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Benefits of Metformin in Attenuating the Hallmarks of Aging

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SUMMARY

Biological aging involves an interplay of conserved and targetable molecular mechanisms, summarized as the hallmarks of aging. Metformin- a biguanide that combats age-related disorders and improves healthspan, is the first drug to be tested for its age-targeting effects in the large clinical trial- TAME (Targeting Aging by MEtformin). This review focuses on metformin's mechanisms in attenuating hallmarks of aging and their interconnectivity, by improving nutrient-sensing, enhancing autophagy and intercellular communication, protecting against macromolecular damage, delaying stem-cell aging, modulating mitochondrial function, regulating transcription, and lowering telomere attrition and senescence. These characteristics make metformin an attractive gerotherapeutic to translate to human trials.

Graphical Abstract

Metformin is the first drug to be tested for its age-targeting effects in a large clinical trial. In this Perspective, Kulkarni et al. review how metformin acts on its primary and secondary targets to attenuate the hallmarks of aging, highlighting its utility as an effective gerotherapeutic intervention.

Introduction

Aging is characterized by a progressive loss of physiological function, which drives the development of chronic morbidities including metabolic, cardiovascular, neoplastic and neurodegenerative disorders as well as geriatric symptoms like frailty and immobility. Aging is accompanied by an inherent biological mechanism that is malleable and can be targeted using therapeutic interventions. Indeed, over the past few decades, scientists have achieved

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Declaration of Interests

The authors declare no competing interests

remarkable progress in extending healthspan and lifespan of model organisms using several genetic, dietary and pharmacological interventions. These advancements have urged the geroscience research community to initiate clinical trials to investigate the efficacy of interventions in targeting human aging, starting with the TAME (Targeting Aging with MEtformin) study (Barzilai 2017, Campisi et al. 2019). The TAME study, soon to be launched in the near future, aims to prove the concept that human aging can be targeted while simultaneously preventing a multitude of major age-related outcomes. Furthermore, TAME is a potential tool to facilitate the FDA to approve “aging” as a target for drug discovery and development. Thus, TAME will pave the way for the development of novel interventions that could target and delay the aging process and improve human healthspan, by modulating the conserved mechanistic pathways involved in aging.

To systematically dissect the biological aging process, Lopez-Otin et al characterized nine major hallmarks of aging, widely accepted by the geroscience research community, namely 1) Genomic Instability, 2) Epigenetic Alterations, 3) Loss of Proteostasis, 4) Deregulated Nutrient-Sensing, 5) Mitochondrial Dysfunction, 6) Cellular Senescence, 7) Stem Cell Exhaustion, 8) Altered Intercellular Communication and 9) Telomere Attrition (López-Otín et al. 2013). These are divided as primary, antagonistic and integrative hallmarks depending on their functional characteristics as causes of damage, responses to damage, and end results of the first two categories, respectively (López-Otín et al. 2013). The hallmarks and their interconnectivity can serve as an evaluation tool to assess and prioritize interventions that can be deemed effective in targeting aging. Although the contribution of each of these hallmarks towards the progression of biological aging is not yet fully elucidated, interventions that can modulate several of these hallmarks, at least in part, need to be studied extensively to provide newer insights into the druggable targets of biological aging.

In humans, metformin has been in clinical use for over 60 years, studied extensively, has a high safety profile and is uniquely positioned to intervene several crucial pathways responsible for aging and age-related diseases (Barzilai et al. 2016). As recommended by the American Diabetes Association, due to its glucose-lowering effects, metformin monotherapy is the preferred first-line pharmacological action against type-2 diabetes (2019). Epidemiological studies have revealed metformin’s gerotherapeutic effect in lowering the incidence of multiple age-related diseases as well as all-cause mortality, in both diabetics and non-diabetics (Campbell et al. 2017, Valencia et al. 2017). Clinical studies including the Diabetes Prevention Program (DPP) in non-diabetics and U.K. Prospective Diabetes Study (UKPDS) in diabetics, support metformin’s role as an effective intervention against diabetes and cardiovascular disease. Association studies suggest a decrease in the incidence of most age-related cancers, Alzheimer’s Disease while clinical studies support metformin’s role in a decrease in cognitive decline and reduced mortality in diabetics taking metformin compared to non-diabetics (Barzilai et al. 2016).

Studies in multiple model organisms and human cell lines have elucidated metformin’s role in targeting multiple mechanisms of aging. In mice and *C. elegans*, metformin extends lifespan and improves several indicators of healthspan (Anisimov et al. 2008, Martin-Montalvo et al. 2013, De Haes et al. 2014, Chen et al. 2017). Intermittent metformin treatment, when initiated in mice, even later in life and administered every other week, has

also shown to provide health benefits such as a reduction in hepatic steatosis through regulation of the liver transcriptome and metabolome (Alfaras et al. 2017). Recently, metformin's extraordinary ability as a gerotherapeutic is established through several experimental, clinical and observational evidence (Novelle et al. 2016, Piskovatska et al. 2018, Glossmann and Lutz 2019, Soukas et al. 2019). Moreover, in older human adults, we recently demonstrated metformin's efficacy in targeting multiple age-associated metabolic and non-metabolic pathways and reverting derangements, using cardiometabolic and transcriptomic outcomes (Kulkarni et al. 2018).

In this review, we aim to determine the influence of metformin's mechanisms of action on the hallmarks of biological aging. We demonstrate that each hallmark of aging, as well as their interconnectivity is attenuated by metformin's direct and/or downstream effects (Figure 1).

Mechanisms of metformin in targeting biological aging

Metformin was introduced to the world in 1957, as an antihyperglycemic agent by the French physician Jean Sterne, and today, it has become one of the most commonly used pharmaceutical interventions and the most prescribed glucose-lowering medication, worldwide (Bailey 2017). Indeed, it is important to note that metformin does not cause hypoglycemia *per se* but rather reduces hepatic glucose production through improved hepatic insulin sensitivity, which results in the reduction of fasting plasma glucose levels (Jackson et al. 1987, DeFronzo et al. 1991). Furthermore, with the epidemiological, preclinical and clinical evidence of metformin exhibiting beneficial effects beyond glycemic control in diabetics, it has been suggested to be repurposed against cancer and its recurrence (Heckman-Stoddard et al. 2017), cardiovascular disease (Rena and Lang 2018), neurodegenerative diseases (Rotermund et al. 2018), autoimmune diseases (Ursini et al. 2018) and most recently, systemic aging as a whole (Barzilai et al. 2016, Barzilai 2017). Despite such widespread use and efficacy, the mechanisms by which metformin regulates fundamental pathways in aging and diseases are not fully elucidated. Metformin's antihyperglycemic role is attributed to its action on glucose metabolism, specifically as a suppressor of hepatic gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase, thereby modifying the hepatocellular redox state to reduce glucose formation from lactate and glycerol (Madiraju et al. 2014). On the other hand, metformin's suppression of hepatic glucose production is shown to be the result of AMP-induced inhibition of fructose-1,6-bisphosphatase-1, a rate-controlling enzyme in gluconeogenesis (Hunter et al. 2018). In addition to these mechanisms, metformin's metabolic action includes a reduction in glucose absorption in the intestine (Wu et al. 2017), restoring insulin secretion in pancreatic beta-cells (Patane et al. 2000) and to a lesser extent increasing insulin-mediated glucose uptake in the peripheral tissues of muscle and adipose (Galuska et al. 1991).

Being a hydrophilic compound charged positively at physiological pH, metformin enters and leaves the cells mainly via organic cationic transporters (OCTs) and multidrug and toxin extrusion transporters (MATEs) (Gong et al. 2012). The hepatic, intestinal and adipocytic uptake of metformin is primarily mediated through the OCT1 (Wang et al. 2002, Moreno-Navarrete et al. 2011) (Figure 1). Furthermore, it has been shown to accumulate in

mitochondria due to the membrane potential across the mitochondrial inner membrane (Owen et al. 2000). One of the main actions of metformin is the inhibition of mitochondrial complex I (NADH:ubiquinone oxidoreductase), that leads to multiple downstream effects both on metabolic and non-metabolic pathways responsible in the aging process (El-Mir et al. 2000, Foretz et al. 2014, Barzilai et al. 2016). The mechanisms by which metformin inhibits mitochondrial complex I remain unsolved. Although, very high concentrations of metformin are needed to directly inhibit complex I activity in isolated mitochondria, micromolar concentrations of the drug are effective in achieving a dose- and time-dependent weak, reversible and selective complex I inhibition (Vial et al. 2019). As a result, the effects of metformin diverge on metabolic and oxidative pathways and their downstream targets. However, some actions of metformin, including its antiproliferative effect can be demonstrated regardless of its effect on mitochondria, especially in Rho0 cells deficient in mitochondrial DNA (Liu et al. 2014).

In parallel to complex I inhibition and its action on the mitochondrial electron transport chain (ETC), metformin's mechanism of action is attributed both to its 5' adenosine monophosphate -activated protein kinase (AMPK)-dependent and AMPK-independent roles (Figure 1). The downstream effect of mitochondrial complex I inhibition is directly evident from the increase in cytoplasmic AMP:ATP and ADP:ATP ratios, which in turn leads to phosphorylation and activation of AMPK (Foretz et al. 2014). The direct activation of AMPK and inhibition of mTORC1 is a result of metformin's action on the lysosomal pathway, requiring v-ATP-ase-AXIN/LKB1, proposed to be a mechanism to increase the lifespan of *C. elegans* (Zhang et al. 2016, Chen et al. 2017). Phosphorylation and activation of AMPK leads to further inhibition of mTORC1, activation of peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC1-α) and mitochondrial biogenesis, activation of SIRT1 and other nutrient-sensing pathways, inhibition of advanced-glycation end products partly by inhibiting Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) and pro-inflammatory cytokines, activation of Ulk1 and regulation of autophagy, among others (Kim et al. 2011, Salminen and Kaarniranta 2012, Barzilai et al. 2016, Zhou et al. 2016, Herzig and Shaw 2018) (Figure 1). Metformin's AMPK-independent mechanisms also contribute to the direct activation of SIRT1, direct inhibition of mTORC1 via Rag-GTPases, suppression of adipogenesis through inhibition of $p70^{S6K}$ pathway, activation of DNA damage-like response via the activation of ATM/Chk2 pathway, and activation of Nuclear factor erythroid 2-Related Factor 2 (Nrf2), all of which result in downregulation of inflammatory responses (Kalender et al. 2010, Vazquez-Martin et al. 2011, Chen et al. 2017, Prasad et al. 2017, Cuyas et al. 2018) (Figure 1). A novel role of metformin was elucidated in modulating the gut microbiota and the availability of branched-chain amino acids to the gut microbiota, which is directly attributable to its effect on nutrient-sensing and aging. This function is further discussed below in metformin's capability to improve intercellular signaling and attenuate inflammation. More recently, metformin's beneficial effects on maintaining energy balance and body weight were shown to be regulated via growth/differentiation factor 15 (GDF15), which further warrants more understanding of metformin's GDF15-mediated targeting of biological aging (Coll et al. 2020).

Although the mechanisms of metformin in targeting fundamental pathways in biological aging are far from completely understood, here we attempt to link its mode of action by highlighting its role on individual hallmarks of biological aging as evidenced in cell lines and model organisms (Table 1, Figure 1).

Effects of metformin on attenuating individual hallmarks of aging

Primary targets of metformin among aging hallmarks

1. **Deregulated nutrient-sensing—**Nutrient availability and sensing are major regulators of cell-signaling pathways that take cues from environmental stimuli and intracellular activity to maintain energy homeostasis within a cell. These pathways determine cellular energy levels and communicate and coordinate with hormonal and nutrient signaling cascades to induce a positive feedback loop whereby an optimal level is achieved. However, with aging, there is a decline in the ability of the cell to maintain metabolic homeostasis, further contributing to the organismal aging phenotype (Bettedi and Foukas 2017). The most important nutrient sensors and signaling cascades that regulate aging and determine longevity are identified from genetic polymorphism studies of long-lived individuals and genetic manipulation screens in model organisms. These include highly conserved nutrient-sensing systems- 1) Somatotropic axis (GH/IGF-1) that elicits a response similar to insulin, regarding the cellular availability of glucose; 2) mTOR signaling responsible for sensing high amino acid concentrations; 3) AMPK that is involved in sensing low energy states via high AMP levels; 4) Sirtuins that complement in sensing low energy states by detecting high NAD+ levels (López-Otín et al. 2013). Although there are inconsistencies in the disease- and age-associated changes in the directionality of the GH/IGF-1 axis, a number of human studies suggest that its attenuation may protect from age-related conditions (Milman et al. 2016). Impaired insulin/IGF-1 signaling (IIS) extends lifespan by 2-fold in *C. elegans daf-2* mutants, by promoting L-proline catabolism and endogenous stress defense response via a transient ROS signal (Zarse et al. 2012). On the other hand, although the effects are tissue- and sex-specific, unequivocal evidence lies in showing that aging and age-related diseases upregulate mTORC1, while AMPK and sirtuins act in the opposite direction to mTOR signaling (López-Otín et al. 2013).

Metformin activates AMPK and SIRT1 and downregulates IIS and mTORC1

Metformin's beneficial effects on energy metabolism and aging are partly a consequence of directly targeting these key energy sensors. Metformin is shown to decrease insulin and IGF-1 levels and downregulate IIS in cancer cells (Sarfstein et al. 2013, Xie et al. 2014, Klement and Fink 2016). Additionally, metformin activates AMPK via LKB1-dependent mechanism, mediating its effect on lifespan and healthspan extension in mice and *C. elegans* (Onken and Driscoll 2010, Martin-Montalvo et al. 2013). AMPK-activation by metformin is also indicative of its suppressive effect on advanced glycation end products (AGEs), thereby mitigating the expression of inflammatory cytokines (Chung et al. 2017) (Figure 1). Metformin is shown to directly inhibit mTORC1 via Rag-GTPase inhibition and indirectly via REDD1 upregulation promoting TSC2 activity and AMPK activation-mediated phosphorylation of S6K1 and 4E-BP1- mTORC1 substrates decreasing its translation (Dowling et al. 2007, Kalender et al. 2010, Ben Sahra et al. 2011, Amin et al. 2019). It is

also revealed to be a direct activator of SIRT1, especially in low NAD+ concentrations (Cuyas et al. 2018).

As an established AMPK activator, metformin's role has been very well studied in modulating nutrient-sensing pathways. However, recent evidence in its direct inhibition of mTORC1 as well as sirtuins can have potential implications downstream of nutrient depletion, mimicking the benefits of dietary restriction. The tissue-specificity and dose-dependency in mediating these nutrient-signaling pathways in the context of human aging need to be further elucidated.

2. **Altered intercellular communication—**Endocrine, neuronal and neuroendocrine pathways provide cues to the cells to respond effectively to environmental changes, pathogens, tissue disruptors and mechanical stressors (López-Otín et al. 2013). Aging leads to a systemic dysregulation of effective cell-cell connectivity and its associated response disrupting the maintenance of intercellular communication. A key consequence of this dysregulation is age-associated chronic, sterile, low-grade inflammatory state called inflammaging, that is usually accompanied by a consistent increase in pro-inflammatory cytokine secretion (TNF, IL-6, and IL-1β), activation of NF-κB and interferon signaling, increased burden of senescent cells and their secretome as well as altered autophagic response (López-Otín et al. 2013, Franceschi et al. 2018). Importantly, inflammation is shown to trigger or respond to multiple other hallmarks including epigenetic alterations, stem cell dysfunction and loss of proteostasis, thereby contributing to the pathogenesis of age-related diseases and accelerated aging (Franceschi et al. 2018, Sanada et al. 2018, Vinatier et al. 2018, Benayoun et al. 2019, Josephson et al. 2019).

Metformin suppresses pro-inflammatory cytokines and inhibits NF-κB pathway

Several studies suggest metformin's role in modulating several key immunoregulatory mechanisms, often with improvement in metabolic parameters (Saisho 2015). In older diabetic individuals, metformin monotherapy lowered the levels of circulating pro-inflammatory cytokines and the associated mortality risk in a recent five-year follow-up study (Tizazu et al. 2019). In primary hepatocytes, using a distinct mechanism to its anti-hyperglycemic effect, metformin suppressed TNF-α dependent NF-κB signaling and expression of IL-6 and IL-1β (Cameron et al. 2016). This suppression of proinflammatory cytokine markers along with senescence-associated secretory phenotype (SASP) is also evident through metformin's inhibition of IKK/NF-κB activation (Moiseeva et al. 2013). A working model of metformin's anti-inflammatory properties also suggests that metformin, in an AMPK-dependent inhibition of STAT3, prevents the differentiation of monocytes to macrophages (Vasamsetti et al. 2015). In addition to direct inhibition of inflammation, metformin's action in lowering body weight and improving insulin metabolism and sensitivity has indirect implications on lowering systemic inflammation as well (Saisho 2015).

Metformin modulates the gut microbiota further improving metabolism and reducing inflammation

There is increasing evidence on the role of gut microbiota in effectively mediating inflammatory responses. Age-associated microbial dysbiosis (alterations in gut microbiota) is shown to promote inflammation, gut permeability and proinflammatory cytokine release (Thevaranjan et al. 2017). Healthy gut microbiome has been shown to be associated with optimal healthy immune function (Seidel and Valenzano 2018). Early evidence of metformin's role in delaying aging and increasing lifespan, via modulating the microbial folate and inducing methionine restriction was demonstrated in *C. elegans* (Cabreiro et al. 2013). Similarly, recent evidence suggests that metformin extends Drosophila lifespan in a dose-dependent manner, only in control OP50 E.coli colonized strains, and not germ-free strains, indicating the role of microbiota in mediating metformin-induced lifespan extension across species (Pryor et al. 2019). Later, animal studies revealed that metformin's glucoregulatory role was an effect of increased *Lactobacilli* abundance in the upper small intestine, while its anti-inflammatory role was associated with increased abundance of *Akkermansia*, *Bacteroides*, *Butyricimonas*, and *Parabacteroides* genera (Bauer et al. 2018, Lee et al. 2018). In human diabetic individuals, metformin was shown to shift gut microbiota composition to predominantly short-chain fatty-acid producing microbes (de la Cuesta-Zuluaga et al. 2017). Furthermore, metformin's role in improving metabolic dysfunction in diabetics was linked to an increase in levels of bile acid glycoursodeoxycholic acid (GUDCA) by inhibiting the growth of *B. fragilis*, thereby altering intestinal Farnesoid X Receptor (FXR) signaling (Sun et al. 2018).

Metformin's role as an anti-inflammatory is directly responsive and consequential to regulating cellular senescence, stem cell function, autophagy, and macromolecular damage. Cell-cell communication and response are maintained, thereby leading to a decrease in the abundance of pro-inflammatory cytokines. It is essential to understand how metformin mitigates inflammatory responses against various stressors. Moreover, understanding the role of gut microbiota in regulating metformin's action on inflammation and intercellular communication is crucial for establishing this drug as a candidate to target aging.

3. Genomic instability—Genomic instability refers to the accumulation of genetic damage throughout one's lifespan, due to a multitude of DNA alterations like mutations, indels and chromosomal rearrangements (Vijg and Suh 2013). Attributed to both endogenous (mitochondrial ROS, DNA replication errors, etc.) and exogenous (environmental and iatrogenic) agents, genomic instability often accompanies biological aging, while its artificial induction can often lead to an accelerated aging phenotype (Niedernhofer et al. 2018). Such genetic damage involves oxidation of constituent DNA bases, crosslinking of DNA and protein as well as accumulation of DNA double-stranded breaks. Although the extent of lesions occurring daily in each somatic cell can be quite high, most of them are countervailed by the vast network of DNA repair mechanisms. However, as evidenced by increased DNA repair deficits in mouse models of accelerated aging and human progeroid syndromes as well as increased nuclear and mitochondrial DNA damage, genomic instability is a pivotal hallmark of biological aging (Vijg and Suh 2013).

Metformin's genome protective effects are a result of increased DNA-damage like response and DNA repair

Several mechanisms have been associated with metformin's response to mitigate genomic instability, in the context of aging and various types of cancers. Studies have shown metformin's genome protective effects via reduction of oxidative stress, DNA damage and DNA damage response, regulation of Ataxia Telangiectasia Mutated (ATM) protein kinase and epigenetic effects, however, there is no consensus regarding the underlying mechanism of the drug in regulating components of genome stability (Najafi et al. 2018). In mouse embryonic fibroblasts (MEFs), metformin is shown to reduce paraquat-associated endogenous ROS levels and associated DNA damage by preventing ROS toxicity (Algire et al. 2012). Furthermore, in a dose-dependent manner, metformin prevents activation of ATM-protein kinase, thereby lowering ROS and γH_2AX-a sensitive marker of DNA-double stranded breaks and genomic instability (Halicka et al. 2011). Similarly, it takes 10-fold lower levels of metformin to scavenge mitochondrial ROS than required to inhibit mitochondrial complex I, without directly impacting respiratory capacity as evidenced in rats (Kane et al. 2010). The genotoxicity-protective effects of metformin are demonstrated in human lymphocytes, normal and diabetic rats, rat and mouse bone marrow cells, mouse kidney cells as evidenced by a reduction in micronuclei and chromosomal aberrations (Sant'Anna et al. 2013, Cheki et al. 2016, Othman et al. 2016, Najafi et al. 2018, Cheki et al. 2019). In T2D patients, metformin induces an antioxidant response further leading to DNA Base Excision Repair system (Dogan Turacli et al. 2018). In the presence of DNA damage, metformin also activates ATM and Checkpoint kinases-2 thereby phosphorylating serine 132 on histone H2AX $\rightarrow$ γH_2AX, and recruiting DNA repair complexes at the DNA double-strand breaks (Vazquez-Martin et al. 2011). Additionally, we have previously shown that short-term metformin treatment in older adults induced BRCA-mediated DNA damage response and DNA repair in skeletal muscle (Kulkarni et al. 2018).

Although there is no consensus on how metformin regulates oxidative damage and attenuates genome instability, the evidence is stronger for metformin stimulating DNA damage responses and protective mechanisms against genotoxic stress. It is essential to further elucidate metformin's role in preventing or reverting age-induced dysregulation in both nuclear and mitochondrial genome stability, chromosomal structure and its interplay with key processes like senescence and gene regulatory changes.

4. Loss of proteostasis—A highly regulated network of > 2000 proteins comprising of molecular chaperones, proteolytic systems and regulators, is crucial to maintain a stable proteome or protein homeostasis (proteostasis) (Hipp et al. 2019). Aging, age-associated pathologies and neurodegenerative diseases including Alzheimer's and Parkinson's diseases, exhibit an impaired network of coordinated proteostasis, with the accumulation of intracellular damage (Kaushik and Cuervo 2015). Three key aspects of the proteostasis network, namely- protein synthesis and folding, maintenance of conformational stability and autophagy-mediated protein degradation are deteriorated with aging, leading to an imbalance in protein abundance (Hipp et al. 2019). Model organism studies have revealed lifespan extensions through alterations within pathways enhancing proteostasis, including Insulin/IGF-1 signaling, mitochondrial ETC, enhanced autophagy, and nutrient-sensing.

Metformin augments autophagy and rescues protein misfolding

Metformin's stabilizing effect on proteostasis is mainly the result of augmented autophagy and inhibited protein synthesis via direct and indirect inhibition of mTOR signaling (Shi et al. 2012). In a Clk1 mutant Parkinson's Disease mouse models, with increased activity of neurotoxin MPP, metformin treatment reversed behavioral impairments, reduced α-synuclein accumulation and enhanced LC3-II-mediated autophagy in dopaminergic neurons and midbrain, in an AMPK-dependent mechanism (Lu et al. 2016, Yan et al. 2017). Autophagy-related proteins LAMP-1 and Beclin-1 are upregulated by metformin treatment, which have been shown to attenuate hyperglycemia-induced apoptosis in cardiomyocytes (Wang et al. 2017). Accumulating evidence also suggests that metformin induces enhanced autophagic responses in δ-Sarcoglycan deficiency-induced dilated cardiomyopathic mouse hearts and enhanced mitophagy in *ob/ob* mice (Song et al. 2016, Kanamori et al. 2019). Furthermore, metformin is also shown to rescue misfolding and trafficking of rhodopsin, downstream of AMPK-activation (Athanasiou et al. 2017).

These studies in model systems reveal metformin's role against impairments in proteostasis. But whether the prevention of protein misfolding and trafficking translates to attenuation of aging in target organs is yet to be elucidated. However, further studies are warranted to understand the drug's effect on rescuing age-associated imbalance in protein abundance and misfolding. An ongoing clinical trial examining metformin's pro-autophagy effects in prediabetic individuals may help elucidate the drug's role in humans (NCT03309007).

Secondary targets of metformin among aging hallmarks

5. Mitochondrial dysfunction—There is longstanding evidence that the decline in mitochondrial function with aging, may contribute to age-associated dysregulation in energy homeostasis and increased predisposition to age-related diseases (Sun et al. 2016). Aging induced disruptions in mitochondrial function is a result of several intra- and extracellular stresses including mtDNA mutations, nuclear genomic instability, reduced mitochondrial biogenesis, defective mitophagy, and altered mitochondrial dynamics and ETC regulation, among others (López-Otín et al. 2013). With an increased understanding of the role of mitochondria in addition to serving as the powerhouse of the cell, its dysfunction with aging can have implications on inflammation, senescence, autophagy and retrograde nuclear signaling (Jang et al. 2018).

Metformin lowers oxidative stress via mitochondrial complex I inhibition and improves mitochondrial biogenesis via PGC-1α

Due to their key role in regulating these vital cellular processes along with energy homeostasis and oxidative stress via ROS, mitochondria are deemed to be an attractive therapeutic target against aging and age-related diseases and pathologies (Madreiter-Sokolowski et al. 2018, Murphy and Hartley 2018). Metformin inhibits complex I (NADH:ubiquinone oxidoreductase) of the mitochondrial ETC, thereby directly impacting mitochondria-induced oxidative stress via lowering ROS or by indirect scavenging mechanism (Kelly et al. 2015, Fontaine 2018, Vial et al. 2019). This inhibition also induced an anti-inflammatory response by specifically inhibiting pro-IL1β production in macrophages (Kelly et al. 2015). Similarly, complex I inhibition by metformin also reduces

ROS mediated IL-6 release in alveolar macrophages, thereby preventing arterial thrombosis in mice (Soberanes et al. 2019). Decreased hepatic gluconeogenesis is also demonstrated to be associated with metformin's direct inhibition of mitochondrial glycerophosphate dehydrogenase (mGP_{DH})- a flavin-linked respiratory chain-linked dehydrogenase, which in turn modulates cytosolic redox state (Madiraju et al. 2014). Metformin increases the expression and protein activity of hepatic and skeletal muscle associated PGC-1α, a co-transcriptional regulator that induces mitochondrial biogenesis (Suwa et al. 2006, Aatsinki et al. 2014). Interestingly, *in vitro* studies have demonstrated the ability of metformin to revert mitochondrial dysfunction associated with Down syndrome, a human model of accelerated aging, via restoration of mitochondrial gene expression network and rescue of mitochondrial morphology (Izzo et al. 2017). Linking its effects on histone methylation and sirtuin activation, metformin was also shown to induce mitochondrial biogenesis and delay cellular senescence by acting through SIRT3 upregulation (Karnewar et al. 2018). Metformin's direct activation of SIRT1 may help combat age-related mitochondrial dysfunction which is attributed to a reduction in SIRT1 activity leading to downregulation of a nuclear-encoded mitochondrial transcription factor A (TFAM) and declines in NAD+ levels (Gomes et al. 2013, Cuyas et al. 2018). Recently, metformin's cardioprotective role in human arterial appendage tissue was elucidated through its dose-dependent mild inhibitory effect on the activity of complexes I, IV and V with a reduced superoxide production and attenuation of mitochondrial permeability transition pore (Emelyanova et al. 2019).

Although there is substantial evidence of the role of metformin in inhibiting mitochondrial complex I and on improving mitochondrial biogenesis by increasing PGC-1α expression, there is no consensus on whether this effect is tissue-specific. For instance, in skeletal muscle, metformin is shown to inhibit aerobic exercise-induced mitochondrial adaptations in older adults (Konopka et al. 2019). But, in mice with AMPK-kinase-dead mutation, even short-term metformin treatment enhances mitochondrial function and biogenesis in skeletal muscle (Kristensen et al. 2013). Hence, it is important to further expand the understanding of metformin's species- and tissue-specific mitochondrial regulation as a monotherapy and in combination with other interventions.

6. Stem cell exhaustion—With aging, there is a systemic decrease in the regenerative capacity of tissues. It is imperative to maintain homeostasis of stem and progenitor cells and their regeneration potential since both a reduced number of stem cells and excessive proliferation can induce aging phenotypes (López-Otín et al. 2013). Age-associated decrease in stem cell function is observed in several stem cell populations, including those of hematopoietic cells, intestinal stem cells, satellite cells, neuronal stem cells, hair follicle cells, melanocytes, and germline cells (Schultz and Sinclair 2016). Currently, there is no consensus regarding the association between the change in stem cell number of various tissue sub-types and aging. However, some studies have demonstrated that hematopoietic and intestinal stem cells increase with age in humans as well as animal models (mice, *Drosophila spp.*) (Schultz and Sinclair 2016). The exact mechanism for decreased stem and progenitor cell capacity to rejuvenate is not known, but the common mechanisms of stem cell aging can be explained by some cell-intrinsic and extrinsic factors including telomere

attrition, molecular damage, epigenetic drift and deregulation of developmental pathways (Ermolaeva et al. 2018).

Metformin induces stem cell rejuvenation capacity and delays stem cell aging

Fortunately, metformin targets several pathways that cause stem cell exhaustion, thereby reinforcing the rationale to understand the role of metformin in addressing age-associated stem cell exhaustion. The previously mentioned gerotherapeutic effect of metformin in activating Gpx7 via Nrf2 is shown to delay cellular attrition and prevent premature aging thereby increasing the lifespan of human mesenchymal stem cells (Fang et al. 2018). The early evidence of metformin delaying stem cell aging was demonstrated in *Drosophila* midgut intestinal stem cells (ISCs), where it inhibited DNA-damage and downstream centrosome amplification via the AKT/TOR pathway, later found out to be also mediated by Atg6- an autophagy-related factor (Na et al. 2013, Na et al. 2015, Na et al. 2018). The anti-aging effect of metformin in stem cell function is not limited to ISCs and can be extended to other stem cell populations. Recently, in female mice, metformin was found to expand the neural stem cell pool, while still promoting neurogenesis and cognitive recovery in both sexes (Ruddy et al. 2019). Interestingly, metformin also reversed age-associated dysregulation of rejuvenating and differentiating capacity of oligodendrocyte progenitor cells, further improving remyelination (Neumann et al. 2019). Metformin-induced retention of self-renewing capacity and neurogenesis are mediated through two distinct mechanisms- the former via a transcription factor TAp73, and the latter via AMPK-activated αPKC-CBP pathway (Fatt et al. 2015). In muscle, aged satellite cells fail to maintain quiescence and the capacity to self-renew once activated. Metformin treatment is shown to delay satellite cell activation and maintain a quiescent, low metabolic state thereby maintaining stem cell numbers (Pavlidou et al. 2019).

Maintenance of stem cell number and function can help combat age-associated loss of rejuvenation potential and thereby promote tissue repair. In several instances, metformin has been effective in targeting stem cell exhaustion, delaying stem cell aging and maintaining stem cell function. It is very important to delineate metformin's role as a gerotherapeutic agent preserving stem cell function, while also preventing abnormal differentiation and leading to tumorigenesis. Furthermore, this role of metformin needs to be investigated further in studies and trials in regenerative medicine.

7. Epigenetic alterations—Loss of histones, imbalance in histone modifications, changes in chromatin architecture, breakdown of the nuclear lamina, as well as DNA and histone methylation changes are characteristics of biological aging (Sen et al. 2016). Histone modifications such as acetylation and phosphorylation can lead to active transcription, loss of cellular homeostasis and age-associated metabolic decline while post-translational modifications like ubiquitination, sumoylation, O-GlcNAcylation, and ADP-ribosylation, can have varying influences on transcriptional dysregulation (Pal and Tyler 2016, Peleg et al. 2016). Furthermore, DNA and histone methylation landscapes change during aging, generally leading to global hypomethylation and promoter-specific hypermethylation (Michalak et al. 2019). These changes are determinants of conserved, tissue-specific, age-

associated transcriptional changes, that contribute to metabolic and inflammatory phenotypes of aging (Benayoun et al. 2015, Benayoun et al. 2019).

Metformin regulates transcriptional activity via histone modifications, DNA methylation, and miRNAs

Due to their modifiable nature, epigenetic and transcriptional changes are generally indicative of the effect of therapeutic interventions on age-related diseases and promoting healthy aging. Metformin's AMPK-dependent and independent mechanisms have been observed to influence histone modifications through phosphorylation of histone acetyltransferases (HATs), inhibition of Class II histone deacetylases (HDACs) and activation of SIRT1 (Bridgeman et al. 2018). In non-diabetic HER2-positive breast cancer patients as well as human breast cancer xenografts injected into nude mice, the drug augmented global H3K27me3 levels, directly targeted demethylase KDM6A/UTX and moreover, reverted age-associated loss of global H3K27me3 in fibroblasts from both healthy older donors and Werner's syndrome patients (Cuyàs et al. 2017). This effect was irrespective of metformin's mitochondrial complex I inhibition, as evidenced in Rho0 cells devoid of mitochondria. Additionally, metformin acts as a metaboloepigenetic regulator by promoting global DNA methylation via the mitochondrial one-carbon metabolism pathway and in cancer cells via the H19/S-adenosylhomocysteine hydrolase (SAHH) axis (Cuyàs et al. 2017, Zhong et al. 2017). Metformin's direct upregulation of global DNA methylation and its downstream tissue-specific (skeletal muscle, subcutaneous adipose tissue, peripheral blood mononuclear cells) transcriptional profiles in healthy and diabetic individuals as well as cancer cell lines (breast and colorectal), reflect its age-targeting and anti-tumoral role (de Kreutzenberg et al. 2015, Yu et al. 2017, Bridgeman et al. 2018, Elbere et al. 2018, Kulkarni et al. 2018, Sabit et al. 2018, Schulten and Bakhashab 2019). Apart from its effect on DNA and histone methylation, metformin also upregulates DICER1 and downstream miRNAs, several of which are reduced with cellular senescence, suggesting metformin's multiple mechanisms to regulate transcription and post-transcription (Noren Hooten et al. 2016). Most recently, metformin, in combination with recombinant human growth hormone. (rhGH) and dehydroepiandrosterone (DHEA) was shown to revert the mean "epigenetic age"- a DNA-methylation based marker of biological age, by 1.5 years after a year of treatment in healthy human adults (Fahy et al. 2019).

There is inherent variability in metformin's transcriptional and epigenetic regulation via histone and DNA modifications and its effects on miRNA. Most of the studies so far have investigated this regulation in the cell lines at supraphamacological doses that may limit their generalizability to clinical studies. It is essential to elucidate these responses further, in the context of pharmacologically relevant doses in tissue and cell-specific manner as well as at systemic levels.

8. Telomere attrition—Telomeres are highly conserved ribonucleoprotein complexes on the termini of eukaryotic chromosomes that play a crucial role in protecting chromosomal ends from degradation caused by an incomplete DNA replication (Casagrande and Hau 2019). With aging and stress exposure, these regions are particularly vulnerable to deterioration, and due to insufficient telomerase in adult cells to replenish this loss, they

undergo progressive attrition with cell proliferation (Blackburn et al. 2015). Progressive telomere shortening is found to be associated with biological aging and age-related morbidities, frailty, and mortality (Armanios et al. 2009, Araujo Carvalho et al. 2019, Whittemore et al. 2019, Zhu et al. 2019). Furthermore, telomere shortening is also shown to trigger cellular senescence, inflammation, and mitochondrial dysfunction via p53-mediated DNA damage response pathways (Zhu et al. 2019).

Metformin activates TERRA and is shown to reduce telomere shortening

There is little evidence on the action of interventions including metformin on telomere length and especially on their consequences on healthy aging and increased lifespan. However, Diman et al. identified downstream targets of AMPK activated by metformin-nuclear respiratory factor 1 and PGC1-α as regulators of human telomere transcription via telomeric repeat-containing RNA (TERRA) (Diman et al. 2016). In diabetic individuals, metformin is suggested to reduce telomere shortening, when compared to those not taking metformin (Robb-MacKay 2018). This is similar to other studies where metformin monotherapy to improve glucose tolerance or insulin sensitivity prevented shortening of leukocyte telomere length in diabetic individuals (de Zegher et al. 2015, Rosa et al. 2018, Liu et al. 2019). In mothers with gestational diabetes, metformin treatment prevented telomere shortening in their male offspring (Garcia-Martin et al. 2018).

Further studies are warranted to understand how metformin may prevent telomere attrition and maintain telomeric integrity. Additionally, metformin's effect on mitochondrial function and cellular senescence may induce protective feedback mechanisms on telomere maintenance. It is also important to delineate metformin's effects on telomere length and integrity in aging versus cancer.

9. Cellular senescence—Senescence, defined as a stable cell cycle arrest when cells reach their replicative potential or are exposed to internal or external stressors, is claimed to be a major cause of aging (Gil 2019). Seminal discoveries, first by Hayflick and Moorehead, and lately by Baker et al., concretized cellular senescence as a major driver of aging with the age-associated accumulation of senescent cells, and attenuation of age-related tissue dysfunction after their removal, even late in life (Hayflick and Moorhead 1961, Baker et al. 2011). Although senescence acts as a protective mechanism against malignancies, serving as a tumor-suppressive mechanism, recent evidence suggests that senescent cells can morph their phenotype to SASP, which can paradoxically cause tumor growth (Coppé et al. 2010). Furthermore, senescent cell accumulation via aging-induced epigenetic, proteotoxic and genotoxic stresses usually exhibits a causal role in many age-related diseases, including glaucoma, cataracts, diabetes mellitus, osteoarthritis, among others (Childs et al. 2015). More recently, senolytics- drugs that selectively induce apoptosis in senescent cells have shown tremendous potential and clinical utility to alleviate aging and age-related phenotypes (Kirkland et al. 2017, Xu et al. 2018, Justice et al. 2019).

Metformin downregulates SASP and lowers senescent cell burden

Metformin- although not exhibiting senolytic properties, has been effective in suppressing cellular senescence and SASP in multiple age-associated dysfunctions. Chronic metformin

administration at low doses delays senescence in human diploid fibroblasts and human mesenchymal stem cells, as evidenced by reduced SA-β-Gal staining, via Nrf2-mediated upregulation of Glutathione peroxidase 7 (GPx7) (Fang et al. 2018). Metformin's senotherapeutic role is also mediated by its anti-inflammatory effect by preventing NF-κB translocation into the nucleus thereby not phosphorylating IκB and its kinase and inhibiting NF-κB pathway altogether (Moiseeva et al. 2013). Inhibition of NF-κB by metformin in ex-vivo cultures of murine olfactory ensheathing cells is also associated with decreased SA-β-Gal activity along with a decreased expression of pro-inflammatory cytokines and oxidative stress markers (Śmieszek et al. 2017). In a DICER1-dependent mechanism, metformin is shown to lower the protein levels of p16 and p21, and RNA levels of SASP hallmarks including IL-6 and IL-8 in human fibroblasts (Noren Hooten et al. 2016). Recently, in human periodontal ligament cells, the protective effect against senescence and oxidative stress was linked to metformin-induced stimulation of autophagy, further highlighting the role of metformin in targeting the interconnectedness of these two hallmarks of aging (Kuang et al. 2019). Similarly, in *in vitro* and *in vivo* models of intervertebral disc degeneration, metformin treatment reduced senescence in nucleus pulposus cells by upregulating AMPK-mediated autophagy (Chen et al. 2016).

It is evident that metformin attenuates the increased burden of senescent cells and upregulation of SASP with aging. Its role as a SASP modulator via Nrf2-Gpx7 activation in mediating oxidative stress, and via NF-κB inhibition in mediating inflammatory response provides a better understanding of its senotherapeutic mechanism. However, in the context of breast cancer and hepatoma, low dose metformin has been previously shown to induce SASP gene expression signature and p53-dependent senescence, respectively (Williams et al. 2013, Yi et al. 2013). Thus, metformin's effect on cellular senescence and SASP is context-dependent (anti-cancer by inducing apoptosis and anti-aging by inhibiting inflammation), probing for further understanding of what stimulates metformin to induce an anti-senescence response in aging tissues as opposed to pro-senescence in cancerous tissues. With the advent of clinical trials with senolytics, it is also crucial to understand how metformin can be best utilized in combination with other drugs to optimize the combinatorial effect on senescence and aging.

Side Effects of Metformin

Clearly, the unexpected positive side effects of metformin include increased healthspan and decreased mortality. Recently, it was evident that diabetic patients with liver, kidney and heart diseases, who did not receive metformin could have benefitted from its use with reduced hospitalization and mortality (Crowley et al. 2017). Hence, the 'black box' warning for metformin has been steadily disappearing, as evidenced by less strict indication for use in patients with reduced kidney function (Bakris and Molitch 2016). As in most drugs, minor side effects occur with metformin, but it is important to understand whether they reveal anything about the mechanism of action of this drug. Most of these minor side effects include gastrointestinal discomfort such as abdominal or stomach pain, early satiety, decreased appetite, and diarrhea, which usually subside after one or two weeks of use (McCreight et al. 2016). If diarrhea persists beyond a week (in ~3% of users), the drug is discontinued. These side effects could be the result of an acute change in the gut

microbiome, as evidenced in nematodes and other smaller organisms (Cabreiro et al. 2013). Microbiome changes may also be secondary to reduced vitamin B12 levels with chronic metformin use, although the mechanism is still unclear and few patients need vitamin B12 replacement (Aroda et al. 2016). A condition termed Metformin Associated Lactic Acidosis (MALA) is associated with metformin use in patients who already have high lactic acid levels as in severe kidney, liver and heart diseases, while also taking metformin (DeFronzo et al. 2016). Few patients may complain about anxiety, sleeplessness, fast breathing, and other symptoms soon after taking metformin and will usually stop the drugs on their own. Metformin increases lactate levels, while maintaining the normal range, but it is possible that those with severe side effects including MALA subjects may be more sensitive to the inhibition of mitochondrial complex-1, attributable to genetic mechanisms.

The TAME trial aims to examine the subjects who stop taking metformin for all the aforementioned reasons and determine if they are prone to different outcomes, with their biological samples available for further mechanistic studies. It is also important to understand that, consistent with its biological effects since metformin is not a hypoglycemic agent it is not likely to cause hypoglycemia unless used in combination with other glucose-lowering drugs in diabetic individuals. Thus, it is very crucial to note that outside of its indication for diabetes, the use of metformin is recommended only in the context of clinical trials, under physician supervision.

Conclusions and Perspectives

Metformin’s efficacy in attenuating hallmarks of biological aging is reflective of its strength and potential as a therapeutic that can target crucial mechanistic pathways involved in aging. The metabolic effects of metformin are mainly through its metabolic arm via activation of AMPK and oxidative arm through inhibition of complex I of the mitochondrial ETC. There are additional direct effects on mTORC1, PGC1-α, Insulin-IGF1 signaling, SIRT1, NF-κB signaling, and pro-inflammatory cytokines thereby allowing us to classify the four hallmarks (deregulated nutrient-sensing, altered intercellular communication, genomic instability and loss of proteostasis) as the primary targets of metformin. The effects on mitochondrial function, DNA and histone modifications, stem cell rejuvenation, preventing telomere shortening and downregulation of senescence and SASP are downstream of the primary targets (Figure 2).

Several clinical trials are currently underway to assess the response of metformin as a monotherapy and in combination with lifestyle interventions like exercise, on clinical and molecular outcomes of individual hallmarks of aging. These are particularly involved in measuring metformin’s effect on frailty and associated inflammatory and SASP candidate biomarkers (NCT03451006), pro-autophagy effects by measuring LC3 levels (NCT03309007), changes in muscle size (NCT03107884), improvements in immune response via the gut microbiota (NCT03713801), and effective influenza vaccine responses (NCT03996538).

An important limitation to studying metformin’s action on aging is its tissue-specificity, dose-dependency and the role of mitochondrial inhibition in contributing to any of its

effects. No doubt, further studies will potentially shed more light on each of these limitations. But as the accumulation of evidence links metformin to all hallmarks of aging, it is very important to remember that metformin's impact on any of these hallmarks has direct consequences on systemic attenuation across several other hallmarks of aging (Figure 2). For example, as highlighted above, evidence suggests that targeting autophagy impacts mitochondrial function, nutrient-sensing, and macromolecular damage. With this line of thought, we suggest that targeting aging at any of the primary or secondary levels, will ultimately make the cells, tissues, and systems more youthful, with a direct effect on the systemic biology of aging (Figure 2). Thus, some effects that are observed are a result of achieving systemic youthfulness and not direct targets of the drug. In fact, reviewing the effects of other interventions like rapalogs and sirtuins also shows that they may be impacting many of the similar hallmarks of aging (Lamming et al. 2013, Grabowska et al. 2017).

However, for any biotechnology or pharmaceutical firm that wants to design metformin-like drugs, it is also possible that all effects of metformin need to be combined for the optimal effect. For instance, targeting complex I is reasonable for the development of anti-cancer drugs and a drug that does not depend on tissue selectivity and gets to the mitochondria in all tissues (that is also relevant for aging) is warranted. But to show that it is superior to metformin in preventing age-related diseases may be risky, not to mention-expensive for patients.

In conclusion, there is extensive epidemiological, basic science, and clinical data highlighting the effectiveness of metformin in targeting several age-related morbidities in humans. Studies in model organisms and cell lines provide compelling evidence on metformin's beneficial effects against crucial pathways in aging. In addition to its known safety profile and long-term use in humans, metformin-induced attenuation of the major hallmarks of biological aging and their interconnectivity makes it a very attractive candidate against aging, which the TAME study is set to prove and change the landscape of healthspan around the world.

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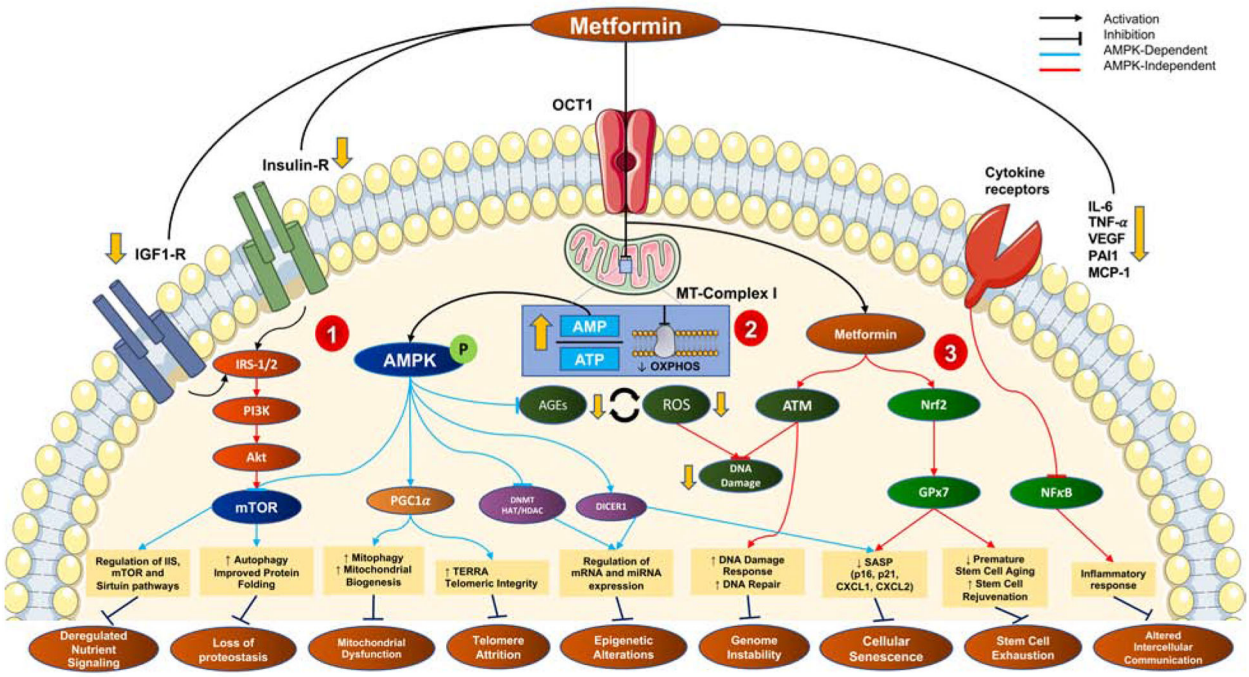

Figure 1- Mechanisms of metformin action in attenuating hallmarks of biological aging

The cellular uptake of metformin is via the organic cationic transporter 1 (OCT1), after which it exerts three arms of action- 1) metabolic; 2) oxidative and 3) inflammatory.

1) Metformin inhibits mitochondrial complex I and thereby oxidative phosphorylation leading to an increased AMP:ATP ratio, causing a direct activation of AMPK. AMPK-dependent mechanisms (blue) contribute to the downstream inhibition of mTORC1 (improved nutrient-sensing and autophagy), activation of PGC-1α (improved mitochondrial biogenesis), transcriptional regulation via DNA/histone modifications and miRNAs. Extracellularly, metformin downregulated Insulin/IGF1 signaling, also leading to mTORC1 inhibition.

2) The inhibition of mitochondrial ETC also leads to AMPK-independent effects (red) including reduced reactive oxygen species (ROS), reduced advanced glycation end-products (AGEs) and thereby reduced macromolecular damage.

3) The AMPK-independent (red) anti-inflammatory and senotherapeutic effects of metformin are evident via the downregulation of pro-inflammatory cytokines, NF-κB signaling, and activation of Nrf2-Gpx7 and ATM-signaling, respectively.

These three arms work to mitigate the aging-induced dysregulation in cells, thereby attenuating hallmarks of aging.

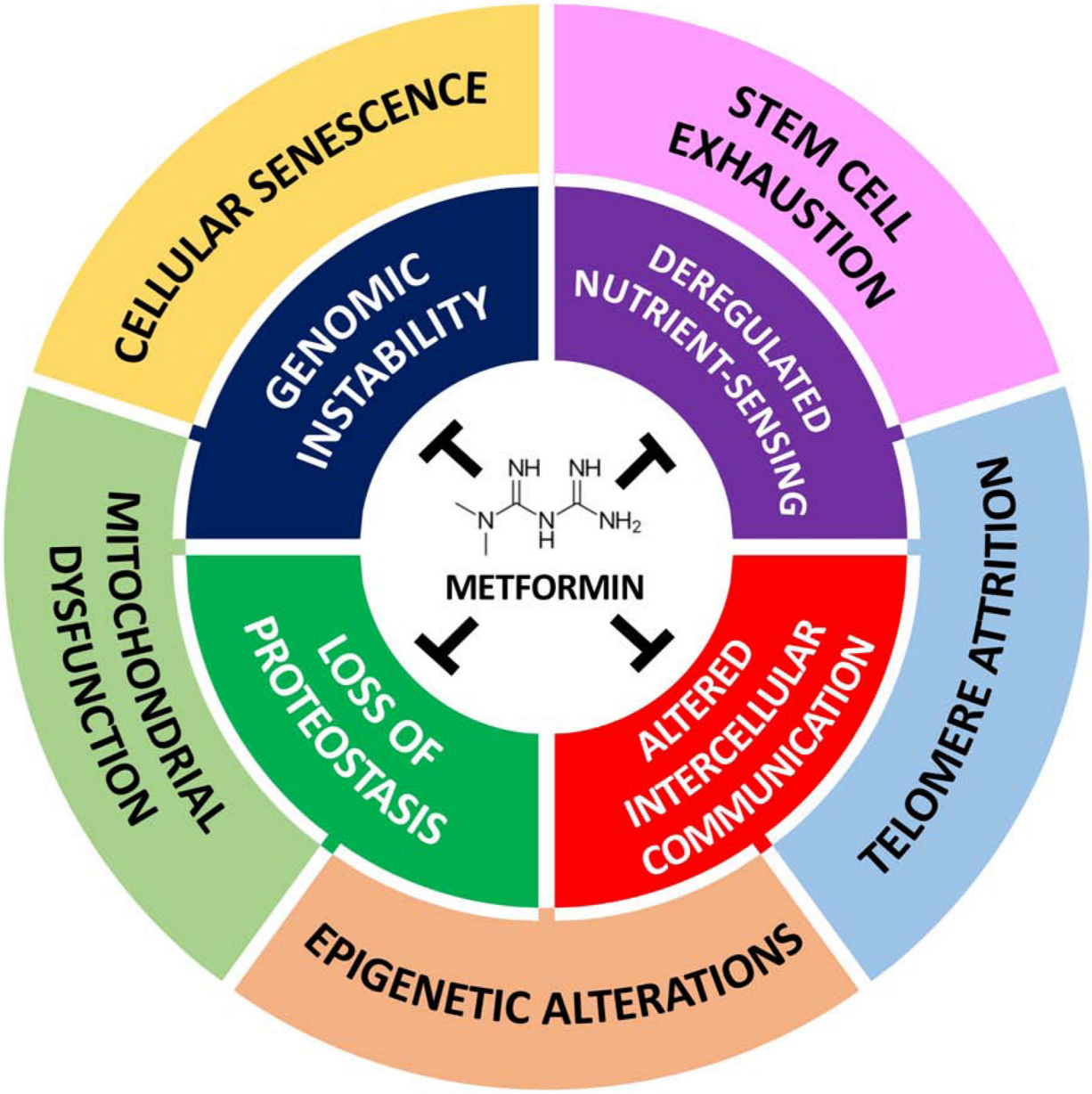

Figure 2- The primary and secondary targets of metformin among the hallmarks of aging

Metformin attenuates hallmarks of aging to varying degrees and in turn, contributes to its gerotherapeutic effects. We classified the hallmarks in the inner circle (deregulated nutrient-sensing, genomic instability, loss of proteostasis and altered intercellular communication) as primary, reflective of them being direct targets of metformin via its action on AMPK, SIRT1, mTORC1, IIS pathways, protection against macromolecular damage, improved autophagy response and reduced inflammation, respectively. The hallmarks in the outer circle (stem cell exhaustion, cellular senescence, mitochondrial dysfunction, epigenetic alterations, telomere attrition) are secondary targets, due to their attenuation being mediated by metformin's role on a primary target. Due to the high degree of interconnectedness

between the hallmarks of aging, metformin's attenuation of any single hallmark has a major influence on several others, thereby leading to a widespread response against aging.

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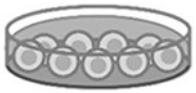
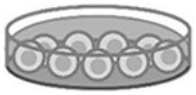
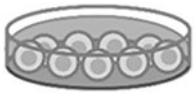
Table 1-

Summary of key targets and pathways impacted by metformin as evidenced by their modulation in cell lines, *C. elegans*, Drosophila, and rodents.

Attenuation of Hallmarks of Aging	Effects of Metformin on key targets and pathways involved in regulating each hallmark of aging			
				
Improved Nutrient Signaling	• AMPK ↑ (Hawley et al. 2002) • SIRT1 ↑ in low NAD+ concentrations (Cuyas et al. 2018) • lnsulin/IGF-1 signaling ↓ (Sarfstein et al. 2013) • AGEs ↓ (Chung et al. 2017) • mTORCI ↓ via Rag-GTPase ↓ (Kalender et al. 2010), via TSC2 ↑ (Dowling et al. 2007) & via REDD1 ↑ (Ben Sahra et al. 2011)	• AMPK ↑ (Onken and Driscoll 2010, Cabreiro et al. 2013) • LKB1 ↑ (Onken and Driscoll 2010) • SKN1 ↑ (Onken and Driscoll 2010) • TORC1 ↓ (Chen et al. 2017)	• AMPK ↑ (no change in lifespan) (Slack et al. 2012) • Lipid stores ↓ (Slack et al. 2012)	• AMPK ↑ (Martin-Montalvo et al. 2013, Howell et al. 2017) • mTORCI ↓ (Howell et al. 2017)
Enhanced Intercellular Communication	• IKKα/β IL-6 ↓, IL-1β ↓, CXCL1/2 ↓ (Cameron et al. 2016) • NF-κB pathway ↓ (Moiseeva et al. 2013) • MMP-9 ↓ & STAT3 ↓ (Vasamsetti et al. 2015) • Activator protein 1 (AP-1) transcription factor network ↑ and cytokine-cytokine receptor interactions modulated (Gillespie et al. 2019)	• Methionine restriction (Cabreiro et al. 2013) • Branched-chain amino acids ↓ (De Haes et al. 2014)	–	• IL-6 ↓, IL-1β ↓ *Lactobacilli* in upper small intestine ↑ (Bauer et al. 2018) • *Bacteroides fragilis* ↓ & FXR signaling ↓ (Sun et al. 2018)
Ameliorated Proteostasis	• LAMP-1 ↑ & Beclin-1 ↑ (Wang et al. 2017) • LC3 ↑ & p-ULK ↑ (Wang et al. 2018) • CEBPD-mediated autophagy ↑ (Tsai et al. 2017)	• HLH-30 translocalized to nucleus → autophagy gene expression ↑ (Chen et al. 2017)	–	• α-synuclein ↓ (Lu et al. 2016) • LC3-II ↑ (enhanced autophagy) (Yan et al. 2017)
Protection against Genomic Instability	• Ataxia-telangiectasia mutated (ATM) dependent DNA-damage response ↑ (Vazquez-Martin et al. 2011) • DNA-damage ↓ (Algire et al. 2012)	–	• DNA damage ↓ & oxidative	• Micronuclei ↓ (Sant'Anna et al. 2013, Cheki et al. 2019)

Attenuation of Hallmarks of Aging	Effects of Metformin on key targets and pathways involved in regulating each hallmark of aging			
				
	• Micronuclei ↓, dicentrics & acentric fragments ↓, nucleoplasmic bridges ↓, rings ↓ (Cheki et al. 2016) • DNA repair ↑ & DNA damage ↓ (Lee et al. 2016)		stress ↓ (Na et al. 2013)	
Regulated Mitochondrial Function	• Mt-Complex I ↓, oxidative phosphorylation ↓ & endogenous ROS ↓ (El-Mir et al. 2000, Algire et al. 2012) • mGP_{DH} ↓ & hepatic glucose production ↓ (Madiraju et al. 2014) • PGC-1α ↑ (Aatsinki et al. 2014)	• Mt-Complex I ↓ (De Haes et al. 2014, Wu et al. 2016) • PRDX-2 ↑ leading to mitohormesis (De Haes et al. 2014)	–	• Mt-Complex I ↓ (Wheaton et al. 2014) • Energy state ↓ & AMP/ATP ↑ (Foretz et al. 2010) • PGC-1α ↑ (Foretz et al. 2010)
Increased Stem Cell rejuvenation capacity	• Gpx7-Nrf2 ↑ leading to increased lifespan in mesenchymal stem cells (Fang et al. 2018) • Rejuvenation ↑, myelination ↑ in aged oligodendrocyte progenitor cells (Neumann et al. 2019) • TAp73 ↑, AMPK-mediated αPKC-CBP pathway ↑ & rejuvenation of stem cells (Fatt et al. 2015)	–	• Intestinal stem cell aging ↓ mediated by Atg6 (Beclin-1) (Na et al. 2018)	• Expansion of neural stem cell pool (Ruddy et al. 2019) • Adult neural precursors (Fatt et al. 2015) • Quiescence ↑ & delayed differentiation of satellite cells (Pavlidou et al. 2019)
Regulated Epigenetic Alterations	• Histone methyltransferase ↑ & Histone acetyltransferase ↑, Class II Histone deacetylase ↓ & Class III Histone deacetylase ↑ (Bridgeman et al. 2018) • Global H3K27me3 ↑ (Cuyàs et al. 2017) • Increased global DNA methylation (Cuyàs et al. 2017) via the H19/S-adenosylhomocysteine hydrolase (SAHH) axis (Zhong et al. 2017)	• S-adenosylmethionine ↓ & S-adenosylhomocysteine ↑ thereby modulating histone methylation (Cabreiro et al. 2013)	–	• H3K27me3 ↑ via directly targeting KDM6A/UTX (Cuyàs et al. 2017) • DICER-1 ↑ & increased regulation of miRNA expression (Noren Hooten et al. 2016) • Regulation of hydroxymethylation via AMPK/Tet2/BDNF pathway (Wang et al. 2019)

Attenuation of Hallmarks of Aging	Effects of Metformin on key targets and pathways involved in regulating each hallmark of aging			
				
Minimized Telomere Attrition	• TERRA & telomeric transcriptional regulation ↑ via Nrf 1 & PGC-1α (Diman et al. 2016)	–	–	–
Attenuated Cellular Senescence	• Senescence ↓ & SASP ↓ via Nrf2 mediated Gpx7 ↑ (Fang et al. 2018) • SASP ↓ via anti-inflammatory properties like NF-κB pathway ↓, (Moiseeva et al. 2013, Śmieszek et al. 2017)	–	–	• p16 ↓ & p21 ↓, IL-6 ↓ & IL-8 ↓ via DICER ↑ (Noren Hooten et al. 2016)