calculated length **(D)** and volume **(E)** of the intestinal villi are shown. Data were expressed as the means ± SD and evaluated using one-way ANOVA followed by the LSD *post-hoc* test. $^{\#\#}P < 0.01$ compared to the normal group; $^{*}P < 0.05$ compared to the HFD group; $^{**}P < 0.01$ compared to the HFD group ($n = 3$).

impact of combined treatment with metformin and HCE in a dysmetabolic animal model induced by HFD. More specifically, the major goal of this study was to examine whether the edible formulation of the medicinal herb HC can exert certain synergic effects on the activity of metformin or relieve the side effects of this antidiabetic drug, as well as to elucidate the underlying mechanism of any overserved effects. Based on the actual clinical dosage calculated by a conversion formula from FDA guidance (26), we selected 100 mg/kg of metformin, 400 mg/kg of HCE and half of this dose of metformin (50 mg/kg) together with half the dose of HCE (200 mg/kg) for this investigation. As a representative anti-hyperglycemia agent, metformin significantly ameliorated the FBG in HFD-treated animals. Similarly, HCE treatment significantly reduced the FBG level in HFD-fed animals; however, combination of the metformin and HCE more effectively lowered the FBG than metformin or HCE alone at their higher doses. OGTT, the most widely used procedure for evaluating whole body glucose tolerance, has often been employed to assess insulin sensitivity (27, 28). Indeed, since last 20 years, various indices of insulin sensitivity/resistance using the data from OGTT are documented (29). In the present study, treatment of HFD-fed animals with metformin and HCE in combination led to a greater improvement in OGTT parameters than higher doses of metformin or HCE alone, suggesting the synergistic beneficial impact of these two therapeutic agents on glucose tolerance as well as insulin sensitivity/resistance. Furthermore, in a previous study, using relevant *in vitro* and *in vivo* models, we showed that treatment with metformin + HCE was more beneficial than metformin alone in the improvement of glucose uptake, insulin secretion, glucose metabolism and insulin sensitivity (23).

Our results revealed that the level of quercetin and quercitrin in its glycoside form in HCE were 0.363 and 0.045 mg/g, respectively. These two compounds are active pharmaceutical ingredients of HCE known to have potential antioxidant and anti-inflammatory activities (30). Quercetin shares a common mechanism with metformin in elevating glucose uptake, which is mediated via AMPK activation and upregulation of GLUT expression (31). Our results indicated that HCE assisted metformin in further phosphorylation and gene expression of AMPK. Exposure of HFD-fed animals to all treatments significantly elevated glucose uptake ability via an increase in gene expression of GLUT2 as well as the hepatic level of transporter protein. Thus, our *in vitro* and *in vivo* findings indicate that the combination of metformin and HCE may ameliorate hyperglycemia and glucose tolerance via cooperative augmentation of glucose uptake. It is worth noting that HCE boosts these effects, which is likely because of the collaborative action of quercetin and quercitrin rather than other components.

As expected, obesity, fatty liver, and fatty kidney pathophysiological states were induced in animals in response to long-term HFD feeding as supported by a noteworthy increase in

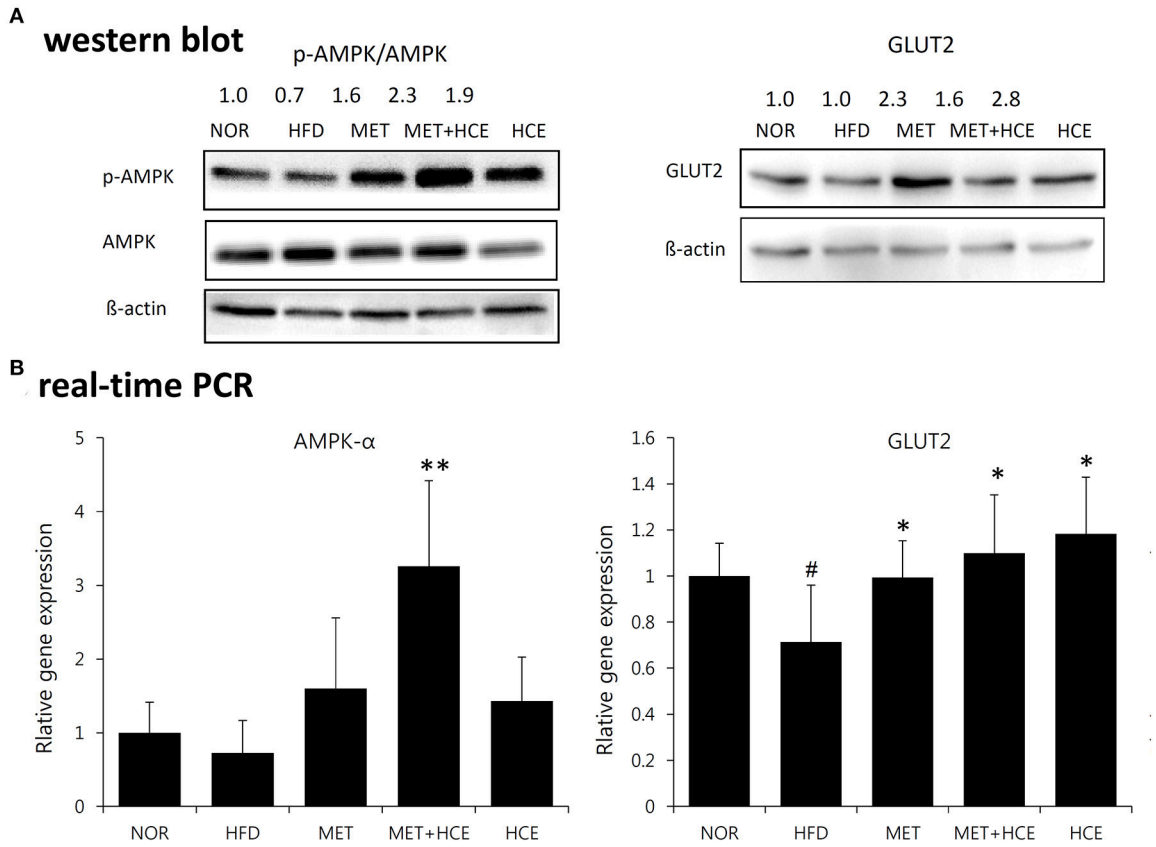

FIGURE 4 | Activation of AMPK and GLUT2**.** Impact of metformin and HCE alone or combination on the activation of hepatic AMPK and GLUT2 as valued by Western blotting **(A)** and real-time PCR **(B)**. $^{\#}P < 0.05$ compared to the normal group; $^{*}P < 0.05$ compared to the HFD group; $^{**}P < 0.01$ compared to the HFD group ($n = 7$).

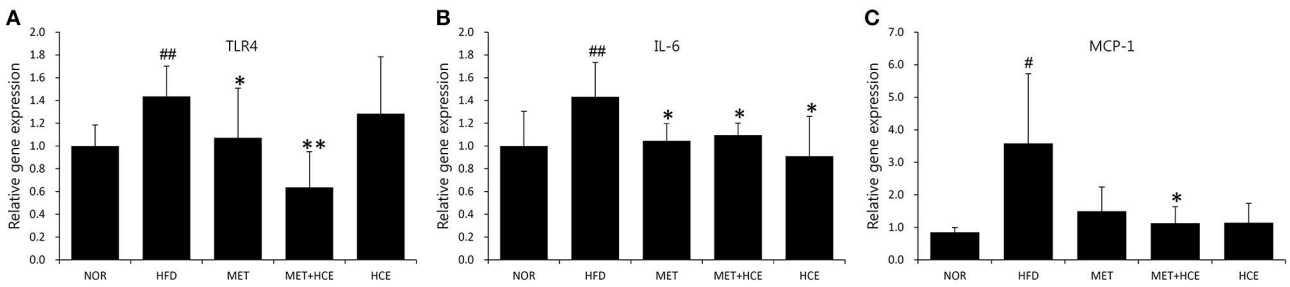

FIGURE 5 | Suppression of inflammatory cytokines. The gene expression of the TLR4 **(A)**, IL-6 **(B)** and MCP-1 **(C)** were analyzed using real-time PCR in liver tissue. Data were expressed as the means ± SD and statistically evaluated using one-way ANOVA followed by the LSD *post-hoc* test. $^{\#}P < 0.05$ compared to the normal group; $^{\#\#}P < 0.01$ compared to the normal group; $^{*}P < 0.05$ compared to the HFD group; $^{**}P < 0.01$ compared to the HFD group ($n = 7$).

body, fat, liver and kidney weights. In parallel, histopathological evidence, such as marked hepatic lipid accumulation and increased adipocyte population in the adipose tissue of HFD-fed animals also indicated that HFD generates grievous lipid dysmetabolism. As in previous studies (23), treatment with either HCE or metformin ameliorated the symptoms of obesity and fatty liver in the present investigation. Meanwhile, HFD destroyed the morphology of intestinal villus; however, these effects were obviously ameliorated by HCE and/or metformin treatment. Interestingly, treatment of HFD-fed animals with HCE and metformin in combination at their half doses was found to be more effective at reducing the body weight, liver weight and fat weight, especially the weight of abdominal and perinephric fats, than treatment with HCE or metformin alone at their original doses. Notably, none of the aforementioned treatments altered the epididymal fat content of HFD-fed animals.

As circulating lipid markers, the levels of serum TG, TC, and HDL indicate the status of holistic lipid metabolism.

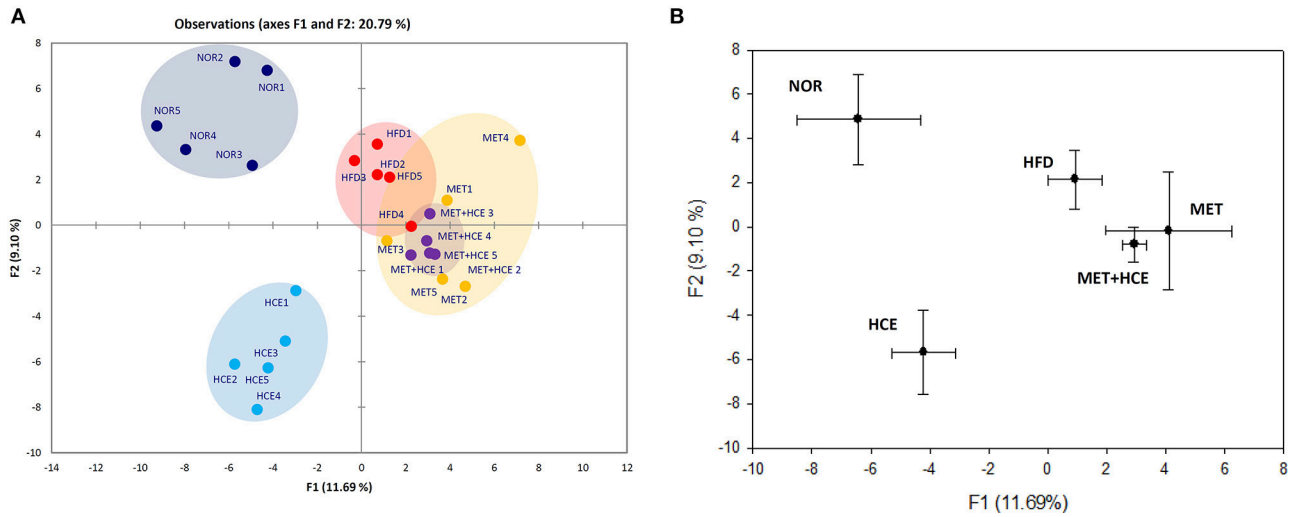

FIGURE 6 | RFLP and PCoA analysis of mice feces. **(A)** Before sacrifice, the mice fecal samples were collected and the microbial communities were analyzed by restriction fragment length polymorphism (RFLP) as described in the Materials and Methods section. **(B)** PCoA analysis of the RFLP data was conducted and diagramed using XLSTAT to further evaluate the similarities between bacterial communities.

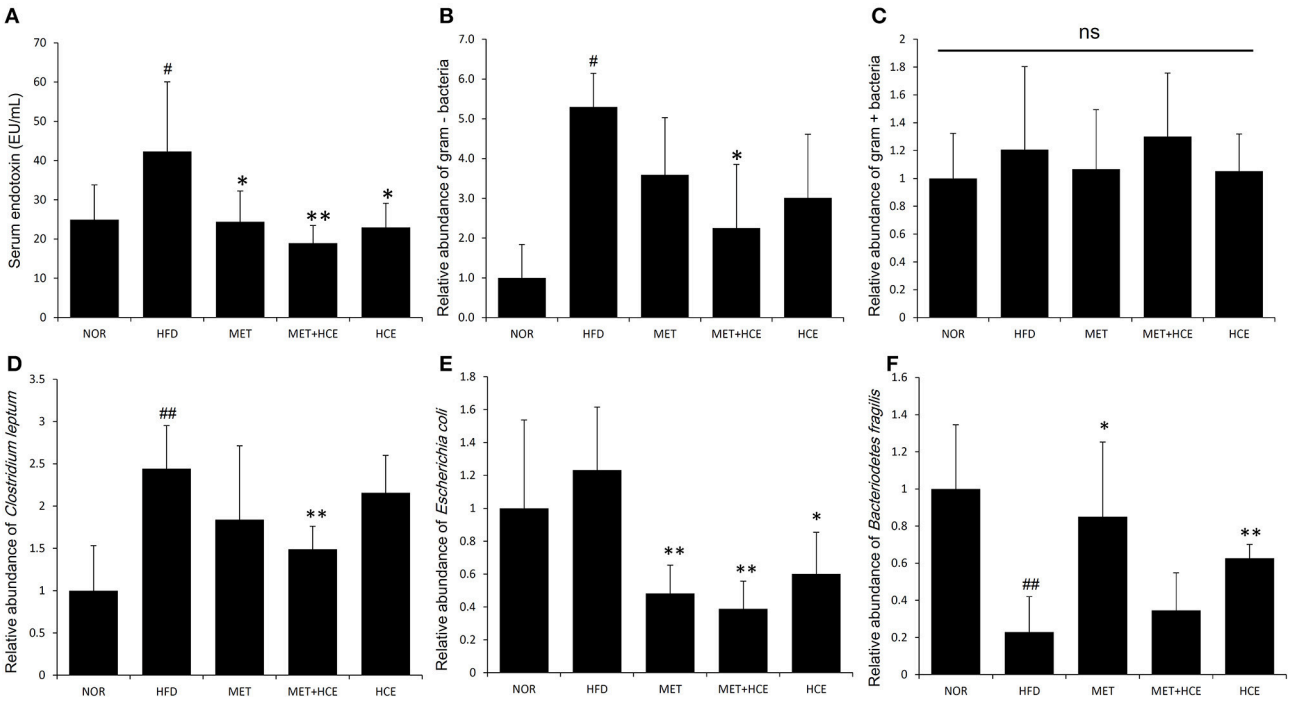

FIGURE 7 | Quantitative determination of systemic endotoxins and relative abundance of gut microbiota. On the final experimental day, blood was collected from the animals and the serum endotoxin level **(A)** was determined as described in the Materials and methods. Stool samples were collected and the abundance of the 16S rRNA gene of the bacterial strains **(B–F)** was determined as described in the Materials and Methods section. The results are expressed as normalized fold values relative to the normal group. Data were expressed as the means ± SD and evaluated using one-way ANOVA followed by the LSD *post-hoc* test. $^{\#}P < 0.05$ compared to the normal group; $^{\#\#}P < 0.01$ compared to the normal group; $^{*}P < 0.05$ compared to the HFD group; $^{**}P < 0.01$ compared to the HFD group ($n = 7$). "ns" means none statistic significant.

Chronic consumption of HFD induces dyslipidemia and the development of fatty liver (32). Previous reports demonstrated that treatment of HFD-fed rats with metformin or HC alone depleted the increased serum levels of TG and TC, and that this was accompanied with increased serum HDL levels (33, 34). Interestingly, in the present study, HCE + metformin treatment

more effectively restored the dysregulated lipid metabolism than HCE or metformin alone in HFD-fed animals. Additionally, as expected, the serum levels of both hepatic transaminases AST and ALT, the sensitive indicators of various liver injuries including fatty liver, were found to be significantly higher in the HFD group than the normal group, which was in keeping with the aberrated histological architecture of the liver in the former group. Overall, our results revealed that treatment of HFD-fed animals with HCE + metformin was more effective than treatment with either compound alone at restoring liver morphology and reducing the serum levels of AST and ALT.

Lipopolysaccharides (LPS), which are also known as endotoxins, exists in the outer membrane of Gram-negative bacteria, where they trigger entotoxemia (5). Metabolic endotoxemia-induced chronic low-grade inflammation has been deemed a vital hallmark of metabolic diseases such as obesity and type 2 diabetes (35). Previous reports have shown that both metformin and HC possess anti-inflammatory activities (36, 37). Furthermore, metformin prevents a number of diseases that are associated with endotoxin insult of Gram-negative bacteria (38–40). In our study, combination of metformin and HCE more significantly attenuated the level of endotoxin in the circulatory system of HFD-fed animals than either compound alone. This is further supported by our findings regarding the significant reduction in abundance of fecal universal Gram-negative bacteria without any modulation in the population of fecal universal Gram-positive bacteria in HFD-fed mice in response to treatment with metformin + HCE, but not with metformin or HCE alone. The significant suppression of gene expression of both proinflammatory cytokine IL-6 and inflammatory chemokine MCP-1, as well as the potent inhibition of TLR4 in HFD-fed mice by metformin + HCE also indicates a feasible mechanism for the cooperative effects of this combination on the anti-inflammatory action against endotoxemia.

For the last few years, the relationship between various diseases and gut commensal microbiota has been widely investigated worldwide (41). Gut microbial composition, which can be altered by HFD (42), plays a vital role in the development of metabolic diseases through regulation of host energy homeostasis and redundancy in fat accumulation (43). Therefore, gut microbial modulation is regarded as a feasible strategy for ameliorating metabolic diseases. Indeed, previous studies have revealed that both metformin and medicinal herbs can ameliorate obesity and related endotoxemia, probably via alteration of the distribution of gut microbiota (22, 44). According to our RFLP analysis, exposure of HFD-fed animals to metformin + HCE caused a more pronounced modulation of the gut microbial population than other treatments. The more similar profile of gut microbiota between the metformin + HCE group and the metformin alone group indicates that metformin potentially restrained the HCE-induced gut microbiota shift. Interestingly, similar to dietary fiber (45), combination of metformin and HCE notably improved glycemia and reduced *Clostridium leptum* in HFD-induced obese animals. Therefore, it is conceivable that HCE together with metformin may exert prebiotic effects leading to significant reduction in the population of gut Gram-negative bacteria, including *Escherichia coli*.

Taken together, our findings suggest that HCE assists metformin in the improvement of obesity, glucose tolerance, hyperglycemia, and hyperlipidemia. This is more likely mediated by reduction of endotoxin and inflammatory stress through regulation of the gut microbial community, particularly *Clostridium leptum* and Gram-negative bacteria including *Escherichia coli*. Thus, it is conceivable that combined treatment with *Houttuynia cordata* and metformin may provide a more efficient strategy for the treatment of patients with metabolic syndrome, particularly T2D and hyperlipidemia. The gut microbiota responsible for contributing the synergistic effects of *Houttuynia cordata* on metformin need to be further explored in future studies.

AUTHOR CONTRIBUTIONS

J-HW wrote manuscript. SB edited and rewrote some parts of manuscript. NS analyzed microbiota data. Y-WC and YC involved in study design and data analysis. HK conceived and designed the study.

ACKNOWLEDGMENTS

This work was supported by a grant from the Convergence of Conventional Medicine and Traditional Korean Medicine R&D program funded by the Ministry of Health and Welfare through the Korea Health Industry Development Institute (HI14C0558). This work was also supported by the National Research Foundation of Korea Grant funded by the Korean Government (NRF-2016R1A2B4014225).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00620/full#supplementary-material

REFERENCES

1. Roglic G. World diabetes congress 2015: the global health challenges stream: assessing global progress and results. *Diabetes Res Clin Pract.* (2015) 108:367–8. doi: 10.1016/j.diabres.2015.03.010
2. Adamo KB, Tesson F. Gene-environment interaction and the metabolic syndrome. *Novartis Found Symp.* (2008) 293:103–19; discussion 119–27.
3. Guo J, Jou W, Gavrilova O, Hall KD. Persistent diet-induced obesity in male C57BL/6 mice resulting from temporary obesigenic diets. *PLoS ONE* (2009) 4:e5370. doi: 10.1371/journal.pone.0005370
4. Schmidt I. Metabolic diseases: the environment determines the odds, even for genes. *News Physiol Sci.* (2002) 17:115–21. doi: 10.1152/nips.01380.2001
5. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity

and diabetes in mice. *Diabetes* (2008) 57:1470–81. doi: 10.2337/db07-1403
6. Kim KA, Gu W, Lee IA, Joh EH, Kim DH. High fat diet-induced gut microbiota exacerbates inflammation and obesity in mice via the TLR4 signaling pathway. *PLoS ONE* (2012) 7:e47713. doi: 10.1371/journal.pone.0047713
7. Kim SK, Ryu SY, No J, Choi SU, Kim YS. Cytotoxic alkaloids from Houttuynia cordata. *Arch Pharm Res.* (2001) 24:518–21. doi: 10.1007/BF02975156
8. Li GZ, Chai OH, Lee MS, Han EH, Kim HT, Song CH. Inhibitory effects of Houttuynia cordata water extracts on anaphylactic reaction and mast cell activation. *Biol Pharm Bull.* (2005) 28:1864–8. doi: 10.1248/bpb.28.1864
9. Chen YY, Liu JF, Chen CM, Chao PY, Chang TJ. A study of the antioxidative and antimutagenic effects of Houttuynia cordata Thunb. using an oxidized frying oil-fed model. *J Nutr Sci Vitaminol. (Tokyo)* (2003) 49:327–33. doi: 10.3177/jnsv.49.327
10. Li W, Fan T, Zhang Y, Zhou P, Niu X, He L. Houttuynia cordata Thunb. volatile oil exhibited anti-inflammatory effects *in vivo* and inhibited nitric oxide and tumor necrosis factor-alpha production in LPS-stimulated mouse peritoneal macrophages *in vitro*. *Phytother Res.* (2013) 27:1629–39. doi: 10.1002/ptr.4905
11. Han EH, Park JH, Kim JY, Jeong HG. Houttuynia cordata water extract suppresses anaphylactic reaction and IgE-mediated allergic response by inhibiting multiple steps of FcepsilonRI signaling in mast cells. *Food Chem Toxicol* (2009) 47:1659–66. doi: 10.1016/j.fct.2009.04.025
12. Tian L, Shi X, Yu L, Zhu J, Ma R, Yang X. Chemical composition and hepatoprotective effects of polyphenol-rich extract from Houttuynia cordata tea. *J Agric Food Chem.* (2012) 60:4641–8. doi: 10.1021/jf3008376
13. Lau KM, Lee KM, Koon CM, Cheung CS, Lau CP, Ho HM, et al. Immunomodulatory and anti-SARS activities of houttuynia cordata. *J Ethnopharmacol.* (2008) 118:79–85. doi: 10.1016/j.jep.2008.03.018
14. Sekita Y, Murakami K, Yumoto H, Mizuguchi H, Amoh T, Ogino S, et al. Anti-bacterial and anti-inflammatory effects of ethanol extract from houttuynia cordata poultice. *Biosci Biotechnol Biochem.* (2016) 80:1205–13. doi: 10.1080/09168451.2016.1151339
15. Miyata M, Koyama T, Yazawa K. Water extract of Houttuynia cordata Thunb. leaves exerts anti-obesity effects by inhibiting fatty acid and glycerol absorption. *J Nutr Sci Vitaminol. (Tokyo)* (2010) 562:150–6.
16. Kumar M, Prasad SK, Krishnamurthy S, Hemalatha S. Antihyperglycemic activity of houttuynia cordata thunb. in streptozotocin-induced diabetic rats. *Adv Pharmacol Sci.* (2014) 2014:809438. doi: 10.1155/2014/809438
17. Prager R, Schernthaner G, Graf H. Effect of metformin on peripheral insulin sensitivity in non insulin dependent diabetes mellitus. *Diab Metab.* (1986) 12:346–50.
18. Radziuk J, Zhang Z, Wiernsperger N, Pye S. Effects of metformin on lactate uptake and gluconeogenesis in the perfused rat liver. *Diabetes* (1997) 46:1406–13. doi: 10.2337/diab.46.9.1406
19. Jackson WP, Coetzee EJ. Side-effects of metformin. *S Afr Med J.* (1979) 56:1113–4.
20. Ota S, Horigome K, Ishii T, Nakai M, Hayashi K, Kawamura T, et al. Metformin suppresses glucose-6-phosphatase expression by a complex I inhibition and AMPK activation-independent mechanism. *Biochem Biophys Res Commun.* (2009) 388:311–6. doi: 10.1016/j.bbrc.2009.07.164
21. Foretz M, Hebrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, et al. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest.* (2010) 120:2355–69. doi: 10.1172/JCI40671
22. Shin NR, Lee JC, Lee HY, Kim MS, Whon TW, Lee MS, et al. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut* (2014) 63:727–35. doi: 10.1136/gutjnl-2012-303839
23. Wang JH, Bose S, Lim S-K, Ansari A, Chin Y-W, Choi HS, et al. Houttuynia cordata facilitates metformin on ameliorating insulin resistance associated with gut microbiota alteration in OLETF Rats. *Genes* (2017) 8:239. doi: 10.3390/genes8100239
24. Stumvoll M, Nurjhan N, Perriello G, Dailey G, Gerich JE. Metabolic effects of metformin in non-insulin-dependent diabetes mellitus. *N Engl J Med.* (1995) 333:550–4. doi: 10.1056/NEJM199508313330903
25. Wang HY, Bao JL. Effect of houttuynia cordata aetherolea on adiponectin and connective tissue growth factor in a rat model of diabetes mellitus. *J Tradit Chin Med.* (2012) 32:58–62. doi: 10.1016/S0254-6272(12)60032-6
26. FDA guidance. *Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers.* Fishers Lane Rockville, MD: U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER) (2005).
27. Stumvoll M, Mitrakou A, Pimenta W, Jenssen T, Yki-Järvinen H, Van Haeften T, et al. Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity. *Diab Care* (2000) 23:295–301. doi: 10.2337/diacare.23.3.295
28. Soonthornpun S, Setasuban W, Thamprasit A, Chayanunnukul W, Rattarasarn C, Geater A. Novel insulin sensitivity index derived from oral glucose tolerance test. *J Clin Endocrinol Metab.* (2003) 88:1019–23. doi: 10.1210/jc.2002-021127
29. Gutch M, Kumar S, Razi SM, Gupta KK, Gupta A. Assessment of insulin sensitivity/resistance. *Ind J Endocrinol Metab.* (2015) 19:160–4. doi: 10.4103/2230-8210.146874
30. Li X, Jiang Q, Wang T, Liu J, Chen D. Comparison of the antioxidant effects of quercitrin and isoquercitrin: understanding the role of the 6"-OH Group. *Molecules* (2016) 21:E1246. doi: 10.3390/molecules21091246
31. Dhanya R, Arya AD, Nisha P, Jayamurthy P. Quercetin, a lead compound against type 2 diabetes ameliorates glucose uptake via AMPK pathway in skeletal muscle cell line. *Front Pharmacol.* (2017) 8:336. doi: 10.3389/fphar.2017.00336
32. D'Souza AM, Beaudry JL., Szigiato AA, Trumble SJ, Snook LA, Bonen A, et al. Consumption of a high-fat diet rapidly exacerbates the development of fatty liver disease that occurs with chronically elevated glucocorticoids. *Am J Physiol Gastrointest Liver Physiol.* (2012) 302:G850–63. doi: 10.1152/ajpgi.00378.2011
33. Kang H, Koppula S. Houttuynia cordata alleviates high-fat diet-induced non-alcoholic fatty liver in experimental rats. *Pharm Biol.* (2015) 53:414–22. doi: 10.3109/13880209.2014.923002
34. Meng XM, Ma XX, Tian YL, Jiang Q, Wang LL, Shi R, et al. Metformin improves the glucose and lipid metabolism via influencing the level of serum total bile acids in rats with streptozotocin-induced type 2 diabetes mellitus. *Eur Rev Med Pharmacol Sci.* (2017) 21:2232–7.
35. Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* (2007) 56:1761–72. doi: 10.2337/db06-1491
36. Koh SJ, Kim JM, Kim IK, Ko SH, Kim JS. Anti-inflammatory mechanism of metformin and its effects in intestinal inflammation and colitis-associated colon cancer. *J Gastroenterol Hepatol.* (2014) 29:502–10. doi: 10.1111/jgh.12435
37. Lee JH, Ahn J, Kim JW, Lee SG, Kim HP. Flavonoids from the aerial parts of Houttuynia cordata attenuate lung inflammation in mice. *Arch Pharm Res.* (2015) 38:1304–11. doi: 10.1007/s12272-015-0585-8
38. Bergheim I, Luyendyk JP, Steele C, Russell GK, Guo L, Roth RA, et al. Metformin prevents endotoxin-induced liver injury after partial hepatectomy. *J Pharmacol Exp Ther.* (2006) 316:1053–61. doi: 10.1124/jpet.105.092122
39. Al-Attas OS, Al-Daghri NM, Al-Rubeaan K, da Silva NF, Sabico SL, Kumar S, et al. Changes in endotoxin levels in T2DM subjects on anti-diabetic therapies. *Cardiovasc Diabetol.* (2009) 8:20. doi: 10.1186/1475-2840-8-20
40. Kalariya NM, Shoeb M, Ansari NH, Srivastava SK, Ramana KV. Antidiabetic drug metformin suppresses endotoxin-induced uveitis in rats. *Invest Ophthalmol Vis Sci.* (2012) 53:3431–40. doi: 10.1167/iovs.12-9432
41. Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis.* (2015) 26:26191. doi: 10.3402/mehd.v26.26191
42. Daniel H, Gholami AM, Berry D, Desmarchelier C, Hahne H, Loh G, et al. High-fat diet alters gut microbiota physiology in mice. *ISME J.* (2014) 8:295–308. doi: 10.1038/ismej.2013.155

43. Musso G, Gambino R, Cassader M. Gut microbiota as a regulator of energy homeostasis and ectopic fat deposition: mechanisms and implications for metabolic disorders. *Curr Opin Lipidol.* (2010) 21:76–83. doi: 10.1097/MOL.0b013e3283347ebb
44. Wang JH, Bose S, Kim GC, Hong SU, Kim JH, Kim JE, et al. Flos Lonicera ameliorates obesity and associated endotoxemia in rats through modulation of gut permeability and intestinal microbiota. *PLoS ONE* (2014) 9:e86117. doi: 10.1371/journal.pone.0086117
45. Eslinger AJ, Eller LK, Reimer RA. Yellow pea fiber improves glycemia and reduces Clostridium leptum in diet-induced obese rats. *Nutr Res.* (2014) 34:714–22. doi: 10.1016/j.nutres.2014.07.016

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 06 November 2018
doi: 10.3389/fendo.2018.00657

Metformin Is a Direct SIRT1-Activating Compound: Computational Modeling and Experimental Validation

Elisabet Cuyàs[1,2†], Sara Verdura[1,2†], Laura Llorach-Parés[3†], Salvador Fernández-Arroyo[4], Jorge Joven[4], Begoña Martin-Castillo[2,5], Joaquim Bosch-Barrera[2,6,7], Joan Brunet[7,8,9], Alfons Nonell-Canals[3], Melchor Sanchez-Martinez[3] and Javier A. Menendez[1,2]*

[1] ProCURE (Program Against Cancer Therapeutic Resistance), Metabolism and Cancer Group, Catalan Institute of Oncology, Girona, Spain, [2] Girona Biomedical Research Institute (IDIBGI), Girona, Spain, [3] Mind the Byte, Barcelona, Spain, [4] Unitat de Recerca Biomèdica, Hospital Universitari de Sant Joan, Institut d'Investigació Sanitària Pere Virgili (IISPV), Rovira i Virgili University, Reus, Spain, [5] Unit of Clinical Research, Catalan Institute of Oncology (ICO), Girona, Spain, [6] Department of Medical Sciences, Medical School, University of Girona, Girona, Spain, [7] Medical Oncology, Catalan Institute of Oncology (ICO), Dr. Josep Trueta University Hospital, Girona, Spain, [8] Hereditary Cancer Programme, Catalan Institute of Oncology (ICO), Bellvitge Institute for Biomedical Research (IDIBELL), L'Hospitalet del Llobregat, Barcelona, Spain, [9] Hereditary Cancer Programme, Catalan Institute of Oncology (ICO), Girona Biomedical Research Institute (IDIBGI), Girona, Spain

Metformin has been proposed to operate as an agonist of SIRT1, a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase that mimics most of the metabolic responses to calorie restriction. Herein, we present an *in silico* analysis focusing on the molecular docking and dynamic simulation of the putative interactions between metformin and SIRT1. Using eight different crystal structures of human SIRT1 protein, our computational approach was able to delineate the putative binding modes of metformin to several pockets inside and outside the central deacetylase catalytic domain. First, metformin was predicted to interact with the very same allosteric site occupied by resveratrol and other sirtuin-activating compounds (STATCs) at the amino-terminal activation domain of SIRT1. Second, metformin was predicted to interact with the NAD^+ binding site in a manner slightly different to that of SIRT1 inhibitors containing an indole ring. Third, metformin was predicted to interact with the C-terminal regulatory segment of SIRT1 bound to the NAD^+ hydrolysis product ADP-ribose, a "C-pocket"-related mechanism that appears to be essential for mechanism-based activation of SIRT1. Enzymatic assays confirmed that the net biochemical effect of metformin and other biguanides such as a phenformin was to improve the catalytic efficiency of SIRT1 operating in conditions of low NAD^+ *in vitro*. Forthcoming studies should confirm the mechanistic relevance of our computational insights into how the putative binding modes of metformin to SIRT1 could explain its ability to operate as a direct SIRT1-activating compound. These findings might have important implications for understanding how metformin might confer health benefits *via* maintenance of SIRT1 activity during the aging process when NAD^+ levels decline.

Keywords: metformin, SIRT1, aging, NAD^+, NAD loss

OPEN ACCESS

Edited by:
Frederic Bost, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Pierre De Meyts, de Duve Institute, Belgium
Yves Combarnous, Centre National de la Recherche Scientifique (CNRS), France

****Correspondence:***
Javier A. Menendez
jmenendez@iconcologia.net; jmenendez@idibgi.org

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *05 July 2018*
Accepted: *19 October 2018*
Published: *06 November 2018*

Citation:
Cuyàs E, Verdura S, Llorach-Parés L, Fernández-Arroyo S, Joven J, Martin-Castillo B, Bosch-Barrera J, Brunet J, Nonell-Canals A, Sanchez-Martinez M and Menendez JA (2018) Metformin Is a Direct SIRT1-Activating Compound: Computational Modeling and Experimental Validation. Front. Endocrinol. 9:657. doi: 10.3389/fendo.2018.00657

INTRODUCTION

A small molecule capable of targeting aging and delaying the onset of aging-related multimorbidity has the potential to radically amend the way we understand (and practice) modern medicine (1). One such molecule is the biguanide metformin, which, 60 years after its introduction in Europe as a first-line therapeutic for type 2 diabetes (2), could have the potential to prevent multiple aging-related disorders (3–5). Against this background, the TAME (Targeting Aging with Metformin) clinical trial has been designed to evaluate the healthspan-promoting effects of metformin by enrolling patients aged 65–79 years diagnosed with one single age-associated condition, and then assessing the global impact of metformin on a composite outcome including cardiovascular events, cancer, dementia, mortality, and other functional and geriatric endpoints (6). Although the current consensus is that metformin has the ability to target multiple pathways of aging, it is still unclear whether such a capacity reflects downstream consequences of a primary action on a single mechanism or whether it involves direct effects on aging regulators (6).

Metformin has been proposed to exert indirect pleiotropy on core metabolic hallmarks of aging such as the insulin/IGF-1 and AMPK/mTOR signaling pathways (4) downstream of its primary inhibitory action on mitochondrial respiratory complex I. Alternatively, but not mutually exclusive, its capacity to operate as a poly-therapeutic anti-aging agent might involve the direct targeting of the biologic machinery of aging *per se*. A systematic chemoinformatics approach established to computationally predict metformin targets recently revealed that the salutary effects of metformin on human cellular aging might involve its direct binding to core chromatin modifiers of the aging epigenome (7, 8), such as the H3K27me3 demethylase KDM6A/UTX (9–11). The ability of metformin to directly interact with TGF-β1, thereby blocking its binding to TβRII and resulting in impaired downstream signaling (12), is another example of how metformin might exert pleiotropic effects on numerous (TGF-β1 hyperfunction-associated) aging diseases such as organ fibrosis and cancer, without necessarily involving changes in cellular bioenergetics.

SIRT1 is a member of the class III (NAD^+-dependent) histone deacetylases (HDACs) that mimics most of the metabolic responses to calorie restriction and contributes to enhanced healthy aging, including a reduced incidence of cardiovascular and metabolic diseases, cancer, and neurodegeneration (13–17). The regulation of SIRT1 by metformin is an archetypal example of its ability to indirectly and directly impact the aging process. Because of its enzymatic requirement for NAD^+, SIRT1 is commonly viewed as a unique energy sensor that couples its function to the NAD^+/NADH ratio of the cell or organism (18–20). Accordingly, metformin-induced metabolic stress has been shown to induce SIRT1 expression and activity as a downstream consequence of AMPK activation-induced augmentation of cellular NAD^+ levels (21–24). Although the striking similarity between the pleiotropic effects of metformin and the physiological consequences of SIRT1 activation might merely represent the overlapping metabolic effects of SIRT1 and AMPK activators (25, 26), we are beginning to uncover evidence on the occurrence of energy crisis (i.e., AMPK/mTOR)-independent agonist effects of metformin on SIRT1 activity (27–31). Nonetheless, both the putative molecular interactions on the atomic scale between metformin and SIRT1 and the mechanism of action of metformin as a direct modulator of SIRT1 activity remain elusive.

Here, we performed an *in silico* docking and molecular dynamics (MD) simulation study of the SIRT1-metformin complex coupled to laboratory-based experimental validation, aiming to interrogate the ability of metformin to directly enhance NAD^+-dependent SIRT1 activity. Our findings present a first-in-class structural basis to understand the behavior of metformin as a direct SIRT1-activating compound.

MATERIALS AND METHODS

Computational Modeling of the Human SIRT1 Protein

To provide *in silico* insights into the binding pattern of metformin with SIRT1, we employed eight different crystal structures of the human SIRT1 protein, namely 4KXQ, 4IF6, 4ZZJ, 4ZZI, 4ZZH, 4I5I, 5BTR, and 4IG9. 4KXQ, and 4IF6 represent the heterodimeric (chains A and B), closed conformation of SIRT1 bound to adenosine-5-diphosphoribose (APR) (32). 4ZZJ represents the heterodimeric (chains A –SIRT1 and B –p53), open conformation of SIRT1 bound to small molecule sirtuin-activating compounds (STATCs) such as the non-hydrolyzable NAD^+ analog carbaNAD (carba nicotinamide adenine dinucleotide) or to the carboxamide SIRT1 inhibitor 4TQ (33). 4ZZI represents the monomeric (chain A), open conformation of SIRT1 bound to the carboxamide SIRT1 inhibitors 4TQ and 1NS, whereas 4ZZH represents the monomeric (chain A), open conformation of SIRT1 bound to the carboxamide SIRT1 inhibitor 4TO (33). 4I5I represents the dimeric (chains A and B) conformation of SIRT1 bound to NAD or, alternatively, to the carboxamide SIRT1 inhibitor 4I5 (34). 5BTR represents the heterotrimeric (chains A, B, and C –SIRT1 and D, E, and F –p53), closed conformation of SIRT1 bound to resveratrol (35). Finally, 4IG9 represents a quaternary complex of SIRT1 with no bound ligand (32).

Docking Calculations

All docking calculations were performed using *Itzamna* and *Kin* (www.mindthebyte.com), classical docking and blind-docking software tools. The above mentioned protein structures from RCSB Protein Data Bank (https://www.rcsb.org) were directly employed for docking calculations using the cavities defined by crystallographic ligands where available. Two runs were carried out for each calculation to avoid false positives.

Molecular Dynamics Simulations

Docking post-processing allowing conformational selections/induced fit events to optimize the interactions were performed via short (1 ns) MD simulations using NAMD version 2.10 over the best-docked complexes, which were selected based on the interaction energy. The Ambers99SB-ILDN and the

GAFF forcefield set of parameters were employed for SIRT1 and metformin, respectively. The GAFF parameters were obtained using Acpype software, whereas the SIRT1 structures were modeled using the leap module of Amber Tools. Simulations were carried out in explicit solvent using the TIP3P water model with the imposition of periodic boundary conditions via a cubic box. Electrostatic interactions were calculated by the particle-mesh Ewald method using constant pressure and temperature conditions. Each complex was solvated with a minimum distance of 10 Å from the surface of the complex to the edge of the simulation box; Na^+ or Cl^- ions were also added to the simulation to neutralize the overall charge of the systems. The temperature was maintained at 300 K using a Langevin thermostat, and the pressure was maintained at 1 atm using a Langevin Piston barostat. The time step employed was 2 fs. Bond lengths to hydrogens were constrained with the SHAKE algorithm. Before production runs, the structure was energy minimized followed by a slow heating-up phase using harmonic position restraints on the heavy atoms of the protein. Subsequently, the system was energy minimized until volume equilibration, followed by the production run without any position restraints.

Binding Free Energy Analysis

Molecular Mechanics/Generalized Borne Surface Area (MM/GBSA) calculations were performed to calculate the alchemical binding free energy (ΔG_{bind}) of metformin against SIRT1. MM/GBSA rescoring was performed using the MMPBSA.py algorithm within AmberTools. The snapshots generated in the 1 ns MD simulation were imputed into the post-simulation MM/GBSA calculations of binding free energy. Graphical representations were prepared using PyMOL program and PLIP version 1.3.0.

Interaction Analysis

The predicted binding site residues of metformin to SIRT1 were defined using evidence-based interaction analyses of known SIRT1 activators/ inhibitors with well-defined binding residues.

SIRT1 Enzymatic Assay

The effects of metformin on SIRT1 activity were assessed using the SIRTainty™ Class III HDAC Assay (Cat. #17-10090, Millipore) and the Epigenase™ Universal SIRT1 Activity/Inhibition Assay Kit (Cat. # P-4027, Epigentek), as per the manufacturers' instructions. In the former assay, purified SIRT1 enzyme, β-NAD, acetylated peptide substrate, metformin, and nicotinamidase enzyme were combined and incubated for 30 min. During this time the acetylated peptide substrate is deacetylated by SIRT1 and produces nicotinamide. In a secondary reaction, the nicotinamidase enzyme converts nicotinamide into nicotinic acid and free ammonia (NH_3^+). To generate a signal for readout, a proprietary developer reagent is added and the signal is read ($420_{ex}/460_{em}$ nm) using a fluorescent plate reader. In the latter assay, an acetylated histone SIRT1 substrate is stably coated onto microplate wells; active SIRT1 binds to the substrate and removes acetyl groups from the substrate and the amount of SIRT1-deacetylated products, which is proportional to the enzyme activity, can be measured using a specific antibody. The ratio or amount of deacetylated product, which is proportional to the enzyme activity, is fluorometrically measured by reading the fluorescence at $530_{ex}/590_{em}$ nm. Metformin, phenformin, and buformin (Sigma-Aldrich Ltd.) were added from aqueous stock solutions, and proguanil (Sigma-Aldrich Ltd.) from stock solutions in DMSO.

RESULTS

Molecular Docking and Molecular Dynamics Simulation Analyses of Metformin With SIRT1

First, rigid docking calculations were performed over the cavities defined by the crystallographic ligands in the 4KXQ, 4IF6, 4ZZJ, 4ZZI, 4ZZH, 4I5I, and 5BTR structures (**Figures 1**, **2**). In the case of the ligandless 4IG9 structure, we performed blind docking calculations involving cavity searching and docking calculations over the found cavities. After simulations, we selected more than one model conformation of metformin to cover all the possible binding models within the crystallographic binding poses of the ligands.

The binding energies obtained from the rigid docking calculations, which were run twice to avoid false positives, are summarized in **Table 1**. This approach predicted the ability of silibinin to directly bind all the above crystal structures of human SIRT1, with binding energy values up to −5.0 kcal/mol for the crystal structure 4I5I. It should be acknowledged that the predicted *in silico* capacity of metformin to poorly interact with SIRT1, with rather high binding energies, could be explained by the small size of metformin and by docking calculations performed against cavities that, in most cases, are biased toward the ligand to which the target structure is co-crystallized. To add protein flexibility to the analysis and to test the stability of the selected metformin-target complexes, we carried out short MD simulations of 1 ns to filter out poorly interacting poses. We then performed MM/GBSA calculations (36) to estimate the free energy of the binding of metformin to biological macromolecules such as SIRT1. This estimation of ligand-binding affinities takes into consideration the dynamic nature of SIRT1 and it is therefore more reliable to provide a realistic view of metformin binding affinity than rigid docking estimations (**Figures 3**, **4**). The energies obtained following MM/GBSA rescoring calculations over MD simulations are summarized in **Table 1**, with the best model highlighted in green. From 30 models of metformin-SIRT1 interactions, 11 of them (which are highlighted in green in **Table 1**) were found to maintain their predicted interacting residues in their corresponding docking poses.

Analysis of the Binding Mode of Metformin to SIRT1

The best binding energies of metformin to SIRT1 using rigid docking calculations were predicted to occur when employing the model 1 in the 4KXQ and 4IF6 crystal structures, which highly resemble each other. A detailed analysis of the metformin-binding mode to 4KXQ and 4IF6 predicted the interaction of metformin with the same group of amino acids in both

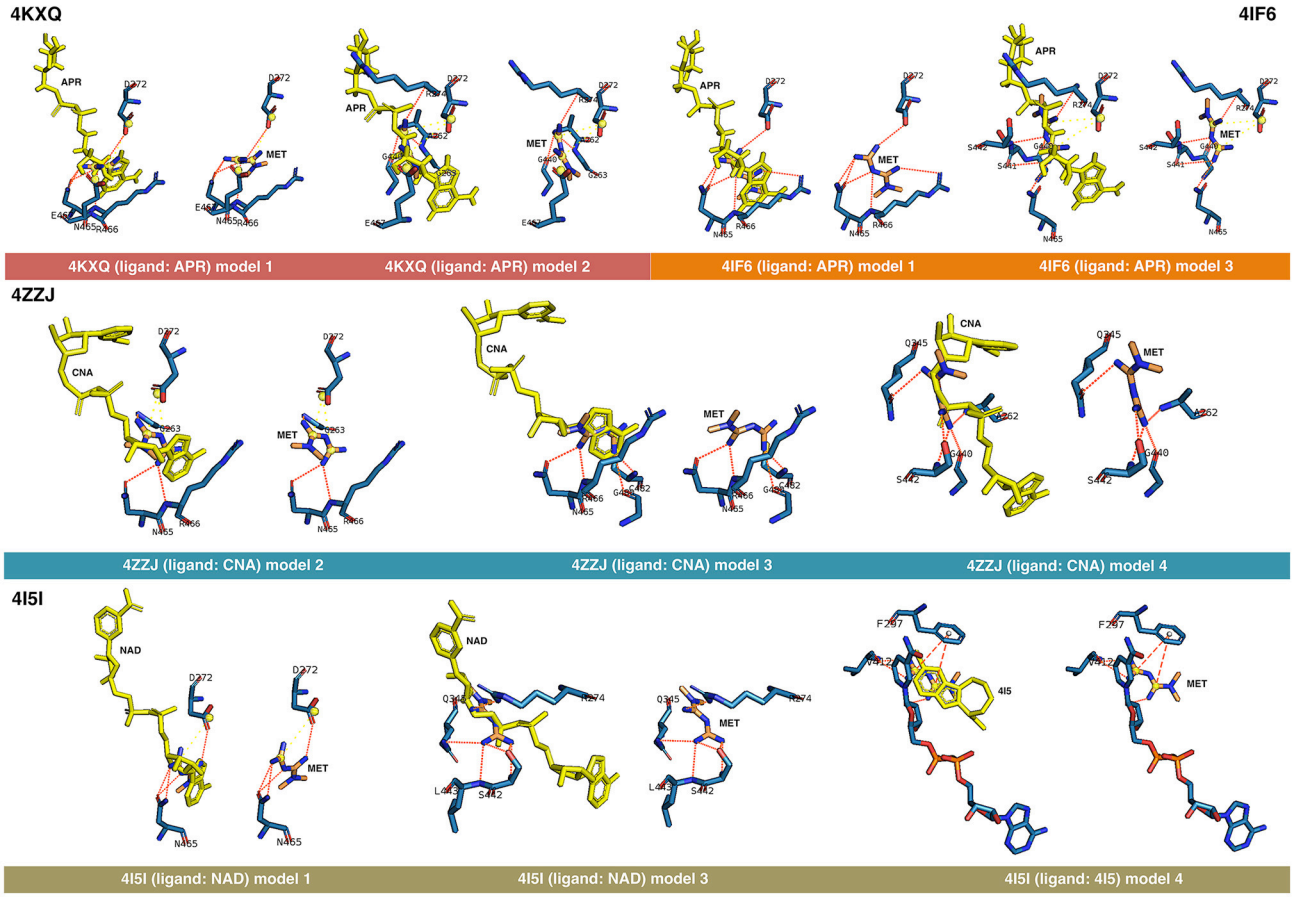

FIGURE 1 | Rigid docking study of the metformin-binding mode to the APR, CNA, NAD^+, and 4I5 binding pockets of SIRT1. Figure shows in sticks all the pharmacophoric interaction residues involved in the *in silico* binding of metformin to the APR, CNA, NAD^+, and 4I5 binding pockets of SIRT1, using PLIP. The main residues involved in silibinin interaction with the protein backbone are shown in black; the residue numbers shown correspond to the original PDB file numbering.

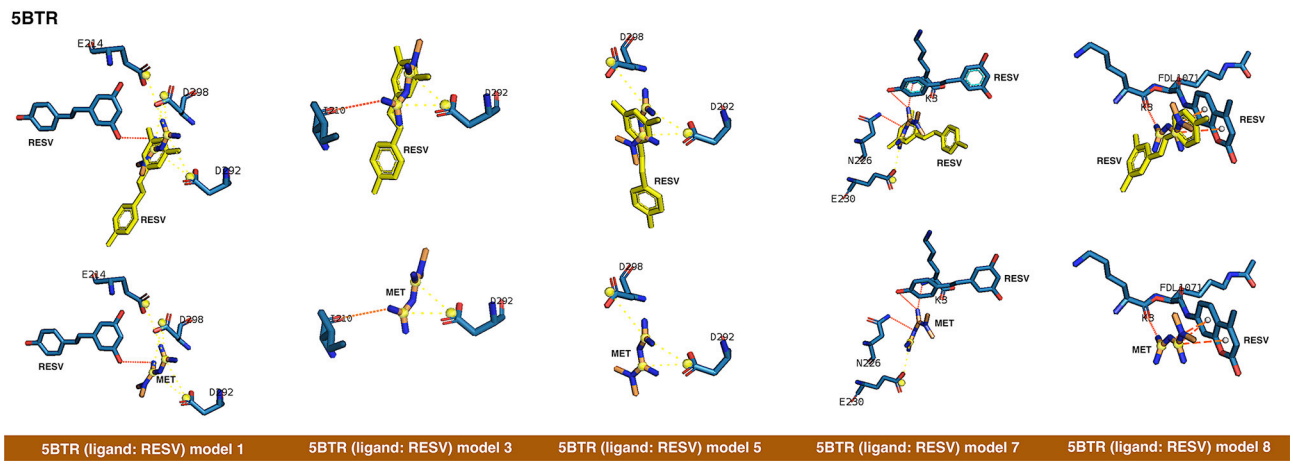

FIGURE 2 | Rigid docking study of the metformin-binding mode to the resveratrol (RESV) binding pocket of SIRT1. Figure shows in sticks all the pharmacophoric interaction residues involved in the *in silico* binding of metformin to the RESV binding pocket of SIRT1, using PLIP. The main residues involved in silibinin interaction with the protein backbone are shown in black; the residue numbers shown correspond to the original PDB file numbering.

TABLE 1 | Docking binding energies and MM/GBSA-based energy rescoring calculations over MD simulations of metformin against SIRT1.

PDB ID	Ligand	Model	Binding Energy (kcal/mol) R0/R1[a]	MM/GBSA energy (kcal/mol)[b]
4KXQ	APR	1	−4.0/−4.6	−18.6175
		2	−3.9/−3.8	−14.6640
		3	−3.3/−2.5	−13.8421
		4	−3.1/−2.0	−4.6926
4IF6	APR	1	−4.2/−4.2	−14.3340
		2	−4.4/−3.8	−12.7689
		3	−3.7/−3.8	−10.4075
		4	−3.1/−2.1	−13.2829
4ZZJ	4TQ	1	−2.3/−2.0	−0.8082
	CNA	2	−3.6/−3.4	−17.8281
		3	−3.5/−4.0	−25.1540
		4	−3.4/−3.4	−21.7529
4ZZI	4TQ	1	−2.1/−2.2	−2.2537
	1NS	2	−4.6/−3.2	−19.0828
4ZZH	4TO	1	−2.1/−1.7	−4.1866
4I5I	NAD	1	−5.0/−5.0	−13.4730
		2	−4.9/−4.9	−5.9833
		3	−4.4/−4.3	−14.8859
	4I5	4	−4.6/−3.7	−16.9806
5BTR	STL-A	1	−3.6/−3.6	−20.8897
	STL-B	2	−3.2/−3.1	−11.2383
		3	−2.9/−2.5	−26.9390
	STL-C	4	−3.2/−3.0	−11.0961
		5	−3.1/−3.2	−16.5834
	STL-D	6	−3.5/−3.7	−10.7575
		7	−3.5/−3.5	−23.6198
	STL-E	8	−3.4/−3.2	−25.0726
	STL-F	9	−3.4/−3.4	−18.9041
4IG9		1	−3.9/−3.9	−2.7150
		2	−4.4/−4.4	−8.3935

The more negative the binding energy, the more plausible the interaction.
[a] *Each docking calculation was performed twice (R0 and R1) to avoid false positives.*
[b] *Energy obtained after MM/GBSA calculations.*
Green, best model per target; Yellow, models better maintaining the binding mode in docking and MD studies.

SIRT1 crystal structures, namely D272, G440, S442, N465, and E467.

When evaluating the binding mode of metformin to the open conformation of the heterodimer 4ZZJ, which has two crystallographic ligands (carbaNAD and 4TQ), we observed that metformin was predicted to share one interacting residue (G263) with those predicted in the 4KXQ crystal structure. It is noteworthy that the carbaNAD structure exhibits a reasonable similarity to APR, which is the crystallographic ligand present in 4KXQ and 4IF6. Even though there were no other matching residues, the other predicted interactions suggested a common binding site for 4KXQ and 4IF6, which can be explained in terms of the large size of the cavity in which the interaction could take place, the small size of metformin as a ligand, and the dynamic nature of the protein. When focusing on the crystallographic ligand 4TQ, which is placed at the N-terminal domain (NTD) of 4ZZJ, we predicted a very low interaction energy following MM/GBSA analyses, which can be explained in terms of the exposure of the NTD region and the lack of predicted interacting residues nearby. Therefore, metformin is not predicted to bind the NTD region in the open state of SIRT1.

The monomeric 4ZZI and 4ZZH crystallographic structures contain the ligands 4TQ and 4TO, respectively, at the NTD region of SIRT1. As above predicted for 4ZZJ, we failed to predict any putative interaction of metformin at the NTD region. However, it should be noted that good binding energies were predicted for the crystallographic ligand 1NS, which is placed in a position that is opposed to the cavity occupied by 4TQ and 4TO and, accordingly, we predicted some residues with which metformin could interact with at the 1NS cavity. To better understand this difference, we performed an alignment using 4I5I as a template, finding that 1NS was placed near the terminal benzene ring of the SIRT1 cofactor NAD and the 4I5 cavity. This a region where metformin is predicted to correctly bind according to the results obtained when employing the 4I5I crystallographic structure (see below).

The monomeric conformation of 4I5I contains NAD and 4I5 as crystallographic ligands. When focusing on the NAD binding site, the model 1 predicted a binding mode equivalent to that predicted by the model 1 in 4KXQ and 4IF6, with a good binding energy. Indeed, the predicted interacting residues were shared with those predicted in the model 1 of 4KXQ and 4IF6, namely D272, G440, N465, and E467. When focusing on the 4I5-binding site, it should be noted that the mechanism of action of 4I5 involves a displacement of NAD from its natural site, as it places near the terminal benzene ring of NAD. Interestingly, the predicted interacting residues of metformin were different to those predicted when employing 4KXQ and 4IF6, but similar to those predicted when evaluating metformin binding to the 1NS cavity at 4ZZI. Moreover, the MM/GBSA-based energy binding of metformin at the 4I5 site was reasonably good (−16.9806 kcal/mol), similar to that for 1NS (−19.0828 kcal/mol; **Table 1**).

The closed conformation of SIRT1 represented by 5BTR with resveratrol as a crystallographic ligand also contains p53 peptides, as in the case of 4ZZJ. Following a detailed analysis of the putative binding modes and predicted residues interactions, we concluded that metformin models 1 for chain A, model 3 for chain B, and model 5 for chain C were placed over the same binding pocket of resveratrol and, importantly, exhibited good binding energies (−20.9987, −26.9390, and −16.5834 kcal/mol, respectively; **Table 1**). It should be noted that in the case of the model 1 for chain A, an extra resveratrol ligand appears and interacts with metformin, as resveratrol was another residue within the cavity. Good interaction energies were also predicted for chains D (model 7, −23.6198 kcal/mol) and E (model 8, −25.0726 kcal/mol), which represent the same resveratrol ligand. A detailed evaluation of the binding mode of metformin predicted a shared interaction in both models involving N226, E230, and K3 (a residue from p53), thereby suggesting that

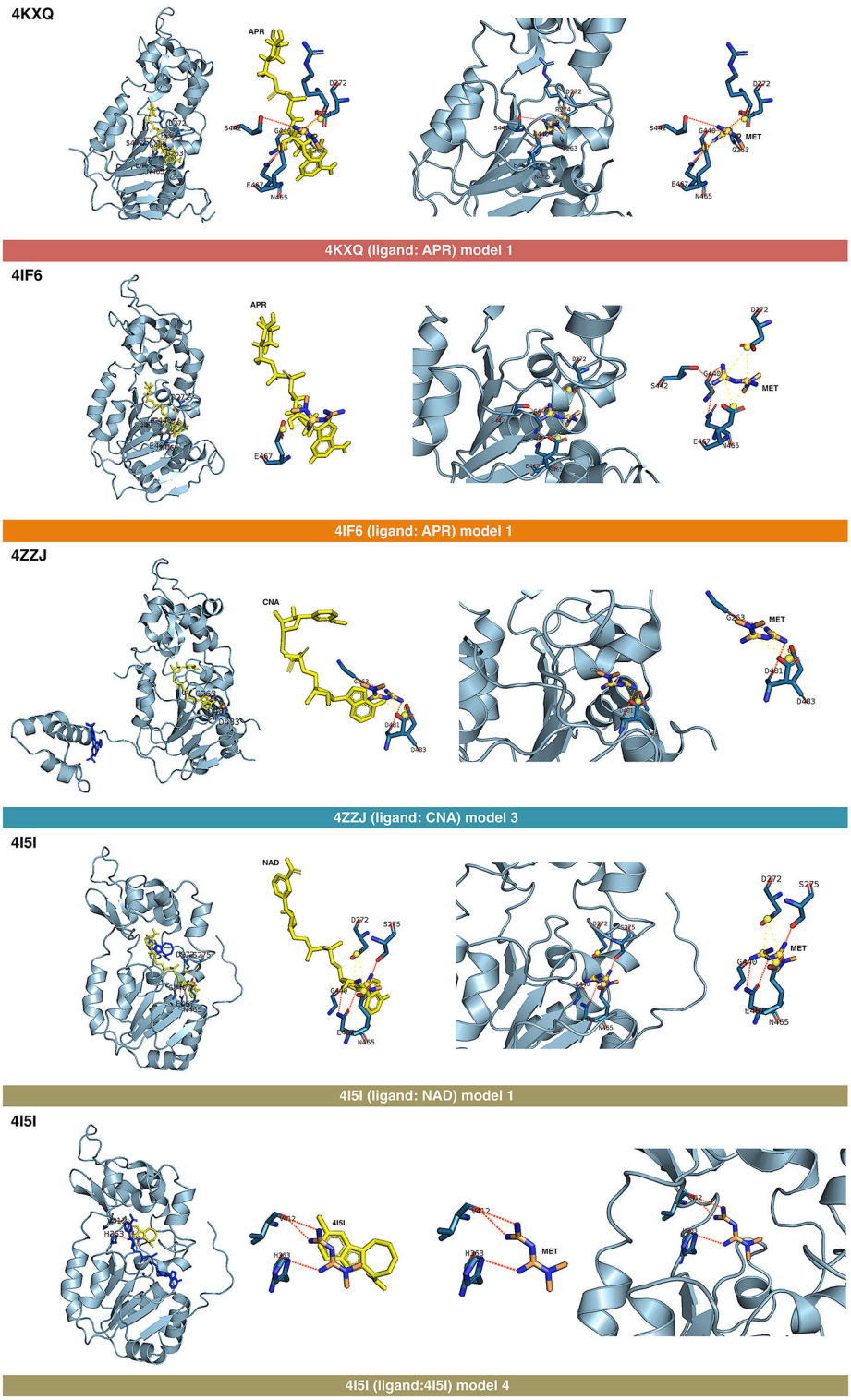

FIGURE 3 | Self-docking poses under molecular dynamics simulations modeling the metformin binding mode to the APR, CNA, NAD^+, and 4I5 binding pockets of SIRT1. Overall structure and views of the interaction between metformin and the APR, CNA, NAD^+, and 4I5 binding pockets of SIRT1. The coordinating residues are numbered.

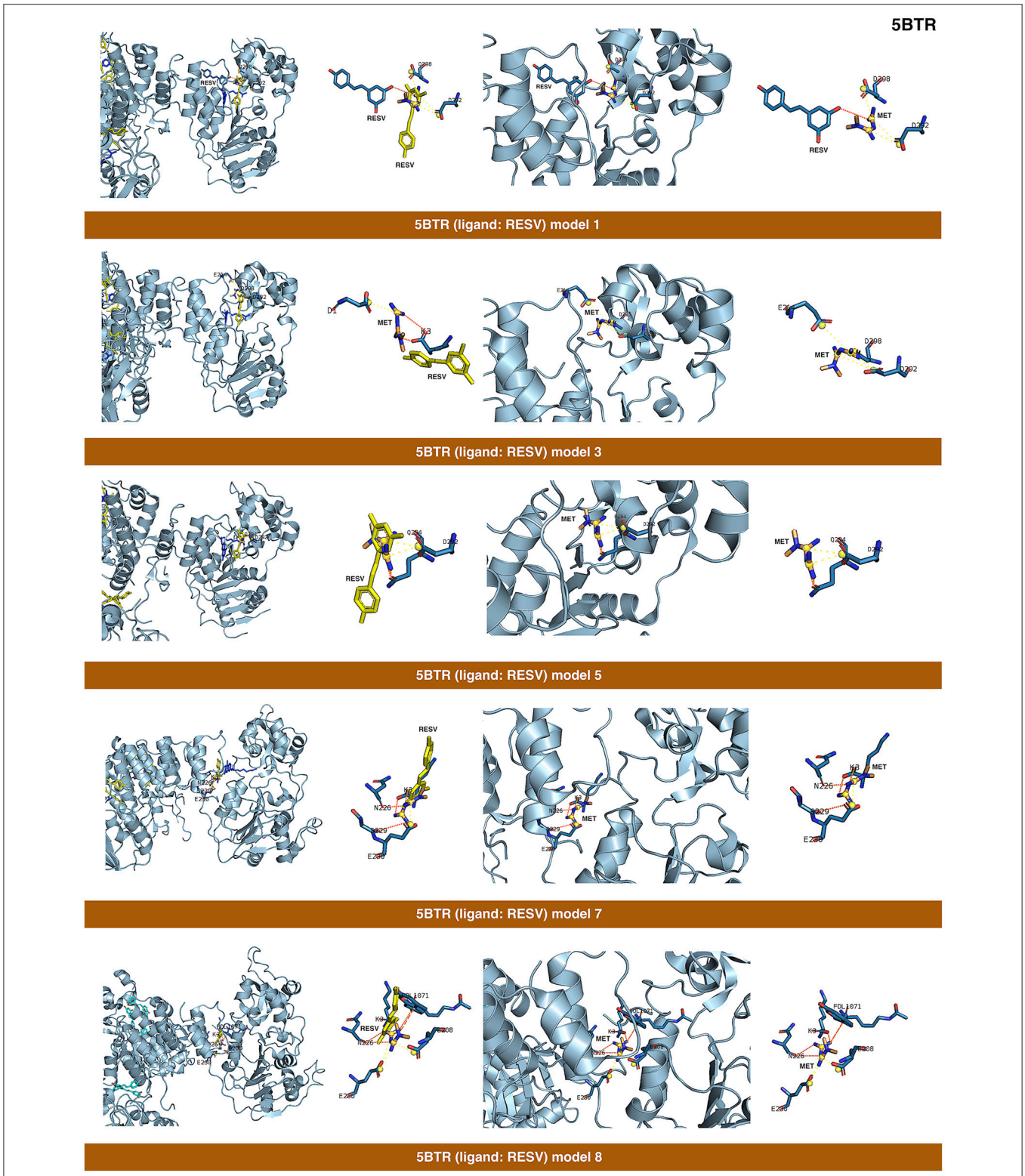

FIGURE 4 | Self-docking poses under molecular dynamics simulations modeling the metformin binding mode to the resveratrol (RESV) binding pocket of SIRT1. Overall structure and views of the interaction between metformin and the RESV binding pockets of SIRT1. The coordinating residues are numbered.

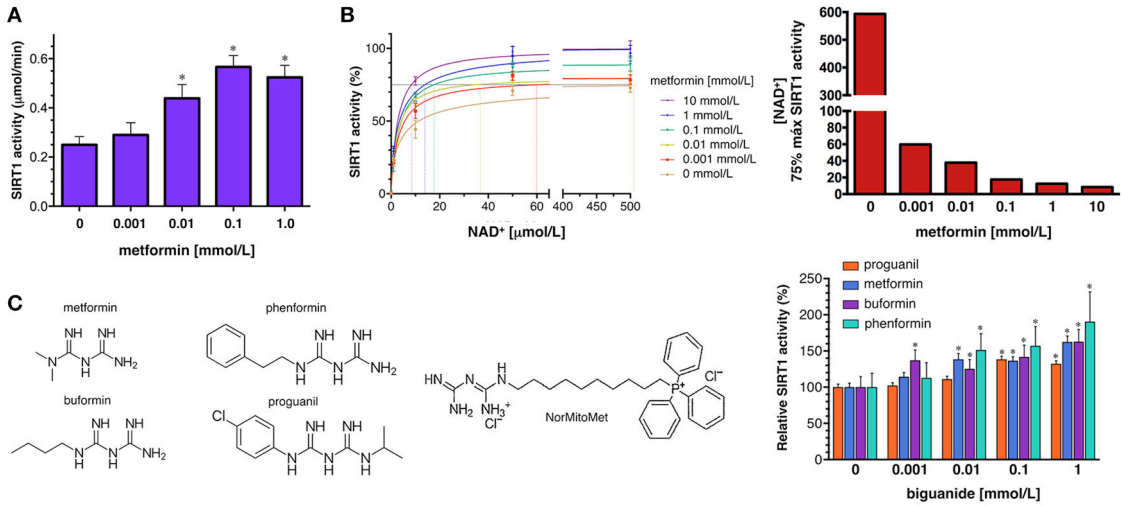

FIGURE 5 | Effects of metformin on the enzymatic activity of SIRT1. **(A)** Dose-response analyses of the effects of graded concentrations of metformin on the activity of SIRT1 using the SIRTainty assay. *Columns* and *error bars* represent mean values and S.D., respectively. Comparisons of means were performed by ANOVA; $P < 0.01$ were considered to be statistically significant (denoted as *). Data points are presented as mean ± SD; three technical replicates per *n*; $n = 2$ biochemical replicates **(B)** *Left.* Human recombinant SIRT1 enzyme was incubated with graded concentrations of NAD^+ and indicated metformin concentrations in a cell-free system using the Epigenase™ Universal SIRT1 Activity/Inhibition Assay Kit (Fluorometric). Data points are presented as mean ± SD; three technical replicates per *n*; $n = 2$ biochemical replicates. Points are connected by best-fit lines using the Michaelis-Menten model (GrahPad Prism software). *Right.* NAD^+ concentrations needed to achieve 75% of the maximal SIRT1 activity in the absence or presence of graded concentrations of metformin. **(C)** *Left.* Structural formulas of the compounds with the biguanide moiety highlighted in red. *Right.* Human recombinant SIRT1 enzyme was incubated with 10 µmol/L NAD^+ in the absence or presence of graded concentrations of biguanides as in **(B)**. Data points are presented as mean ± SD; three technical replicates per *n*; $n = 2$ biochemical replicates.

metformin might bind the closed conformation of SIRT1 at the resveratrol-binding cavity.

The binding mode of metformin to the 4IG9 crystal structure of SIRT1 required a careful and detailed analysis. Following the blind docking calculations, we selected the two models that seemed to better place in the NAD-binding site, which was identified upon structural overlapping. Despite the low interaction energies predicted by MM/GBSA (**Table 1**), a comprehensive analysis of the interacting residues confirmed the accuracy of the selected cavities and models. Metformin was predicted to move from the docking binding area to a better position near the NAD^+-binding site. Interestingly, at the end of each MD simulation, metformin was predicted to interact with those residues that seemed to be relevant for defining the binding mode of metformin to SIRT1. The model 1 predicted that the interacting residues after blind docking were R274, F297, and V412. However, following the MD simulation, the residues predicted to be involved in the metformin-binding mode were D292, Q294, and F414. It should be noted that the interacting residues D292 and Q294 were shared also with the binding mode of metformin on the chain C of 5BTR, with D292 emerging as a key residue involved in the metformin-binding mode to the 5BTR crystal. In the model 2 of 4IG9, the sole interacting residue predicted after blind docking was D348. Following MD simulation, however, the residues predicted to be involved in the metformin-binding mode were A262, P271, D272, and F273, with D272 as a key residue involved in the metformin-binding mode to 4KXQ, 4IF6, and 4I5I. Once again, this suggests metformin's capacity to bind not only the inhibitor pocket but also the cofactor cavity of SIRT1.

The displacement of metformin observed when using the ligandless 4IG9 crystal structure of SIRT1 was found to take place also in the model 3 of the 4KXQ crystal, in which the predicted interacting residues in the metformin-binding mode after blind docking were A262, R274, Q345, H363, G440, and S441. By contrast, after MD simulation, the predicted residues were D272, G440, N465, and E467 (i.e., the same group of residues predicted to be involved in the model 1 of 4KXQ crystal). The fact that three of the models that fail to maintain the pose (i.e., model 3 in the 4KXQ crystal, and models 1 and 2 in the 4IG9 crystal) finally move to a better binding site seems to validate the binding modes of metformin observed in other SIRT1 crystal structures.

Metformin Directly Enhances SIRT1 Enzymatic Activity

To confirm the ability of metformin to directly enhance SIRT1 activity, we first used the SIRTainty™ Class III HDAC Assay, which employs nicotinamidase to measure nicotinamide generated upon cleavage of NAD^+ during SIRT1-mediated substrate deacetylation, and provides a direct assessment of SIRT1 activity. The production of nicotinamide during the 30 min that the acetylated peptide substrate is acted on by SIRT1 was dose-dependently increased by the concomitant presence of graded concentrations of metformin until a saturating plateau

TABLE 2 | Docking binding energies of metformin-related biguanides against SIRT1.

PDB ID	Ligand	Biguanide	Binding Energy (kcal/mol) R0/R1[a]
4KXQ	APR	Proguanil	−6.8/−6.7
		Cycloguanil	–
		Buformin	−5.5/−5.6
		Phenformin	−7.2/−7.2
		NorMitoMet	−5.6/−5.0
4IF6	APR	Proguanil	−6.2/−6.2
		Cycloguanil	−5.3/−5.3
		Buformin	−5.7/−5.7
		Phenformin	−7.1/−6.9
		NorMitoMet	−5.6/−4.6
4ZZI	4TQ	Proguanil	−3.7/−3.7
		Cycloguanil	−4.7/−4.7
		Buformin	−2.5/−2.6
		Phenformin	−4.5/−4.4
		NorMitoMet	−5.2/−3.8
4ZZI	1NS	Proguanil	−6.9/−6.9
		Cycloguanil	−7.7/−7.7
		Buformin	−5.7/−5.5
		Phenformin	−7.4/−7.2
		NorMitoMet	−9.0/−8.4
4I5I	4I51	Proguanil	−7.3/−6.5
		Cycloguanil	−7.8/−7.3
		Buformin	−6.2/−6.2
		Phenformin	−6.9/−6.8
		NorMitoMet	1.2/1.1
4I51	NAD	Proguanil	−7.3/−6.2
		Cycloguanil	−7.8/−7.4
		Buformin	−6.2/−5.7
		Phenformin	−6.4/−6.4
		NorMitoMet	−3.8/−3.6
4ZZJ	4TQ	Proguanil	−3.9/−3.1
		Cycloguanil	−4.2/−4.0
		Buformin	−2.8/−2.2
		Phenformin	−2.7/−1.9
		NorMitoMet	−5.1/−4.9
4ZZJ	CNA	Proguanil	−7.4/−7.4
		Cycloguanil	−7.3/−7.3
		Buformin	−5.5/−5.6
		Phenformin	−6.9/−6.0
		NorMitoMet	−8.5/−7.5
4ZZH	4TO	Proguanil	−4.1/−4.0
		Cycloguanil	−4.0/−4.1
		Buformin	−3.4/−3.4
		Phenformin	−3.7/−3.7
		NorMitoMet	−3.7/−3.5
4IG9	–	Proguanil	−5.0/−4.9
		Cycloguanil	−5.7/−5.7
		Buformin	−4.3/−4.3
		Phenformin	−5.5/−5.5
		NorMitoMet	−1.4/−1.2
5BTR	STL-A	Proguanil	−6.9/−6.9
		Cycloguanil	−6.9/−6.9
		Buformin	−5.4/−5.5
		Phenformin	−7.2/−7.1
		NorMitoMet	−7.3/−7.3
5BTR	STL-B	Proguanil	−7.3/−6.8
		Cycloguanil	−7.3/−7.3
		Buformin	−5.3/−5.3
		Phenformin	−7.3/−7.3
		NorMitoMet	−6.8/−6.9
5BTR	STL-C	Proguanil	−6.9/−6.9
		Cycloguanil	−7.6/−7.6
		Buformin	−4.8/−4.8
		Phenformin	−7.0/−6.5
		NorMitoMet	−7.3/−7.6
5BTR	STL-D	Proguanil	−7.5/−7.5
		Cycloguanil	−7.5/−7.5
		Buformin	−5.5/−4.9
		Phenformin	−6.9/−6.4
		NorMitoMet	−7.1/−6.9
5BTR	STL-E	Proguanil	−8.5/−8.6
		Cycloguanil	−6.3/−6.3
		Buformin	−5.6/−5.6
		Phenformin	−8.0/−8.0
		NorMitoMet	6.5/4.2

The more negative the binding energy, the more plausible the interaction.
[a] *Each docking calculation was performed twice (R0 and R1) to avoid false positives.*

level of SIRT1 activity was reached at 1 mmol/L metformin (**Figure 5A**).

To characterize further how metformin might directly regulate SIRT1 functioning under different NAD^+ concentrations in a cell-free system, we used the Epigenase™ Universal SIRT Activity/Inhibition Assay Kit. The activation curves of recombinant SIRT1 functioning under different NAD^+ concentrations in the absence or presence of metformin are shown in **Figure 5B**. Treatment with graded concentrations of metformin significantly reduced the K_M for NAD^+ while the V_{max} of SIRT1 was slightly increased (up to 30%) in the presence of the highest concentration of metformin tested (10 mmol/L). The metformin-induced leftward-shift of the SIRT1 activation curve, was more evident when evaluating the concentration of NAD^+ (in terms of relative K_M) required to achieve ¾ of the maximal SIRT1 activity in the presence of metformin, which was increased by 70-fold—from 8.5 μmol/L NAD^+ in the presence of 10 mmol/L metformin to >500 μmol/L in the absence of metformin (**Figure 5B**). Perhaps more importantly, the ability of metformin to enhance the capacity of SIRT1 to operate at lower NAD^+ concentrations similarly occurred at physiological/therapeutic concentrations of metformin; thus, metformin concentrations as low as 1 μmol/L were sufficient to reduce by 7-fold the

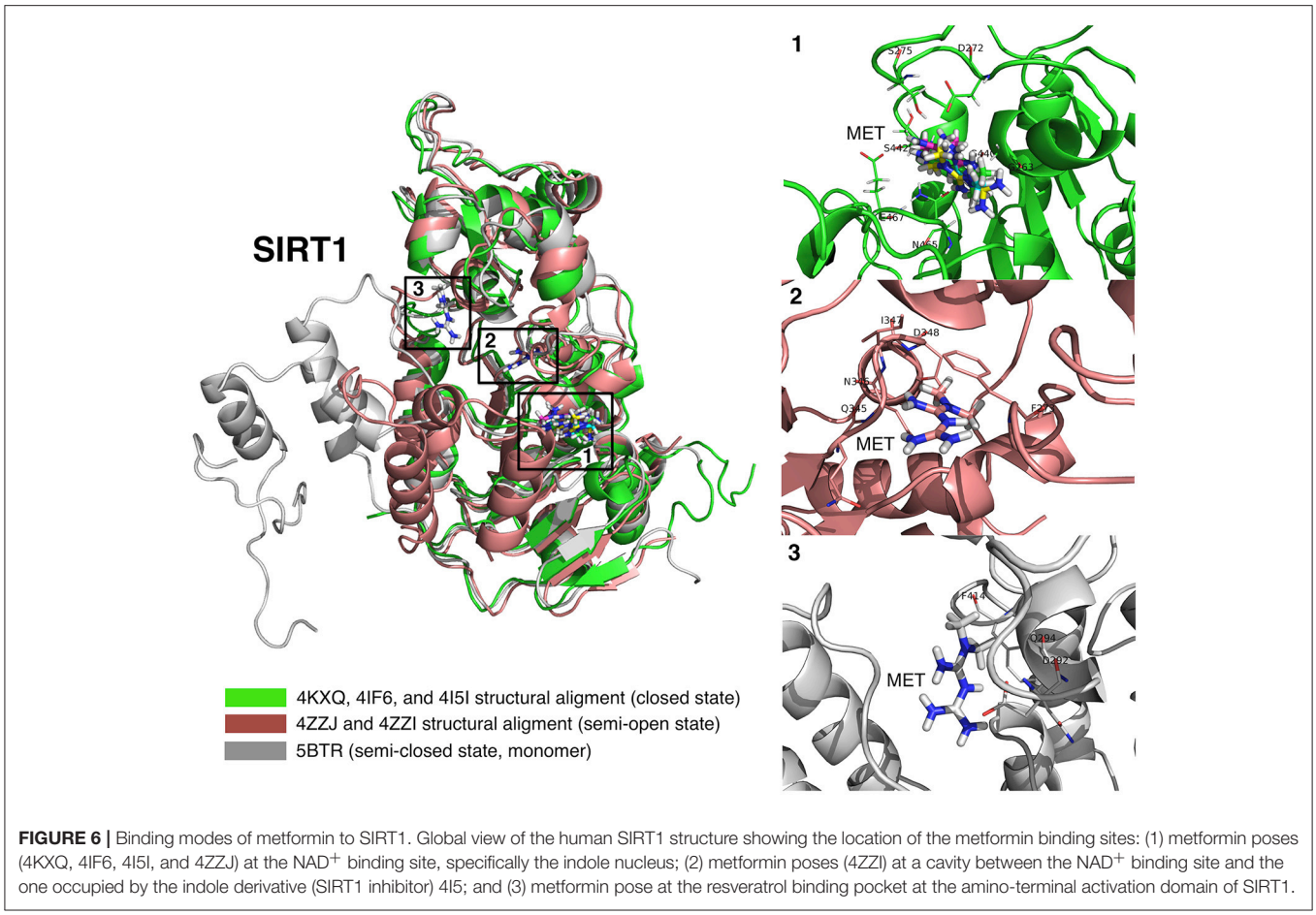

FIGURE 6 | Binding modes of metformin to SIRT1. Global view of the human SIRT1 structure showing the location of the metformin binding sites: (1) metformin poses (4KXQ, 4IF6, 4I5I, and 4ZZJ) at the NAD^+ binding site, specifically the indole nucleus; (2) metformin poses (4ZZI) at a cavity between the NAD^+ binding site and the one occupied by the indole derivative (SIRT1 inhibitor) 4I5; and (3) metformin pose at the resveratrol binding pocket at the amino-terminal activation domain of SIRT1.

amount of NAD^+ required to allow a near-maximal activity of SIRT1.

To evaluate whether pharmacologically relevant biguanides might be viewed as a new family of pharmacologically active SIRT1 activators, we re-evaluated the docking binding energies of several metformin-related biguanides including the anti-malarial biguanides proguanil and cycloguanil, the anti-diabetic biguanides phenformin and buformin, as well as norMitoMet, a novel metformin derivative tagged with the mitochondrial vector triphenylphosphonium (TPP^+) (37) (**Table 2**). The open conformations of SIRT1 bound to SIRT1 inhibitors (i.e., 4ZZI-4TQ, 4ZZJ-4TQ, and 4ZZH-4TO) yielded the worst energy binding predictions for all the biguanides. The predicted binding behavior of buformin and proguanil was relatively similar across all the cavities, with the exception of 5BTR (STL-E), which appeared as the preferred one for proguanil. Our molecular docking approach was incapable of predicting the binding energy of cycloguanil to cofactor cavity 4KXQ-APR; very poor energy binding energies were also predicted for norMitoMet and the 4I5I-4I5, 4IG9, 5BTR (STL-E), and 5BTR (STL-F), most likely because of its large size. Phenformin emerged as a good SIRT1-interacting candidate among all the biguanides, exhibiting relatively high binding energies across all the SIRT1 cavities tested, especially against those representing the closed conformation of SIRT1 binding. We then selected proguanil, buformin, and phenformin to experimentally validate the computational predictions. **Figure 5C** shows that SIRT1 activity was augmented in a dose-dependent manner in the presence of different biguanides, with 1 mmol/L phenformin being capable of enhancing the catalytic activity of SIRT1 by 90% when forced to operate at a NAD^+ concentration as low as 10 μmol/L.

DISCUSSION

We performed a first-in-class computational study aimed to disentangle the putative binding modes of metformin to the SIRT1 enzyme. Our approach reveals that, whereas metformin is predicted to interact with several pockets of SIRT1 inside and outside the central deacetylase catalytic domain (**Figure 6**), the net biochemical effect is to improve the catalytic efficiency of SIRT1 when it operates at low NAD^+ conditions *in vitro* (**Figure 7**). These findings altogether appear to confirm the ability of metformin to operate as a direct SIRT1-activating compound.

When used at low-millimolar concentrations that are incapable of activating the energy-sensing AMPK/mTOR pathway, metformin was previously shown to operate as a

bona fide SIRT1 agonist to block Th17 cell differentiation, similar to well-known SIRT1 activators such as resveratrol and SRT1720 (27). The capacity of metformin to operate as a direct pharmacological SIRT1 activator, which was defined by the selective targeting of SIRT1 and not the AMPK/mTOR pathway *in vitro* and *in vivo* by therapeutic doses in mice and humans (27, 38), has been further bolstered by the finding that the combination metformin and leucine allows SIRT1 to operate at lower NAD^+ concentrations in cell-free systems (28–31). Thus, some of the effects of metformin on SIRT1 activation have been proposed to occur *via* its actions as a direct activator of SIRT1, capable of reducing the K_M for NAD^+. We here confirm that physiological/therapeutic concentrations of metformin mimic the effects of calorie restriction by directly promoting an optimal use of NAD^+ and improving the reaction speed of SIRT1. Importantly, our computational characterization of the putative binding modes of metformin to the regulatory and catalytic pockets of SIRT1 provides new insights into how metformin might directly enhance NAD^+-dependent SIRT1 deacetylation activity.

Resveratrol and other STACs have been found to facilitate SIRT1 activation by establishing key molecular interactions within a specific STAC-binding allosteric site located at the NTD of SIRT1 (33, 39, 40). Mutagenesis screenings and crystallographic structure studies have provided some information of the interface governing the allosteric binding of STACs. This includes glutamic acid 230 (E230), which appears to be critical for allosteric stimulation of SIRT1 activity by chemically diverse STACs including resveratrol (40) via formation or stabilization of the activated conformation of SIRT1 (33). In addition, asparagine 226 (N226) and aspartate 292 (D292) appear to directly interact with resveratrol and are crucial for the resveratrol-stimulated SIRT1 activity on the substrate (33, 35). It is noteworthy that some of the best SIRT1-metformin complex conformations and SIRT1-metformin binding sites, in terms of binding energies, took place outside of the active site of SIRT1 but involved those residues ostensibly controlling the common mechanism of SIRT1 regulation by allosteric activators, such as E230, N226, and D292. Our biochemical assays showed that metformin sensitizes SIRT1 activity by left-shifting the response of SIRT1 to NAD^+, which is characteristic of positive allosteric modulators. Besides sensitization, metformin also produces a small but consistent increase in the maximum response of SIRT1 at saturating doses of NAD^+, which resembles the estimated intracellular content of NAD^+ in mammals [200–500 μmol/L, (41–44)]. It is therefore tempting to suggest that a concerted allosteric change might occur between the activation domain and the catalytic domain in SIRT1 bound to metformin, thereby allowing SIRT1 to operate at low NAD^+ concentrations, which mirrors the NAD^+ deficits occurring during aging [(45–47); **Figure 7**]. The unforeseen capacity of metformin to interact with the STAC-binding allosteric site of SIRT1, which was predicted to solely occur at the substrate-bound closed state, together with the sensitized NAD^+-SIRT1 activity curve shifting leftwards in the presence of metformin, strongly suggests an allosteric behavior of metformin toward SIRT1. Nevertheless, we acknowledge that our study did not directly evaluate how the binding of metformin to the very same binding pocket of resveratrol at the amino-terminal activation domain might increase NAD^+-dependent deacetylation of specific substrates. A model of assisted allosteric activation of SIRT1 activation has been proposed, in which STATCs increased the binding affinity for the substrate and *vice versa* (48, 51). Accordingly, it will be interesting to test whether the activation mechanism by metformin is analogous to that of STATCs, lowering the K_m for the substrate and requiring the region around E230. The use of primary cells reconstituted with activation-defective SIRT1 might

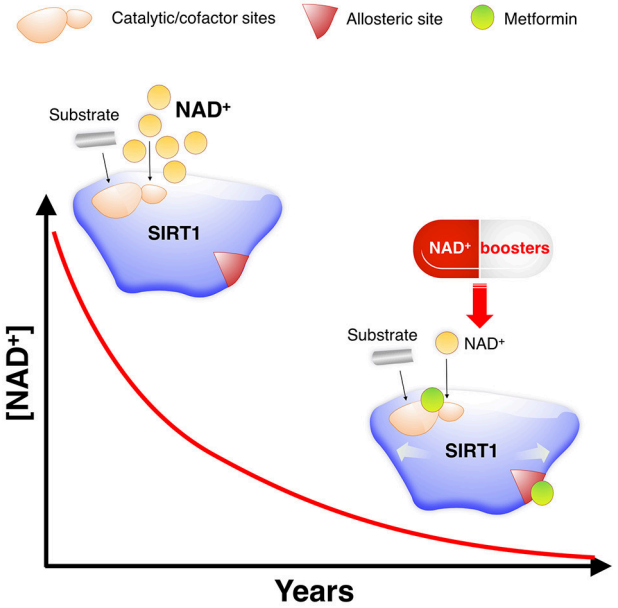

FIGURE 7 | Metformin as a direct SIRT1-activating compound: A new anti-aging role of metformin by modulating NAD^+-sensing enzymes. SIRT1 activity often declines during aging for reasons other than substrate depletion, namely NAD^+ decrements. NAD^+ levels have been described to decrease during aging, mostly due to changes in metabolic pathways leading to NAD^+ synthesis. Such NAD^+ deficit is beginning to be viewed as a central mechanism connecting aging and aging-related diseases, including cancer. However, nearly all known STACs target SIRT1 and operate with a limited number of substrates by binding outside of the activate/catalytic site to an allosteric domain of SIRT1 that is not shared with the other sirtuin family members (SIRT2–7) (48). This substrate-dependent, allosteric activation of SIRT1 exerted by the vast majority of STACs cannot compensate for the reduction in NAD^+ levels. Accordingly, a variety of physiological and pharmacological strategies aimed to boost NAD^+ levels or inhibit NAD^+ consumption is being rapidly pursued for nutraceutical and pharmaceutical development to control SIRT1 activity and thereby achieve healthy benefits (44–46, 49). Given the valuable physiological effects of improving the catalytic efficiency of SIRT1 under NAD^+ depletion in a substrate-independent manner, a preferred general strategy for activation of sirtuins including SIRT1 would be to lower the K_m for NAD^+. K_m, which would have a similar activating effect to that of NAD^+ supplementation, could provide specific activation of sirtuin isoforms, and might be achievable without the need to alter the binding affinity of NAD^+ (50). Our molecular study of the SIRT1-metformin complex coupled to laboratory-based experimental validation strongly suggests that metformin would functionally mimic NAD^+ boosters by operating as a direct SIRT1-activating compound that ensures health quality during aging via sensitization of SIRT1 to NAD^+.

clarify whether metformin directly activates SIRT1 through an allosteric mechanism capable of decreasing the dissociation constant for specific substrates, which is a common mode of action of other chemically diverse STACs.

Our *in vitro* discovery that metformin allows SIRT1 to operate efficiently at low concentrations of NAD^+ might alternatively suggest that metformin operates as a mechanism-based enzyme activating compound (MB-STAC) by targeting (and accelerating) the unique NAD^+-dependent deacetylation turnover mechanism of SIRT1. Although some information is available regarding mechanism-based sirtuin inhibitors (MB-SI) such as Ex-527 and Sir-Real2 (52–54), very little is known about the mechanistic functioning of putative MB-STACs. It has been postulated that a prerequisite for a given modulator to operate as a MB-STAC is the requirement for co-binding with the SIRT1 substrates NAD^+ and acetylated peptide. Accordingly, crystal structures of SIRT1:MB-SI complexes have shown that MB-SI occupy the nicotinamide site and a neighboring pocket to contact the ribose of NAD^+ or of the coproduct $2'$-O-acetyl-ADP ribose. Interestingly, whereas metformin was predicted to bind the SIRT1 cofactor/inhibitor catalytic regions regardless of the conformational status of SIRT1, it remains to be clarified whether the predicted interacting residues might alter the binding and orientation of the NAD^+ cofactor, catalytically required to extract a proton from the activated NAD^+, or are involved in the capture of the released nicotinamide from NAD^+ (32). Indeed, it should be acknowledged that metformin was predicted to establish interactions with F414, a residue that has been suggested to interact with NAD^+ (34) and mediate the interaction of the SIRT1 active site with the substrate peptide (32, 33); with N465, a residue that seems to participate in the establishment of an inhibitor-extended conformation of NAD^+ that sterically prevents productive binding of substrate (34); and also with F273, a key residue involved in the steric blockade of the binding of NAD^+ in the active conformation of SIRT1 (34). Perhaps more importantly, metformin was predicted to interact with the C-terminal regulatory segment of SIRT1 bound to the NAD^+ hydrolysis product APR, a "C-pocket"-related mechanism that appears to be essential for MB activation (55, 56). All these elements could be taken to suggest that at low, therapeutic concentrations, metformin might partially mimic the behavior of MB-SI (e.g., by satisfying the requirement of co-binding with substrates) but possessing additional critical attributes necessary to operate as an MB-STAC, including the ability to modulate the local degrees of freedom of the NAD^+ cofactor and various intermediates and products in the active site. Correspondingly, it could positively alter the balance of productive vs. non-productive SIRT1:NAD^+ complexes. Conversely, supraphysiological concentrations of metformin might be predicted to force the NAD^+ cofactor to adopt an inactive binding mode and/or sterically block substrate binding, thereby behaving as a MB-SI. In this vein, metformin concentrations >50 mmol/L were found to significantly reduce SIRT1 enzymatic activity (data not shown). Moreover, our discovery that other metformin-related compounds containing the biguanide functional group (i.e., two guanidiniums joined by common nitrogen) could enhance also SIRT1 activity highlight the importance of considering the biguanides as a new molecular family of weak to moderate direct activators of SIRT1. An enhanced understanding of the molecular pharmacology and mechanisms of biguanide-SIRT1 interactions might enable the design and investigation of novel, more potent metformin-related compounds as direct SIRT1 activators. Nonetheless, our findings provide mechanistic support for recent clinical initiatives conducted to evaluate advantage of the direct activation of SIRT1 by metformin (28–31, 57).

Future studies should confirm the mechanistic relevance of our *in silico* insights into how the putative binding modes of metformin to SIRT1 could explain its ability to operate as a direct SIRT1-activating compound (**Figure 7**). These findings might have important implications in understanding how metformin could confer health benefits via maintenance of SIRT1 when NAD^+ levels decline during the aging process.

AUTHOR CONTRIBUTIONS

EC and SV examined the chemoinformatics data, performed the enzymatic assays, and critically read the manuscript. LL-P, MS-M, and AN-C performed virtual profiling, docking and molecular dynamics calculations, and Molecular Mechanics-Generalized Born/Surface Area scoring. SF-A, JJ, BM-C, JB-B, and JB provided intellectual insight and essential materials necessary for the study. JM conceived the idea, directed the project, and wrote the manuscript.

FUNDING

This work was supported by grants from the Ministerio de Ciencia e Innovación (Grant SAF2016-80639-P to JM), Plan Nacional de I+D+I, Spain, and the Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) (Grant 2014 SGR229 to JM). This study was supported also by an unrestricted research grant from the Fundació Oncolliga Girona (Lliga catalana d'ajuda al malalt de càncer, Girona) to JM and JB. JB-B is supported by SEOM, Pfizer (Grant WI190764), Boehringer Ingelheim, Meda Pharma, and Pla strategic de recerca i innovació en salut 2016–2020 de la Generalitat de Catalunya (SLT006/17/114). EC is supported by a Sara Borrell post-doctoral contract (CD15/00033) from the Ministerio de Sanidad y Consumo, Fondo de Investigación Sanitaria (FIS), Spain.

ACKNOWLEDGMENTS

We would like to thank Dr. Kenneth McCreath for editorial support.

REFERENCES

1. Espeland MA, Crimmins EM, Grossardt BR, Crandall JP, Gelfond JA, Harris TB, et al. Clinical trials targeting aging and age-related multimorbidity. *J Gerontol A Biol Sci Med Sci.* (2017) 72:355–61. doi: 10.1093/gerona/glw220
2. Bailey CJ. Metformin: historical overview. *Diabetologia* (2017) 60:1566–76. doi: 10.1007/s00125-017-4318-z
3. Campbell JM, Bellman SM, Stephenson MD, Lisy K. Metformin reduces all-cause mortality and diseases of ageing independent of its effect on diabetes control: a systematic review and meta-analysis. *Ageing Res Rev.* (2017) 40:31–44. doi: 10.1016/j.arr.2017.08.003
4. López-Otín C, Galluzzi L, Freije JMP, Madeo F, Kroemer G. Metabolic control of longevity. *Cell* (2016) 166:802–21. doi: 10.1016/j.cell.2016.07.031
5. Novelle MG, Ali A, Diéguez C, Bernier M, de Cabo R. Metformin: a hopeful promise in aging research. *Cold Spring Harb Perspect Med.* (2016) 6:a025932. doi: 10.1101/cshperspect.a025932
6. Barzilai N, Crandall JP, Kritchevsky SB, Espeland MA. Metformin as a tool to target aging. *Cell Metab.* (2016) 23:1060–5. doi: 10.1016/j.cmet.2016.05.011
7. Shah PP, Donahue G, Otte GL, Capell BC, Nelson DM, Cao K, et al. Lamin B1 depletion in senescent cells triggers large-scale changes in gene expression and the chromatin landscape. *Genes Dev.* (2013) 27:1787–99. doi: 10.1101/gad.223834.113
8. Ito T, Teo YV, Evans SA, Neretti N, Sedivy JM. Regulation of cellular senescence by polycomb chromatin modifiers through distinct DNA damage- and histone methylation-dependent pathways. *Cell Rep.* (2018) 22:3480–92. doi: 10.1016/j.celrep.2018.03.002
9. Jin C, Li J, Green CD, Yu X, Tang X, Han D, et al. Histone demethylase UTX-1 regulates C. elegans life span by targeting the insulin/IGF-1 signaling pathway. *Cell Metab.* (2011) 14:161–72. doi: 10.1016/j.cmet.2011.07.001
10. Maures TJ, Greer EL, Hauswirth AG, Brunet A. The H3K27 demethylase UTX-1 regulates C. elegans lifespan in a germline-independent, insulin-dependent manner. *Aging Cell* (2011) 10:980–90. doi: 10.1111/j.1474-9726.2011.00738.x
11. Cuyàs E, Verdura S, Llorach-Pares L, Fernández-Arroyo S, Luciano-Mateo F, Cabré N, et al. Metformin directly targets the H3K27me3 demethylase KDM6A/UTX. *Aging Cell* (2018) 17:e12772. doi: 10.1111/acel.12772
12. Xiao H, Zhang J, Xu Z, Feng Y, Zhang M, Liu J, et al. Metformin is a novel suppressor for transforming growth factor (TGF)-β1. *Sci Rep.* (2016) 6:28597. doi: 10.1038/srep28597
13. Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol.* (2010) 5:253–95. doi: 10.1146/annurev.pathol.4.110807.092250
14. Herranz D, Muñoz-Martin M, Cañamero M, Mulero F, Martinez-Pastor B, Fernandez-Capetillo O, et al. Sirt1 improves healthy ageing and protects from metabolic syndrome-associated cancer. *Nat Commun.* (2010) 1:3. doi: 10.1038/ncomms1001
15. Imai S, Guarente L. Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases. *Trends Pharmacol Sci.* (2010) 31:212–20. doi: 10.1016/j.tips.2010.02.003
16. Hubbard BP, Sinclair DA. Small molecule SIRT1 activators for the treatment of aging and age-related diseases. *Trends Pharmacol Sci.* (2014) 35:146–54. doi: 10.1016/j.tips.2013.12.004
17. Bonkowski MS, Sinclair DA. Slowing ageing by design: the rise of NAD^+ and sirtuin-activating compounds. *Nat Rev Mol Cell Biol.* (2016) 17:679–90. doi: 10.1038/nrm.2016.93
18. Gambini J, Gomez-Cabrera MC, Borras C, Valles SL, Lopez-Grueso R, Martinez-Bello VE, et al. Free [NADH]/[NAD^+] regulates sirtuin expression. *Arch Biochem Biophys.* (2011) 512:24–9. doi: 10.1016/j.abb.2011.04.020
19. German NJ, Haigis MC. Sirtuins and the metabolic hurdles in cancer. *Curr Biol.* (2015) 25:R569–83. doi: 10.1016/j.cub.2015.05.012
20. Madsen AS, Andersen C, Daoud M, Anderson KA, Laursen JS, Chakladar S, et al. Investigating the sensitivity of NAD^+-dependent sirtuin deacylation activities to NADH. *J Biol Chem.* (2016) 291:7128–41. doi: 10.1074/jbc.M115.668699
21. Cantó C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, et al. AMPK regulates energy expenditure by modulating NAD^+ metabolism and SIRT1 activity. *Nature* (2009) 458:1056–60. doi: 10.1038/nature07813
22. Caton PW, Nayuni NK, Kieswich J, Khan NQ, Yaqoob MM, Corder R. Metformin suppresses hepatic gluconeogenesis through induction of SIRT1 and GCN5. *J Endocrinol.* (2010) 205:97–106. doi: 10.1677/JOE-09-0345
23. Caton PW, Kieswich J, Yaqoob MM, Holness MJ, Sugden MC. Metformin opposes impaired AMPK and SIRT1 function and deleterious changes in core clock protein expression in white adipose tissue of genetically-obese db/db mice. *Diab Obes Metab.* (2011) 13:1097–104. doi: 10.1111/j.1463-1326.2011.01466.x
24. Song YM, Lee YH, Kim JW, Ham DS, Kang ES, Cha BS, et al. Metformin alleviates hepatosteatosis by restoring SIRT1-mediated autophagy induction via an AMP-activated protein kinase-independent pathway. *Autophagy* (2015) 11:46–59. doi: 10.4161/15548627.2014.984271
25. Hou X, Xu S, Maitland-Toolan KA, Sato K, Jiang B, Ido Y, et al. SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem.* (2008) 283:20015–26. doi: 10.1074/jbc.M802187200
26. Price NL, Gomes AP, Ling AJ, Duarte FV, Martin-Montalvo A, North BJ, et al. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. *Cell Metab.* (2012) 15:675–90. doi: 10.1016/j.cmet.2012.04.003
27. Limagne E, Thibaudin M, Euvrard R, Berger H, Chalons P, Végan F, et al. Sirtuin-1 activation controls tumor growth by impeding Th17 differentiation via STAT3 deacetylation. *Cell Rep.* (2017) 19:746–59. doi: 10.1016/j.celrep.2017.04.004
28. Bruckbauer A, Zemel MB. Synergistic effects of metformin, resveratrol, and hydroxymethylbutyrate on insulin sensitivity. *Diab Metab Syndr Obes.* (2013) 6:93–102. doi: 10.2147/DMSO.S40840
29. Bruckbauer A, Zemel MB. Synergistic effects of polyphenols and methylxanthines with Leucine on AMPK/Sirtuin-mediated metabolism in muscle cells and adipocytes. *PLoS ONE* (2014) 9:e89166. doi: 10.1371/journal.pone.0089166
30. Fu L, Bruckbauer A, Li F, Cao Q, Cui X, Wu R, et al. Leucine amplifies the effects of metformin on insulin sensitivity and glycemic control in diet-induced obese mice. *Metabolism* (2015) 64:845–56. doi: 10.1016/j.metabol.2015.03.007
31. Banerjee J, Bruckbauer A, Zemel MB. Activation of the AMPK/Sirt1 pathway by a leucine-metformin combination increases insulin sensitivity in skeletal muscle, and stimulates glucose and lipid metabolism and increases life span in *Caenorhabditis elegans*. *Metabolism* (2016) 65:1679–91. doi: 10.1016/j.metabol.2016.06.011
32. Davenport AM, Huber FM, Hoelz A. Structural and functional analysis of human SIRT1. *J Mol Biol.* (2014) 426:526–41. doi: 10.1016/j.jmb.2013.10.009
33. Dai H, Case AW, Riera TV, Considine T, Lee JE, Hamuro Y, et al. Crystallographic structure of a small molecule SIRT1 activator-enzyme complex. *Nat Commun.* (2015) 6:7645. doi: 10.1038/ncomms8645
34. Zhao X, Allison D, Condon B, Zhang F, Gheyi T, Zhang A, et al. The 2.5 Å crystal structure of the SIRT1 catalytic domain bound to nicotinamide adenine dinucleotide (NAD^+) and an indole (EX527 analogue) reveals a novel mechanism of histone deacetylase inhibition. *J Med Chem.* (2013) 56:963–9. doi: 10.1021/jm301431y
35. Cao D, Wang M, Qiu X, Liu D, Jiang H, Yang N, et al. Structural basis for allosteric, substrate-dependent stimulation of SIRT1 activity by resveratrol. *Genes Dev.* (2015) 29:1316–25. doi: 10.1101/gad.265462.115
36. Genheden S, Ryde U. The MM/PBSA and MM/GBSA methods to estimate ligand-binding affinities. *Expert Opin Drug Discov.* (2015) 10:449–61. doi: 10.1517/17460441.2015.1032936
37. Boukalova S, Stursa J, Werner L, Ezrova Z, Cerny J, Bezawork-Geleta A, et al. Mitochondrial targeting of metformin enhances its activity against pancreatic cancer. *Mol Cancer Ther.* (2016) 15:2875–86. doi: 10.1158/1535-7163.MCT-15-1021
38. Zhao D, Long XD, Lu TF, Wang T, Zhang WW, Liu YX, et al. Metformin decreases IL-22 secretion to suppress tumor growth in an orthotopic mouse model of hepatocellular carcinoma. *Int J Cancer* (2015) 136:2556–65. doi: 10.1002/ijc.29305
39. Gut P, Verdin E. Rejuvenating SIRT1 activators. *Cell Metab.* (2013) 17:635–7. doi: 10.1016/j.cmet.2013.04.016

40. Hubbard BP, Gomes AP, Dai H, Li J, Case AW, Considine T, et al. Evidence for a common mechanism of SIRT1 regulation by allosteric activators. *Science* (2013) 339:1216–9. doi: 10.1126/science.1231097
41. Schmidt MT, Smith BC, Jackson MD, Denu JM. Coenzyme specificity of Sir2 protein deacetylases: implications for physiological regulation. *J Biol Chem.* (2004) 279:40122–9. doi: 10.1074/jbc.M407484200
42. Houtkooper RH, Cantó C, Wanders RJ, Auwerx J. The secret life of NAD^+: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev.* (2010) 31:194–223. doi: 10.1210/er.2009-0026
43. Bai P, Cantó C, Oudart H, Brunyánszki A, Cen Y, Thomas C, et al. PARP-1 inhibition increases mitochondrial metabolism through SIRT1 activation. *Cell Metab.* (2011) 13:461–8. doi: 10.1016/j.cmet.2011.03.004
44. Hong J, Kim BW, Choo HJ, Park JJ, Yi JS, Yu DM, et al. Mitochondrial complex I deficiency enhances skeletal myogenesis but impairs insulin signaling through SIRT1 inactivation. *J Biol Chem.* (2014) 289:20012–25. doi: 10.1074/jbc.M114.560078
45. Fang EF, Lautrup S, Hou Y, Demarest TG, Croteau DL, Mattson MP, et al. NAD^+ in aging: molecular mechanisms and translational implications. *Trends Mol Med.* (2017) 23:899–916. doi: 10.1016/j.molmed.2017.08.001
46. Garrido A, Djouder N. NAD^+ Deficits in age-related diseases and cancer. *Trends Cancer* (2017) 3:593–610. doi: 10.1016/j.trecan.2017.06.001
47. Katsyuba E, Auwerx J. Modulating NAD^+ metabolism, from bench to bedside. *EMBO J.* (2017) 36:2670–83. doi: 10.15252/embj.201797135
48. Sauve AA. Pharmaceutical strategies for activating sirtuins. *Curr Pharm Des.* (2009) 15:45–56. doi: 10.2174/138161209787185797
49. Rajman L, Chwalek K, Sinclair DA. Therapeutic potential of NAD-boosting molecules: the *in vivo* evidence. *Cell Metab.* (2018) 27:529–47. doi: 10.1016/j.cmet.2018.02.011
50. Guan X, Upadhyay A, Munshi S, Chakrabarti R. Biophysical characterization of hit compounds for mechanism-based enzyme activation. *PLoS ONE* (2018) 13:e0194175. doi: 10.1371/journal.pone.0194175
51. Sinclair DA, Guarente L. Small-molecule allosteric activators of sirtuins. *Annu Rev Pharmacol Toxicol.* (2014) 54:363–80. doi: 10.1146/annurev-pharmtox-010611-134657
52. Smith BC, Denu JM. Mechanism-based inhibition of Sir2 deacetylases by thioacetyl-lysine peptide. *Biochemistry* (2007) 46:14478–86. doi: 10.1021/bi7013294
53. Gertz M, Fischer F, Nguyen GT, Lakshminarasimhan M, Schutkowski M, Weyand M, et al. Ex-527 inhibits Sirtuins by exploiting their unique NAD^+-dependent deacetylation mechanism. *Proc Natl Acad Sci USA.* (2013) 110:E2772–81. doi: 10.1073/pnas.1303628110
54. Rumpf T, Schiedel M, Karaman B, Roessler C, North BJ, Lehotzky A, et al. Selective Sirt2 inhibition by ligand-induced rearrangement of the active site. *Nat Commun.* (2015) 6:6263. doi: 10.1038/ncomms7263
55. Avalos JL, Boeke JD, Wolberger C. Structural basis for the mechanism and regulation of Sir2 enzymes. *Mol Cell* (2004) 13:639–48. doi: 10.1016/S1097-2765(04)00082-6
56. Hawse WF, Wolberger C. Structure-based mechanism of ADP-ribosylation by sirtuins. *J Biol Chem.* (2009) 284:33654–61. doi: 10.1074/jbc.M109.024521
57. Chalasani N, Vuppalanchi R, Rinella M, Middleton MS, Siddiqui MS, Barritt AS IV, et al. Randomised clinical trial: a leucine-metformin-sildenafil combination (NS-0200) vs placebo in patients with non-alcoholic fatty liver disease. *Aliment Pharmacol Ther.* (2018) 47:1639–51. doi: 10.1111/apt.14674

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 22 November 2018
doi: 10.3389/fendo.2018.00675

Metformin in Reproductive Biology

Melanie Faure[1†], Michael J. Bertoldo[2†], Rita Khoueiry[3], Alice Bongrani[1], François Brion[4], Cecilia Giulivi[5,6], Joelle Dupont[1] and Pascal Froment[1]*

[1] Unité de Physiologie de la Reproduction et des Comportements, Centre Val de Loire, Institut National de la Recherche Agronomique, UMR85, Nouzilly, France, [2] Discipline of Obstetrics and Gynaecology, School of Women's and Children's Health, University of New South Wales, Sydney, NSW, Australia, [3] Department of Development and Regeneration, Stem Cell Institute, KU Leuven, Leuven, Belgium, [4] INERIS, Direction des Risques Chroniques, Pole VIVA, Unite d'ecotoxicologie in vitro et in vivo, BP2, Verneuil-en-Halatte, France, [5] Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, Davis, CA, United States, [6] Medical Investigations of Neurodevelopmental Disorders Institute, University of California, Davis, Davis, CA, United States

Initially produced in Europe in 1958, metformin is still one of the most widely prescribed drugs to treat type II diabetes and other comorbidities associated with insulin resistance. Metformin has been shown to improve fertility outcomes in females with insulin resistance associated with polycystic ovary syndrome (PCOS) and in obese males with reduced fertility. Metformin treatment reinstates menstrual cyclicity, decreases the incidence of cesareans, and limits the number of premature births. Notably, metformin reduces steroid levels in conditions associated with hyperandrogenism (e.g., PCOS and precocious puberty) in females and improves fertility of adult men with metabolic syndrome through increased testosterone production. While the therapeutical use of metformin is considered to be safe, in the last 10 years some epidemiological studies have described phenotypic differences after prenatal exposure to metformin. The goals of this review are to briefly summarize the current knowledge on metformin focusing on its effects on the female and male reproductive organs, safety concerns, including the potential for modulating fetal imprinting via epigenetics.

Keywords: testis, ovary, metformin, oocytes, spermatogenesis

OPEN ACCESS

Edited by:
Benoit Viollet, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Linus R. Shao, University of Gothenburg, Sweden
Daniel Rappolee, Wayne State University School of Medicine, United States

***Correspondence:**
Pascal Froment
pascal.froment@inra.fr

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *04 May 2018*
Accepted: *29 October 2018*
Published: *22 November 2018*

Citation:
Faure M, Bertoldo MJ, Khoueiry R, Bongrani A, Brion F, Giulivi C, Dupont J and Froment P (2018) Metformin in Reproductive Biology. Front. Endocrinol. 9:675. doi: 10.3389/fendo.2018.00675

INTRODUCTION

Brief History of Metformin

The insulin-response sensitizer metformin (*N*,*N*-dimethylbiguanide) has been an important drug for the treatment of diabetes since the 1950's, being one of the most widely prescribed anti-hyperglycemic compounds. Metformin belongs to the biguanide family of anti-diabetic compounds that are related to galegine, a guanidine derivative from the French lilac (*Galga officinalis*). In the beginning of the twentieth century, a chemical study of active molecules contained in *Galga officinalis* demonstrated anti-hyperglycemic properties in diabetic patients (1). Metformin synthesized in 1958, showed similarities with galegine and lowered blood glucose in initial tests on animals (2, 3). Metformin decreases the glycemia through a reduction in hepatic gluconeogenesis and intestinal glucose absorption, with a general improvement in tissue insulin sensitivity and peripheral glucose uptake (4). It is a stable, low molecular weight hydrophilic compound, which upon administration to patients, it reaches numerous tissues including muscle, liver, pancreas, adipose tissue, hypothalamus, pituitary, and the gonads.

Cellular Targets of Metformin

The exact molecular mechanism of metformin's action remains unclear. In the first publications, metformin's actions at the cellular level have been attributed to inhibition of Complex I of the mitochondrial respiratory chain, albeit at relatively high concentrations (mM) (5–10). This inhibition results in a decline in ATP production by mitochondria and an increase in the [adenosine monophosphate to ATP ratio ([AMP]/[ATP]) leading to the activation of the AMP-activated protein kinase (AMPK) complex (11). AMPK is a critical cellular energy sensor that maintains cellular energy homeostasis. Following its activation, AMPK initiates energy-producing catabolic pathways including facilitation of cellular glucose uptake and stimulation of glucose transporter expression, glycolysis, fatty acid beta-oxidation, oxidative phosphorylation and mitochondrial biogenesis. This compensatory mechanism aims at restoring sufficient energy to maintain cellular homeostasis (12–15) **Figure 1**. AMPK activity often counteracts the actions of the mammalian target of rapamycin (mTOR), a central cell-growth factor controlled by extracellular growth triggers and nutrients.

However, the metformin-dependent mitochondrial Complex I inhibition can not account for all of metformin's effects suggesting that metformin may act in an AMPK-independent manner (12–14, 16–20). For instance, it has been described that metformin inhibits the mitochondrial redox shuttle glycerophosphate dehydrogenase. The limited conversion of lactate and glycerol to glucose results in lower hepatic gluconeogenesis (21, 22) (**Figure 2**). Other studies identified H3K27me3 demethylase, KDM6A/UTX as a metformin target based on a structure- and ligand-based bioinformatic analysis (23). Some of the antidiabetic effects of metformin seem to be mediated in part to changes in gut microbiota, thereby promoting the growth of short chain fatty acid-producing bacteria (24, 25). Other studies reported the effect of metformin on the mitochndrial permeability transition pore (26) whereas others on the effects of this drug on cell death (27).

ABSORPTION AND DISTRIBUTION OF METFORMIN

Metformin is used at daily doses of 30–50 mg/kg body weight to treat type II diabetes, reaching serum levels of 10–40 μM (28, 29). It is absorbed through the small intestine, with peak concentrations 1–2 h after oral administration. Its plasma half-life is about 1–6 h. No metabolites of metformin have been identified, and is excreted as such in the urine within 12 h (29).

Species-specific differences show that mice are ∼10 times less sensitive to metformin than humans (20, 29, 30). In mice, after daily administrations at 50 mg/kg body weight, the serum concentrations of metformin are 1.5 and 30 μM for 500 mg/kg (30, 31). In human, 10–40 μM in blood level is reached with 30–50 mg metformin/kg (28, 29). As such, daily administrations of 250–300 mg/kg of metformin to diabetic mice are significantly higher than those used in humans in order to obtain similar therapeutic benefits (30). These species-specific metformin examples are relevant, not only when doses are compared across species but also when interpreting potential effects and targets.

Once in plasma/serum, metformin reaches the intracellular milieu via a limited passive diffusion, while other studies indicated that cationic transporters (Organic Cation Transporter 1: OCT1, OCT2, and MATE1) are able to transport metformin intracellularly (32). It has been claimed that genetic polymorphisms in the genes coding for these transporters may alter the tissue distribution and pharmacological effect of metformin (33).

METFORMIN'S POTENTIAL IMPACT ON INFERTILITY

The bioenergetic, metabolic processes indicated above are critical to sustain a physiological function of the male and female gonads, therefore, in the following sections, we summarize the current knowledge on metformin in regard to its effects on the reproductive processes of males and females in humans and across species. We also discuss the safety of metformin and its potential epigenetic consequences for fetal imprinting.

Clinical and Molecular Impact of Metformin in the Female Reproductive System

Since polycystic ovary syndrome (PCOS) is often associated with obesity, metabolic syndrome, gestational diabetes, and T2DM and cardiovascular risk factors, it is not surprising that PCOS patients with insulin resistance and hyperandrogenism are treated with metformin. PCOS is a major health issue affecting ∼5–20% of reproductive age women, representing the most common ovarian pathology in the world. Based on the criteria of the 2003 Rotterdam Consensus, PCOS is characterized by at least two of the following three criteria: (a) oligo- or anovulation, (b) clinical and/or biochemical signs of hyperandrogenism, (c) presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter and/or increased ovarian volume (>10 ml) and the exclusion of other etiologies (34, 35). The immediate and short-term effects of metformin in women affected with PCOS are in general beneficial. Metformin's treatment improves ovarian cyclicity and reduces gestational diabetes with no impact on the incidence of cesareans or premature births (36–38). While in some countries, metformin is prescribed during pregnancy to women suffering PCOS and gestational diabetes (39–41), the US Food and Drug Administration (FDA) indicated that the safety of metformin during pregnancy is still unclear (28, 42).

However, metformin's efficacy on ovulation and birth rate alone or in combination with clomiphene citrate, compared to clomiphene citrate treatment alone, is still a matter of debate (43–46). For example, an analysis of nine randomized trials including 816 women with PCOS has shown that metformin increased clinical pregnancy rates and decreased the risk of ovarian hyperstimulation syndrome, although there was no clear beneficial evidence for increased rates of live births (47, 48) or increasing the risk for birth

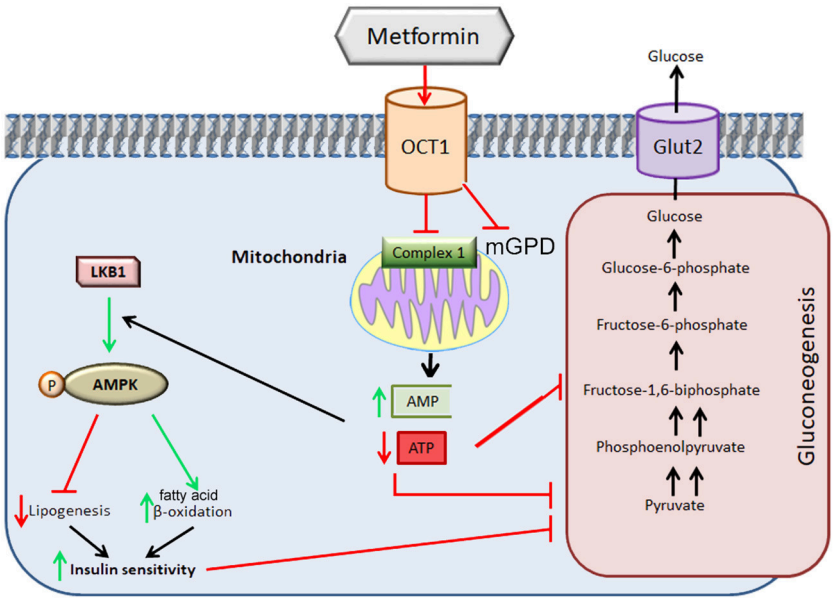

FIGURE 1 | Metformin-induced inhibition of mitochondrial Complex I. The direct inhibition of Complex I by metformin decreases the production of ATP ensuing in increases in AMP. The increase in the [AMP] to [ATP] ratio signals energy resulting in inhibition of high-energy demanding gluconeogenesis process. This ratio leads to the activation of the AMPK complex leading to a decrease in lipogenesis, increase in fatty acid beta-oxidation with an improvement in insulin sensitivity which allows the restoration of gluconeogenesis. The inhibition of metformin on mGDP prevents the use of lactate or glycerol for gluconeogenesis. OCT1: Organic Cation Ttransporter 1; LKB1: Liver Kinase B1; Glut2: GLUcose Transporter 2. mGPD: mitochondrial glycerophosphate dehydrogenase. Adapted from (20).

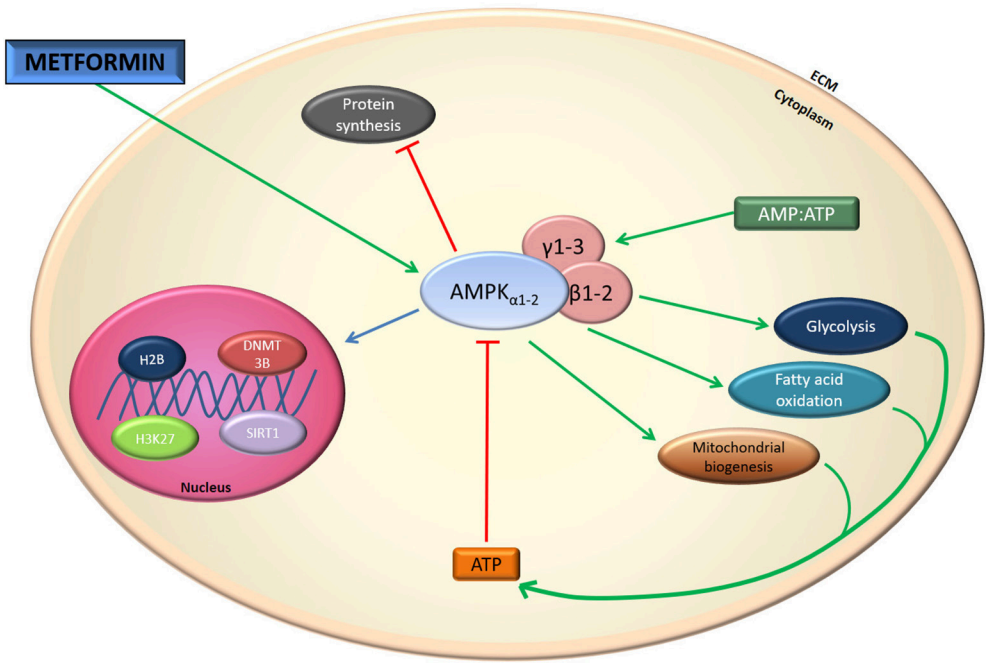

FIGURE 2 | Metformin effect on AMPK. Indirectly metformin activates AMPK. This activation results in mitochondrial biogenesis and glycolysis.

defects (49). The premature birth incidence was higher under metformin therapy (45). A recent meta-analysis described that ovulation rate was significantly higher under a combination of metformin and letrozole treatments than with other treatments (metformin alone, letrozole alone, metformin and clomiphene citrate, FSH, laparoscopic ovarian drilling) in

females affected with PCOS resistant to clomiphene citrate treatment (50).

At the cellular level, metformin has been observed to regulate oocyte maturation. During bovine and porcine oocyte maturation, metformin is able to impede meiotic progression (51, 52). In the bovine oocyte, meiotic arrest was associated with an increase in AMPK activity, a reduction in MAPK ERK1/2 phosphorylation in both oocytes and cumulus cells, and the latency of ribosomal protein 6 and EEF2 (Eukaryotic elongation factor 2), two critical factors regulating protein synthesis in oocytes. Moreover, these effects were only evident in cumulus-oocyte complexes and not in oocytes that had the cumulus compartment removed, indicating that at least in the bovine, cumulus cells are key for metformin to access the oocyte (52).

In a mouse model of PCOS, metformin treatment was explored to alleviate the negative influence of hyperandrogenism on oocyte quality (53). Metformin treatment of PCOS-affected dams was also able to partially reverse ovulatory dysfunction and improve oocyte quality and embryo development outcomes (53). These metformin-mediated improvements were associated with a reduction in oocyte lipid content and reactive oxygen species content, and improved mitochondrial function and glutathione levels (53), consistent with the effect of metformin on the oocyte-specific AMPK knockout mouse model (50). While AMPK is expressed in all ovarian compartments across different species (cow, goat, ewe, sow, hen, rat) including women, deletion of the AMPKα1 subunit specifically in oocytes of mice results in a 27% reduction in litter size (54), highlighting the importance of the AMPK complex to oocyte developmental competence and fertility. Moreover, following *in vitro* fertilization of oocyte-specific AMPK KO mice, a 68% reduction in the number of embryos passing the 2-cell stage was observed (54). This decrease in fertility could be partly explained to defective mitochondrial morphology and ATP synthesis (54). These results suggest that metformin could reverse the negative effects of hyperandrogenism on oocytes in PCOS individuals.

Mouse embryos exposed to metformin from the 2-cell to the blastocyst stage *in vitro* are smaller in size with lower cell numbers (55). The cell-to cell contact with trophectoderm was also altered because of an increase in tight junction permeability (55). *In vivo*, metformin reduced apoptosis in blastocysts of obese mice (56) possibly through an increase in NAMPT expression, but induced early bovine embryo arrest (57). Taken together, these studies demonstrate metformin's important contribution in the cross-talk between somatic cells and oocytes for the normal development of high-quality female germ cells and embryo developmental competence.

Metformin and Male Reproductive Biology

In males, metformin is prescribed for the treatment of T2DM. It is well-known that T2DM alters spermatogenesis in males, decreasing both sperm number and quality (58–60), resulting in reduced fertility. Furthermore, *in utero* exposure to metformin reduces fetal testicular size and the population of Sertoli cells (SC) (61). It is possible that these processes are driven by metformin-mediated increase in lactate production with a decrease in testosterone secretion (61). Metformin impacts the cell cycle by decreasing FSH-induced proliferation and increasing Cyclin-Dependent Kinase Inhibitor (CDKI) and inhibiting cyclin D in primary cultures of mouse Sertoli cells (62) (see **Figure 3**).

However, administration of metformin (for 4 or 8 weeks at doses of 100 or 500 mg/kg) to adult, non-obese rats did not impact sperm number, sperm motility, or the percentage of abnormal spermatozoa (63) (see **Table 1**). In contrast, obesity induced an increase in the number of sperm abnormalities and a decrease in the spermatozoa concentration and motility which was rescued by metformin administration (63). In obese patients, metformin treatment improves sperm concentration and motility in the same way as observed in obese rats (63–66) as judged by the decreased number of morphological defects, with higher concentration and motility of sperm (64). In humans, it appears that a treatment for several months with metformin (850 mg/day during the first week, 1,700 mg/day during the second week, and 2,550 mg/day until the end of 6 months of treatment) can increase the serum testosterone and LH pulsatility of obese individuals (64). This suggests that metformin can modulate and improve pituitary LH pulsatility and regulate Leydig cell steroidogenesis in testis. Recently, it has been shown that metformin decreases sperm motility in pigs (67), correlated with an increase in the viability of the spermatozoa after 24 h storage (see **Table 1**). In a model of testicular ischemia—stress-triggered apoptosis—metformin pre-treatment reduced both oxidative stress and the loss of germ cells, thereby limiting injury on sperm production, suggesting that metformin has cytoprotective effects (68).

Other studies have exposed rodents (rat, rabbit) to metformin in different metabolic models. Either diabetes or obesity induced by fatty acid-rich diet have shown that metformin could limit the decrease in testicular weight, and the thickness of the seminiferous epithelium (66, 69, 70). Depending on the report, the testosterone concentrations and sperm concentrations were improved following the treatment (63, 65, 66, 71). In rabbit, the metformin treatment showed a negative effect on concentration, mobility and number of morphological abnormalities of spermatozoa (71).

In birds, metformin (1 mM) treatment increased viability, mobility and acrosomal response of chicken sperm (72). During chicken seminiferous tubule culture, 48 h of metformin treatment (5 mM) induced a decrease in the rate of proliferative germ cells number (73). This effect was opposite to that observed in equines. Metformin (up to 10 mM) did not induce any effects on viability and mobility of sperm or phosphorylation of AMPK in horses (74).

In regards to the mechanism leading to decreased male fertility, and considering that in mice both the AMPK α1 and α2 subunits are expressed in Leydig, Sertoli and male germ cells (75) with a predominant expression of α1 subunit over α2 (75 vs. 25%) (76), it is possible that metformin impacts spermatogenesis and steroidogenesis in the testes partly through the AMPK pathway. It has been shown that AMPK activation by metformin in rat cultured Sertoli cells induces an increase in lactate production without changes in LDH (lactate dehydrogenase) activity. However, a decrease in the expression

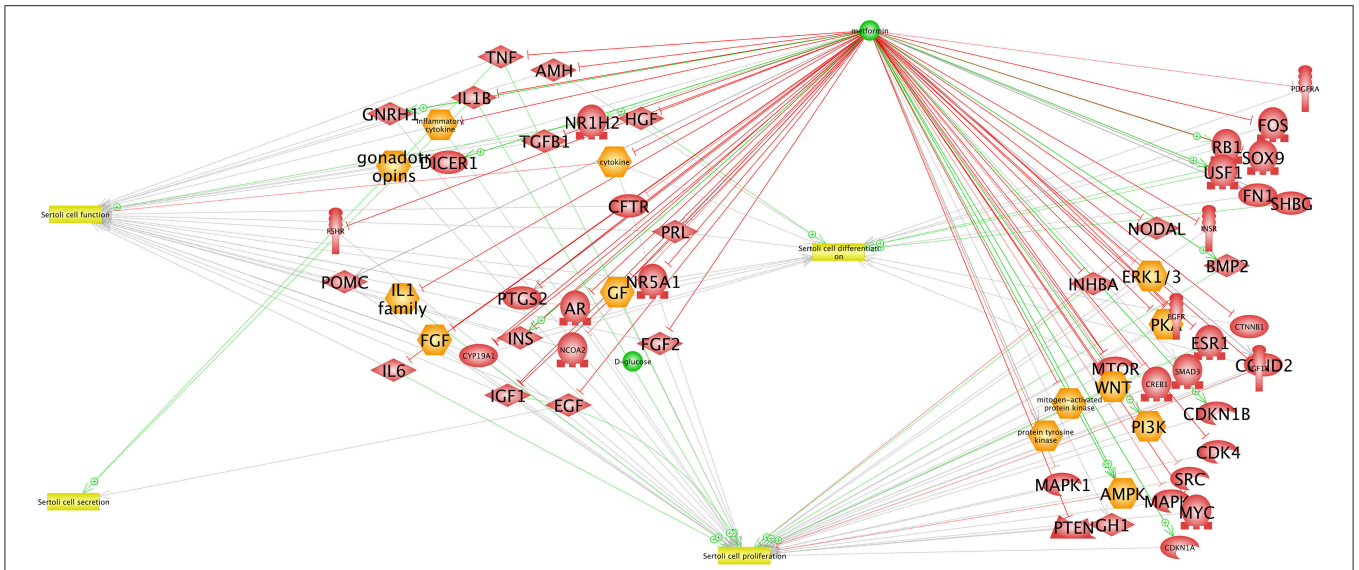

FIGURE 3 | Interaction network between AMPK and proteins expressed in Sertoli cells. The network was created using the Elsevier Pathway Studio program. Green arrows indicate activation while red arrows indicate inhibition.

of MCT4 (monocarboxylate transporter 4), GLUT1, GLUT3 and an increase in the concentrations of alanine and acetate were also reported (77) suggesting activation of glycolysis without concomitant increase in mitochondrial bioenergetics.

Collectively, these studies suggested that obesity (or the metabolic changes associated with this condition) or a high fat diet may set the basis of an increased susceptibility to infertility issues. Metformin (in a dose, biological sample, and species-specific manner) treatment has the potential to activate targets resulting in an overall improvement of fertility.

METFORMIN AND STEROIDOGENESIS

In females, the impact of metformin on androgen synthesis is controversial (78–80). It is argued that metformin may reduce androgen levels indirectly through the resumption of ovulation. Several studies have shown that treatment with metformin induces a reduction in the hyperinsulinemia and hyperandrogenism that is associated with PCOS in obese and non-obese patients (81, 82). It has been suggested that metformin reduces hyperandrogenism through its ability to modulate both ovarian and adrenal androgen output, reducing LH secretion and increasing in some cases, sex hormone binding globulin. The ability of metformin to reduce the androgen levels seems to be variable according to the studies (83, 84).

Metformin has been shown to regulate steroidogenesis through a number of different mechanisms and cell types. Culture of luteinized granulosa cells exposed to the pre-ovulatory LH surge and with metformin lowers progesterone and estradiol syntheses in the same manner as non-luteinized granulosa cells exposed to either FSH or insulin (85). Rice et al. and Fuhrmeister et al. have demonstrated, that metformin induces a decrease in estradiol synthesis via the inhibition of aromatase expression by the MAPK signaling pathway (86, 87). However, metformin also induces activation of insulin-dependent AMPK pathways involved in lactate production by human granulosa cells (88, 89). Incubation of a human theca cell line with metformin induces a decline in androstenedione synthesis (90), possibly via the activation of AMPK.

In males, Tartarin et al. demonstrated that exposure of mice and human fetal testes to metformin decreases testosterone production. *In vivo*, administration of metformin resulted in a decrease in testosterone secretion, however, this effect was reversed when metformin administration was stopped (61). Conversely in humans, it appears that several months of treatment with metformin (850 mg/day during the first week, 1,700 mg/day during the second week, and 2,550 mg/day until the end of treatment period at 6 months) can increase the serum testosterone and LH levels of obese individuals.

In vitro studies have demonstrated that metformin significantly perturbs both androstenedione and testosterone syntheses in theca cells (90). Incubation of primary cultures of rat Leydig cells in the presence of an activator of AMPK, resveratrol, decreases hCG-induced testosterone synthesis by inhibition of P450c17 and StAR (91). Moreover, it have been demonstrated that metformin decreases the capacity of Leydig cells to secrete progesterone (92). In rats and cows, the incubation of granulosa cells with metformin also induces a decline in steroid synthesis that is correlated with an increase in AMPK phosphorylation. It appears that progesterone synthesis falls in the presence of metformin alone, but also during stimulation with FSH, IGF-1, or both (93, 94). This decrease can be explained by a decrease in the expression of some steroidogenic enzymes (3-β HSD in the rat and 3-β HSD, CYP11A1, and StAR in the cow), an effect which is not observed in rats (95).

TABLE 1 | Effect of *in vivo* metformin treatment on male sperm.

Species			**Rat**	**Rat**	**Rat**	**Rat**	**Rat**
Pathology			Diabetic (streptozotocin)	Control	Control	Diabetic (streptozotocin)	Diabetic (streptozotocin)
Metformin treatment			Yes	Yes	Yes	No	Yes
Comparison type			Comparison untreated with metformin	Comparison untreated with metformin	Comparison untreated with metformin	Comparison diabetic with non-diabetic	Comparison untreated with metformin
Administration mode			Gavage 50 mg/kg/day during 4 weeks	Gavage 30 mg/kg/day during 21 days	Gavage 500 mg/kg during 4 or 8 weeks		Gavage 500 mg/kg during 4 or 8 weeks
Sperm	*In vivo*	Morphological defects	↓ (−20%)	ND	NS	↑ (−67%)	↓ (−70%)
		Number/concentration	↑ (+6%)	↓ (−30%)	NS	↓ (−20%)	↑ (+60%)
		Motility	ND	↓ (−30%)	NS	↓ (−20%)	↑ (+40%)
		Viability	ND	NS	ND	ND	ND
References			(65)	(129)	(63)	(63)	(63)
Species			**Rat**	**Rabbit**	**Rabbit**	**Fish**	
Pathology			Obesity (HFD)	Control	Diabetic (alloxan)	Control	
Metformin treatment			Yes	Yes	Yes	Yes	
Comparison type			Comparison HFD with HFD + metformin	Comparison untreated with metformin	Comparison untreated diabetic with metformin diabetic	Comparison untreated with metformin	
Administration mode			Gavage 100 mg/kg during 8 weeks	Gavage 120 mg/kg/day during 3 months	Gavage 120 mg/kg/day during 3 months	Water with 40 μg/L metformin (exposition: 3 days after hatching and for 365 days)	
Sperm	*in vivo*	Morphological defects	↓ (+7%)	↑ (+44%)	↑ (+60%)	Low sperm production due to intersex gonad (partly convert in ovary with oocytes)	
		Number/concentration	↑ (+10%)	↓ (−30%)	↓ (−87%)	↓ clutch size (−80%)	
		Motility	↑ (+20%)	↓ (−12%)	↓ (−40%)	ND	
		Viability	↑ (+20%)	↑ (+85% dead)	NS	ND	
	References		(66)	(71)	(71)	(112)	

NS, not significant; HFD, high fat diet; ND, not determined.

In human primary breast adipose tissue, consequences of metformin exposure revealed a significant decrease in the forskolin/phorbol ester induced aromatase expression (96).

Thus, based on the available literature, metformin acting via a number of mechanisms has the ability to modulate steroid levels both *in vivo* and *in vitro*.

In utero EXPOSURE AND GONADAL DEVELOPMENT

Metformin is the treatment of choice in cases of pregnancy disorders, such as gestational diabetes mellitus or preeclampsia (97). One hypothesis is that metformin regulates preeclampsia via mitochondrial function especially in the placenta and expression of antiangiogenic factors (97). However, the maternal administration of metformin reaches the fetus with umbilical cord concentrations (on average 457 ± 335 μg/L, equivalent to 3 μM) similar to those found in the maternal circulation (730 ± 440 μg/L, equivalent to 5 μM) (98, 99). Salomaki et al. reported serum concentrations of only 0.174 and 0.130 μM in the mother and fetus, respectively, at 24 h post-administration of daily doses of 300 mg/kg throughout pregnancy (100), suggesting that metformin may accumulate in certain tissues at higher concentrations than in plasma (101).

Considering that there are no reports of metformin inducing teratogenicity, the long-term health consequences of *in utero* metformin exposure remains elusive, primarily due to limited study designs (102, 103). *In utero* metformin-exposure resulted in children that were heavier, and with larger head size at 18 m of age (104). Recently, Hanem et al. demonstrated that *in utero* exposure to metformin resulted in children with a higher body mass index (BMI) and increased prevalence of overweight/obesity at 4 years of age compared to children of the placebo group (105), indicating that in humans, metformin administered during pregnancy has the ability to alter anthropometrics in the offspring.

But what is known on the effects of metformin on gonadal development during *in utero* exposure? Very limited studies exist, focusing mainly on the effects of metformin on male offspring. From these, no effect on testicular size was reported for young boys between 2.5 and 7 years of age born to mothers affected with gestational diabetes treated with either insulin or metformin and insulin (106). However, testicular size was not compared to boys born from placebo mothers and to individuals that had not yet reached the age of puberty. Tartarin et al. observed that embryonic exposure of mice to metformin during the first half of pregnancy had a negative impact on the testicular size of young mice and number of Sertoli cells (at 16.5 dpc and 1 dpp) (61). At 16.5 dpc, a decrease in testicular testosterone concentration and Leydig cell count was also observed but was no longer found at birth. This suggests that while metformin is able to modulate mammalian testis development, some plasticity in the ability for the testis to recover exists during perinatal periods. Nonetheless, the long-term effects on fertility are yet to be determined.

In females with early symptoms of precocious puberty associated with hyperinsulinemia, metformin administration has been shown to delay the onset of clinical puberty and the pubertal increase in IGF1 levels (107, 108). Moreover, there was also a metformin-associated delay of menarche (108). The mode of action whereby metformin is able to delay pubertal onset and progression in girls remains to be understood. The observed delay of menarche appeared to be associated with falls in adiposity and insulin, leptin and IGF1 concentrations (107), suggesting that the effects of metformin on the ovary seemed to be indirect.

While exposure to metformin is usually through therapeutical administration, in the last decade, due to the increased use of metformin, accumulation of this drug has been reported in wastewater, drinking water and cosmetics, making it one of the 14 most active pharmacological molecules in the environment (109–111) with concentration reaching between 10 and 100 μg/L (1 μM) (112). Thus, it is possible that ingestion of metformin-containing water and/or use of metformin-containing cosmetics may elicit unwanted effects on humans as well as aquatic species exposed to metformin. Indeed, a 360-days long exposure of male fish (Pimephales promelas) to metformin (40 μg/l) leads to the appearance of an "intersex" gonads (113), with no intersex phenotype observed in mammals (61, 106). Gonadal estrogen and aromatase function play an important role in the gonad determinism in fish and avian species, thus, disruption of steroid production could lead to modification on gonadal development in these species. However, the exposure of cyp19a1b-GFP zebrafish model (GFP-driven promoter of aromatase in the central nervous system) to metformin (0.3–30 μM) or its derivative guanylurea (0.08–7 μM) did not result in changes in GFP expression suggesting a specific regulation of different aromatase transcripts by metformin depending on the promoter and tissue (gonad vs. central nervous system).

METFORMIN AND EPIGENETICS

In utero metformin exposure has been described safe for the fetus. Some evidence suggests that the beneficial effects of metformin are partly AMPK-dependent to counteract stress (114). However, it is important to follow the development of the offspring until adulthood to evaluate the risk to develop metabolic disorders through epigenetic information (114). Thus, if metformin is provided with a high-fat diet, then a significant increase in visceral fat depot of the offspring is observed during adulthood (99). In rodents, prenatal exposure to metformin modifies the hepatic fetal imprinting resulting in changes in the expression of several genes involved in the metabolism of cholesterol, lipids, fatty acids and steroids. Moreover, it decreases the expression of insulin-sensitive glucose transporter, GLUT4, in epididymal adipose tissue suggesting long-term effects, such as glucose intolerance in the testis (99). In a follow-up study of metformin in a gestational diabetes trial showed that prenatal exposure had a change in the pattern of fat distribution in children at 2 years-old (same body fat mass but more subcutaneous fat (102). Taken

together these studies point to a putative epigenetic effect of metformin which could be exerted during perinatal periods.

As possible mechanisms involved in epigenetics, indicated the section of Cellular Targets of Metformin, metformin directly targets the H3K27me3 demethylase KDM6A/UTX resulting in global augmentation of H3K27me3 levels in cultured cells *in vitro* and *in vivo* (23). Moreover, several studies reported DNA hypermethylation following metformin treatment via its effect on one-carbon metabolism (115, 116). This increase in DNA methylation is probably due to an increase in the activity of *S*-adenosylhomocysteine hydrolase (SAHH). This enzyme hydrolyzes *S*-adenosylhomocysteine (SAH), a strong feedback inhibitor of *S*-adenosyl-L-methionine-dependent methyltransferases including DNA methyltransferases (DNMTs). Treating endometrial or ovarian cancer cells with metformin results in a decrease of the histone H19 levels and enables DNMT3B to increase DNA methylation (115). However, no statistically significant effect of metformin was observed in plasma homocysteine (metabolite that plays a critical role in DNA methylation) concentrations in PCOS patients with or without metformin treatment (117). A subgroup analyses suggested that metformin might induce Hcy accumulation when administered without folic acid or B-group vitamins supplementation (117). Further studies are warranted at demonstrating the links amongst metformin, B-group vitamins, and DNA methylation in patients with PCOS or infertility.

Alternatively, metformin could regulate epigenetic reprogramming through the activation of AMPK. A recent review reported the different mechanisms involved in the histone modifications in response to metformin-induced activation of AMPK phosphorylation of HATs (histone acetyltransferases), increased SIRT1 activity, and inhibition of class II HDACs (histone deacetylases) (118–120). AMPK has been shown to phosphorylate histone H2B by regulating HDAC in mouse embryonic fibroblasts (121). Metformin was shown to inhibit ovarian cancer via decreasing H3K27 trimethylation in an AMPK-dependent manner (122). Studies on Sertoli cells lacking AMPKα1 have highlighted the role of the α1 subunit of AMPK (123) metabolic activity and the secretion of many metabolites, such as glycine, malonate, succinate and alanine, which may act on the enzymes modifying epigenetic marks. Oocytes lacking α1AMPK, a hyperacetylation of histone H3 and a decrease in the activity of SIRT1 is detected (123). Interestingly, the effects of metformin on HDACs are dissimilar because it increases the expression and/or activity of the class III HDAC SIRT1 (124–127) and pharmacological doses of metformin in the cryopreservation media of mouse sperm induced SIRT1 activity (128).

Taken together, while these studies show a link between metformin and DNA methylation status, many precise aspects of this link still need to be clarified.

CONCLUSION

After half a century, metformin has established itself as a first defense against insulin-dependent morbidities and undoubtedly has become a useful drug for improving fertility outcomes in both male and female patients. Metformin can modify testis and ovary function directly through AMPK-dependent and independent mechanisms. Its effects include improved sperm function and fertilization rates, oocyte quality and embryo development and reduction in miscarriage rates. The general consensus in the literature is that metformin is considered safe to use during pregnancy in regards to perinatal outcomes. However, adverse effects of metformin in the germ cell populations of offsprings exposed *in utero* and those on subsequent generations are less clear. While our understanding of the effects of metformin is continually progressing, further research is needed to have a more complete understanding of metformin's impact on fertility.

AUTHOR CONTRIBUTIONS

MF, MB, RK, AB, FB, CG, JD, and PF: redaction of different parts of the review.

FUNDING

This work received support from the Region Centre Val de Loire through the project OXYFERTI and by Institut National de la Recherche Agronomique. Alice Bongrani was supported by a French fellowship from the French Region Centre Val de Loire.

REFERENCES

1. Simonet H, Tanret G. Sur les propriétés hypoglycemiantes du sulfate de galegine. In: *Bull Soc Chim Biol.* Paris (1927). p. 908–27.
2. Passik C. *Glucophage: 40 ans au Service de la Diabétologie*. Paris (1997).
3. Rinaldi D. *La Metformine, une Vieille Molécule Pleine d'espoir*. Doctoral Diss Fac Pharm, Univ de Lorraine (2012).
4. 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* (2006) 49:434–41. doi: 10.1007/s00125-006-0141-7
5. El-Mir M-Y, Nogueira V, Fontaine E, Avéret N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem.* (2000) 275:223–8. doi: 10.1074/jbc.275.1.223
6. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J.* (2000) 614:607–14. doi: 10.1042/bj3480607
7. 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.* (2002) 63:1259–72. doi: 10.1016/S0006-2952(02)00858-4
8. Guigas B, Bertrand L, Taleux N, Foretz M, Wiernsperger N, Vertommen D, et al. 5-Aminoimidazole-4-carboxamide-1-B-D-ribofuranoside and metformin inhibit hepatic glucose phosphorylation by an AMP-activated protein kinase–independent effect on glucokinase translocation. *Diabetes* (2006) 55:865–74. doi: 10.2337/diabetes.55.04.06.db05-1178
9. Cameron AR, Logie L, Patel K, Erhardt S, Bacon S, Middleton P, et al. Metformin selectively targets redox control of complex I energy transduction. *Redox Biol.* (2018) 14:187–97. doi: 10.1016/j.redox.2017.08.018

10. Wu C, Qiu S, Zhu X, Lin H, Li L. OCT1-mediated metformin uptake regulates pancreatic stellate cell activity. *Cell Physiol Biochem.* (2018) 47:1711–20. doi: 10.1159/000491003
11. Hardie DG, Ross FA, Hawley SA. AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol.* (2012) 13:251–62. doi: 10.1038/nrm3311
12. Nascimben L, Ingwall JS, Lorell BH, Pinz I, Schultz V, Tornheim K, et al. Mechanisms for increased glycolysis in the hypertrophied rat heart. *Hypertension* (2004) 44:662–7. doi: 10.1161/01.HYP.0000144292.69599.0c
13. Jager S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1a. *Proc Natl Acad Sci USA.* (2007) 104:12017–22. doi: 10.1073/pnas.0705070104
14. Coughlan KA, Valentine RJ, Ruderman NB, Saha AK. AMPK activation: a therapeutic target for type 2 diabetes? *Diabetes Metab Syndr Obes.* (2014) 7:241–53. doi: 10.2147/DMSO.S43731
15. Ke R, Xu Q, Li C, Luo L, Huang D. Mechanisms of AMPK in the maintenance of ATP balance during energy metabolism. *Cell Biol Int.* (2004) 28:229–36. doi: 10.1016/j.cellbi.2003.12.004
16. Gamble J, Lopaschuk GD. Insulin Inhibition of 5′adenosine monophosphate-activated protein kinase in the heart results in activation of acetyl coenzyme A carboxylase and inhibition of fatty acid oxidation. *Metabolism* (1997) 46:1270–4.
17. Hardie DG. Sensing of energy and nutrients by AMP-activated protein kinase 1–4. *Am J Clin Nutr.* (2011) 93:891S—6. doi: 10.3945/ajcn.110.001925.1
18. Kukidome D, Nishikawa T, Sonoda K, Imoto K, Fujisawa K, Yano M, et al. Activation of AMP-activated protein kinase reduces hyperglycemia-induced mitochondrial reactive oxygen species production and promotes mitochondrial biogenesis in human umbilical vein endothelial cells. *Diabetes* (2006) 55:120–7. doi: 10.2337/diabetes.55.01.06.db05-0943
19. Karnewar S, Neeli PK, Panuganti D, Kotagiri S, Mallappa S, Jain N, et al. Metformin regulates mitochondrial biogenesis and senescence through AMPK mediated H3K79 methylation: relevance in age-associated vascular dysfunction. *Biochim Biophys Acta* (2018) 1864:1115–28. doi: 10.1016/j.bbadis.2018.01.018
20. Foretz M, Hébrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, et al. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest.* (2010) 120:2355–69. doi: 10.1172/JCI40671DS1
21. Madiraju AK, Erion DM, Rahimi Y, Zhang X-M, Braddock DT, Albright RA, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature* (2014) 510:542–6. doi: 10.1038/nature13270
22. Madiraju AK, Qiu Y, Perry RJ, Rahimi Y, Zhang X-M, Zhang D, et al. Metformin inhibits gluconeogenesis via a redox-dependent mechanism *in vivo*. *Nat Med.* (2018) 24:1384–94. doi: 10.1038/s41591-018-0125-4
23. Cuyàs E, Verdura S, Llorach-Pares L, Fernández-Arroyo S, Luciano-Mateo F, Cabré N, et al. Metformin directly targets the H3K27me3 demethylase KDM6A/UTX. *Aging Cell* (2018) 17:e12772. doi: 10.1111/acel.12772
24. Wu H, Esteve E, Tremaroli V, Khan MT, Caesar R, Mannerås-Holm L, 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.* (2017) 23:850–8. doi: 10.1038/nm.4345
25. Bauer PV, Duca FA, Waise TMZ, Rasmussen BA, Abraham MA, Dranse HJ, et al. Metformin alters upper small intestinal microbiota that impact a glucose-SGLT1-sensing glucoregulatory pathway. *Cell Metab.* (2017) 27:101–17. doi: 10.1016/j.cmet.2017.09.019
26. Lablanche S, Cottet-Rousselle C, Lamarche F, Benhamou PY, Halimi S, Leverve X, et al. Protection of pancreatic INS-1 β-cells from glucose- and fructose-induced cell death by inhibiting mitochondrial permeability transition with cyclosporine A or metformin. *Cell Death Dis.* (2011) 2:e134–6. doi: 10.1038/cddis.2011.15
27. El-Mir MY, Detaille D, R-Villanueva G, Delgado-Esteban M, Guigas B, Attia S, et al. Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons. *J Mol Neurosci.* (2008) 34:77–87. doi: 10.1007/s12031-007-9002-1
28. Royal Australian College of General Practitioners. Gestational diabetes mellitus. In: *General Practice Management of Type 2 Diabetes: 2016–18*. (2014). Available online at: www.racgp.org.au
29. Graham GG, Punt J, Arora M, Day RO, Doogue MP, Duong JK, et al. Clinical pharmacokinetics of metformin. *Clin Pharmacokinet.* (2011) 50:81–98. doi: 10.2165/11534750-000000000-00000
30. Hou M, Venier N, Sugar L, Musquera M, Pollak M, Kiss A, et al. Protective effect of metformin in CD1 mice placed on a high carbohydrate-high fat diet. *Biochem Biophys Res Commun.* (2010) 397:537–42. doi: 10.1016/j.bbrc.2010.05.152
31. Gras V, Bouffandeau B, Montravers PH, Lalau JD. Effect of metformin on survival rate in experimental sepsis. *Diabetes Metab.* (2006) 32:147–50. doi: 10.1016/S1262-3636(07)70261-6
32. Gong L, Goswami S, Giacomini KM, Altman RB, Klein TE. Metformin pathways: pharmacokinetics and pharmacodynamics. *Chemosphere* (2012) 104:8897–901. doi: 10.1097/FPC.0b013e3283559b22
33. Shu Y, Brown C, Castro RA, Shi RJ, Lin ET, Owen RP, et al. Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics. *Clin Pharmacol Ther.* (2008) 83:273–80. doi: 10.1038/sj.clpt.6100275
34. Fauser BCJM. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod.* (2004) 19:41–7. doi: 10.1093/humrep/deh098
35. Cimino I, Casoni F, Liu X, Messina A, Parkash J, Jamin SP, et al. Novel role for anti-mullerian hormone in the regulation of GnRH neuron excitability and hormone secretion. *Nat Commun.* (2016) 7:1–12. doi: 10.1038/ncomms10055
36. Bertoldo MJ, Faure M, Dupont J, Froment P. Impact of metformin on reproductive tissues: an overview from gametogenesis to gestation. *Ann Transl Med.* (2014) 2:1–13. doi: 10.3978/j.issn.2305-5839.2014.06.04
37. Vanlalhruaii, Dasgupta R, Ramachandran R, Mathews JE, Regi A, Thomas N, et al. How safe is metformin when initiated in early pregnancy? A retrospective 5-year study of pregnant women with gestational diabetes mellitus from India. *Diabetes Res Clin Pract.* (2018) 137:47–55. doi: 10.1016/j.diabres.2018.01.002
38. Brown J, Grzeskowiak L, Williamson K, Downie Michelle R, Crowther Caroline A. Insulin for the treatment of women with gestational diabetes. *Cochrane Database Syst Rev.* (2016). 11:1–294. doi: 10.1002/14651858.CD012037
39. New Zealand Ministry of Health. *Screening, Diagnosis and Management of Gestational Diabetes in New Zealand: A Clinical Practice Guideline*. (2014). Available online at: www.health.govt.nz
40. Network SIG. *Management of Diabetes: A National Clinical Guideline (SIGN Publication o. 116.)* (2008) SIGN. Available online at: www.sign.ac.uk/guidelines/fulltext/
41. National Institute for Health and Care Excellence (NICE). *Diabetes in Pregnancy: Management From Preconception to the Postnatal Period* (2015). Available online at: https://www.nice.org.uk/guidance/ng3
42. Association AD. Management of diabetes in pregnancy. *Diabetes Care* (2017) 40:S114–9. doi: 10.2337/dc17-S016
43. Palomba S, Pasquali R, Orio F Jr., Nestler JE. Clomiphene citrate, metformin or both as first-step approach in treating anovulatory infertility in patients with polycystic ovary syndrome (PCOS): a systematic review of head-to-head randomized controlled studies and meta-analysis. *Clin Endocrinol (Oxf).* (2009) 70:311–21. doi: 10.1111/j.1365-2265.2008.03369.x
44. Tang T, Lord J, Norman R, Yasmin E, Balen A. Insulin-sensitising drugs (metformin, rosiglitazone, pioglitazone, D-chiro-inositol) forwomenwith polycystic ovary syndrome, oligo amenorrhoea and subfertility. *Cochrane Database Syst Rev.* (2012) 5–7:CD003053. doi: 10.1002/14651858.CD003053.pub5
45. Petersen KB, Pedersen NG, Pedersen AT, Lauritsen MP, Freiesleben N la C. Mono-ovulation in women with polycystic ovary syndrome: a clinical review on ovulation induction. *Reprod Biomed.* (2016) 32:563–83. doi: 10.1016/j.rbmo.2016.03.006
46. Misso ML, Costello MF, Garrubba M, Wong J, Hart R, Rombauts L, et al. Metformin versus clomiphene citrate for infertility in non-obese women with polycystic ovary syndrome: a systematic review and meta-analysis. *Hum Reprod.* (2013) 19:2–11. doi: 10.1093/humupd/dms036

47. Tso LO, Costello MF, Albuquerque LET, Andriolo RB, Macedo CR. Metformin treatment before and during IVF or ICSI in women with polycystic ovary syndrome. *Cochrane Database Syst Rev.* (2009) 2:CD006105. doi: 10.1002/14651858.CD006105.pub3
48. Tso LO, Costello MF, Albuquerque LET, Andriolo RB, Marjoribanks J, MacEdo CR. Metformin treatment before and during *in vitro* fertilization or intracytoplasmic sperm injection in women with polycystic ovary syndrome: summary of a cochrane review. *Fertil Steril.* (2015) 104:542–4. doi: 10.1016/j.fertnstert.2015.05.038
49. Cassina M, Dona M, Di Gianantonio E, Litta P, Clementi M. First-trimester exposure to metformin and risk of birth defects: a systematic review and meta-analysis. *Hum Reprod.* (2014) 20:656–69. doi: 10.1093/humupd/dmu022
50. Yu Y, Fang L, Zhang R, He J, Xiong Y, Guo X, et al. Comparative effectiveness of 9 ovulation-induction therapies in patients with clomiphene citrate-resistant polycystic ovary syndrome: a network meta-analysis. *Sci Rep.* (2017) 7:1–12. doi: 10.1038/s41598-017-03803-9
51. Mayes MA, Laforest MF, Guillemette C, Gilchrist RB, Richard FJ. Adenosine 5′-monophosphate kinase-activated protein kinase (PRKA) activators delay meiotic resumption in porcine oocytes. *Biol Reprod.* (2007) 76:589–97. doi: 10.1095/biolreprod.106.057828
52. Tosca L, Uzbekova S, Chabrolle C, Dupont J. Possible role of 5′AMP-activated protein kinase in the metformin-mediated arrest of bovine oocytes at the germinal vesicle stage during *in vitro* maturation. *Biol Reprod.* (2007) 77:452–65. doi: 10.1095/biolreprod.107.060848
53. Huang Y, Yu Y, Gao J, Li R, Zhang C. Impaired oocyte quality induced by dehydroepiandrosterone is partially rescued by metformin treatment. *PLoS ONE* (2015) 10:e0122370. doi: 10.1371/journal.pone.0122370
54. Bertoldo MJ, Guibert E, Faure M, Ramé C, Foretz M, Viollet B, et al. Specific deletion of AMP-activated protein kinase (α1AMPK) in murine oocytes alters junctional protein expression and mitochondrial physiology. *PLoS ONE* (2015) 10:e0119680. doi: 10.1371/journal.pone.0119680
55. Calder MD, Edwards NA, Betts DH, Watson AJ. Treatment with AICAR inhibits blastocyst development, trophectodermdifferentiation and tight junction formation and function in mice. *Mol Hum Reprod.* (2017) 23:771–85. doi: 10.1093/MOLEHR/GAX050
56. Louden ED, Luzzo KM, Jimenez PT, Chi T, Chi M, Moley KH. TallyHO obese female mice experience poor reproductive outcomes and abnormal blastocyst metabolism which is reversed by metformin. *Reprod Fertil Dev.* (2014) 1:31–9. doi: 10.1071/RD14339
57. Pikiou O, Vasilaki A, Leondaritis G, Vamvakopoulos N, Messinis IE. Effects of metformin on fertilisation of bovine oocytes and early embryo development: possible involvement of AMPK3-mediated TSC2 activation. *Zygote* (2014) 23:58–67. doi: 10.1017/S0967199413000300
58. Sermondade N, Dupont C, Faure C, Boubaya M, Cédrin-Durnerin I, Chavatte-Palmer P, et al. Body mass index is not associated with sperm-zona pellucida binding ability in subfertile males. *Asian J Androl.* (2013) 15:626–9. doi: 10.1038/aja.2013.10
59. Kort HI, Massey JOEB, Elsner CW, Mitchell-leef D, Shapiro DB, Witt MA, et al. Impact of body mass index values on sperm quantity and quality. *J Androl.* (2006) 27:1150–2. doi: 10.2164/jandrol.05124
60. Dupont C, Faure C, Sermondade N, Boubaya M, Eustache F, Clément P, et al. Obesity leads to higher risk of sperm DNA damage in infertile patients. *Asian J Androl.* (2013) 15:622–5. doi: 10.1038/aja.2013.65
61. Tartarin P, Moison D, Guibert E, Dupont J, Habert R, Rouiller-Fabre V, et al. Metformin exposure affects human and mouse fetal testicular cells. *Hum Reprod.* (2012) 27:3304–14. doi: 10.1093/humrep/des264
62. Riera MF, Regueira M, Galardo MN, Pellizzari EH, Meroni SB, Cigorraga SB. Signal transduction pathways in FSH regulation of rat Sertoli cell proliferation. *Am J Physiol Endocrinol Metab.* (2012) 302:E914–23. doi: 10.1152/ajpendo.00477.2011
63. Attia SM, Helal GK, Alhaider AA. Assessment of genomic instability in normal and diabetic rats treated with metformin. *Chem Biol Interact.* (2009) 180:296–304. doi: 10.1016/j.cbi.2009.03.001
64. Morgante G, Tosti C, Orvieto R, Musacchio MC, Piomboni P, De Leo V. Metformin improves semen characteristics of oligo-terato-asthenozoospermic men with metabolic syndrome. *Fertil Steril.* (2011) 95:2150–2. doi: 10.1016/j.fertnstert.2010.12.009
65. Rabbani SI, Devi K, Khanam S. Role of pioglitazone with metformin or glimepiride on oxidative stress-induced nuclear damage and reproductive toxicity in diabetic rats. *J Med Sci.* (2010) 17:3–11.
66. Yan W, Mu Y, Yu N, Yi T, Zhang Y, Pang X, et al. Protective effects of metformin on reproductive function in obese male rats induced by high-fat diet. *J Assist Reprod Genet.* (2015) 32:1097–104. doi: 10.1007/s10815-015-0506-2
67. Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ, Bragado MJ. AMP-activated kinase AMPK is expressed in boar spermatozoa and regulates motility. *PLoS ONE* (2012) 7:e38840. doi: 10.1371/journal.pone.0038840
68. Ghasemnejad-Berenji M, Ghazi-Khansari M, Yazdani I, Nobakht M, Abdollahi A, Ghasemnejad-Berenji H, et al. Effect of metformin on germ cell-specific apoptosis, oxidative stress and epididymal sperm quality after testicular torsion/detorsion in rats. *Andrologia* (2017) 50:1–8. doi: 10.1111/and.12846
69. Nasrolahi O, Khaneshi F, Rahmani F, Razi M. Honey and metformin ameliorated diabetes-induced damages in testes of rat; correlation with hormonal changes. *Iran J Reprod Med.* (2013) 11:1013–20.
70. Ayuob NN, Hussam ASM, Soad SA. Impaired expression of sex hormone receptors in male reproductive organs of diabetic rat in response to oral antidiabetic drugs. *Folia Histochem Cytobiol.* (2015) 53:35–48. doi: 10.5603/FHC.a2015.0005
71. Naglaa ZHE, Hesham AM, Fadil HA, Motal ASM. Impact of metformin on immunity and male fertility in rabbits with alloxan-induced diabetes. *J Am Sci.* (2010) 6:417–26.
72. Nguyen TMD, Alves S, Grasseau I, Métayer-Coustard S, Praud C, Froment P, et al. Central role of 5′-AMP-activated protein kinase in chicken sperm functions. *Biol Reprod.* (2014) 91:1–15. doi: 10.1095/biolreprod.114.121855
73. Faure M, Guibert E, Alves S, Pain B, Ramé C, Dupont J, et al. The insulin sensitiser metformin regulates chicken Sertoli and germ cell populations. *Reproduction* (2016) 151:527–38. doi: 10.1530/REP-15-0565
74. Córdova A, Strobel P, Vallejo A, Valenzuela P, Ulloa O, Burgos RA, et al. Cryobiology use of hypometabolic TRIS extenders and high cooling rate refrigeration for cryopreservation of stallion sperm: presence and sensitivity of 5 0 AMP-activated protein kinase (AMPK). *Cry* (2014) 69:473–81. doi: 10.1016/j.cryobiol.2014.10.008
75. Tartarin P, Guibert E, Touré A, Ouiste C, Leclerc J, Sanz N, et al. Inactivation of AMPKα1 induces asthenozoospermia and alters spermatozoa morphology. *Endocrinology* (2012) 153:3468–81. doi: 10.1210/en.2011-1911
76. Cheung PCF, Salt IP, Davies SP, Hardie DG, Carling D. Characterization of AMP-activated protein kinase γ-subunit isoforms and their role in AMP binding. *Biochem* (2000) 669:659–69. doi: 10.1042/bj3460659
77. Alves MG, Martins AD, Vaz CV, Correia S, Moreira PI, Oliveira PF, et al. Metformin and male reproduction: effects on Sertoli cell metabolism. *Br J Pharmacol.* (2014) 171:1033–42. doi: 10.1111/bph.12522
78. Arlt W, Auchus RJ, Miller WL. Thiazolidinediones but not metformin directly inhibit the steroidogenic enzymes P450c17 and 3β-hydroxysteroid dehydrogenase. *J Biol Chem.* (2001) 276:16767–71. doi: 10.1074/jbc.M100040200
79. Bailey C, Puah JA. Effect of metformin on glucose metabolism in mouse soleus muscle. *Diabete Metab.* (1986) 12:212–8.
80. Lashen H. Review: role of metformin in the management of polycystic ovary syndrome. *Ther Adv Endocrinol Metab.* (2010) 1:117–28. doi: 10.1177/2042018810380215
81. Velazquez EM, Sosa F, Glueck CJ. Metformin therapy in polycystic ovary syndrome reduces hyperinsulinemia, insulin resistance, hyperandrogenemia, and systolic blood pressure, while facilitating normal menses and pregnancy. *Metabolism* (1994) 43:647–54. doi: 10.1016/0026-0495(94)90209-7
82. Nestler JE, Jakubowicz DJ. Lean women with polycystic ovary syndrome respond P450c17a activity and serum androgens. *J Clin Endocrinol Metab.* (1997) 82:4075–9.
83. Bailey C, Turner R. Metformin. *N Engl J Med.* (1998) 334:574–9. doi: 10.1056/NEJM199602293340906

84. Harborne L, Fleming R, Lyall H, Sattar N, Norman J. Metformin or antiandrogen in the treatment of hirsutism in polycystic ovary syndrome. *J Clin Endocrinol Metab.* (2003) 88:4116–23. doi: 10.1210/jc.2003-030424
85. Mansfield R, Galea R, Brincat M, Hole D, Mason H. Metformin has direct effects on human ovarian steroidogenesis. *Fertil Steril.* (2003) 79:956–62. doi: 10.1016/S0015-0282(02)04925-7
86. Rice S, Pellatt L, Ramanathan K, Whitehead SA, Mason HD. Metformin inhibits aromatase via an extracellular signal-regulated kinase-mediated pathway. *Endocrinology* (2009) 150:4794–801. doi: 10.1210/en.2009-0540
87. Fuhrmeister I, Branchini G, Pimentel A, Ferreira G, Capp E, Brum I, et al. Human granulosa cells: insulin and insulin-like growth factor-1 receptors and aromatase expression modulation by metformin. *Gynecol Obs Invest.* (2014) 77:156–62. doi: 10.1159/000358829
88. Richardson MC, Ingamells S, Simonis CD, Cameron IT, Sreekumar R, Vijendren A, et al. Stimulation of lactate production in human granulosa cells by metformin and potential involvement of adenosine 5 monophosphate-activated protein kinase. *J Clin Endocrinol Metab.* (2009) 94:670–7. doi: 10.1210/jc.2008-2025
89. Pellatt LJ, Rice S, Mason HD. Phosphorylation and activation of AMP-activated protein kinase (AMPK) by metformin in the human ovary requires insulin. *Reprod Dev.* (2011) 152:1112–8. doi: 10.1210/en.2009-1429
90. Attia GR, Rainey WE, Carr BR. Metformin directly inhibits androgen production in human thecal cells. *Fertil Steril.* (2001) 76:517–24. doi: 10.1016/S0015-0282(01)01975-6
91. Svechnikov K, Spatafora C, Svechnikova I, Tringali C, Söder O. Effects of resveratrol analogs on steroidogenesis and mitochondrial function in rat Leydig cells *in vitro*. *J Appl Toxicol.* (2009) 29:673–80. doi: 10.1002/jat.1456
92. Rone MB, Midzak AS, Martinez-arguelles DB, Fan J, Ye X, Papadopoulos V. Steroidogenesis in MA-10 mouse leydig cells is altered via fatty acid import into the mitochondria. *Biol Res Nurs.* (2014) 91:1–14. doi: 10.1095/biolreprod.114.121434
93. Tosca L, Froment P, Solnais P, Ferre P, Foufelle F. Adenosine 5-monophosphate-activated protein kinase regulates progesterone secretion in rat granulosa cells. *Endocrinology* (2005) 146:4500–13. doi: 10.1210/en.2005-0301
94. Tosca L, Chabrolle C, Uzbekova S, Dupont J. Effects of metformin on bovine granulosa cells steroidogenesis: possible involvement of adenosine 5′ monophosphate-activated protein kinase (AMPK). *Biol Reprod.* (2007) 76:368–78. doi: 10.1095/biolreprod.106.055749
95. Mesbah F, Moslem M, Vojdani Z, Mirkhani H. Does metformin improve *in vitro* maturation and ultrastructure of oocytes retrieved from estradiol valerate polycystic ovary syndrome-induced rats. *J Ovarian Res.* (2015) 8:1–10. doi: 10.1186/s13048-015-0203-x
96. Samarajeewa NU, Ham S, Yang F, Simpson ER, Brown KA. Promoter-specific effects of metformin on aromatase transcript expression. *Steroids* (2011) 76:768–71. doi: 10.1016/j.steroids.2011.02.041
97. Romero R, Erez O, Hüttemann M, Maymon E, Panaitescu B, Conde-Agudelo A, et al. Metformin, the aspirin of the 21st century: its role in gestational diabetes mellitus, prevention of preeclampsia and cancer, and the promotion of longevity. *Am J Obstet Gynecol.* (2017) 217:282–302. doi: 10.1016/j.ajog.2017.06.003
98. Tertti K, Laine K, Ekblad U, Rinne V, Ronnemaa T. The degree of fetal metformin exposure does not influence fetal outcome in gestational diabetes mellitus. *Acta Diabetol.* (2014) 51:731–8. doi: 10.1007/s00592-014-0570-6
99. Vanky E, Zahlsen K, Spigset O, Carlsen SM. Placental passage of metformin in women with polycystic ovary syndrome. *Fertil Steril.* (2005) 83:1575–8. doi: 10.1016/j.fertnstert.2004.11.051
100. Salomäki H, Vähätalo LH, Laurila K, Jäppinen NT, Penttinen A-M, Ailanen L, et al. Prenatal metformin exposure in mice programs the metabolic phenotype of the offspring during a high fat diet at adulthood. *PLoS ONE* (2013) 8:e56594. doi: 10.1371/journal.pone.0056594
101. Wilcock C, Bailey CJ. Accumulation of metformin by tissues of the normal and diabetic mouse. *Xenobiotica* (1994) 24:49–57. doi: 10.3109/00498259409043220
102. Rowan JA, Rush EC, Obolonkin V, Battin M, Wouldes T, Hague WM. Metformin in gestational diabetes: the offspring follow-up (MiG TOFU): body composition at 2 years of age. *Diabetes Care* (2011) 34:2279–84. doi: 10.2337/dc11-0660
103. Ijäs H, Vääräsmäki M, Saarela T, Keravuo R, Raudaskoski T. A follow-up of a randomised study of metformin and insulin in gestational diabetes mellitus: growth and development of the children at the age of 18 months. *BJOG* (2015) 122:994–1000. doi: 10.1111/1471-0528.12964
104. Hjorth-Hansen A, Salvesen Ø, Hanem LGE, Eggebø T, Salvesen KA, Vanky E, et al. Fetal growth and birth anthropometrics in metformin-exposed offspring born to mothers with PCOS. *J Clin Endocrinol Metab.* (2018) 103:740–7. doi: 10.1210/jc.2017-01191
105. Hanem LGE, Stridsklev S, Júlíusson PB, Salvesen Ø, Roelants M, Carlsen SM, et al. Metformin use in PCOS pregnancies increases the risk of offspring overweight at 4 years of age; follow-up of two RCTs. *J Clin Endocrinol Metab.* (2018) 103:1612–21. doi: 10.1210/jc.2017-02419
106. Tertti K, Toppari J, Virtanen HE, Sadov S, Rönnemaa T. Metformin treatment does not affect testicular size in offspring born to mothers with gestational diabetes. *Rev Diabet Stud.* (2016) 13:59–65. doi: 10.1900/RDS.2016.13.59
107. Ibáñez L, López-Bermejo A, Díaz M, Marcos MV, de Zegher F. Early metformin therapy (age 8-12 years) in girls with precocious pubarche to reduce hirsutism, androgen excess, and oligomenorrhea in adolescence. *J Clin Endocrinol Metab.* (2011) 96:E1262–7. doi: 10.1210/jc.2011-0555
108. Ibáñez L, Ong K, Valls C, Marcos MV, Dunger DB, de Zegher F. Metformin treatment to prevent early puberty in girls with precocious pubarche. *J Clin Endocrinol Metab.* (2006) 91:2888–91. doi: 10.1210/jc.2006-0336
109. Trautwein C, Kümmerer K. Incomplete aerobic degradation of the antidiabetic drug Metformin and identification of the bacterial dead-end transformation product Guanylurea. *Chemosphere* (2011) 85:765–73. doi: 10.1016/j.chemosphere.2011.06.057
110. Khan U, Nicell J. Human health relevance of pharmaceutically active compounds in drinking water. *AAPS J.* (2015) 17:558–85. doi: 10.1208/s12248-015-9729-5
111. Blair BD, Crago JP, Hedman CJ, Klaper RD. Pharmaceuticals and personal care products found in the Great Lakes above concentrations of environmental concern. *Chemosphere* (2013) 93:2116–23. doi: 10.1016/j.chemosphere.2013.07.057
112. Ter Laak T, Baken K. The occurrence, fate and ecological and human health risks of metformin and guanylurea in the water cycle–a literature review. *KWR* (2014) 1–24.
113. Niemuth NJ, Klaper RD. Emerging wastewater contaminant metformin causes intersex and reduced fecundity in fish. *Chemosphere* (2015) 135:38–45. doi: 10.1016/j.chemosphere.2015.03.060
114. Puscheck EE, Bolnick A, Awonuga A, Yang Y, Abdulhasan M, Li Q, et al. Why AMPK agonists not known to be stressors may surprisingly contribute to miscarriage or hinder IVF/ART. *J Assist Reprod Genet.* (2018) 35:1359–66. doi: 10.1007/s10815-018-1213-6
115. Zhong T, Men Y, Lu L, Geng T, Zhou J, Mitsuhashi A, et al. Metformin alters DNA methylation genome-wide via the H19/SAHH axis. *Oncogene* (2017) 36:2345–54. doi: 10.1038/onc.2016.391
116. Cuyàs E, Fernández-Arroyo S, Verdura S, García RÁF, Stursa J, Werner L, et al. Metformin regulates global DNA methylation via mitochondrial one-carbon metabolism. *Oncogene* (2018) 37:963–70. doi: 10.1038/onc.2017.367
117. Zhang Q, Li S, Li L, Li Q, Ren K, Sun X, et al. Metformin treatment and homocysteine: a systematic review and meta-analysis of randomized controlled trials. *Nutrients* (2016) 8:E798: doi: 10.3390/nu8120798
118. Bridgeman SC, Ellison GC, Melton PE, Newsholme P, Mamotte CDS. Epigenetic effects of metformin: from molecular mechanisms to clinical implications. *Diabetes Obes Metab.* (2018) 20:1553–62. doi: 10.1111/dom.13262
119. Banerjee P, Surendran H, Chowdhury DR, Prabhakar K, Pal R. Metformin mediated reversal of epithelial to mesenchymal transition is triggered by epigenetic changes in E-cadherin promoter. *J Mol Med.* (2016) 94:1397–409. doi: 10.1007/s00109-016-1455-7
120. Khan S, Jena G. Sodium butyrate reduces insulin-resistance, fat accumulation and dyslipidemia in type-2 diabetic rat: a comparative study with metformin. *Chem Biol Interact.* (2016) 254:124–34. doi: 10.1016/j.cbi.2016.06.007
121. Bungard D, Fuerth BJ, Zeng P-Y, Faubert B, Maas NL, Viollet B, et al. Signaling kinase AMPK activates stress-promoted transcription

via histone H2B phosphorylation. *Science* (2010) 329:1201–5. doi: 10.1126/science.1191241

122. Tang G, Guo J, Zhu Y, Huang Z, Liu T, Cai J, et al. Metformin inhibits ovarian cancer via decreasing H3K27 trimethylation. *Int J Oncol.* (2018) 52:1899–911. doi: 10.3892/ijo.2018.4343
123. Bertoldo MJ, Guibert E, Faure M, Guillou F, Ramé C, Nadal-Desbarats L, et al. Specific deletion of AMP-activated protein kinase (α1AMPK) in mouse Sertoli cells modifies germ cell quality. *Mol Cell Endocrinol.* (2016) 423:96–112. doi: 10.1016/j.mce.2016.01.001
124. Vigili de Kreutzenberg S, Ceolotto G, Cattelan A, Pagnin E, Mazzucato M, Garagnani P, et al. Metformin improves putative longevity effectors in peripheral mononuclear cells from subjects with prediabetes. A randomized controlled trial. *Nutr Metab Cardiovasc Dis.* (2015) 25:686–93. doi: 10.1016/j.numecd.2015.03.007
125. Caton PW, Nayuni NK, Kieswich J, Khan NQ, Yaqoob MM, Corder R. Metformin suppresses hepatic gluconeogenesis through induction of SIRT1 and GCN5. *J Endocrinol.* (2010) 205:97–106. doi: 10.1677/JOE-09-0345
126. Zhang E, Guo Q, Gao H, Xu R, Teng S, Wu Y. Metformin and resveratrol inhibited high glucose-induced metabolic memory of endothelial senescence through SIRT1/p300/p53/p21 pathway. *PLoS ONE* (2015) 10:e0143814. doi: 10.1371/journal.pone.0143814
127. Lennox R, Porter DW, Flatt PR, Holscher C, Irwin N, Gault VA. Comparison of the independent and combined effects of sub-chronic therapy with metformin and a stable GLP-1 receptor agonist on cognitive function, hippocampal synaptic plasticity and metabolic control in high-fat fed mice. *Neuropharmacology* (2014) 86:22–30. doi: 10.1016/j.neuropharm.2014.06.026
128. Bertoldo MJ, Guibert E, Tartarin P, Guillory V, Froment P. Effect of metformin on the fertilizing ability of mouse spermatozoa. *Cryobiology* (2014) 68:262–8. doi: 10.1016/j.cryobiol.2014.02.006
129. Adaramoye O, Akanni O, Adesanoye O, Labo O. Evaluation of toxic effects of metformin hydrochloride and glibenclamide on some organs of male rats. *Niger J Physiol Sci.* (2012) 27:137–44.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 17 December 2018
doi: 10.3389/fendo.2018.00753

Metformin-Induced Mitochondrial Complex I Inhibition: Facts, Uncertainties, and Consequences

*Eric Fontaine**

INSERM, LBFA, Université Grenoble Alpes, Grenoble, France

Metformin is the most widely prescribed drug to treat patients with type II diabetes, for whom retrospective studies suggest that metformin may have anticancer properties. However, in experiments performed with isolated cells, authors have reported both pro- and anti-apoptotic effects of metformin. The exact molecular mechanism of action of metformin remains partly unknown despite its use for over 60 years and more than 17,000 articles in PubMed. Among the various widely recognized or recently proposed targets, it has been reported consistently that metformin is capable of inhibiting mitochondrial respiratory chain Complex I. Since most of the effects of metformin have been replicated by other inhibitors of Complex I, it has been suggested that the mechanism of action of metformin involved the inhibition of Complex I. However, compared to conventional Complex I inhibitors, the metformin-induced inhibition of Complex I has unique characteristics. Among these, the most original one is that the concentrations of metformin required to inhibit Complex I are lower in intact cells than in isolated mitochondria. Experiments with isolated mitochondria or Complex I were generally performed using millimolar concentrations of metformin, while plasma levels remain in the micromolar range in both human and animal studies, highlighting that metformin concentration is an important issue. In order to explain the effects in animals based on observations in cells and mitochondria, some authors proposed a direct effect of the drug on Complex I involving an accumulation of metformin inside the mitochondria while others proposed an indirect effect (the drug no longer having to diffuse into the mitochondria). This brief review attempts to: gather arguments for and against each hypothesis concerning the mechanism by which metformin inhibits Complex I and to highlight remaining questions about the toxicity mechanism of metformin for certain cancer cells.

Keywords: metformin, mitochondria, Complex I, pharmacokinetic, cell death, cancer, permeability transition

OPEN ACCESS

Edited by:
Frederic Bost,
Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Gayathri K. Natarajan,
Medical College of Wisconsin, United States
Adam Mickiewicz,
University in Poznan, Poland

***Correspondence:**
Eric Fontaine
eric.fontaine@univ-grenoble-alpes.fr

Specialty section:
This article was submitted to Cellular Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *27 April 2018*
Accepted: *27 November 2018*
Published: *17 December 2018*

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

INTRODUCTION

Metformin is a drug with pleiotropic effects. It takes part in glucose homeostasis, mainly by inhibiting liver glucose production (1). It also modifies the production of reactive oxygen species and affects cell death processes (2, 3). Most of these effects have been traced to the inhibition of mitochondrial respiratory chain Complex I for two main reasons: First, over the past 20 years, different laboratories have reproducibly observed that metformin inhibits mitochondrial respiratory chain Complex I (4–20). Second, these pleiotropic effects have been reproduced by well identified Complex I inhibitors [gluconeogenesis (21, 22), cell death (18, 23–28)].

However, the mechanism by which metformin affects the activity of Complex I remains debated. In order to clarify whether the different conclusions found in the literature may be due to methodological differences, this review compares results obtained *in vivo* or with intact cells, to results obtained with isolated mitochondria or isolated Complex I. In this last case, authors tend to assume that metformin accumulates in mitochondria, here we will discuss evidence supporting or not this assumption. Finally, since pro- and anti-apoptotic effects of metformin are observed in intact cells, we will examine the role of metformin concentrations as a potential cause of these conflicting observations.

METFORMIN PHARMACOKINETICS

Metformin is a hydrophilic compound charged positively at physiological pH. Its hydrophilicity limits its permeability through lipid membranes. Metformin enters and leaves cells by the presence of several transporters including Organic Cation Transporters (OCTs) and multidrug and toxin extrusion (MATE) transporters (29). This leads to a steady-state concentration of metformin inside cells, depending on both the amount and activity of such transporters as well as metformin plasma concentration.

The pharmacological inhibition or the genetic ablation of OCTs reduce the distribution of metformin to the liver, small intestine and kidney (30–32) while the overexpression of OCT1 in HEK293 and CHO cells increases metformin uptake (30, 33). The pharmacological inhibition or the genetic ablation of MATE1 cause hepatic and kidney accumulation of metformin (32, 34). In humans, the genomic variations of metformin transporters can affect its pharmacokinetics (concentration, clearance, volume of distribution) (35, 36) suggesting that such genomic variations affect metformin concentration in tissues.

Whether the activities of the metformin transporters (i.e., the metformin concentration in tissues) affect the metabolic effects of metformin is not systematically reported in the literature. On the one hand, metformin failed to reduce fasting plasma glucose concentration in OCT1-knockout mice submitted to a high-fat diet for 8 weeks and failed to suppress glucagon-stimulated glucose production in OCT1$^{-/-}$ hepatocytes (30). On the other hand, the effect of metformin on glucose tolerance tests was similar in animal controls and OCT1/2-knockout animals (31). A broad variation in clinical efficacy of metformin has long been recognized as well as a reduced function polymorphism of OCT1 in humans. However, if some authors reported a decreased effect of metformin in type-2 diabetes patients carrying reduced function polymorphism of OCT1 (30, 36), others did not observe such a correlation (37, 38).

To the best of my knowledge, no study correlating metformin concentration in tissue (or cells) and metformin-induced Complex I inhibition was ever published.

Drugs that are extensively sequestered in organelles have a very large apparent volume of distribution and a prolonged half-life *in vivo* (39). Metformin is not metabolized and is secreted by the kidneys with a half-life of 1.74–7.3 h in humans depending on the studies (35, 40–42). With a volume of distribution of 1.12 ± 0.08 L/kg in healthy volunteers (40), metformin is not supposed to accumulate dramatically in tissues. The amount of metformin in the liver ranges from 2 to 5 times that of plasma -depending on the studies (32, 35, 42, 43)- and increases up to 10 times that of plasma in small intestinal walls (32).

Thus, the pharmacokinetic studies indicate that metformin enters but does not accumulate in large amounts in cells. Whether its metabolic activity depends on its diffusion inside the cells is supported by several but not all studies.

Once in the cell, as metformin inhibits Complex I it is tempting to speculate that metformin penetrates the mitochondria. The composition of the mitochondrial matrix (the space delimited by the inner mitochondrial membrane) is different from that of the cytosol. In order to maintain such a different metabolite composition, the inner membrane is impermeable to almost all hydrophilic molecules which enter or leave the mitochondria through specific transporters. Among the numerous recognized mitochondrial carriers, no specific carrier for metformin has been identified yet.

Despite this, many authors have hypothesized that metformin accumulates in mitochondria (5, 13, 15, 44). This scenario may reconcile the observation that millimolar concentrations of metformin are necessary to inhibit Complex I in isolated mitochondria (see below) while, when used at the therapeutic dose, the plasma metformin concentration remains in the micromolar range in both humans and animals (31, 36, 42).

From a theoretical point of view, this hypothesis is plausible. Indeed, because the mitochondrial respiratory chain transfers protons from the matrix to the intermembrane space, mitochondria build up and maintain an electrical mitochondrial membrane potential that drives the accumulation of positively charged molecules into mitochondria, provided the molecule crosses the membrane. In these conditions, Nernst equation indicates that for a physiological mitochondrial membrane potential of −180 mV the thermodynamic equilibrium is reached after a 1,000-fold accumulation of a positively charged molecule if the molecule has one charge. Since metformin is a positively charged molecule and assuming the presence of a still unknown carrier for metformin in the inner membrane, its mitochondrial concentration could reach the millimolar range despite a cytosolic concentration within the micromolar range (see **Figure 1**). In addition, assuming a plasma membrane potential of −36 mV and the absence of kinetic constraints on metformin transporters (OCT and MATE), the cytosolic concentration of metformin would be 4 times that of plasma.

However, the hypothesis that metformin accumulates in mitochondria contradicts several observations.

First of all, the accumulation of numerous positive charges in the matrix compensated by proton extrusion by the respiratory chain, should lead to a collapse of mitochondrial membrane potential associated with an increase in delta pH. However, note that metformin did not depolarize isolated mitochondria (8).

Secondly, assuming that the total mitochondrial volume represents approximately 20% of hepatocytes, a 1,000-fold accumulation of metformin inside mitochondria would represent an approximately 200-fold accumulation of metformin in liver

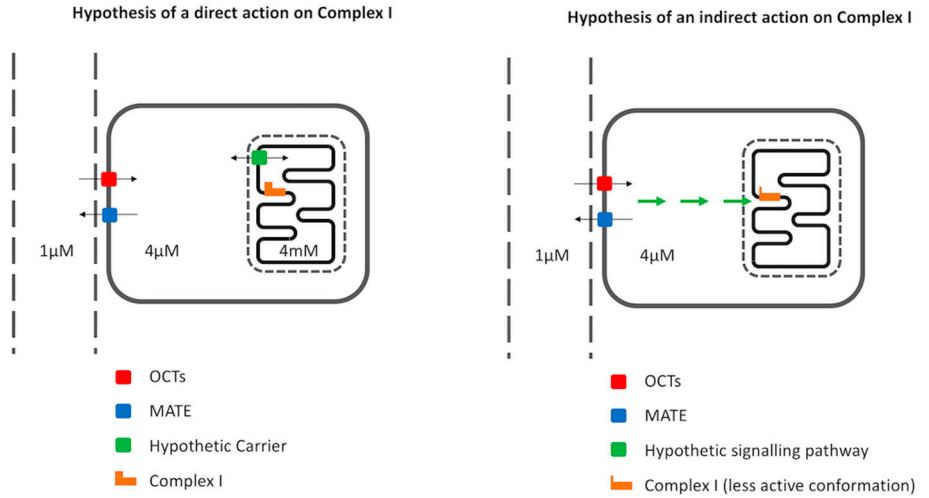

FIGURE 1 | Mechanism of action of metformin on complex I: the direct hypothesis and the indirect hypothesis. Metformin enters cells via Organic Cation Transporters (OCTs) and leaves cells via multidrug and toxin extrusion (MATE) transporters. Assuming a plasma membrane potential of 35 mV and a passive mechanism of OCTs and MATE, the cytosolic metformin concentration is expected to be 4 times that of the plasma concentration. In the direct hypothesis **(left)**, metformin is supposed to enter mitochondria via a hypothetical carrier reaching a matrix concentration 1,000 times that of the cytosol for mitochondrial membrane potential of 180 mV. In the indirect hypothesis **(right)**, metformin does not enter mitochondria but stimulates a hypothetical signaling pathway that eventually modifies Complex I conformation, making it less active.

(without accounting for accumulation in the cytosol). Such an accumulation is 2 orders of magnitude higher than that measured by several groups (32, 35, 42, 43).

Thirdly, a large mitochondrial accumulation is not compatible with the low volume of distribution of metformin and its short half-live (see above).

Fourthly, using radioactive [^{14}C] metformin, the radioactivity was not found to accumulate in liver mitochondria of rats treated orally with metformin (45) and no radioactivity was measured inside mitochondria when *Xenopus laevis* oocytes were exposed to concentrations of metformin that led to Complex I inhibition (6). Importantly, Complex I remained inhibited after mitochondrial isolation. Although this result does not definitively exclude a possible accumulation of metformin in mitochondria as a cause of Complex I inhibition (accumulated metformin may diffuse during the isolation procedure), it rules out the hypothesis that the inhibition of Complex I by metformin requires metformin inside mitochondria.

In summary, unlike the less hydrophilic biguanides (46), the accumulation of metformin inside the mitochondria is not supported by direct measurements, is not consistent with the pharmacokinetic data, and would require a transporter that has not yet been discovered.

Derivatives combining a molecule of metformin at different alkyl chain lengths containing a triphenylphosphonium cation (a liposoluble cation known to accumulate in mitochondria according to membrane potential) have been synthesized (47, 48) in order to increase the anti-cancer effect of metformin (see below). These different compounds accumulate in cells (47), depolarize mitochondria (48) and inhibit Complex I with an IC_{50} in the micromolar range (47, 48), which according to Nernst equation is consistent with the accumulation of compounds in the mitochondrial matrix at a concentration in the millimolar range. If metformin accumulated spontaneously in the mitochondria, the addition of molecules targeting the mitochondria would be unnecessary, which is clearly not the case.

CHARACTERISTICS OF COMPLEX I INHIBITION ACCORDING TO THE MODELS USED

Although this may seem odd, it has been reported by several different laboratories that the concentrations required to inhibit Complex I are lower for intact cells than for isolated mitochondria (4–6, 13, 19). Note however that the characteristics of Complex I inhibition reveals some differences depending on whether metformin acts on intact cells (animal models, infused organs, isolated cells) or directly on isolated mitochondria or isolated Complex I (see **Table 1**).

The incubation of isolated Complex I or submitochondrial particles in the presence of millimolar concentrations of metformin leads to an inhibition of Complex I that can be complete (13) with an IC_{50} ranging from 19 to 79 mM depending on laboratories (5, 9, 13). It should be noted that there is no membrane potential in these particular conditions of incubation, thus no possibility of metformin accumulation. In other words, the concentrations tested are the actual concentrations to which Complex I is exposed.

The incubation of isolated mitochondria in the presence of millimolar concentrations of metformin leads to a rather fast (within a few minutes) inhibition of Complex I with an "apparent" IC_{50} also in the millimolar range (5).

TABLE 1 | Main differences in the characteristics of Complex I inhibition according to the model used.

	Live animal, perfused organ, intact cells		Isolated mitochondria, isolated Complex I	
		References		References
Speed of inhibition	Time dependent	(4) (5)	Immediate (minutes)	(14) (13)
Type of inhibition	Partial	(4) (5) (18)	Total	(13)
Affinity	Apparent IC_{50} approximately 1 mM after 30 min in isolated rat hepatocytes	(4)	Apparent IC_{50} 15 mM in isolated mitochondria	(5)
	Apparent IC_{50} 250 μM and 330 μM after 6 h incubation in 143B and HepG2 cells respectively	(13)	IC_{50} 79 mM in sub-mitochondrial particles IC_{50} 66 mM in immunocaptured Complex I IC_{50} 19 mM in isolated Complex I	(5) (9) (13)
Inhibition in State-3?	Yes	(5) (11) (12) (14) (15)	Yes	(5) (8) (9) (10) (12) (14) (15)
Inhibition in State-4?	Yes	(4) (7) (14)	No	(4) (8) (10) (15)
Inhibition after uncoupling	Yes	(4) (7) (11) (14) (19)	No	(4) (15) (19)
Inhibition after the removal of metformin	Yes	(4) (14) (20)	No for metformin concentration ≤ 2 mM	(13)
$NADH/NAD^+$	Increases	(4) (5) (18)	Decreases	(19)

This observation is not easily reconcilable with the proposal that metformin accumulates in mitochondria. Indeed, assuming that metformin did accumulate in mitochondria, Complex I inhibition would have been observed at micromolar concentrations of metformin (corresponding to millimolar concentrations inside mitochondria), which has not been reported. One could argue that at millimolar concentrations of metformin, the inhibition of Complex I would depolarize mitochondria, preventing metformin accumulation. However, it has been shown that millimolar concentrations of metformin did not depolarize isolated mitochondria (8).

In these particular conditions of incubation (isolated mitochondria exposed to millimolar concentrations of metformin), it has to be noted that the inhibition of Complex I is observed almost exclusively during ATP synthesis (also called State 3) and disappears when mitochondria are depolarized (uncoupled State) or at rest (also called State 4) (4, 8, 15). Such behavior is not observed with rotenone (the reference inhibitor of Complex I) but is typical of biguanide-induced inhibition of Complex I in isolated mitochondria (49).

It has been proposed that the reason why the inhibition is not observed after uncoupling might be due to the fact that the driving force for metformin accumulation within mitochondria disappears in these particular conditions. Although, as stated above, the accumulation of metformin in mitochondria is not supported by any evidence, this hypothesis does not explain the lack of inhibition in State 4, a situation in which the driving force (the membrane potential) is higher than in State 3.

Curiously, it has been reported that the inhibition of oxygen consumption in isolated mitochondria is accompanied by NADH oxidation (19). This observation is not expected in case of a simple Complex I inhibition, suggesting an uncoupling effect of metformin in this particular condition.

The incubation of intact cells in the presence of metformin leads to a slower inhibition of Complex I depending on metformin concentration (hours are required for micromolar concentrations of metformin) (5, 50). Contrary to what is observed in isolated Complex I, the inhibition is not total and plateaus at approximately 40% of the Vmax (4). Consistent with a pure effect on Complex I, the inhibition leads to an increase in the $NADH/NAD^+$ ratio (as assessed by the Lactate/pyruvate and 3-hydroxybutyrate/ acetoacetate ratios) (4, 5). Importantly, once cells are permeabilized (i.e., once mitochondria can be studied as if they were isolated) the inhibition is observed in State 3, but also in State 4 and after uncoupling (4, 11, 19). Finally, Complex I remains inhibited in mitochondria isolated from either rat exposed to metformin or liver perfused with metformin, even after uncoupling (4, 14) or when NADH:quinone oxidoreductase activity (i.e., Complex I activity) is studied directly using broken mitochondria (4). Note that the isolation procedure removes most of (if not all) the free metformin, while uncoupling (either chemical or after inner membrane rupture) would release the putative accumulated metformin. Although these results do not exclude a possible binding of metformin in mitochondrial membrane, they rule out the hypothesis that the inhibition of Complex I by metformin could depend on membrane potential.

OTHER MITOCHONDRIAL EFFECTS OF METFORMIN

In intact cells the inhibition of oxygen consumption is strictly located on Complex I. This conclusion comes from the observation that metformin has no effect on oxygen consumption when electrons feed the respiratory chain downstream Complex I (using succinate for example) regardless of the respiratory State (3, 4 and uncoupled) (4).

On the contrary, using isolated mitochondria and millimolar concentrations of metformin, some authors reported inhibitory effects on complexes III and IV (16). High concentrations of metformin have been reported to inhibit ATP hydrolysis but not

ATP synthesis (13), suggesting an unconventional effect on the ATP synthase.

Some evidence suggests that Complex I can interact with ATP synthase (51). So we may infer that in this particular condition of incubation (isolated mitochondria exposed to millimolar concentrations of metformin), ATP synthesis possibly sensitizes Complex I to metformin. Although speculative, this personal suggestion could account for the observation that millimolar concentrations of metformin inhibit Complex I almost exclusively in State 3.

In summary, on intact cells metformin acts slowly but the effect is visible at micromolar concentrations. The inhibition affects only Complex I in all the respiratory states and does not depend on mitochondrial membrane potential. On isolated mitochondria (or isolated Complex I), metformin acts rapidly but the effect requires millimolar concentrations. The inhibition does not only affect Complex I and Complex I inhibition is not observed in all the respiratory states.

WHERE DOES METFORMIN ACT ON COMPLEX I?

The respiratory chain is a sequence of redox reactions which couple an electron flux with a vectorial transfer of protons. Mammalian respiratory chain complex I is a large protein complex with at least 45 subunits. It includes a hydrophobic part embedded in the inner membrane involved in proton transfer and a hydrophilic part protruding into the matrix in which electrons pass from NADH to ubiquinone via a succession of redox reactions. Complex I inhibitors rotenone and piericidin bind at, or close to, the ubiquinone binding site, inhibiting both electron flux and proton extrusion. Using artificial electron acceptors, a rotenone-insensitive NADH oxidation which is not coupled with proton pumping (i.e., a non-physiological pathway) can occur in Complex I.

Using isolated Complex I and millimolar concentrations of metformin, it has been shown that metformin does not inhibit NADH oxidation due to artificial electron acceptors, behaves as a non-competitive inhibitor of the physiological electron pathway and preferentially binds Complex I when the enzyme is in its "deactive" conformation (13). However, the exact localization where metformin acts in this condition of incubation remains unknown. Moreover, the exact mechanism leading to the inhibition of Complex I in intact cells using micromolar concentrations of metformin and where exactly it inhibits the electron flux in Complex I has not been reported.

HYPOTHETICAL MECHANISMS OF ACTION

To account for the fact that the concentration of metformin required to observe the inhibition of Complex I on whole cells is lower than the concentration required to observe the inhibition on mitochondria, two hypotheses have been proposed in the literature (see **Figure 1**).

The first one (in chronological order, but second in popularity) proposes that *in vivo* and in intact cells, metformin triggers a signaling pathway that in turn induces the inhibition of Complex I (4). Although such a signaling pathway is yet unknown, it has been reported that Complex I exists in two different functional conformations (active and inactive) (52), while reactive thiols of several Complex I subunits have been identified as targets for post-translational modifications (53, 54). However, whether metformin affects reactive thiols in Complex I has not been published yet.

The second hypothesis necessarily involves an accumulation of metformin in the mitochondria that would be driven by mitochondrial membrane potential. Although proposed by several authors, this hypothesis is not yet supported by any evidence (see above).

EFFECTS OF METFORMIN-INDUCED COMPLEX I INHIBITION ON CELL DEATH PROCESSES

Apparently contradictory effects are found in the literature regarding the effects of metformin on cell death. Some authors have put forward its protective effects against cell death (3) while others have reported its induction of cell death especially in cancer cells (2). Yet, all of them have concluded that the observed effects are due to the inhibition of Complex I (see below).

METFORMIN PREVENTS CELL DEATH WHEN IT IS DUE TO PTP OPENING

The permeability transition pore (PTP) is a channel located in the inner membrane normally closed in order to maintain a high mitochondrial membrane potential required for ATP synthesis. Once permanently opened, the membrane potential collapses (55), leading to a drastic inhibition of ATP synthesis. Beyond this uncoupling effect, PTP opening has many other effects: It allows the thermodynamic equilibrium of the mitochondrial and cytosolic redox potentials, leading to an increase in cytosolic NAD(P)H concentration (56). It partly inhibits Complex I (57), reallocating the electron flux for the production of reactive oxygen species (58). Finally, it leads to the release of mitochondrial pro-apoptotic proteins both in isolated mitochondria (secondary to mitochondrial swelling leading to the rupture of the outer membrane) (59) and in intact cells (most probably by a distinct but still unknown mechanism) (56, 60–62).

As there are several signaling pathways involved in cell death, there are many factors activating these pathways. To discriminate whether a given condition leading to cell death involves PTP opening or not, experiments are performed in the presence or absence of a recognized PTP inhibitor (generally cyclosporine A, but not exclusively). Using this approach, it has been reproducibly observed that PTP opening occurs when cell death is triggered by calcium overload or oxidative stress (63).

The molecular nature of the PTP has long been a subject of dispute but recent and compelling data from different laboratories suggest that the PTP might involve ATP synthase (51, 64). Surprisingly, the reference Complex I inhibitor rotenone has been shown to inhibit PTP opening in all the tested cells and tissues (either spontaneously or in the presence of cyclosporine A) (23, 65). Although rotenone induces an energetic stress, it also prevents cell death in the same models as cyclosporine A (23) and does inhibit Complex I and PTP opening with a similar concentration dependence (65). Piericidin, another well recognized Complex I inhibitor also inhibits PTP opening (23). Thus, the activity of Complex I can be said to be a regulator of PTP opening. Moreover, several ubiquinone analogs (known to bind with Complex I among others) have been proved to regulate PTP opening and cell death (57, 66–69).

Knowing that metformin partly inhibits Complex I, we tested whether it also inhibited PTP opening and related cell death. We found that, metformin was less potent than rotenone but also inhibited PTP opening (50). Suggesting a common mechanism of action with rotenone, the effect of metformin was not additive with that of rotenone, whereas it was additive with that of cyclosporine A (65). At present, metformin has been shown to prevent PTP opening in endothelial cells (50), KB cells (7), INS-1 insulinoma cells (61), HeLa cells (65), LNCaP cells (70), A375 cells (70), primary cortical neurons (71) and kidney mitochondria (72). Accordingly, metformin prevents cell death induced by oxidative stress in endothelial cells (50) and KB cells (7), etoposide in primary neurons (71), gentamicin in kidneys (72), hyperglycemia in endothelial (50) and INS-1 cells (61), hyperfructosemia in INS-1 cells (61) and ischemia reperfusion in INS-1 cells (73). Many other works have found a protective effect of metformin (particularly during oxidative stress or ischemia reperfusion injury) without having studied the role of the PTP (18, 74–77).

ANTI-NEOPLASTIC EFFECTS OF METFORMIN

Although PTP opening irremediably leads to cell death, PTP opening is not mandatory to kill cells as cells can die with a closed PTP. Although Complex I inhibition prevents PTP opening-related cell death (see above), it can also induce cell death in several models. Indeed, it has been repetitively reported that rotenone (25) or biguanides (including metformin) can induce cell death, especially in cancer cells (15, 17, 20, 78).

Cancer cells are known to be generally highly glycolytic (Warburg effect) and are thus not supposed to be very sensitive to mitochondrial poison. But is it so simple? As soon as cells consume oxygen at the mitochondrial level, they are supposed to produce mitochondrial ATP. Thus, even if the proportion of mitochondrial ATP production is reduced in cancer cells, this mitochondrial ATP production exists and its reduction could be toxic. Supporting this proposal, it has been reported that metformin inhibits the proliferation of HCT116 $p53^{-/-}$ cancer cells in the presence of glucose, while it induces cell death in case of glucose deprivation (15). Moreover, the effect of metformin is totally prevented by the overexpression of a metformin-resistant *Saccharomyces cerevisiae* NADH dehydrogenase NDI1 (15), very elegantly demonstrating that the toxicity of metformin is due to its effect on Complex I.

The suggestion that metformin's toxicity is related to an energetic stress raises several questions: Why is metformin less toxic in non-cancer cells that are yet more dependent on mitochondrial ATP production? How can metformin protect against PTP-induced cell death despite its effect on ATP production? In other words, what triggers that a same inhibition of Complex I either prevents or induces cell death?

Again, part of the answer could be found in the comparison of metformin concentrations. While millimolar concentrations of metformin are generally used to induce cell death *in vitro*, micromolar concentrations are sufficient to prevent PTP-opening induced cell death. Although it has been shown that cellular energy status is inversely correlated with metformin concentrations (11, 79), a 24-h incubation with 100 μM metformin did not affect the AMP/ATP ratio in primary cultured hepatocytes (11). This suggests that the metformin concentration used to prevent PTP opening (100 μM, overnight) was not sufficient to induce a lethal decrease in energy status. On the contrary, this confirms that the concentrations used to kill cells dramatically affect the energy status. Note however that some authors have reported an anti-apoptotic effect even at millimolar concentrations of metformin, suggesting that some cells are able to overcome energy stress (75, 80).

However, if the mechanism by which metformin kills isolated cells can be traced to a collapse in energy status, the concentrations that prevent cancer growth in animal models are in the micromolar range. The practical assumption of metformin accumulation in mitochondria has obviously been retained, but one can wonder: why are normal cells preserved? Alternative or complementary explanations must exist. Among them, it has been proposed that the effect of metformin in animal models is indirect (for example due to a decrease in blood insulin concentration) (2). It is also possible that the accumulation of metformin or the sensitivity of Complex I to metformin is higher in cancer cells than in normal tissues (personal hypothesis). As far as I know, these assumptions have not yet been tested.

CONCLUSIONS AND PROPOSAL

As explained several times in this manuscript, the concentration with which experiments were conducted is the main misleading point regarding the effect of metformin on Complex I. On the one hand, it is obvious that the assumption that metformin accumulates in mitochondria suits many authors. This hypothesis can bridge the gap between concentrations measured *in vivo* and those used *in vitro*. On the other hand, two different laboratories that attempted to measure such an accumulation put forward a total absence of metformin accumulation in mitochondria (6, 45) in which Complex I was nevertheless inhibited (6). Furthermore, although the pharmacokinetic data are indirect evidence, they are not compatible with an accumulation of metformin in mitochondria.

Facing the facts, one must admit that there is either a technical mistake in the studies that did not find metformin accumulation in mitochondria or there is absolutely no experiment performed at millimolar concentrations of metformin that reflect what occurs *in vivo*. This includes a lot of articles both on its antidiabetic role and on its anticancer effect. There is an urgent need to solve this problem for good, and this could be performed easily by fast cell fractionation coupled to mass spectrometry (or other technics to detect metformin) in order to confirm if metformin is found in large amount in mitochondria of cells exposed to metformin. Currently, the published evidence does not support the generally accepted hypothesis of metformin accumulation in mitochondria.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

I thank Christophe Cottet and Emily Tubbs for the corrections of the English version of this paper.

REFERENCES

1. Li M, Li X, Zhang H, Lu Y. Molecular mechanisms of metformin for diabetes and cancer treatment. *Front Physiol.* (2018) 9:1039. doi: 10.3389/fphys.2018.01039
2. Yu X, Mao W, Zhai Y, Tong C, Liu M, Ma L, et al. Anti-tumor activity of metformin: from metabolic and epigenetic perspectives. *Oncotarget* (2017) 8:5619–28. doi: 10.18632/oncotarget.13639
3. Rotermund C, Machetanz G, Fitzgerald JC. The therapeutic potential of metformin in neurodegenerative diseases. *Front Endocrinol (Lausanne).* (2018) 9:400. doi: 10.3389/fendo.2018.00400
4. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem.* (2000) 275:223–8. doi: 10.1074/jbc.275.1.223
5. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J.* (2000) 348:607–14. doi: 10.1042/bj3480607
6. 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.* (2002) 63:1259–72. doi: 10.1016/S0006-2952(02)00858-4
7. Guigas B, Detaille D, Chauvin C, Batandier C, De Oliveira F, Fontaine E, et al. Metformin inhibits mitochondrial permeability transition and cell death: a pharmacological *in vitro* study. *Biochem J.* (2004) 382:877–84. doi: 10.1042/BJ20040885
8. Carvalho C, Correia S, Santos MS, Seica R, Oliveira CR, Moreira PI. Metformin promotes isolated rat liver mitochondria impairment. *Mol Cell Biochem.* (2008) 308:75–83. doi: 10.1007/s11010-007-9614-3
9. Dykens JA, Jamieson J, Marroquin L, Nadanaciva S, Billis PA, Will Y. Biguanide-induced mitochondrial dysfunction yields increased lactate production and cytotoxicity of aerobically-poised HepG2 cells and human hepatocytes *in vitro*. *Toxicol Appl Pharmacol.* (2008) 233:203–10. doi: 10.1016/j.taap.2008.08.013
10. Palenickova E, Cahova M, Drahota Z, Kazdova L, Kalous M. Inhibitory effect of metformin on oxidation of NADH-dependent substrates in rat liver homogenate. *Physiol Res.* (2011) 60:835–9.
11. Stephenne X, Foretz M, Taleux N, van der Zon GC, Sokal E, Hue L, et al. Metformin activates AMP-activated protein kinase in primary human hepatocytes by decreasing cellular energy status. *Diabetologia* (2011) 54:3101–10. doi: 10.1007/s00125-011-2311-5
12. Hirsch A, Hahn D, Kempna P, Hofer G, Nuoffer JM, Mullis PE, et al. Metformin inhibits human androgen production by regulating steroidogenic enzymes HSD3B2 and CYP17A1 and complex I activity of the respiratory chain. *Endocrinology* (2012) 153:4354–66. doi: 10.1210/en.2012-1145
13. Bridges HR, Jones AJ, Pollak MN, Hirst J. Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria. *Biochem J.* (2014) 462:475–87. doi: 10.1042/BJ20140620
14. Wessels B, Ciapaite J, van den Broek NM, Nicolay K, Prompers JJ. Metformin impairs mitochondrial function in skeletal muscle of both lean and diabetic rats in a dose-dependent manner. *PLoS ONE* (2014) 9:e100525. doi: 10.1371/journal.pone.0100525
15. Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, et al. Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *Elife (Cambridge)* (2014) 3:e02242. doi: 10.7554/eLife.02242
16. Matsuzaki S, Humphries KM. Selective inhibition of deactivated mitochondrial complex I by biguanides. *Biochemistry* (2015) 54:2011–21. doi: 10.1021/bi501473h
17. Boukalova S, Stursa J, Werner L, Ezrova Z, Cerny J, Bezawork-Geleta A, et al. Mitochondrial targeting of metformin enhances its activity against pancreatic cancer. *Mol Cancer Ther.* (2016) 15:2875–86. doi: 10.1158/1535-7163.MCT-15-1021
18. Ling S, Shan Q, Liu P, Feng T, Zhang X, Xiang P, et al. Metformin ameliorates arsenic trioxide hepatotoxicity via inhibiting mitochondrial complex I. *Cell Death Dis.* (2017) 8:e3159. doi: 10.1038/cddis.2017.482
19. Cameron AR, Logie L, Patel K, Erhardt S, Bacon S, Middleton P, et al. Metformin selectively targets redox control of complex I energy transduction. *Redox Biol.* (2018) 14:187–97. doi: 10.1016/j.redox.2017.08.018
20. Wu Y, Gao WN, Xue YN, Zhang LC, Zhang JJ, Lu SY, et al. SIRT3 aggravates metformin-induced energy stress and apoptosis in ovarian cancer cells. *Exp. Cell Res.* (2018) 367:137–49 doi: 10.1016/j.yexcr.2018.03.030
21. Pryor HJ, Smyth JE, Quinlan PT, Halestrap AP. Evidence that the flux control coefficient of the respiratory chain is high during gluconeogenesis from lactate in hepatocytes from starved rats. Implications for the hormonal control of gluconeogenesis and action of hypoglycaemic agents. *Biochem J.* (1987) 247:449–57. doi: 10.1042/bj2470449
22. Hou WL, Yin J, Alimujiang M, Yu XY, Ai LG, Bao YQ, et al. Inhibition of mitochondrial complex I improves glucose metabolism independently of AMPK activation. *J Cell Mol Med.* (2018) 22:1316–28. doi: 10.1111/jcmm.13432
23. Chauvin C, De Oliveira F, Ronot X, Mousseau M, Leverve X, Fontaine E. Rotenone inhibits the mitochondrial permeability transition-induced cell death in U937 and KB cells. *J Biol Chem.* (2001) 276:41394–8. doi: 10.1074/jbc.M106417200
24. Li N, Ragheb K, Lawler G, Sturgis J, Rajwa B, Melendez JA, et al. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. *J Biol Chem.* (2003) 278:8516–25. doi: 10.1074/jbc.M210432200
25. Deng YT, Huang HC, Lin JK. Rotenone induces apoptosis in MCF-7 human breast cancer cell-mediated ROS through JNK and p38 signaling. *Mol Carcinog.* (2010) 49:141–51. doi: 10.1002/mc.20583
26. Xu A, Szczepanek K, Maceyka MW, Ross T, Bowler E, Hu Y, et al. Transient complex I inhibition at the onset of reperfusion by extracellular acidification decreases cardiac injury. *Am J Physiol Cell Physiol.* (2014) 306:C1142–53. doi: 10.1152/ajpcell.00241.2013
27. Hu W, Tian H, Yue W, Li L, Li S, Gao C, et al. Rotenone induces apoptosis in human lung cancer cells by regulating autophagic flux. *IUBMB Life* (2016) 68:388–93. doi: 10.1002/iub.1493
28. Rekuviene E, Ivanoviene L, Borutaite V, Morkuniene R. Rotenone decreases ischemia-induced injury by inhibiting mitochondrial

permeability transition in mature brains. *Neurosci Lett.* (2017) 653:45–50. doi: 10.1016/j.neulet.2017.05.028
29. Gong L, Goswami S, Giacomini KM, Altman RB, Klein TE. Metformin pathways: pharmacokinetics and pharmacodynamics. *Pharmacogenet Genomics* (2012) 22:820–7. doi: 10.1097/FPC.0b013e3283559b22
30. Shu Y, Sheardown SA, Brown C, Owen RP, Zhang S, Castro RA, et al. Effect of genetic variation in the organic cation transporter 1 (OCT1) on metformin action. *J Clin Invest.* (2007) 117:1422–31. doi: 10.1172/JCI 30558
31. Higgins JW, Bedwell DW, Zamek-Gliszczynski MJ. Ablation of both organic cation transporter (OCT)1 and OCT2 alters metformin pharmacokinetics but has no effect on tissue drug exposure and pharmacodynamics. *Drug Metab Dispos.* (2012) 40:1170–7. doi: 10.1124/dmd.112.044875
32. Jensen JB, Sundelin EI, Jakobsen S, Gormsen LC, Munk OL, Frokiaer J, 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* (2016) 65:1724–30. doi: 10.2337/db16-0032
33. Wang DS, Jonker JW, Kato Y, Kusuhara H, Schinkel AH, Sugiyama Y. Involvement of organic cation transporter 1 in hepatic and intestinal distribution of metformin. *J Pharmacol Exp Ther.* (2002) 302:510–5. doi: 10.1124/jpet.102.034140
34. Tsuda M, Terada T, Mizuno T, Katsura T, Shimakura J, Inui K. Targeted disruption of the multidrug and toxin extrusion 1 (mate1) gene in mice reduces renal secretion of metformin. *Mol Pharmacol.* (2009) 75:1280–6. doi: 10.1124/mol.109.056242
35. Shu Y, Brown C, Castro RA, Shi RJ, Lin ET, Owen RP, et al. Effect of genetic variation in the organic cation transporter 1, OCT1, on metformin pharmacokinetics. *Clin Pharmacol Ther.* (2008) 83:273–80. doi: 10.1038/sj.clpt.6100275
36. Christensen MM, Brasch-Andersen C, Green H, Nielsen F, Damkier P, Beck-Nielsen H, et al. The pharmacogenetics of metformin and its impact on plasma metformin steady-state levels and glycosylated hemoglobin A1c. *Pharmacogenet Genomics* (2011) 21:837–50. doi: 10.1097/FPC.0b013e32834c0010
37. Shikata E, Yamamoto R, Takane H, Shigemasa C, Ikeda T, Otsubo K, et al. Human organic cation transporter (OCT1 and OCT2) gene polymorphisms and therapeutic effects of metformin. *J Hum Genet.* (2007) 52:117–22. doi: 10.1007/s10038-006-0087-0
38. Zhou K, Donnelly LA, Kimber CH, Donnan PT, Doney AS, Leese G, et al. Reduced-function SLC22A1 polymorphisms encoding organic cation transporter 1 and glycemic response to metformin: a GoDARTS study. *Diabetes* (2009) 58:1434–9. doi: 10.2337/db08-0896
39. Funk RS, Krise JP. Cationic amphiphilic drugs cause a marked expansion of apparent lysosomal volume: implications for an intracellular distribution-based drug interaction. *Mol Pharm.* (2012) 9:1384–95. doi: 10.1021/mp200641e
40. Pentikainen PJ, Neuvonen PJ, Penttila A. Pharmacokinetics of metformin after intravenous and oral administration to man. *Eur J Clin Pharmacol.* (1979) 16:195–202. doi: 10.1007/BF00562061
41. Tucker GT, Casey C, Phillips PJ, Connor H, Ward JD, Woods HF. Metformin kinetics in healthy subjects and in patients with diabetes mellitus. *Br J Clin Pharmacol.* (1981) 12:235–46. doi: 10.1111/j.1365-2125.1981.tb01206.x
42. Scheen AJ. Clinical pharmacokinetics of metformin. *Clin Pharmacokinet.* (1996) 30:359–71. doi: 10.2165/00003088-199630050-00003
43. Gormsen LC, Sundelin EI, Jensen JB, Vendelbo MH, Jakobsen S, Munk OL, et al. *In vivo* imaging of human 11c-metformin in peripheral organs: dosimetry, biodistribution, and kinetic analyses. *J Nucl Med.* (2016) 57:1920–6. doi: 10.2967/jnumed.116.177774
44. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. *Diabetologia* (2017) 60:1577–85. doi: 10.1007/s00125-017-4342-z
45. Wilcock C, Wyre ND, Bailey CJ. Subcellular distribution of metformin in rat liver. *J Pharm Pharmacol.* (1991) 43:442–4. doi: 10.1111/j.2042-7158.1991.tb03507.x
46. Davidoff F. Effects of guanidine derivatives on mitochondrial function. 3. The mechanism of phenethylbiguanide accumulation and its relationship to *in vitro* respiratory inhibition. *J Biol Chem.* (1971) 246:4017–27.
47. Cheng G, Zielonka J, Ouari O, Lopez M, McAllister D, Boyle K, et al. Mitochondria-targeted analogues of metformin exhibit enhanced antiproliferative and radiosensitizing effects in pancreatic cancer cells. *Cancer Res.* (2016) 76:3904–15. doi: 10.1158/0008-5472.CAN-15-2534
48. Boyle KA, Van Wickle J, Hill RB, Marchese A, Kalyanaraman B, Dwinell MB. Mitochondria-targeted drugs stimulate mitophagy and abrogate colon cancer cell proliferation. *J Biol Chem.* (2018) 293:14891–904. doi: 10.1074/jbc.RA117.001469
49. Schafer G. Site-specific uncoupling and inhibition of oxidative phosphorylation by biguanides. II. *Biochim Biophys Acta* (1969) 172:334–7. doi: 10.1016/0005-2728(69)90077-2
50. Detaille D, Guigas B, Chauvin C, Batandier C, Fontaine E, Wiernsperger N, et al. Metformin prevents high-glucose-induced endothelial cell death through a mitochondrial permeability transition-dependent process. *Diabetes* (2005) 54:2179–87. doi: 10.2337/diabetes.54.7.2179
51. Giorgio V, von Stockum S, Antoniel M, Fabbro A, Fogolari F, Forte M, et al. Dimers of mitochondrial ATP synthase form the permeability transition pore. *Proc Natl Acad Sci USA.* (2013) 110:5887–92. doi: 10.1073/pnas.1217823110
52. Vinogradov AD, Grivennikova VG. The mitochondrial complex I: progress in understanding of catalytic properties. *IUBMB Life* (2001) 52:129–34. doi: 10.1080/15216540152845920
53. Galkin A, Meyer B, Wittig I, Karas M, Schagger H, Vinogradov A, et al. Identification of the mitochondrial ND3 subunit as a structural component involved in the active/deactive enzyme transition of respiratory complex I. *J Biol Chem.* (2008) 283:20907–13. doi: 10.1074/jbc.M803190200
54. Hurd TR, Requejo R, Filipovska A, Brown S, Prime TA, Robinson AJ, et al. Complex I within oxidatively stressed bovine heart mitochondria is glutathionylated on Cys-531 and Cys-704 of the 75-kDa subunit: potential role of CYS residues in decreasing oxidative damage. *J Biol Chem.* (2008) 283:24801–15. doi: 10.1074/jbc.M803432200
55. Petronilli V, Penzo D, Scorrano L, Bernardi P, Di Lisa F. The mitochondrial permeability transition, release of cytochrome c and cell death. Correlation with the duration of pore openings *in situ*. *J Biol Chem.* (2001) 276:12030–4. doi: 10.1074/jbc.M010604200
56. Dumas JF, Argaud L, Cottet-Rousselle C, Vial G, Gonzalez C, Detaille D, et al. Effect of transient and permanent permeability transition pore opening on NAD(P)H localization in intact cells. *J Biol Chem.* (2009) 284:15117–25. doi: 10.1074/jbc.M900926200
57. Fontaine E, Eriksson O, Ichas F, Bernardi P. Regulation of the permeability transition pore in skeletal muscle mitochondria. Modulation by electron flow through the respiratory chain complex i. *J Biol Chem.* (1998) 273:12662–8. doi: 10.1074/jbc.273.20.12662
58. Batandier C, Leverve X, Fontaine E. Opening of the mitochondrial permeability transition pore induces reactive oxygen species production at the level of the respiratory chain complex I. *J Biol Chem.* (2004) 279:17197–204. doi: 10.1074/jbc.M310329200
59. Kantrow SP, Piantadosi CA. Release of cytochrome c from liver mitochondria during permeability transition. *Biochem Biophys Res Commun.* (1997) 232:669–71. doi: 10.1006/bbrc.1997.6353
60. De Giorgi F, Lartigue L, Bauer MK, Schubert A, Grimm S, Hanson GT, et al. The permeability transition pore signals apoptosis by directing Bax translocation and multimerization. *FASEB J.* (2002) 16:607–9. doi: 10.1096/fj.01-0269fje
61. Lablanche S, Cottet-Rousselle C, Lamarche F, Benhamou PY, Halimi S, Leverve X, et al. Protection of pancreatic INS-1 beta-cells from glucose- and fructose-induced cell death by inhibiting mitochondrial permeability transition with cyclosporin A or metformin. *Cell Death Dis.* (2011) 2:e134. doi: 10.1038/cddis.2011.15
62. Lamarche F, Carcenac C, Gonthier B, Cottet-Rousselle C, Chauvin C, Barret L, et al. Mitochondrial permeability transition pore inhibitors prevent ethanol-induced neuronal death in mice. *Chem Res Toxicol.* (2013) 26:78–88. doi: 10.1021/tx300395w
63. Rasola A, Bernardi P. Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis. *Cell Calcium* (2011) 50:222–33. doi: 10.1016/j.ceca.2011.04.007
64. Alavian KN, Beutner G, Lazrove E, Sacchetti S, Park HA, Licznerski P, et al. An uncoupling channel within the c-subunit ring of the F1FO ATP synthase is the mitochondrial permeability transition pore. *Proc Natl Acad Sci USA.* (2014) 111:10580–5. doi: 10.1073/pnas.14015 91111

65. Li B, Chauvin C, De Paulis D, De Oliveira F, Gharib A, Vial G, et al. Inhibition of complex I regulates the mitochondrial permeability transition through a phosphate-sensitive inhibitory site masked by cyclophilin D. *Biochim Biophys Acta* (2012) 1817:1628–34. doi: 10.1016/j.bbabio.2012.05.011
66. Walter L, Nogueira V, Leverve X, Heitz MP, Bernardi P, Fontaine E. Three classes of ubiquinone analogs regulate the mitochondrial permeability transition pore through a common site. *J Biol Chem.* (2000) 275:29521–7. doi: 10.1074/jbc.M004128200
67. Walter L, Miyoshi H, Leverve X, Bernard P, Fontaine E. Regulation of the mitochondrial permeability transition pore by ubiquinone analogs. A progress report. *Free Radic Res.* (2002) 36:405–12. doi: 10.1080/10715760290021252
68. Devun F, Walter L, Belliere J, Cottet-Rousselle C, Leverve X, Fontaine E. Ubiquinone analogs: a mitochondrial permeability transition pore-dependent pathway to selective cell death. *PLoS ONE* (2010) 5:e11792. doi: 10.1371/journal.pone.0011792
69. Belliere J, Devun F, Cottet-Rousselle C, Batandier C, Leverve X, Fontaine E. Prerequisites for ubiquinone analogs to prevent mitochondrial permeability transition-induced cell death. *J Bioenerg Biomembr.* (2012) 44:207–12. doi: 10.1007/s10863-012-9406-7
70. Loubiere C, Clavel S, Gilleron J, Harisseh R, Fauconnier J, Ben-Sahra I, et al. The energy disruptor metformin targets mitochondrial integrity via modification of calcium flux in cancer cells. *Sci Rep.* (2017) 7:5040. doi: 10.1038/s41598-017-05052-2
71. El-Mir MY, Detaille D, R-Villanueva G, Delgado-Esteban M, Guigas B, Attia S, et al. Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons. *J Mol Neurosci.* (2008) 34:77–87. doi: 10.1007/s12031-007-9002-1
72. Morales AI, Detaille D, Prieto M, Puente A, Briones E, Arevalo M, et al. Metformin prevents experimental gentamicin-induced nephropathy by a mitochondria-dependent pathway. *Kidney Int.* (2010) 77:861–9. doi: 10.1038/ki.2010.11
73. Lablanche S, Cottet-Rousselle C, Argaud L, Laporte C, Lamarche F, Richard MJ, et al. Respective effects of oxygen and energy substrate deprivation on beta cell viability. *Biochim Biophys Acta* (2015) 1847:629–39. doi: 10.1016/j.bbabio.2015.04.002
74. Elmadhun NY, Sabe AA, Lassaletta AD, Chu LM, Sellke FW. Metformin mitigates apoptosis in ischemic myocardium. *J Surg Res.* (2014) 192:50–8. doi: 10.1016/j.jss.2014.05.005
75. Cahova M, Palenickova E, Dankova H, Sticova E, Burian M, Drahota Z, et al. Metformin prevents ischemia reperfusion-induced oxidative stress in the fatty liver by attenuation of reactive oxygen species formation. *Am J Physiol Gastrointest Liver Physiol.* (2015) 309:G100–11. doi: 10.1152/ajpgi.00329.2014
76. Chen D, Xia D, Pan Z, Xu D, Zhou Y, Wu Y, et al. Metformin protects against apoptosis and senescence in nucleus pulposus cells and ameliorates disc degeneration *in vivo*. *Cell Death Dis.* (2016) 7:e2441. doi: 10.1038/cddis.2016.334
77. Wang X, Yang L, Kang L, Li J, Yang L, Zhang J, et al. Metformin attenuates myocardial ischemia-reperfusion injury via up-regulation of antioxidant enzymes. *PLoS ONE* (2017) 12:e0182777. doi: 10.1371/journal.pone.0182777
78. Gong J, Robbins LA, Lugea A, Waldron RT, Jeon CY, Pandol SJ. Diabetes, pancreatic cancer, and metformin therapy. *Front Physiol.* (2014) 5:426. doi: 10.3389/fphys.2014.00426
79. Foretz M, Hebrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, et al. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest.* (2010) 120:2355–69. doi: 10.1172/JCI40671
80. Bibi F, Ullah I, Kim MO, Naseer MI. Metformin attenuate PTZ-induced apoptotic neurodegeneration in human cortical neuronal cells. *Pak J Med Sci.* (2017) 33:581–5. doi: 10.12669/pjms.333.11996

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 07 May 2019
doi: 10.3389/fendo.2019.00294

Role of Mitochondria in the Mechanism(s) of Action of Metformin

Guillaume Vial[1,2†], *Dominique Detaille*[3†] *and Bruno Guigas*[4*]

[1] *Laboratoire Hypoxie-Physiopathologies Cardiovasculaires et Respiratoires HP2, Faculté de Médecine et de Pharmacie, INSERM U1042, La Tronche, France,* [2] *Laboratoire Hypoxie-Physiopathologies Cardiovasculaires et Respiratoires HP2, Faculté de Médecine et de Pharmacie, Université Grenoble-Alpes, La Tronche, France,* [3] *Centre de Recherche Cardio-Thoracique de Bordeaux, INSERM U1045, Université de Bordeaux, Bordeaux, France,* [4] *Department of Parasitology, Leiden University Medical Center, Leiden, Netherlands*

Metformin is a drug from the biguanide family that is used for decades as the first-line therapeutic choice for the treatment of type 2 diabetes. Despite its worldwide democratization, owing to its clinical efficacy, high safety profile and cheap cost, the exact mechanism(s) of action of this anti-hyperglycemic molecule with pleiotropic properties still remains to be fully elucidated. The concept that metformin would exert some of its actions though modulation of the mitochondrial bioenergetics was initially forged in the 50s but undeniably revived at the beginning of the twenty-first century when it was shown to induce a weak but specific inhibition of the mitochondrial respiratory-chain complex 1. Furthermore, metformin has been reported to reduce generation of reactive oxygen species at the complex 1 and to prevent mitochondrial-mediated apoptosis, suggesting that it can protect against oxidative stress-induced cell death. Nevertheless, despite some recent progress and the demonstration of its key role in the inhibition of hepatic gluconeogenesis, the exact nature of the mitochondrial interaction between the drug and the complex 1 is still poorly characterized. Recent studies reported that metformin may also have anti-neoplastic properties by inhibiting cancer cell growth and proliferation, at least partly through its mitochondrial action. As such, many trials are currently conducted for exploring the repositioning of metformin as a potential drug for cancer therapy. In this mini-review, we discuss both historical and more recent findings on the central role played by the interaction between metformin and the mitochondria in its cellular mechanism of action.

Keywords: biguanides, respiratory-chain complex 1, bioenergetics, AMPK, cancer

OPEN ACCESS

Edited by:
Frederic Bost, Centre National de la Recherche Scientifique (CNRS), France

Reviewed by:
Bilikere S. Dwarakanath, Shanghai Proton and Heavy Ion Center (SPHIC), China
Nathalie M. Mazure, Centre National de la Recherche Scientifique (CNRS), France

***Correspondence:**
Bruno Guigas
b.g.a.guigas@lumc.nl

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Diabetes, a section of the journal Frontiers in Endocrinology

Received: *12 December 2018*
Accepted: *24 April 2019*
Published: *07 May 2019*

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

INTRODUCTION

Historically, the origins of metformin (dimethylbiguanide) came from the Middle Age where medieval doctors used extract from the French Lilac *Galega officinalis* to treat various diseases (1). At the beginning of the twentieth century, the plant was found to be rich in guanidine, an active ingredient that was later reported to have potent anti-hyperglycemic properties. Guanidine derivatives gave rise to the biguanide family, among which metformin is currently the only therapeutic survivor for the treatment of type 2 diabetes. Indeed, after withdrawal of buformin and phenformin at the end of the 70′s, metformin hydrochloride gradually became the most widely prescribed oral antidiabetic agent, due to its efficient glucose-lowering effect, weight-neutral characteristic, high safety profile associated with low risk of hypoglycemia, and cost-effectiveness as

a generic drug (2). Since then, metformin is well recognized for its ability to lower hyperglycemia by decreasing hepatic glucose production while reducing glucotoxicity in different tissues, a feature that might explain some of its cardioprotective benefits (2, 3). However, despite its worldwide democratization, the exact mechanism(s) of action of this molecule with apparent pleiotropic properties still remains to be fully elucidated. As many drugs, the cellular effects of metformin rely on its unique physicochemical characteristics, which include a high hydrophilicity, some metal-binding properties and a pKa within the physiological pH range, implying that the molecule exists solely in its positively charged cationic form (4). Due to its poor lipophilicity, metformin does not cross cell membranes by simple passive diffusion and its bio-distribution relies on tissue-specific transporters, including plasma membrane monoamine transporter (PMAT) in the intestine, organic cation transporter 1 (OCT1) in the liver, and both organic cation transporter 2 (OCT2) and multidrug and toxin extruder (MATE)1/2 in the kidneys (4, 5). By contrast, phenformin exhibits a higher lipophilicity than metformin, owing to its larger phenylethyl side chain, and is therefore crossing more easily lipid membrane bilayer, a property that might explain their differences in terms of selectivity and potency. Various underlying mechanisms have been suggested for metformin throughout the six decades following its first commercialization but a consensus only started to emerge during the last years, placing mitochondria at the heart of metformin's cellular actions.

THE MITOCHONDRIAL RESPIRATORY-CHAIN COMPLEX 1 AS PRIMARY TARGET OF METFORMIN

At the beginning of 2000, the group of Xavier Leverve was the first to report that metformin selectively inhibits the mitochondrial respiratory-chain complex 1 and, as a result, decreases NADH oxidation, reduces proton gradient across the inner mitochondrial membrane, and decreases oxygen consumption rate (6) (**Figure 1**). This major breakthrough was rapidly complemented by a supportive study from Halestrap's group published couple of months later (7). Although the inhibitory effect of metformin on complex 1 was first evidenced in rat hepatocytes in these two seminal studies, it was thereafter confirmed in various species and plenty of biological models, including lately in cancer cells (**Table 1**). Importantly, metformin only exerts a weak and reversible selective inhibition of complex 1 (IC_{50} ~20 mM), making it a peculiar type of inhibitor that does not resemble the canonical ones like rotenone and piericidin A (IC_{50} ~2 μM), which are both uncharged and highly hydrophobic molecules (24). It is worth mentioning that, although the discovery of complex 1 inhibition by metformin undoubtedly constituted a major advance in the understanding of its cellular mode of action, some inhibitory effects of biguanides on mitochondrial oxidative phosphorylation (OXPHOS) were already reported by Gunnar Hollunger, a Swedish scientist at the University of Lund, as early as 1955 (25), and by the German biochemist Günter Schäfer in the 60's (26).

Targeting the Mitochondria for Selective Inhibition of Mitochondrial Complex 1

How exactly metformin gets into the mitochondria and whether it inhibits complex 1 directly or not remains unclear and is still a matter of debate (27). Very high concentrations of metformin (20–100 mM) were reported to directly inhibit complex 1 activity in isolated mitochondria or in inside-out structured sub-mitochondrial particles (SMPs) whereas clinically relevant drug concentrations (<100 μM) did not (**Table 1**). By contrast, micromolar concentrations of the drug are sufficient to achieve a dose- and time-dependent *in situ* inhibition of mitochondrial complex 1 in various cell types (6, 10, 15, 28, 29) or *in vivo* in skeletal muscle from healthy and diabetic rats (30). Among the possible explanations, the positive charge of metformin was proposed to account for its slow accumulation within the matrix of energized mitochondria of intact cells, driven by their transmembrane electrochemical potential ($\Delta\Psi$) (7, 14). Indeed, according to thermodynamic laws and the Nernst equation, a ~1,000-fold accumulation of a positively charged molecule might theoretically occur in energized mitochondria with a physiologically relevant $\Delta\Psi$, suggesting that metformin could reach millimolar concentration in the organelle despite a cytoplasmic level within the micromolar range (27). Furthermore, a $\Delta\Psi$-driven mitochondrial import of the biguanide might also provide an explanation for its weak inhibitory effect on complex 1, the reduction in mitochondrial membrane potential induced by the drug limiting its subsequent buildup. However, no accumulation of radioactively-labeled metformin was observed in mitochondria isolated from *Xenopus laevis* oocytes and exposed to concentrations of the drug that inhibit complex 1 (10). Therefore, even if a direct effect of metformin on complex 1 turns out possible, it seems to be highly facilitated in intact cells regardless of the exact mechanism involved in this process. Although the low accumulation of metformin into mitochondria could primarily be explained by the slow permeation of the drug across the plasma membrane, some studies have also suggested the existence of a specific transport system mediating its mitochondrial import. As such, the observation that the inhibitory effect of metformin on complex 1 was temperature-dependent in intact *Xenopus laevis* oocytes and that low concentration (50 μM) was able to directly inhibit complex 1 activity in isolated mitochondria when delivered as a liposomal-encapsulated form that can eventually fuse with the organelle led to the hypothesis of an endocytic vesicular routing of the drug from the plasma membrane to the mitochondria (10). However, the molecular components involved in this putative process still remain obscure and would deserve extensive investigation, including in mammalian cells. On the other hand, it has also been reported that intra-mitochondrial accumulation of phenformin, another biguanide, could at least partly be mediated by the mitochondrial organic cation/carnitine transporter 1 (OCTN1) (31). More recently, a protein-mediated mitochondrial import of the biguanide was also suggested based on the fact that direct conjugation of a phenyl group and bis-substitution of the biguanide moiety on the molecule prevent its uptake into mitochondria, irrespective of the compound hydrophobicity (32). However, whether this could

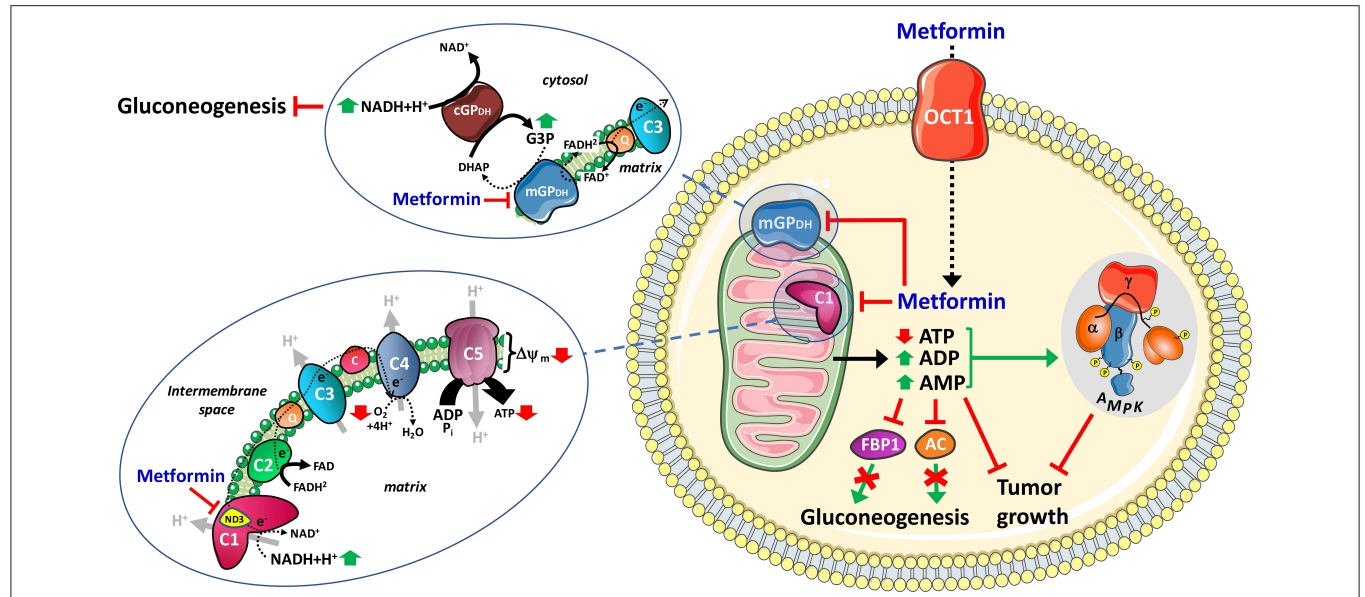

FIGURE 1 | Mitochondrial mechanisms of action of metformin. After cellular uptake, mainly through OCT1 in hepatocytes, the mitochondria is the primary target of metformin which exerts specific inhibition on the respiratory-chain complex 1, presumably through direct interaction with the ND3 core subunit, and on mitochondrial glycerophosphate dehydrogenase (mGP_{DH}). The inhibition of complex 1 decreases NADH oxidation, proton pumping across the inner mitochondrial membrane and oxygen consumption rate, resulting in lower proton gradient ($\Delta\psi$) and reduction of proton-driven ATP synthesis from ADP and inorganic phosphate (Pi). The inhibition of mGP_{DH} modulates cytosolic and mitochondrial redox state resulting in increased cytosolic NADH. FBP1, fructose-1,6-bisphosphatase-1. AC, adenylate cyclase.

also occur for metformin was not assessed and remains therefore to be investigated.

Molecular Interaction Between Metformin and the Respiratory-Chain Complex 1

The mammalian respiratory-chain complex 1 (NADH:ubiquinone oxidoreductase) is a large L-shaped membrane-bound redox enzyme composed of at least 45 different subunits that couples the transfer of electrons from NADH to the ubiquinone pool with a transfer of protons from the mitochondrial matrix toward the intermembrane space (33). The complex 1 exists in two distinct forms: a fully competent active one and a so-called "deactive" D-form where the enzyme is catalytically incompetent but can be activated by a slow reaction of NADH oxidation coupled to ubiquinone reduction (34). As metformin inhibits NADH oxidation by complex 1 in isolated mitochondria from bovine heart, yeast *Pichia pastoris*, bacterium *Escherichia coli* (14, 32), as well as from *C. elegans* (35), it is likely that the molecule binds to some of the phylogenetically conserved "core" subunits of the complex rather than to mammalian-specific accessory ones (33). While it has been shown that metformin did not alter the structural integrity of the whole complex (14), the exact molecular interactions between the drug and the complex 1 remain to be elucidated. In order to investigate how metformin, and other biguanides, could interact with complex 1 for regulating its activity, the group of Judy Hirst has elegantly dissected the effects of the drug at different levels of the catalytic cycle of the enzyme. They demonstrated that metformin is a reversible non-competitive inhibitor that probably binds to some amphipathic regions of the enzyme, i.e., where some hydrophilic and hydrophobic amino acids are in close proximity, and inhibits a rate-limiting step coupled to ubiquinone reduction, but does not competitively bind to the ubiquinone-binding site on complex 1 (14). Moreover, metformin rather stimulates the NADH:FeCN oxidoreduction reaction and does not alter the thermal stability of the flavin site, except at extremely high non-relevant concentration (200 mM), indicating that NADH oxidation occurring at the flavin site is probably not involved in the inhibition of complex 1 by the drug. Similarly, metformin does not modulate the FeS cluster of the NADH-reduced complex 1, suggesting that the intramolecular electron transfer is not impaired (14). However, using SMPs, the authors showed that inhibition of NADH oxidation by metformin is immediate when the drug is added prior to the initiation of catalysis but is delayed once catalysis has already started (14). Altogether, this strongly suggests that the inhibition depends on the catalytic status of complex 1, occurring primarily when the enzyme is in its "deactive" conformation with redox and proton transfer domains no longer efficiently coupled (14). Ultimately, the authors proposed that the Cys39-containing matrix loop of subunit ND3 located within the amphipathic region between the redox and proton-transfer domains might be the binding site for metformin on complex 1, stabilizing the enzyme in an open-loop deactive conformation state (14). It is worth mentioning that most of the above-mentioned mechanistic studies were performed using isolated organelles and high concentrations of the drug and that such experimental *in vitro* conditions might not always reflect the physiological *in situ* environment. For instance, complex 1 forms respiratory-chain supercomplexes together

with complexes 3 and 4 (36), a supramolecular organization that is lost in SMPs and may affect the interaction of metformin with complex 1 and/or the regulatory effect of the drug on mitochondrial OXPHOS.

Modulation of ROS Production at Complex 1 by Metformin

Besides their central role in cellular energy homeostasis, mitochondria are also the main source of reactive oxygen species (ROS) which, on top of potentially causing oxidative damages, could also play a key role as signaling molecules in various pathways (37). Superoxide anions are primarily generated by the mitochondria, mostly at complexes 1 and 3 of the electron transfer chain (ETC) where electrons are leaking and could react with oxygen. It is now well recognized that complex 1 can produce superoxide by both forward (site I_F) and reverse electron (site I_Q) fluxes, depending on substrates used to fuel the ETC (38). As such, rotenone can either increase or decrease mitochondrial ROS production at complex 1, depending on whether glutamate-malate (forward) or succinate (reverse) are provided as respiratory-chain substrates, respectively. By contrast, it has been shown that metformin specifically decreases the ROS production driven by the reverse electron transfer (RET) but without increasing ROS generation through the forward direction (39). Interestingly, a similar lowering effect on ROS production at the complex 1 than the one observed with metformin was also recently reported for imeglimin, a molecule belonging to the tetrahydrotriazine-containing novel class of oral glucose-lowering agents (40), suggesting that inhibition of this RET-mediated ROS production may play a role in the mechanisms of action of the two antidiabetic drugs, notably by conferring protection against oxidative stress-related cell death (15, 28, 29). In line with this, a new generation of oxidative stress inhibitors that specifically neutralize ROS produced *via* RET at the I_Q site within complex 1 has been shown to lower oxidative damage, inhibit cellular stress signaling and protect against ischemia-reperfusion heart injury (41). Furthermore, it has also been suggested that some of the anti-inflammatory effect of metformin observed in lipopolysaccharide-stimulated bone-marrow derived macrophages could result from the specific inhibition of RET-derived ROS production at the complex 1 (42). Taken together, these findings suggest that targeting RET-linked ROS occurring at the mitochondrial respiratory-chain complex 1 using metformin or metformin-like molecules might be therapeutically relevant in the context of both cardiometabolic and inflammatory diseases.

MITOCHONDRIAL EFFECTS OF METFORMIN AND REGULATION OF HEPATIC GLUCONEOGENESIS

Metformin exerts its anti-hyperglycemic action primarily through reduction of hepatic glucose production (3). A major breakthrough occurred in 2001 when Zhou and colleagues reported that metformin increased the AMP-activated protein kinase (AMPK) activity in hepatocytes, a feature associated with inhibition of gluconeogenesis (43). AMPK is a protein kinase that functions as energy gauge which constantly senses the cellular energy status by monitoring AMP, ADP, and ATP levels (44, 45). Once activated in response to decrease in ATP and concomitant rise in intracellular ADP and AMP levels, AMPK inhibits ATP-consuming anabolic processes and promotes ATP-generating catabolic pathways by direct phosphorylation of a broad range of downstream effectors that are involved in the regulation of various metabolic processes, ultimately leading to restoration of cellular energy balance (44). It took a decade of controversy before the general acceptance that AMPK activation by metformin results from increased ADP:ATP and AMP:ATP ratios secondary to inhibition of the mitochondrial respiratory-chain complex 1 (3). Of note, only biguanides with physicochemical characteristics allowing them to enter the mitochondria and to inhibit complex 1 were shown to activate AMPK (32). However, using liver-specific AMPK knockout mice, Foretz and colleagues demonstrated that metformin lowers gluconeogenesis by an AMPK-independent mechanism involving a decrease in cellular energy state, a strong correlation being observed between the increase in cellular [AMP]:[ATP] and the inhibition of gluconeogenesis (46). Altogether, although metformin can activate AMPK, it is therefore neither necessary nor sufficient for inducing acute inhibition of gluconeogenesis (**Figure 1**). In line with this, two other studies also demonstrated that metformin can inhibit hepatic glucose production through AMPK-independent mechanisms: one by AMP-mediated inhibition of adenylate cyclase and subsequent reduction in glucagon-increased cyclic adenosine monophosphate (cAMP) levels (47); the other one through modulation of cytosolic redox state *via* direct inhibition of the mitochondrial glycerol-3-phosphate dehydrogenase (mGP_{DH}) (8) (**Figure 1**). mGP_{DH} is a flavin-linked respiratory-chain dehydrogenase belonging to the glycerol phosphate shuttle that couples the oxidation of glycerol-3-phosphate to dihydroxyacetone with reduction of FAD to $FADH_2$ and the transfer of electrons to coenzyme Q of the ETC, contributing as such to the maintenance of the redox potential across the inner mitochondrial membrane (48). Remarkably, Shulman's group reported that metformin exerts an *in vitro* non-competitive inhibition of the enzyme, with a K_i value ($\sim$40 μM) within the clinical range of drug concentrations, leading to increased hepatic cytosolic $NADH/NAD^+$ ratio and impaired gluconeogenesis from redox-dependent substrates, such as lactate and glycerol, in rats (8). In a recent follow-up study, they showed that both acute and chronic treatment with metformin also inhibit hepatic gluconeogenesis in a redox-dependent manner in diabetic rats, without apparent changes in mitochondrial citrate synthase flux and hepatic nucleotide concentrations (49). By contrast, Sakamoto's group recently provided new supportive elements strengthening the key role of mitochondria-mediated modulation of cellular energy homeostasis in the inhibition of hepatic gluconeogenesis by metformin. Indeed, in an elegant study using knock-in mice, the authors demonstrated that a

TABLE 1 | *Ex vivo* and *in vitro* mitochondrial effects of metformin.

	Cell type	Metformin (mM)	Duration	Effects	Reference
Healthy cells/organelles	Primary rat hepatocytes	0.1–10	45 min	Inhibition of JO_2 Inhibition of C1-linked mJO_2	(6)
		0.05	2 h	Non-competitive inhibition of mGPDH activity Inhibition of mGPDH-linked JO_2	(8)
	Mouse hepatocytes	5	30 min	Inhibition of JO_2 Inhibition of C1-linked mJO_2	(9)
	Human hepatocytes	5	30 min	Inhibition of JO_2 Inhibition of C1-linked mJO_2	(9)
	Xenopus laevis oocytes	0.05	4–18 h	Inhibition of C1	(10)
		10	0.5–3 h	Inhibition of C1	(10)
	Rat liver mitochondria	0.5–5	1 min	No effect	(11)
		5–20	?	Inhibition of C1-linked JO_2	(8)
		8–10	1 min	Inhibition of JO_2 and RCR Decrease in $\Delta\Psi$m	(11)
		10	30 min	No effect	(6)
		2–11	5 min	Inhibition of C1-linked JO_2 Decrease in NADH oxidation	(12)
		>10	5 min	No effect	(7)
		1–10	4h (cold)	Inhibition of JO_2 Inhibition of C1-linked JO_2	(7)
	Mouse muscle mitochondria	2–5	30 min	Inhibition of C1-linked JO_2 Inhibition of TCA cycle activity	(13)
	Xenopus laevis mitochondria	0.05–10	3 h	No effect	(10)
	Rat/liver heart SMPs	5–50	Immediate	Inhibition of C1 activity	(7)
	Bovine heart SMPs	100	Immediate	Inhibition of NADH oxidation	(14)
Cancer cells	Rat hepatoma H4IIE cells	0.05–0.1	24–60 h	Inhibition of C1-linked mJO_2	(7)
		2	2 h30	Inhibition of JO_2	(12)
	Mouse breast NT2196 cells	0.5–5	24–48 h	Inhibition of JO_2 Increase in uncoupled JO_2	(13)
	Human liver hepatoma HepG2 cells	2	0.5–8 h	Inhibition of JO_2	(14)
	Human oral squamous carcinoma KB cells	0.1–10	0.5–24 h	Inhibition of JO_2 Inhibition of C1-linked mJO_2 Inhibition of isolated C1 activity	(15)
	Human colorectal HCT116, prostate LNCaP, squamous SCC-74B and colon POP-092S carcinoma cells	0.2–10	1–8 h	Inhibition of JO_2	(16)
	Human breast MCF7 cells	0.5–5	24 h	Inhibition of JO_2 Increase in uncoupled JO_2 Inhibition of TCA cycle activity	(13)
		2.5–5	5 h	No effect on JO_2 Inhibition of CYP3A4 AA Epoxygenase activity	(17)
	Human thyroid FTC133 and BCAP carcinoma cells	5	48 h	Inhibition of JO_2 Lower mGPDH expression	(18)
		1–5	10 min	Inhibition of mGPDH activity	(18)
	Human lung A549 and cervical HeLa carcinoma cells	1	5–10 min	Inhibition of C1-linked mJO_2 No effect on mG3PDH-linked mJO_2	(19)
	Human pancreatic PDAC stem cells	3–10	1 h	Inhibition of JO_2 Inhibition of C1-linked mJO_2	(20)
	Human HCT116 $p53^{-/-}$ colorectal carcinoma cells	0.25–1	24 h	Inhibition of JO_2 Inhibition of C1-linked mJO_2	(21)
	Human pancreatic PANC-1 carcinoma cells	0.5–10	48 h	Inhibition of C1-linked mJO_2	(22)
		1–10	24 h	Inhibition of JO_2 Inhibition of C1-linked mJO_2	(23)
	Human pancreatic MiaPaCa-2 carcinoma cells	1–10	24 h	Inhibition of JO_2 Inhibition of C1-linked mJO_2	(23)

C1, mitochondrial respiratory-chain complex 1; $mG3P_{DH}$, mitochondrial glycerol-3-phosphate dehydrogenase; JO_2, oxygen consumption rate; mJO_2, mitochondrial oxygen consumption rate; RCR, respiratory control ratio; ROS, reactive oxygen species; TCA, tricarboxylic acid; SMPs, sub-mitochondrial particles.

point mutation in the gluconeogenic enzyme fructose-1,6-bisphosphatase-1 (F1BP) which impairs its allosteric inhibition by AMP reduced the anti-hyperglycemic effect of metformin in diabetic mice (50). Altogether, this strongly suggests that the transient rise in intracellular AMP levels resulting from the weak and reversible inhibition of the respiratory-chain complex 1 by metformin is crucial for inhibiting hepatic gluconeogenesis, either by modulating adenylate cyclase or FBP1 activity (**Figure 1**).

INHIBITION OF COMPLEX 1 BY METFORMIN AND METABOLIC REPROGRAMMING IN CANCER CELLS

A growing body of epidemiological and clinical studies reported that metformin reduces cancer risk in patients with type 2 diabetes and improves survival outcome of cancer patients with breast, ovarian, liver and colorectal tumors (51). Although an extensive overview on this topic can be found elsewhere [for recent reviews see (51–54)], it is striking that the mitochondrial effect of metformin could again play a crucial role in the anti-tumorigenic effect of the drug. Indeed, the inhibition of complex 1 was observed in many cancer cells (**Table 1**) and usually leads to reduced mitochondrial OXPHOS and ATP depletion, ultimately resulting in AMPK-mediated activation of catabolic pathways and inhibition of anabolic processes through its regulation of mechanistic target of rapamycin complex 1 (mTORC1) (54). While some AMPK- and mTORC1-independent mechanisms can also co-exist (55), this metabolic reprogramming lowers growth and proliferation of cancer cells, at least partly due to inhibition of protein and lipid synthesis. It also promotes cell cycle arrest and apoptosis in cells that cannot cope with the energetic stress (54). Wheaton and colleagues clearly showed that the reduction of tumor growth by metformin was prevented in cancer cells expressing NDI1, a metformin-resistant yeast analog of complex 1, highlighting the central role played by inhibition of this mitochondrial target in the antineoplastic effect of the drug (21). This is also consistent with another study showing that phenformin exerts its anti-tumorigenic effects by inhibiting complex 1 (56). Nevertheless, most of the effects of metformin were generally observed at supratherapeutic concentrations and the drug bioavailability in cancer cells is still questionable. Interestingly, more lipophilic derivatives of metformin targeting the mitochondria are currently under investigation with the aim of developing analogs with higher bioavailability and antitumor activity than metformin. Remarkably, some of these newly synthetized molecules were recently reported to be nearly 1,000-fold more potent than metformin in inhibiting mitochondrial complex 1 activity and to exert both anti-proliferative and radiosensitizing effects in pancreatic cancer cells (23). Altogether, developing such kind of mitochondria-targeted metformin-like drugs could pave the way for promising new therapeutic strategies that might also be relevant for various other pathologies than cancer (57).

CONCLUDING REMARKS

Although the interest around metformin has been significantly revived during the last years, principally due to the potential repositioning of this antidiabetic drug for the treatment of cancer, it still remains crucial to better decipher the mechanism by which it inhibits the mitochondrial respiratory-chain complex 1, notably the exact nature of their interaction. Elucidating this aspect may advance our understanding of how metformin regulates cellular energetics and be decisive for optimizing future drug development and therapeutic interventions, notably for cancer patients.

AUTHOR CONTRIBUTIONS

GV performed literature search and drafted the manuscript. DD performed literature search and drafted the manuscript. BG performed literature search, designed the figure, wrote, and edited the manuscript.

ACKNOWLEDGMENTS

BGs' group was/is supported by funding from the Société Francophone du Diabète (SFD), the European Federation for the Study of Diabetes (EFSD), the DiabetesFonds, and the Dutch Organization for Scientific Research (ZonMW).

REFERENCES

1. Bailey CJ. Metformin: historical overview. *Diabetologia.* (2017) 60:1566–76. doi: 10.1007/s00125-017-4318-z
2. Sanchez-Rangel E, Inzucchi SE. Metformin: clinical use in type 2 diabetes. *Diabetologia.* (2017) 60:1586–93. doi: 10.1007/s00125-017-4336-x
3. Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B. Metformin: from mechanisms of action to therapies. *Cell Metab.* (2014) 20:953–66. doi: 10.1016/j.cmet.2014.09.018
4. Graham GG, Punt J, Arora M, Day RO, Doogue MP, Duong JK, et al. Clinical pharmacokinetics of metformin. *Clin Pharmacokinet.* (2011) 50:81–98. doi: 10.2165/11534750-000000000-00000
5. Gormsen LC, Sundelin EI, Jensen JB, Vendelbo MH, Jakobsen S, Munk OL, et al. *In vivo* imaging of human 11C-metformin in peripheral organs: dosimetry, biodistribution, and kinetic analyses. *J Nucl Med.* (2016) 57:1920–6. doi: 10.2967/jnumed.116.177774
6. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem.* (2000) 275:223–8. doi: 10.1074/jbc.275.1.223
7. Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J.* (2000) 348(Pt 3):607–14. doi: 10.1042/bj3480607
8. Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature.* (2014) 510:542–6. doi: 10.1038/nature13270
9. Stephenne X, Foretz M, Taleux N, van der Zon GC, Sokal E, Hue L, et al. Metformin activates AMP-activated protein kinase in primary

human hepatocytes by decreasing cellular energy status. *Diabetologia.* (2011) 54:3101–110. doi: 10.1007/s00125-011-2311-5
10. 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.* (2002) 63:1259–72. doi: 10.1016/S0006-2952(02)00858-4
11. Carvalho C, Correia S, Santos MS, Seica R, Oliveira CR, Moreira PI. Metformin promotes isolated rat liver mitochondria impairment. *Mol Cell Biochem.* (2008) 308:75–83. doi: 10.1007/s11010-007-9614-3
12. Cameron AR, Logie L, Patel K, Erhardt S, Bacon S, Middleton P, et al. Metformin selectively targets redox control of complex I energy transduction. *Redox Biol.* (2018) 14:187–97. doi: 10.1016/j.redox.2017.08.018
13. Andrzejewski S, Gravel SP, Pollak M, St-Pierre J. Metformin directly acts on mitochondria to alter cellular bioenergetics. *Cancer Metab.* (2014) 2:12. doi: 10.1186/2049–3002-2-12
14. Bridges HR, Jones AJ, Pollak MN, Hirst J. Effects of metformin and other biguanides on oxidative phosphorylation in mitochondria. *Biochem J.* (2014) 462:475–87. doi: 10.1042/BJ20140620
15. Guigas B, Detaille D, Chauvin C, Batandier C, De Oliveira F, Fontaine E, et al. Metformin inhibits mitochondrial permeability transition and cell death: a pharmacological *in vitro* study. *Biochem J.* (2004) 382:877–84. doi: 10.1042/BJ20040885
16. Zannella VE, Dal Pra A, Muaddi H, McKee TD, Stapleton S, Sykes J, et al. Reprogramming metabolism with metformin improves tumor oxygenation and radiotherapy response. *Clin Cancer Res.* (2013) 19:6741–50. doi: 10.1158/1078-0432.CCR-13-1787
17. Guo Z, Sevrioukova IF, Denisov IG, Zhang X, Chiu TL, Thomas DG, et al. Heme binding biguanides target cytochrome P450-dependent cancer cell mitochondria. *Cell Chem Biol.* (2017) 24:1259–75 e1256. doi: 10.1016/j.chembiol.2017.08.009
18. Thakur S, Daley B, Gaskins K, Vasko VV, Boufraqech M, Patel D, et al. Metformin targets mitochondrial glycerophosphate dehydrogenase (mGPDH) to control rate of oxidative phosphorylation and growth of thyroid cancer *in vitro* and *in vivo*. *Clin Cancer Res.* (2018) 24:4030–43. doi: 10.1158/1078-0432.CCR-17-3167
19. Gui DY, Sullivan LB, Luengo A, Hosios AM, Bush LN, Gitego N, et al. Environment dictates dependence on mitochondrial complex I for NAD+ and aspartate production and determines cancer cell sensitivity to metformin. *Cell Metab.* (2016) 24:716–27. doi: 10.1016/j.cmet.2016.09.006
20. Sancho P, Burgos-Ramos E, Tavera A, Bou Kheir T, Jagust P, Schoenhals M, et al. MYC/PGC-1α balance determines the metabolic phenotype and plasticity of pancreatic cancer stem cells. *Cell Metab.* (2015) 22:590–605. doi: 10.1016/j.cmet.2015.08.015
21. Wheaton WW, Weinberg SE, Hamanaka RB, Soberanes S, Sullivan LB, Anso E, et al. Metformin inhibits mitochondrial complex I of cancer cells to reduce tumorigenesis. *Elife.* (2014) 3:e02242. doi: 10.7554/eLife.02242
22. Boukalova S, Stursa J, Werner L, Ezrova Z, Cerny J, Bezawork-Geleta A, et al. Mitochondrial targeting of metformin enhances its activity against pancreatic cancer. *Mol Cancer Ther.* (2016) 15:2875–86. doi: 10.1158/1535-7163.MCT-15-1021
23. Cheng G, Zielonka J, Ouari O, Lopez M, McAllister D, Boyle K, et al. Mitochondria-targeted analogues of metformin exhibit enhanced antiproliferative and radiosensitizing effects in pancreatic cancer cells. *Cancer Res.* (2016) 76:3904–15. doi: 10.1158/0008-5472.CAN-15-2534
24. Degli Esposti M. Inhibitors of NADH-ubiquinone reductase: an overview. *Biochim Biophys Acta.* (1998) 1364:222–35. doi: 10.1016/S0005-2728(98)00029-2
25. Hollunger G. Guanidines and oxidative phosphorylations. *Acta Pharmacol Toxicol* (*Copenh*). (1955) 11:1–84. doi: 10.1111/j.1600-0773.1955.tb02972.x
26. Schafer G. Site-specific uncoupling and inhibition of oxidative phosphorylation by biguanides. II. *Biochim Biophys Acta.* (1969) 172:334–7. doi: 10.1016/0005-2728(69)90077-2
27. Fontaine E. Metformin and respiratory chain complex I: the last piece of the puzzle? *Biochem J.* (2014) 463:e3–5. doi: 10.1042/BJ20141020
28. Detaille D, Guigas B, Chauvin C, Batandier C, Fontaine E, Wiernsperger N, et al. Metformin prevents high-glucose-induced endothelial cell death through a mitochondrial permeability transition-dependent process. *Diabetes.* (2005) 54:2179–87. doi: 10.2337/diabetes.54.7.2179
29. El-Mir MY, Detaille D, R-Villanueva G, Delgado-Esteban M, Guigas B, Attia S, et al. Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons. *J Mol Neurosci.* (2008) 34:77–87. doi: 10.1007/s12031-007-9002-1
30. Wessels B, Ciapaite J, van den Broek NM, Nicolay K, Prompers JJ. Metformin impairs mitochondrial function in skeletal muscle of both lean and diabetic rats in a dose-dependent manner. *PLoS ONE.* (2014) 9:e100525. doi: 10.1371/journal.pone.0100525
31. Shitara Y, Nakamichi N, Norioka M, Shima H, Kato Y, Horie T. Role of organic cation/carnitine transporter 1 in uptake of phenformin and inhibitory effect on complex I respiration in mitochondria. *Toxicol Sci.* (2013) 132:32–42. doi: 10.1093/toxsci/kfs330
32. Bridges HR, Sirvio VA, Agip AN, Hirst J. Molecular features of biguanides required for targeting of mitochondrial respiratory complex I and activation of AMP-kinase. *BMC Biol.* (2016) 14:65. doi: 10.1186/s12915-016-0287-9
33. Hirst J. Mitochondrial complex I. *Annu Rev Biochem.* (2013) 82:551–75. doi: 10.1146/annurev-biochem-070511-103700
34. Vinogradov AD. Catalytic properties of the mitochondrial NADH-ubiquinone oxidoreductase (complex I) and the pseudo-reversible active/inactive enzyme transition. *Biochim Biophys Acta.* (1998) 1364:169–85. doi: 10.1016/S0005-2728(98)00026-7
35. Wu L, Zhou B, Oshiro-Rapley N, Li M, Paulo JA, Webster CM, et al. An ancient, unified mechanism for metformin growth inhibition in *C. elegans* and Cancer. *Cell.* (2016) 167:1705–18 e1713. doi: 10.1016/j.cell.2016.11.055
36. Letts JA, Sazanov LA. Clarifying the supercomplex: the higher-order organization of the mitochondrial electron transport chain. *Nat Struct Mol Biol.* (2017) 24:800–8. doi: 10.1038/nsmb.3460
37. Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. *Mol Cell.* (2012) 48:158–67. doi: 10.1016/j.molcel.2012.09.025
38. Wong HS, Dighe PA, Mezera V, Monternier PA, Brand MD. Production of superoxide and hydrogen peroxide from specific mitochondrial sites under different bioenergetic conditions. *J Biol Chem.* (2017) 292:16804–9. doi: 10.1074/jbc.R117.789271
39. Batandier C, Guigas B, Detaille D, El-Mir MY, Fontaine E, Rigoulet M, et al. The ROS production induced by a reverse-electron flux at respiratory-chain complex 1 is hampered by metformin. *J Bioenerg Biomembr.* (2006) 38:33–42. doi: 10.1007/s10863-006-9003-8
40. Detaille D, Vial G, Borel AL, Cottet-Rouselle C, Hallakou-Bozec S, Bolze S, et al. Imeglimin prevents human endothelial cell death by inhibiting mitochondrial permeability transition without inhibiting mitochondrial respiration. *Cell Death Discov.* (2016) 2:15072. doi: 10.1038/cddiscovery.2015.72
41. Brand MD, Goncalves RL, Orr AL, Vargas L, Gerencser AA, Borch Jensen M, et al. Suppressors of Superoxide-H_2O_2 Production at Site IQ of Mitochondrial Complex I Protect against Stem Cell Hyperplasia and Ischemia-Reperfusion Injury. *Cell Metab.* (2016) 24:582–92. doi: 10.1016/j.cmet.2016.08.012
42. Kelly B, Tannahill GM, Murphy MP, O'Neill LA. Metformin inhibits the production of reactive oxygen species from NADH: ubiquinone oxidoreductase to limit induction of interleukin-1β (IL-1β) and boosts interleukin-10 (IL-10) in lipopolysaccharide (LPS)-activated macrophages. *J Biol Chem.* (2015) 290:20348–59. doi: 10.1074/jbc.M115.662114
43. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest.* (2001) 108:1167–74. doi: 10.1172/JCI200113505
44. Hardie DG. AMP-activated protein kinase: maintaining energy homeostasis at the cellular and whole-body levels. *Annu Rev Nutr.* (2014) 34:31–55. doi: 10.1146/annurev-nutr-071812-161148
45. Guigas B, Viollet B. Targeting AMPK: from ancient drugs to new small-molecule activators. *Exp Suppl.* (2016) 107:327–350. doi: 10.1007/978-3-319-43589-3_13
46. Foretz M, Hebrard S, Leclerc J, Zarrinpashneh E, Soty M, Mithieux G, et al. Metformin inhibits hepatic gluconeogenesis in mice independently of the LKB1/AMPK pathway via a decrease in hepatic energy state. *J Clin Invest.* (2010) 120:2355–69. doi: 10.1172/JCI40671

47. Miller RA, Chu Q, Xie J, Foretz M, Viollet B, Birnbaum MJ. Biguanides suppress hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature.* (2013) 494:256–60. doi: 10.1038/nature11808
48. Mracek T, Drahota Z, Houstek J. The function and the role of the mitochondrial glycerol-3-phosphate dehydrogenase in mammalian tissues. *Biochim Biophys Acta.* (2013) 1827:401–10. doi: 10.1016/j.bbabio.2012.11.014
49. Madiraju AK, Qiu Y, Perry RJ, Rahimi Y, Zhang XM, Zhang D, et al. Metformin inhibits gluconeogenesis via a redox-dependent mechanism *in vivo*. *Nat Med.* (2018) 24:1384–94. doi: 10.1038/s41591-018-0125-4
50. Hunter RW, Hughey CC, Lantier L, Sundelin EI, Peggie M, Zeqiraj E, et al. Metformin reduces liver glucose production by inhibition of fructose-1-6-bisphosphatase. *Nat Med.* (2018) 24:1395–406. doi: 10.1038/s41591-018-0159-7
51. Morales DR, Morris AD. Metformin in cancer treatment and prevention. *Annu Rev Med.* (2015) 66:17–29. doi: 10.1146/annurev-med-062613-093128
52. Heckman-Stoddard BM, DeCensi A, Sahasrabuddhe VV, Ford LG. Repurposing metformin for the prevention of cancer and cancer recurrence. *Diabetologia.* (2017) 60:1639–47. doi: 10.1007/s00125-017-4372-6
53. Mallik R, Chowdhury TA. Metformin in cancer. *Diabetes Res Clin Pract.* (2018) 143:409–19. doi: 10.1016/j.diabres.2018.05.023
54. Vancura A, Bu P, Bhagwat M, Zeng J, Vancurova I. Metformin as an anticancer agent. *Trends Pharmacol Sci.* (2018) 39:867–78. doi: 10.1016/j.tips.2018.07.006
55. Kalender A, Selvaraj A, Kim SY, Gulati P, Brule S, Viollet B, et al. Metformin, independent of AMPK, inhibits mTORC1 in a rag GTPase-dependent manner. *Cell Metab.* (2010) 11:390–401. doi: 10.1016/j.cmet.2010.03.014
56. Birsoy K, Possemato R, Lorbeer FK, Bayraktar EC, Thiru P, Yucel B, et al. Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. *Nature.* (2014) 508:108–12. doi: 10.1038/nature13110
57. Murphy MP, Hartley RC. Mitochondria as a therapeutic target for common pathologies. *Nat Rev Drug Discov.* (2018). 17:865–86. doi: 10.1038/nrd.2018.174

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 18 October 2019
doi: 10.3389/fendo.2019.00704

Effect of Metformin on Short-Term High-Fat Diet-Induced Weight Gain and Anxiety-Like Behavior and the Gut Microbiota

Shuqin Ji[1†], *Lingwei Wang*[2†] *and Lei Li*[1*]

[1] *Guangdong Provincial Key Laboratory of Brain Connectome and Behavior, The Brain Cognition and Brain Disease Institute (BCBDI), Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China,* [2] *Shenzhen Key Laboratory of Respiratory Diseases, Shenzhen Key Laboratory of Pathogenic Microorganisms and Bacterial Resistance, Department of Respiratory and Critical Care Medicine, Shenzhen Institute of Respiratory Diseases, Shenzhen People's Hospital, First Affiliated Hospital of Southern Science and Technology University, Second Clinical Medical College of Jinan University, Shenzhen, China*

The pathogenic factors of the complex epidemic disorder–obesity, have expanded from genetic background, endocrine factors, abnormal feeding behaviors, and direct neural control of adipose tissue physiology. As a chronic metabolic disease, it is important to find new potential therapeutic targets and locate a sensitive time window for intervention. In this study, we focus on the early stage of a high-fat diet mouse model: a short-term 3-week treatment. Our results showed that this short-term 3-week HFD can already induce significant body weight gain, increased adipocyte size and surprisingly, anxiety-like behavior of the animals. Then we tried the early intervention with metformin, already reported for its effects in long-term HFD induced obesity. For a short-term 3-week co-treatment, metformin alleviated the HFD-induced increase in body weight, the increase in adipocyte size and furthermore, the anxiety-like behavior. Differences were noted among the normal diet (ND), HFD, and HFD with metformin co-treatment groups in gut microbiota, including its composition and diversity. The possible involvement of gut microbiota cannot be ruled out. Intense phospho-AMPK staining was found in the metformin treatment group in the habenular nuclei, hippocampus and basal ganglia of the brain compared with the HFD group, implying that the anxiolytic effect of metformin could be due to the direct activation of the AMPK pathway in the anxiety-related brain nuclei.

Keywords: high-fat diet, metformin, anxiety, gut microbiota, obesity

OPEN ACCESS

Edited by:
Graham Rena,
University of Dundee, United Kingdom

Reviewed by:
Yuan Zhou,
Peking University, China
Alison Delamere McNeilly,
University of Dundee, United Kingdom

***Correspondence:**
Lei Li
saralilei@siat.ac.cn

[†]*Co-first authors*

Specialty section:
This article was submitted to Clinical Diabetes, a section of the journal Frontiers in Endocrinology

Received: *10 January 2019*
Accepted: *30 September 2019*
Published: *18 October 2019*

Citation:
Ji S, Wang L and Li L (2019) Effect of Metformin on Short-Term High-Fat Diet-Induced Weight Gain and Anxiety-Like Behavior and the Gut Microbiota. Front. Endocrinol. 10:704. doi: 10.3389/fendo.2019.00704

INTRODUCTION

The rising epidemic of obesity calls for increased effort to identify new therapeutic targets/strategies for the treatment of this metabolic disorder. The pathogenic factors of obesity have expanded from genetic background and endocrine factors to central nervous control, including abnormal feeding behaviors and direct neural control of adipose tissue physiology (1, 2). Considerable progress has been made over the past few decades; however, more research is needed to solve the questions surrounding this disorder.

Metformin (dimethylbiguanide) has become a first-line oral blood glucose-lowering agent for patients with type 2 diabetes mellitus (T2DM) (3, 4). In patients with T2DM, who are also obese, metformin also plays a clinical role in obesity (5, 6). The detailed functional mechanism of its action in obesity needs further investigation. Metformin is derived from galegine, a natural product from a medieval European herbal medicine plant *Galega officinalis*. Established as a safe and effective therapy, metformin has multiple modes of actions and its molecular mechanisms are not fully deciphered, despite its clinical usage for over 60 years (3, 4, 7). One of the major molecular targets of metformin is the cellular energy sensor Adenosine monophosphate (AMP)-activated protein kinase (AMPK) (7, 8). Besides hepatic gluconeogenesis, accumulating tissue/organ targets were found for metformin, including white and brown adipose tissue (9), lung (10), and the central nervous system (11).

Although it is known to have a background in genetic and environmental factors, one direct cause of obesity remains an imbalance between caloric intake and expenditure.In this study, a high-fat diet (HFD) mouse model was used. For its chronic characteristics and the complexity of energy expenditure, we focused on the early phase of HFD feeding in relation to body weight gain. Thus, the HFD treatment lasted for 3 weeks. Considering the current implications of the alteration of gut bacteria in both obesity (12) and metformin-treated diabetes (13), the changes in gut microbiota under the short-term HFD and metformin co-treatment was also investigated. In addition, possible central nervous effects of the HFD were evaluated by a free-moving behavioral test in the Elevated plus maze (EPM) and the Open field test (OFT). Considering the reported activation of the AMPK pathway by metformin, the immunostaining of phospho-AMPK was carried out in WAT and the brain.

MATERIALS AND METHODS

Mice

Adult (6 weeks) male C57BL/6J mice (Beijing Vital River Laboratory Animal Technology Co., Ltd. China) were group-housed, given access to food pellets and water *ad libitum*, and maintained on a 12:12-h light/dark cycle. All husbandry and experimental procedures in this study were approved by the Animal Care and Use Committees of the Shenzhen Institute of Advanced Technology (SIAT), Chinese Academy of Sciences (CAS), China.

High-Fat Diet (HFD), Normal Diet (ND), and Metformin Treatment

Three groups of mice were subjected to different treatments: one group received the normal diet (ND); one group received the high-fat diet (HFD); and a third group received the HFD diet and co-treatment with metformin via oral gavage (300 mg/kg/day, Sigma-Aldrich, BP227, St. Louis, MO). Saline was administered to the ND and HFD groups via oral gavage. For HFD, 60% of the energy was derived from fat, while in the ND, 10% of the energy was derived from fat (Trophic Animal Feed High-Tech, China; TP23300 for HFD, TP23302 for ND). The formula for the HFD was as follows: casein (267 g/kg), maltodextrin (157 g/kg), sucrose (89 g/kg), soybean oil (33 g/kg), lard oil (301 g/kg), cellulose (67 g/kg), mineral mix M1020 (66 g/kg), vitamin mix V1010 (13 g/kg), L-cystine (4 g/kg), choline bitartrate (3 g/kg), TNHQ (0.067 g/kg).

All treatments lasted for 3 weeks. Body weight gain was recorded for each mouse daily until day 21. On the morning of day 21, body weights of mice were recorded, fecal samples were collected and then the mice were subjected to Elevated plus maze test (EPM). On day 22, Open field test (OFT) was performed, then the mice were deep anesthetized and sacrificed. Epididymal white adipose tissues and the brain from three mice of each group were subjected for further histological study. Fecal samples of the mice were collected into sterile tubes, snap-frozen, and then stored at −80°C until the day of analysis.

Histological Study

While under deep anesthesia, mice were transcardially perfused with 4% paraformaldehyde (PFA) in PBS, epididymal white adipose tissues (WAT) and the brain were harvested and posted-fixed in 4% PFA. The WAT tissue samples were embedded in paraffin, and 4 μm sections were cut on a microtome. The sections were subjected to hematoxylin and eosin (H&E) staining for morphological examination and immune fluorescent staining for phospho-AMPK (pAMPK). For adipocyte size analysis, the longer diameter of each adipocyte was measured. To avoid clustered analysis, the readings from one mouse were first averaged and used its mean as a single value for further comparison between different treatment groups. To evaluate the adipocyte enlargement, the mean of all adipocytes counted were calculated and adipocyte with a larger diameter than the mean of all cells measured in the ND group was defined as "Large adipocyte." The number and the percentage of Large adipocyte were calculated as a parameter of adipocyte enlargement.

Also, brains harvested were post-fixed with 4% PFA, cryoprotected in 30% sucrose in PBS and cut on a cryostat in 30 μm slices. Immunohistochemistry was performed to map the pAMPK expression in the brain. Antibody staining was performed on single-well floating tissue sections. Sections were incubated for 24 h in primary antibodies at 4°C followed by overnight incubation with secondary antibodies at 4°C. The primary antibody used was rabbit anti-pAMPK (#2535, Cell Signaling Technology;1:50). Suitable secondary antibodies were chosen to reveal different fluorescent colors. For counterstaining, sections were incubated for 10 min with 40, 6-diamidin-2-phenylindol (DAPI, 0.4 mg/mL, Sigma). All the images were captured with a Zesis LSM 880 confocal microscope or an Olympus VS120 virtual microscopy slide scanning system.

DNA Extraction, 16S Ribosome RNA V4 Region Sequencing and Analysis

DNA extraction was carried out according to the manufacturer's instructions–MOBIO PowerSoil® DNA Isolation Kit 12888-100. DNA was stored at −80°C in Tris-EDTA buffer solution. To enable amplification of the V4 region of the 16S rRNA gene and add barcode sequences, unique fusion

primers were designed based on the universal primer set, 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′), along with barcode sequences. PCR mixtures contained 1 μL of each forward and reverse primers (10 μM), 1 μL of template DNA, 4 μL of dNTPs (2.5 mM), 5 μL of 10 × EasyPfu Buffer, 1 μL of Easy Pfu DNA Polymerase (2.5 U/μL), and 1 μL of double-distilled water in a total 50 μL reaction volume. Thermal cycling consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 40 s, with a final extension step at 72°C for 4 min. The expected band size for 515f-806r is ∼300–350 bp checked by agarose gel. Quantify amplicons with Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher/Invitrogen cat. no. P11496). The amplicon library for high-throughput sequencing on the Illumina MiSeq platform by Promegene, China was combined an equal amount and subsequently quantified (KAPA Library Quantification Kit KK4824) according to manufacturer's instructions. Using the Quantitative Insights into Microbial Ecology (QIIME) 1.8.0 pipeline 1, the raw sequences were processed to concatenate reads into tags according to the overlapping relationship, then, reads belonging to each sample were separated with barcodes and low-quality reads were removed. The processed tags were clustered into the operational taxonomic units (OTUs) at the commonly used 97% similarity threshold. The OTUs were assigned to taxa by matching to the Greengenes database (Release 13.8). A phylogenetic tree of representative sequences was built. Alpha and beta diversity analyses were performed. Distances were calculated with R (3.3.1, flexmix package).

Elevated Plus Maze (EPM) and Behavioral Analysis

Mice were placed on a four-arm plus maze with two open arms and two closed arms (white PVC, 30 cm in length per arm × 5 cm in width), which was raised 50 cm above the ground for a 15 min session. The EPM was cleaned between mice with 20% ethanol solution. The number of entries to the open arms, time spent in the open arms, and distance traveled in the open arms were recorded and analyzed by Anymaze® software (Stoelting Co., IL, USA).

Open Field Test (OFT) and Behavioral Analysis

An open field arena (50 cm × 50 cm × 50 cm) made of white PVC was used to assess both locomotor activity and anxiety-like behavior of the animals. The entries to the Center, the time in the Center, the distance traveled in the Center, total distance traveled in the OF, average speed in the OF for a 5 min session were recorded and then analyzed by the Anymaze®software (Stoelting). The open field was cleaned between mice with 20% ethanol solution.

Statistical Analysis

Data were expressed as mean ± SEM (**Figures 1–3**) and Box and Whiskers (**Figure 5**). Statistical significance was set at $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$) and power analysis (Cohen's d) was carried out. All n values represent the number of mice used in each experiment. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons test or permutation test was used as appropriate.

RESULTS

Three-Week High-Fat Diet (HFD) Induced Significant Body Weight Gain and an Increase in the Adipocyte Size, Which Were Alleviated by Metformin Co-treatment

Three groups of mice were subjected to different treatments: one group to the normal diet (ND + saline); one group to the high-fat diet (HFD + saline); and a third group to the HFD diet and co-treatment with metformin (HFD + Met) via oral gavage. All treatments lasted 3 weeks. Body weight gain was recorded daily until day 21 (**Figure 1A**). The average body weight of animals in each group showed no significant differences before treatment on day 0 (20.20 ± 0.45 g of the ND group, 20.59 ± 0.36 g of HFD group, 21.31 ± 0.39 g for HFD + Met group; n=5 for each group, One-way ANOVA with Tukey's test, ND vs. HFD, $p = 0.776$, Cohen's $d = 0.48$; HFD vs. HFD + Met, $p = 0.429$, Cohen's $d = 1.25$). At the end of 3 weeks, mice in the HFD group showed an increase in body weight gain, compared to the ND group, whereas this increase was alleviated by metformin co-treatment (**Figures 1B–D**, $n = 5$ for each group, each dot represents one animal, data presented as body weight gain/original body weight; **Figure 1B**, ND vs. HFD, $p > 0.05$, Cohen's d= 0.43; HFD vs. HFD + Met, $p < 0.01$, Cohen's $d = 3.09$; **Figure 1C**, ND vs. HFD, $p < 0.05$, Cohen's d=1.85; HFD vs. HFD + Met, $p < 0.01$, Cohen's $d = 2.75$; **Figure 1D**, ND vs. HFD, $p < 0.05$, Cohen's $d = 1.94$; HFD vs. HFD + Met, $p < 0.01$, Cohen's $d = 2.93$). Besides, the difference between HFD and HFD + Met was significant already as early as day 7, suggesting the early onset of metformin's effect on body weight gain.

Representative images of epididymal white adipose tissue (WAT) stained with H&E (**Figure 2A**) showed that the changes in body weight gain were accompanied by morphological changes in the adipocytes, with enlarged adipocytes in the HFD group, compared to the ND group and HFD + Met group. The longer diameter of each adipocyte was measured to quantify the change in adipocyte size. The result showed that HFD group had a larger averaged adipocyte size compared with ND, which was rescued by metformin co-treatment (**Figure 2B**, $n = 5$ for each group, each dot represents one animal, for ND vs. HFD, $p < 0.05$, Cohen's $d = 1.90$, for HFD vs. HFD + Met, $p < 0.01$, Cohen's $d = 2.38$). Defining "Large adipocyte" as cells with a diameter larger than the mean of all cell measured in ND group, we found that HFD group had more Large adipocyte cell number and cell percentage than ND, which were also rescued by metformin co-treatment ($n = 5$ for each group, each dot represents one animal, **Figure 2C**, for ND vs. HFD, $p < 0.01$, Cohen's $d = 3.05$, for HFD vs. HFD + Met, $p < 0.01$, Cohen's $d = 2.81$; **Figure 2D**, for ND vs. HFD, $p < 0.01$, Cohen's $d = 3.26$, for HFD vs. HFD + Met, p

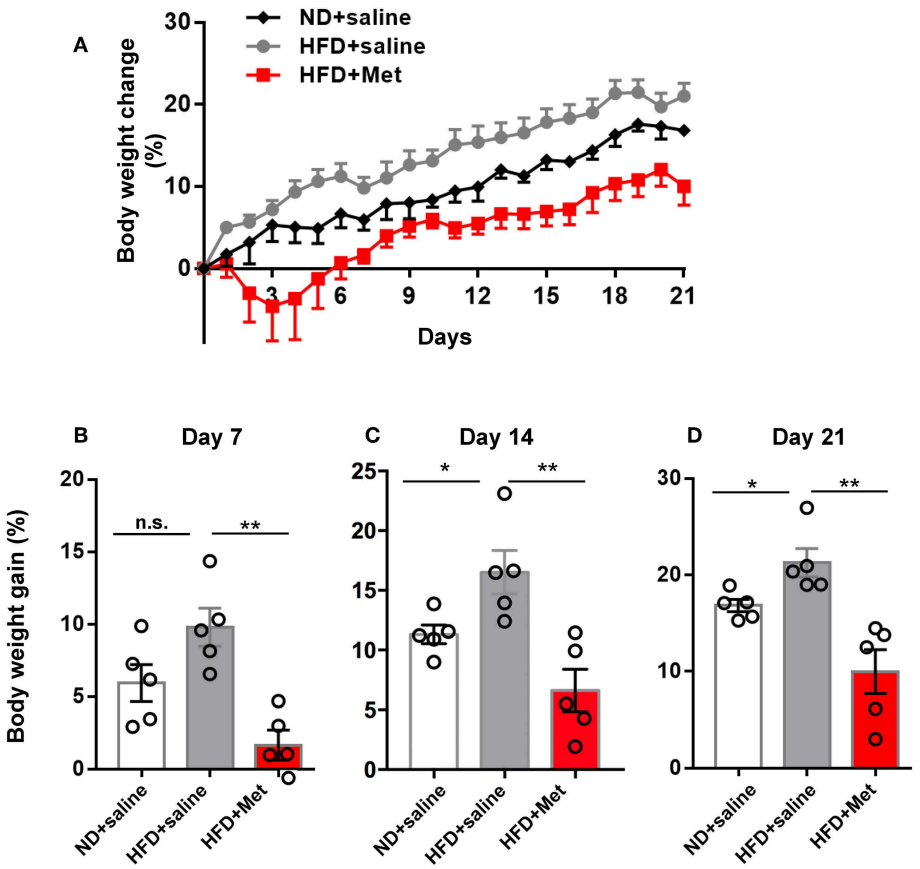

FIGURE 1 | High-Fat Diet (HFD) for 3 weeks induced significant body weight gain compared with Normal Diet (ND) group, which was rescued by metformin co-treatment. **(A)** Three groups of mice were subjected to different treatments, one group for normal diet (ND + saline), one group for high fat diet (HFD + saline) and a third group for HFD diet with oral garage co-treatment of metformin (HFD + Met), the gain of body weight as percentage of original body weight were demonstrated; **(B)** on day 7, HFD + Met group showed a low body weight gain compared with HFD group; **(C,D)** on day 14 and day 21, HFD group showed higher body weight gain compared with ND, which was rescued by metformin co-treatment. For **(A–D)**, data were presented as mean ± SEM, $n = 5$ per group, each dot represents one mouse; One-way ANOVA with Tukey's test, $^{*}p < 0.05$, $^{**}p < 0.01$.

< 0.01 Cohen's $d = 3.79$). These data suggest that metformin co-treatment had a suppressive effect on HFD-induced body weight gain and adipocyte enlargement.

Three-Week HFD Induced Anxiety-Like Behaviors, Which Were Alleviated by Metformin Co-treatment; Metformin Increased pAMPK Levels in the WAT and in the Habenular Nuclei, Hippocampus and Basal Ganglia of the Brain

Considering clinical reports about the interaction between obesity and anxiety (14), we further evaluated the possible variance in anxiety-like behavior in OPT and EPM. In OPT, the entries to the Center, the time in the Center and the distance in the Center all indicated that HFD induced anxiety-like behaviors. There was a trend for metformin to rescue this effect, but not significant (**Figure 3B**, $n = 5$ for each group, each dot represents one animal; for Entries to Center, ND vs. HFD, $p < 0.05$, Cohen's $d = 2.45$, HFD vs. HFD + Met, $p > 0.05$, Cohen's $d = 0.83$; for Time in Center, ND vs. HFD, $p < 0.05$, Cohen's $d = 2.84$, HFD vs. HFD + Met, $p > 0.05$, Cohen's $d = 1.36$; for Distance in Center, ND vs. HFD, $p < 0.01$, Cohen's $d = 3.43$, HFD vs. HFD + Met, $p > 0.05$, Cohen's $d = 1.11$). In EPM, the HFD showed its effect in inducing anxiety like behavior and rescued by the metformin co-treatment. The distance traveled in the open arm decreased in HFD compared with ND, which was alleviated by metformin co-treatment (**Figure 3C**, $n = 5$ for each group, each dot represents one animal; ND vs. HFD, $p < 0.05$, Cohen's $d = 2.10$, HFD vs. HFD + Met, $p < 0.05$, Cohen's $d = 2.21$). The locomotor of the animal was not affected by the 3-week HFD treatment or HFD-Met treatment (**Figure 3A**, $n = 5$ for each group, each dot represents one animal; for total activity, $ps > 0.05$, Cohen's $ds = 0.52$ and 0.86; for average speed, $ps > 0.05$, Cohen's $ds = 0.53$ and 0.87). Though there was no correlation between the body weight changes on day 21 and the parameters of OFT and EPM tests (all R squared <0.26, all $ps > 0.05$), while the parameters of OFT and EPM tests were strongly correlated (for Distance in Open Arms of EPM vs. Distance in Center of OFT, R squared = 0.43, $p < 0.01$).

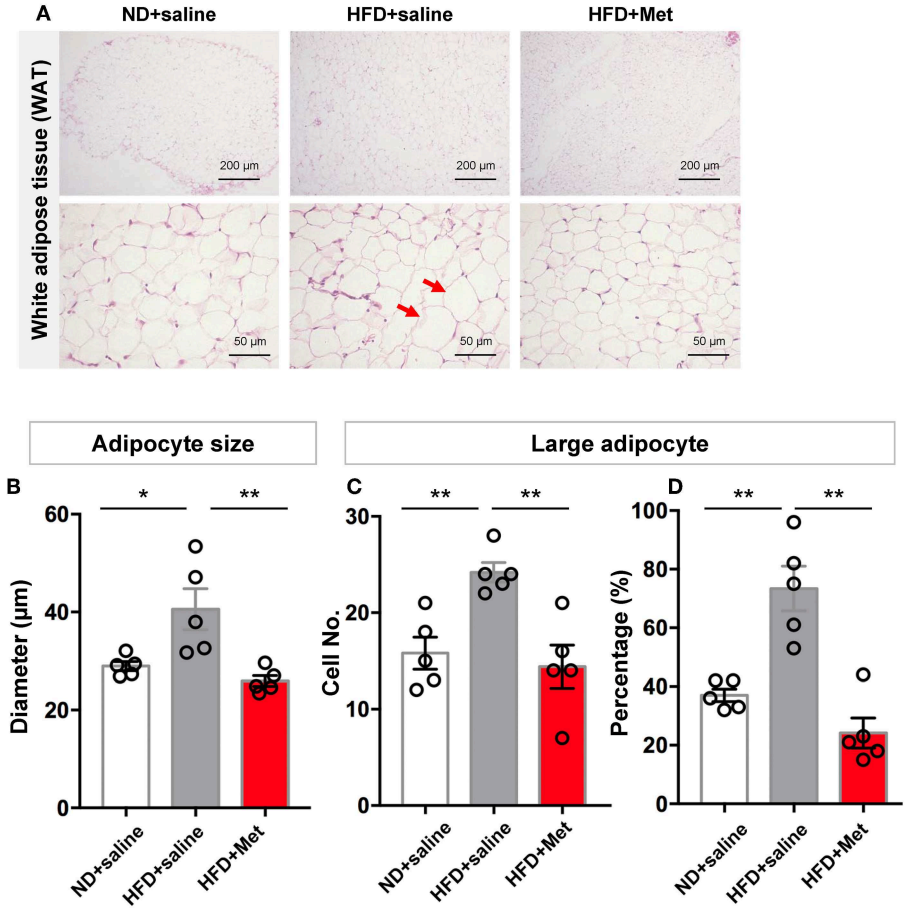

FIGURE 2 | Three-week HFD induced morphological change in white adipose tissue (WAT) and adipocyte size increase, which were alleviated by metformin co-treatment. **(A)** Representative H&E staining figures show that the changes in gain of body weight was accompanied by morphological change in the adipocytes of WAT (arrow heads indicate enlarged adipocytes; scale bars, 200 and 50 μm, respectively); **(B)** HFD group had a larger averaged adipocyte size compared with ND, which was rescued by metformin co-treatment; **(C,D)** for large adipocytes (defined as cells with a diameter larger than the mean of all cells in ND group), HFD group showed higher value in cell number and percentage of large adipocytes. This increase was also rescued by metformin co-treatment. For **(A–D)**, $n = 5$ per group; for **(B–D)**, data were presented as mean ± SEM, each dot represents one mouse; One-way ANOVA with Tukey's test, $^{*}p < 0.05$, $^{**}p < 0.01$.

We next want to know whether the anxiolytic effect of metformin could be partially due to its direct action in the brain. As metformin is a well-known AMPK activator and has been reported for its central function in the brain (15) and AMPK is a primary sensor of cellular energy states and regulates cellular energy metabolism, we examined the possible activation of AMPK pathway by metformin treatment. Stronger pAMPK immunostaining was found in the metformin co-treatment group, not only in the WAT (**Figure 4A**), but also in the brain, located in the habenular nuclei, hippocampus and basal ganglia, compared with the HFD group (**Figures 4B–D**). Habenular nuclei, especially lateral habenular nucleus (LHb), hippocampus, and basal ganglia are all involved with anxiety-related disorders (16–21). Our data suggested that the anxiolytic effect of metformin co-treatment could be due to the direct activation of the AMPK pathway in the anxiety-related brain nuclei.

The HFD and HFD + Metformin Treatments Changed Microbiota Diversity and Altered Its Composition

The close association between altered gut microbiota and obesity (12) or long-term high-fat diet (22, 23) has been established. Considering the significant effect of metformin in rescuing the 3-week HFD-induced body weight gain, we accessed the impact of HFD and HFD + Met on the gut microbiota through 16S rRNA gene sequencing of the fecal contents of the animals (**Figures 5A–H**, $n = 5$ for each group). The bacterial abundance of each group varied at genus levels (**Figure 5A**). With *Firmicutes* and *Bacteroidetes* dominant at phylum level, the top dominant species included *Lactobacillus, Allobaculum, Streptococcus, Oscillospira, Bifidobacterium, Lactococcus, Ruminococcus, Leuconostoc, Prevotella*, among which *Streptococcus, Oscillospira, Ruminococcus, Leuconostoc* and *Prevotella* showed significant difference between groups (**Figure 5B**, all Cohen's *d*s > 0.8). Beta

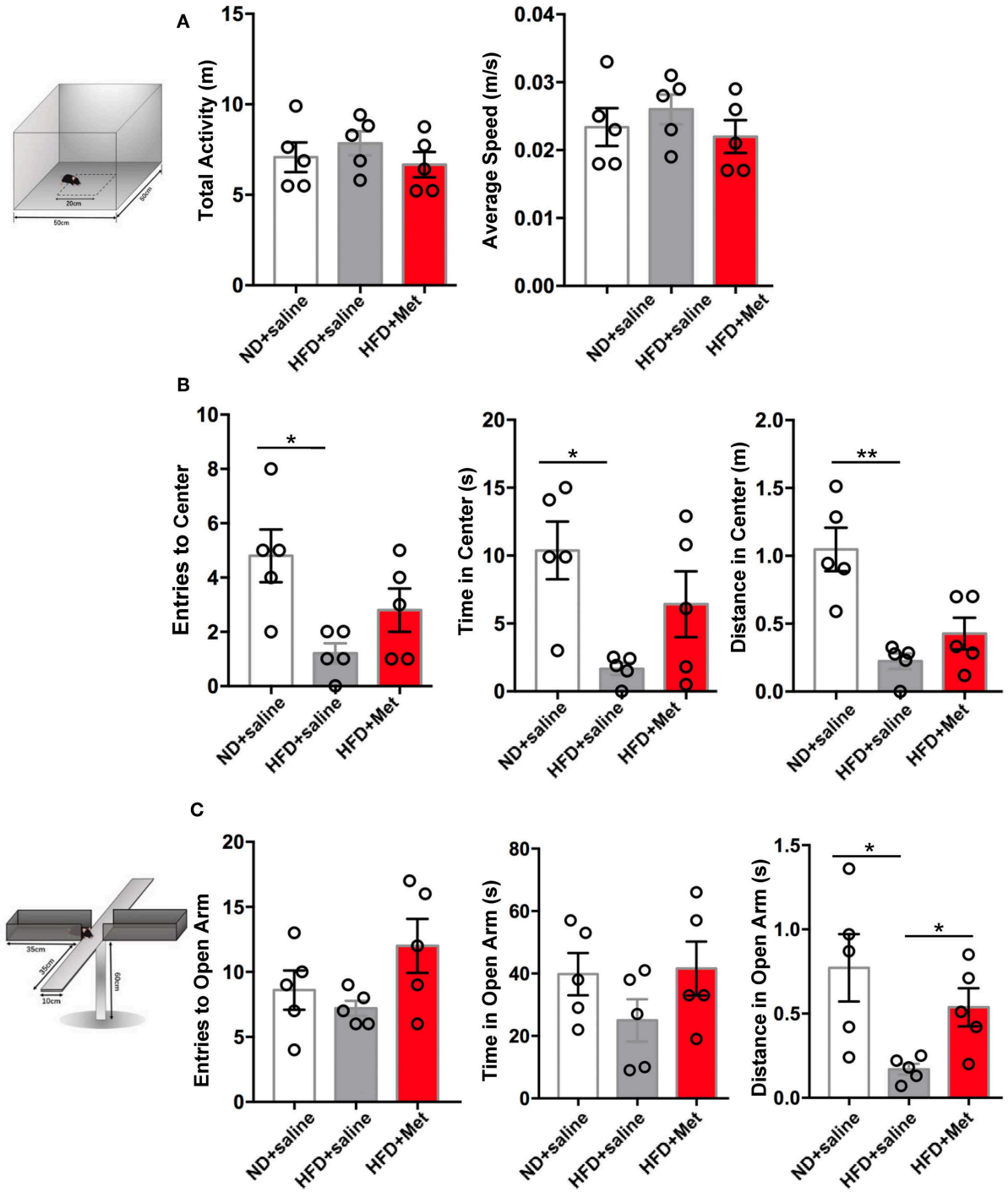

FIGURE 3 | Three-week HFD induced anxiety-like behavior, which were alleviated by metformin co-treatment. **(A)** The total activity and average speed of the animals were not affected by HFD or HFD + Met in the open field; **(B)** in the Open field test (OFT), Entries to Center, Time in Center, and Distance in Center decreased in HFD group compared with ND group; the difference between HFD vs. HFD + Met was not significant; **(C)** in the Elevated plus maze test (EPM), Distance in Open arm decreased in HFD compared with ND, which was alleviated by metformin co-treatment. For **(A–C)**, data were presented as mean ± SEM, $n = 5$ per group, each dot represents one mouse; One-way ANOVA with Tukey's test, $^{*}p < 0.05$, $^{**}p < 0.01$.

diversity analysis showed significant difference between groups after Bray-curtis dissimilarity (**Figure 5C**), unweighted UniFrac (**Figure 5D**) and weighted UniFrac analysis (**Figure 5E**, $p <$ 0.0001 for all tests), though no significant difference was shown by alpha diversity analysis, either by evenness or Shannon's index (**Figures 5F,G**). The difference in gut bacteria composition was

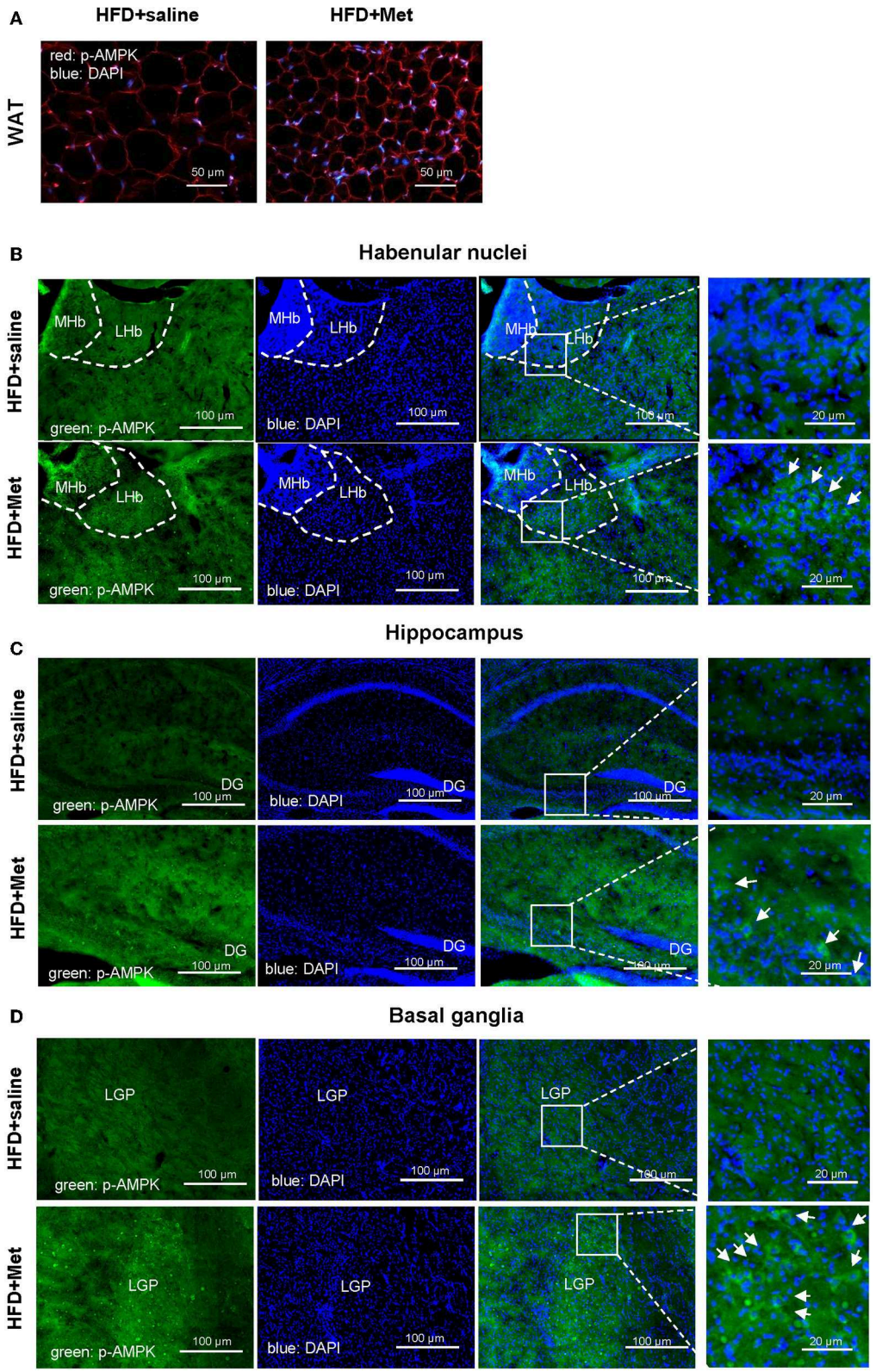

FIGURE 4 | Metformin co-treatments showed stronger pAMPK immunostaining in the WAT and in the habenular nuclei, hippocampus and basal ganglia of the brain. Compared with HFD group, HFD + Met group showed stronger pAMPK staining in the adipocytes (**A**; red, pAMPK, blue, DAPI; scale bars, 50 μm) and in the habenular nuclei, hippocampus and basal ganglia [for **(B–D)**, green, pAMPK, blue, DAPI, scale bars, 100 and 20 μm, respectively; MHb, medial habenula nucleus; LHb, lateral habenular nucleus; DG, dentate gyrus; LGP, lateral globus pallidus; for **(A–D)**, $n = 3$ per group].

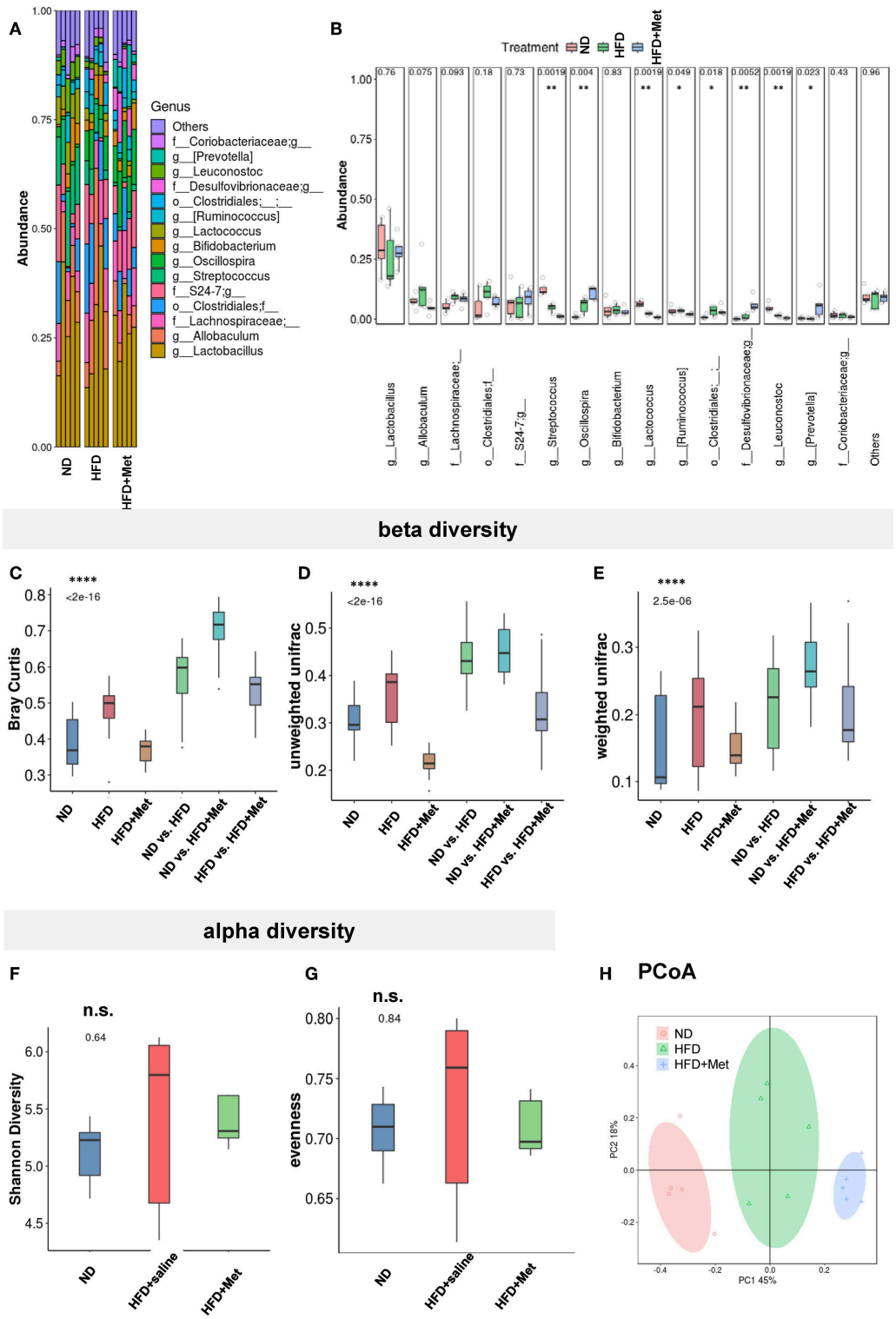

FIGURE 5 | The HFD and HFD + metformin treatments changed microbiota diversity and altered its composition. **(A,B)** The abundance of species varied among ND, HFD, and HFD + Met group with significant difference for *Streptococcus, Oscillospira, Ruminococcus, Leuconostoc,* and *Prevotella*; **(C–E)** beta diversity analysis showed significant difference between groups after Bray-curtis dissimilarity, unweighted UniFracand weighted UniFrac analysis (****$p < 0.0001$); **(F,G)** no significant difference was shown by alpha diversity analysis of Shannon's index or evenness; **(H)** Principal Co-ordinates Analysis (PCoA) revealed the difference in gut bacteria composition in the three distinct groups. For **(A-H)**, n = 5 for each group, box and whiskers was plotted; One-way ANOVA with permutation test, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, n.s. not significant.

revealed by Principal Co-ordinates Analysis (PCoA) plotted into three distinct groups (**Figure 5H**). Though no species was identified by LEfSe with LDA larger than 2, when comparison was made at species level, the *Enterococcus*, *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Blautia, Coprococcus*, *Oscillospira*, *Anaerovorax*, and *Flexispira* were significant different between ND and HFD groups (**Supplementary Table 1A**, ND vs. HFD, all $ps < 0.05$). Among all the above species modified by HFD, the *Enterococcus*, *Lactococcus*, *Streptococcus* demonstrated difference after metformin co-treatment (**Supplementary Table 1B**, HFD vs. HFD + Met, all $ps < 0.05$). The presence of *Akkermansia* was reported to inversely correlates with body weight in rodents and humans (24). Interestingly, we found that though the level of Species *Akkermansia* was not influenced by HFD diet compared with ND (ND vs. HFD, $p = 0.438$, Cohen's $d = 0.27$), it was different after metformin treatment (HFD vs. HFD + Met, $p < 0.05$, Cohen's $d = 0.34$).

DISCUSSION

Obesity is associated with an increased risk of metabolic disorders and cardiovascular diseases, as well as emotional disorders (14, 25–28). In addition, mice fed on HFD displayed learning and memory impairment (29), as well as emotional related disorders, such as depressive-like (30–35) and anxiety-like behavior (36–38). However, the previous all focused on the related effects after long-term HFD treatment, such as 12–18 weeks (30, 31, 33–37, 39, 40), or even 24 weeks (38). As a chronic metabolic disease, it is important to find new potential therapeutic targets and locate a sensitive time window for intervention. In this study, we focus on the early stage of an HFD model: a short-term 3-week treatment. We found that even short-term 3-week-HFD treatment caused significant body weight gain, increased adipocyte size and induced anxiety-like behavior in the animals. This is indicative of a quicker onset of the effects of dietary change on emotional states, which had not been revealed by previous studies. The anti-obesity role of metformin in the HFD model usually adopt a longer treatment period too, for example, 10-week metformin treatment in a 28-week HFD-induced obesity model (41). In our study, the rescue effect of metformin was proved at 3-week period, evident already on day 7 of co-treatment, suggesting the early on-set of the beneficial drug effect.

It is also exciting to find out that 3-week metformin co-treatment can already alleviated not only the metabolic body weight gain effect of HFD, but also the emotional aspect of anxiety-like behavior. Actually, the central roles of metformin have been reported, which includes the promotion of neurogenesis through the atypical protein kinase C-CREB-binding protein (PKC-CBP) pathway (15), learning and memory improvement in association with glucagon-like peptide-1 (42) and AMPK dependent autophagic pathway in an ischemia model (34). Current results imply that the model adopted in the present study could be a candidate animal model for further study of the central effects of metformin in diet-induced anxiety disorders. In our study, we found that pAMPK levels in anxiety-related brain regions like LHb (16, 17), hippocampus (18, 19), and basal ganglia (20, 21) were increased compared with the HFD group. This anxiolytic effect of metformin through the AMPK pathway was in line with a previous model of transient forebrain ischemia model (34).

The association between gut microbiota and obesity has been intensively studied in both clinical and animal studies (12, 24, 43). The effects of metformin in improving T2DM (13) and obesity (41) have been proposed to be partially mediated by modifications in the gut microbiota. Here, we showed that the short-term HFD-induced body weight gain is associated with a shift in the composition of microbiota and increased anxiety. Considering the emerging evidence of the connection between gut microbiota and mental disorders, including autism (44–46), depression (47–49), and anxiety disorders (50, 51), and the impact of HFD and HFD-Met on the anxiety-like behavior of the animals, we also studied the changes in gut microbiota under the short-term HFD and metformin co-treatment. Our data showed that differences were noted among the normal diet (ND), HFD, and HFD with metformin co-treatment groups in gut microbiota, including its composition and diversity. We found that the level of *Akkermansia* increased after metformin treatment in our study, which was in line with previous reports (52, 53). A recent study demonstrated beneficial outcomes from the administration of *Akkermansia* in overweight/obese insulin-resistant volunteers (54). Considering that, it is intriguing to study the detailed action and pathways of *Akkermansia* in the rescue of body weight gain of HFD by metformin, especially at the early stage of the process in the future study.

The beneficial effect of metformin is evident at an early stage of HFD-induced obesity development in aspects of white adipose tissue cellular morphology, the anxiety level of the animals. Also, the possible involvement of gut microbiota cannot be ruled out. And the anxiolytic effect of metformin co-treatment could be due to the direct activation of the AMPK pathway in the anxiety-related brain nuclei. Deciphering further details regarding the gut-brain-axis would foster a better understanding of the mechanisms associated with HFD and anxiety-like behaviors.

ETHICS STATEMENT

Adult (6 weeks) male C57BL/6J mice (Beijing Vital River Laboratory Animal Technology Co., Ltd. China) were group-housed, given access to food pellets and water *ad libitum*, and maintained on a 12:12-h light/dark cycle. All husbandry and experimental procedures in this study were approved by Animal Care and Use Committees at the Shenzhen Institute of Advanced Technology (SIAT), Chinese Academy of Sciences (CAS), China.

AUTHOR CONTRIBUTIONS

LL designed the study. SJ, LW, and LL performed experiments. LL wrote the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China NSFC31971072, NSFC81671444, NSFC31471109. Shenzhen Science Technology, and Innovative Commission Grant JCYJ20180508152336419, KQJSCX20160301144002, and Guangdong Provincial Key Laboratory of Brain Connectome and Behavior 2017B030301017 and Shenzhen Discipline Construction Project for Neurobiology DRCSM [2016]1379.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00704/full#supplementary-material

REFERENCES

1. Luppino FS, de Wit LM, Bouvy PF, Stijnen T, Cuijpers P, Penninx BW, et al. Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies. *Arch Gen Psychiatry*. (2010) 67:220–9. doi: 10.1001/archgenpsychiatry.2010.2
2. Zeng W, Pirzgalska RM, Pereira MM, Kubasova N, Barateiro A, Seixas E, et al. Sympathetic neuro-adipose connections mediate leptin-driven lipolysis. *Cell*. (2015) 163:84–94. doi: 10.1016/j.cell.2015.08.055
3. Bailey CJ, Turner RC. Metformin. *N Engl J Med*. (1996) 334:574–9. doi: 10.1056/NEJM199602293340906
4. Bailey CJ. Metformin: historical overview. *Diabetologia*. (2017) 60:1566–76. doi: 10.1007/s00125-017-4318-z
5. Giugliano D, De Rosa N, Di Maro G, Marfella R, Acampora R, Buoninconti R, et al. Metformin improves glucose, lipid metabolism, and reduces blood pressure in hypertensive, obese women. *Diabetes Care*. (1993) 16:1387–90. doi: 10.2337/diacare.16.10.1387
6. Adeyemo MA, McDuffie JR, Kozlosky M, Krakoff J, Calis KA, Brady SM, et al. Effects of metformin on energy intake and satiety in obese children. *Diabetes Obes Metab*. (2015) 17:363–70. doi: 10.1111/dom.12426
7. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. *Diabetologia*. (2017) 60:1577–85. doi: 10.1007/s00125-017-4342-z
8. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest*. (2001) 108:1167–74. doi: 10.1172/JCI13505
9. Klein J, Westphal S, Kraus D, Meier B, Perwitz N, Ott V, et al. Metformin inhibits leptin secretion via a mitogen-activated protein kinase signalling pathway in brown adipocytes. *J Endocrinol*. (2004) 183:299–307. doi: 10.1677/joe.1.05646
10. Rangarajan S, Bone NB, Zmijewska AA, Jiang S, Park DW, Bernard K, et al. Metformin reverses established lung fibrosis in a bleomycin model. *Nat Med*. (2018) 24:1121–7. doi: 10.1038/s41591-018-0087-6
11. Beiroa D, Imbernon M, Gallego R, Senra A, Herranz D, Villarroya F, et al. GLP-1 agonism stimulates brown adipose tissue thermogenesis and browning through hypothalamic AMPK. *Diabetes*. (2014) 63:3346–58. doi: 10.2337/db14-0302
12. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA*. (2005) 102:11070–5. doi: 10.1073/pnas.0504978102
13. Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. *Nature*. (2015) 528:262–6. doi: 10.1038/nature15766
14. Gariepy G, Nitka D, Schmitz N. The association between obesity and anxiety disorders in the population: a systematic review and meta-analysis. *Int J Obes (Lond)*. (2010) 34:407–19. doi: 10.1038/ijo.2009.252
15. Wang J, Gallagher D, DeVito LM, Cancino GI, Tsui D, He L, et al. Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation. *Cell Stem Cell*. (2012) 11:23–35. doi: 10.1016/j.stem.2012.03.016
16. Okamoto H, Aizawa H. Fear and anxiety regulation by conserved affective circuits. *Neuron*. (2013) 78:411–3. doi: 10.1016/j.neuron.2013.04.031
17. Tovote P, Fadok JP, Luthi A. Neuronal circuits for fear and anxiety. *Nat Rev Neurosci*. (2015) 16:317–31. doi: 10.1038/nrn3945
18. Kheirbek MA, Drew LJ, Burghardt NS, Costantini DO, Tannenholz L, Ahmari SE, et al. Differential control of learning and anxiety along the dorsoventral axis of the dentate gyrus. *Neuron*. (2013) 77:955–68. doi: 10.1016/j.neuron.2012.12.038
19. McHugh SB, Deacon RM, Rawlins JN, Bannerman DM. Amygdala and ventral hippocampus contribute differentially to mechanisms of fear and anxiety. *Behav Neurosci*. (2004) 118:63–78. doi: 10.1037/0735-7044.118.1.63
20. Wu JC, Buchsbaum MS, Hershey TG, Hazlett E, Sicotte N, Johnson JC. PET in generalized anxiety disorder. *Biol Psychiatry*. (1991) 29:1181–99. doi: 10.1016/0006-3223(91)90326-H
21. Marchand WR. Cortico-basal ganglia circuitry: a review of key research and implications for functional connectivity studies of mood and anxiety disorders. *Brain Struct Funct*. (2010) 215:73–96. doi: 10.1007/s00429-010-0280-y
22. Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes*. (2008) 57:1470–81. doi: 10.2337/db07-1403
23. Moreira AP, Texeira TF, Ferreira AB, Peluzio Mdo C, Alfenas Rde C. Influence of a high-fat diet on gut microbiota, intestinal permeability and metabolic endotoxaemia. *Br J Nutr*. (2012) 108:801–9. doi: 10.1017/S0007114512001213
24. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et al. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proc Natl Acad Sci USA*. (2013) 110:9066–71. doi: 10.1073/pnas.1219451110
25. Gariepy G, Wang J, Lesage AD, Schmitz N. The longitudinal association from obesity to depression: results from the 12-year National Population Health Survey. *Obesity (Silver Spring)*. (2010) 18:1033–8. doi: 10.1038/oby.2009.333
26. Onyike CU, Crum RM, Lee HB, Lyketsos CG, Eaton WW. Is obesity associated with major depression? Results from the Third National Health and Nutrition Examination Survey. *Am J Epidemiol*. (2003) 158:1139–47. doi: 10.1093/aje/kwg275
27. Dong C, Sanchez LE, Price RA. Relationship of obesity to depression: a family-based study. *Int J Obes Relat Metab Disord*. (2004) 28:790–5. doi: 10.1038/sj.ijo.0802626
28. Stunkard AJ, Faith MS, Allison KC. Depression and obesity. *Biol Psychiatry*. (2003) 54:330–7. doi: 10.1016/S0006-3223(03)00608-5
29. Cordner ZA, Tamashiro KL. Effects of high-fat diet exposure on learning & memory. *Physiol Behav*. (2015) 152:363–71. doi: 10.1016/j.physbeh.2015.06.008
30. Sharma S, Fulton S. Diet-induced obesity promotes depressive-like behaviour that is associated with neural adaptations in brain reward circuitry. *Int J Obes (Lond)*. (2013) 37:382–9. doi: 10.1038/ijo.2012.48
31. Sharma S, Fernandes MF, Fulton S. Adaptations in brain reward circuitry underlie palatable food cravings and anxiety induced by high-fat diet withdrawal. *Int J Obes (Lond)*. (2013) 37:1183–91. doi: 10.1038/ijo.2012.197
32. Hryhorczuk C, Sharma S, Fulton SE. Metabolic disturbances connecting obesity and depression. *Front Neurosci*. (2013) 7:177. doi: 10.3389/fnins.2013.00177
33. Gupta D, Radhakrishnan M, Kurhe Y. Ondansetron, a 5HT3 receptor antagonist reverses depression and anxiety-like behavior in streptozotocin-induced diabetic mice: possible implication of serotonergic system. *Eur J Pharmacol*. (2014) 744:59–66. doi: 10.1016/j.ejphar.2014.09.041
34. Kurhe Y, Mahesh R. Ondansetron attenuates co-morbid depression and anxiety associated with obesity by inhibiting the biochemical alterations and

improving serotonergic neurotransmission. *Pharmacol Biochem Behav.* (2015) 136:107–16. doi: 10.1016/j.pbb.2015.07.004
35. Kurhe Y, Mahesh R, Gupta D. Effect of a selective cyclooxygenase type 2 inhibitor celecoxib on depression associated with obesity in mice: an approach using behavioral tests. *Neurochem Res.* (2014) 39:1395–402. doi: 10.1007/s11064-014-1322-2
36. Zemdegs J, Quesseveur G, Jarriault D, Penicaud L, Fioramonti X, Guiard BP. High-fat diet-induced metabolic disorders impairs 5-HT function and anxiety-like behavior in mice. *Br J Pharmacol.* (2016) 173:2095–110. doi: 10.1111/bph.13343
37. Andre C, Dinel AL, Ferreira G, Laye S, Castanon N. Diet-induced obesity progressively alters cognition, anxiety-like behavior and lipopolysaccharide-induced depressive-like behavior: focus on brain indoleamine 2,3-dioxygenase activation. *Brain Behav Immun.* (2014) 41:10–21. doi: 10.1016/j.bbi.2014.03.012
38. Kang SS, Jeraldo PR, Kurti A, Miller ME, Cook MD, Whitlock K, et al. Diet and exercise orthogonally alter the gut microbiome and reveal independent associations with anxiety and cognition. *Mol Neurodegener.* (2014) 9:36. doi: 10.1186/1750-1326-9-36
39. Almeida-Suhett CP, Graham A, Chen Y, Deuster P. Behavioral changes in male mice fed a high-fat diet are associated with IL-1beta expression in specific brain regions. *Physiol Behav.* (2017) 169:130–40. doi: 10.1016/j.physbeh.2016.11.016
40. Almeida-Suhett CP, Scott JM, Graham A, Chen Y, Deuster PA. Control diet in a high-fat diet study in mice: Regular chow and purified low-fat diet have similar effects on phenotypic, metabolic, and behavioral outcomes. *Nutr Neurosci.* (2019) 22:19–28. doi: 10.1080/1028415X.2017.1349359
41. Lee H, Ko G. Effect of metformin on metabolic improvement and gut microbiota. *Appl Environ Microbiol.* (2014) 80:5935–43. doi: 10.1128/AEM.01357-14
42. Lennox R, Porter DW, Flatt PR, Holscher C, Irwin N, Gault VA. Comparison of the independent and combined effects of sub-chronic therapy with metformin and a stable GLP-1 receptor agonist on cognitive function, hippocampal synaptic plasticity and metabolic control in high-fat fed mice. *Neuropharmacology.* (2014) 86:22–30. doi: 10.1016/j.neuropharm.2014.06.026
43. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature.* (2006) 444:1027–31. doi: 10.1038/nature05414
44. Malkki H. Neurodevelopmental disorders: human gut microbiota alleviate behavioural symptoms in a mouse model of autism spectrum disorder. *Nat Rev Neurol.* (2014) 10:60. doi: 10.1038/nrneurol.2013.267
45. De Angelis M, Francavilla R, Piccolo M, De Giacomo A, Gobbetti M. Autism spectrum disorders and intestinal microbiota. *Gut Microbes.* (2015) 6:207–13. doi: 10.1080/19490976.2015.1035855
46. Mangiola F, Ianiro G, Franceschi F, Fagiuoli S, Gasbarrini G, Gasbarrini A. Gut microbiota in autism and mood disorders. *World J Gastroenterol.* (2016) 22:361–8. doi: 10.3748/wjg.v22.i1.361
47. Zheng P, Zeng B, Zhou C, Liu M, Fang Z, Xu X, et al. Gut microbiome remodeling induces depressive-like behaviors through a pathway mediated by the host's metabolism. *Mol Psychiatry.* (2016) 21:786–96. doi: 10.1038/mp.2016.44
48. Luna RA, Foster JA. Gut brain axis: diet microbiota interactions and implications for modulation of anxiety and depression. *Curr Opin Biotechnol.* (2015) 32:35–41. doi: 10.1016/j.copbio.2014.10.007
49. Naseribafrouei A, Hestad K, Avershina E, Sekelja M, Linlokken A, Wilson R, et al. Correlation between the human fecal microbiota and depression. *Neurogastroenterol Motil.* (2014) 26:1155–62. doi: 10.1111/nmo.12378
50. Fond G, Boukouaci W, Chevalier G, Regnault A, Eberl G, Hamdani N, et al. The "psychomicrobiotic": Targeting microbiota in major psychiatric disorders: a systematic review. *Pathol Biol* (*Paris).* (2015) 63:35–42. doi: 10.1016/j.patbio.2014.10.003
51. Jiang HY, Zhang X, Yu ZH, Zhang Z, Deng M, Zhao JH, et al. Altered gut microbiota profile in patients with generalized anxiety disorder. *J Psychiatr Res.* (2018) 104:130–6. doi: 10.1016/j.jpsychires.2018.07.007
52. Shin NR, Lee JC, Lee HY, Kim MS, Whon TW, Lee MS, et al. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut.* (2014) 63:727–35. doi: 10.1136/gutjnl-2012-303839
53. de la Cuesta-Zuluaga J, Mueller NT, Corrales-Agudelo V, Velasquez-Mejia EP, Carmona JA, Abad JM, 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.* (2017) 40:54–62. doi: 10.2337/dc16-1324
54. Depommier C, Everard A, Druart C, Plovier H, Van Hul M, Vieira-Silva S, et al. Supplementation with Akkermansia muciniphila in overweight and obese human volunteers: a proof-of-concept exploratory study. *Nat Med.* (2019) 25:1096–103. doi: 10.1038/s41591-019-0495-2

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership