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Geroprotector review: Metformin

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FACTS ABOUT METFORMIN

- ***What:*** A drug for the treatment of type 2 diabetes and polycystic ovary syndrome
- ***Lifespan:*** Increase in yeast, roundworm, and rodents. No effect on lifespan in fruit flies at low- to moderate concentration and a decrease at high concentrations.
- ***Cancer:*** Decreased cancer risk in cohort studies, not supported so far by RCTs. Animal studies suggest that metformin can reduce cancer risk. Metformin sensitizes cancer cells to radio- and chemotherapy.
- ***Heart disease:*** Metformin improves lipoprotein profile and is associated with reduced cardiovascular mortality in type 2 diabetes patients. Metformin seems to reduce the severity of aneurysms in cohort studies. Metformin shows beneficial effects on stroke in animal models.
- ***Autoimmune diseases:*** Metformin ameliorates several autoimmune diseases (at least in animal studies)
- ***Mechanisms:*** AMPK activation, mTOR inhibition, SIRT1 activation, decreased inflammation, decreased glycation, changes in bacterial metabolism, mitohormesis, decreases progerin levels, and reduces oxidative stress. Possible beneficial effects on DNA damage, cell senescence, and autophagy.
- ***Side effects:*** Several minor side effects (such as diarrhea), lactic acidosis, vitamin B12 deficiency, decrease in folic acid levels, and decrease in testosterone. Contradictory data exist on cognitive decline and and β-cell apoptosis

Introduction

Metformin (1,1-dimethylbiguanide) is an oral drug used for the treatment of type 2 diabetes and polycystic ovary syndrome (PCOS). It belongs to a category of drugs known as

biguanides but metformin is the only one of this class that remains in clinical use. Metformin is, unless contra-indicated, the first line treatment for type 2 diabetes to which other drugs can be added if needed to achieve the desired level of blood sugar control (Inzucchi *et al.*, 2015). In addition metformin has been included in the World Health Organization's (WHO) list of essential medicines. Nearly 120 million metformin prescriptions are filled worldwide each year making metformin one of the most sold drugs (Dowling *et al.*, 2011). Metformin is so popular because it's relatively safe, efficient, and costs only cents per dose.

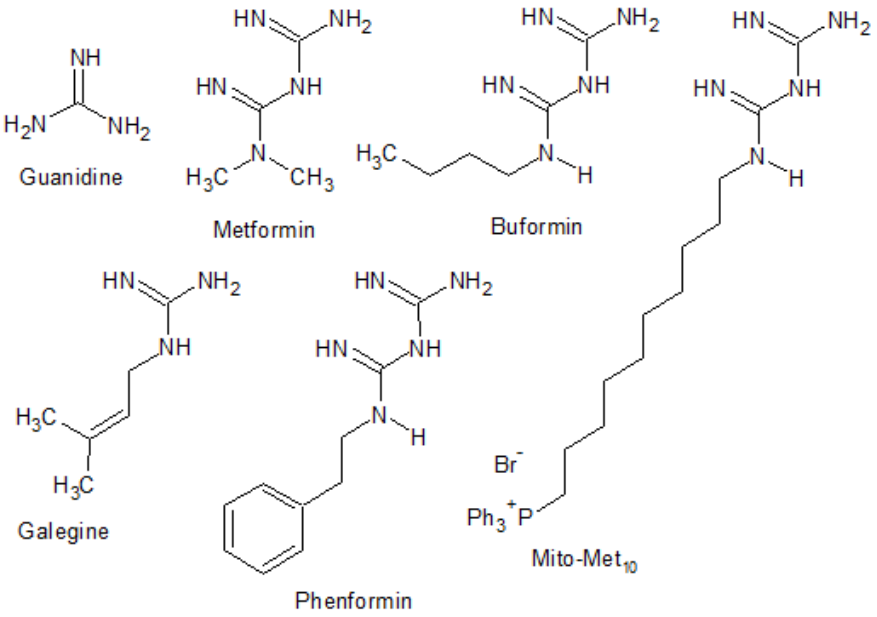

Figure 1 Some of the molecules mentioned in this article. Notice that all molecules share the same guanidine moiety.

During the Middle Ages physicians prescribed *Galega officinalis*, better known as goat's rue, the French lilac, Italian fitch, Spanish sanfoin or false indigo, to treat the intense urination in people suffering from type 2 diabetes (Fig. 2). The active ingredient in this plant is galegine or isoamylene guanidine but this is too toxic for therapeutic use. In fact the name goat's rue refers to the fact that this plant can be deadly when eaten by grazing sheep or goats. In 1926 two synthetic molecules were discovered that have chemical similarity to the active ingredient of *G. officinalis*, termed synthalins A and B. These two synthetic molecules were better tolerated and more efficient but still had some toxicity. The discovery of insulin eventually lead to a discontinuation of the synthalins in the early 1930s. In 1929 several biguanides were synthesised including metformin but it was not until 1956 until the antidiabetic properties of these compounds would be investigated by French researcher Jean Sterne. Sterne proposed the name 'Glucophage', which is still a brand name of metformin until this day. In the following years two more

Figure 2 *Galega officinalis.* Credit JoJan on Wikimedia Commons (https://commons.wikimedia.org/wiki/File:Galegaofficinalis03.jpg).

biguanides were developed: buformin and phenformin (Witters, 2001; Bailey and Day, 2004). Buformin and phenformin have been withdrawn from the market due to concerns about the increased risk of lactic acidosis (see below). The concern about lactic acidosis kept metformin from the US market until it was finally approved in 1995.

The first paper in Pubmed that contains the search term "metformin" was published in 1959. Remarkably, it took until 1991 before the milestone of 50 papers/year was reached. Just 5 years later this had grown to 100 papers a year and from then on it kept growing, reaching 1717 papers in 2016 (Fig. 3). That's almost 5 new papers every day!

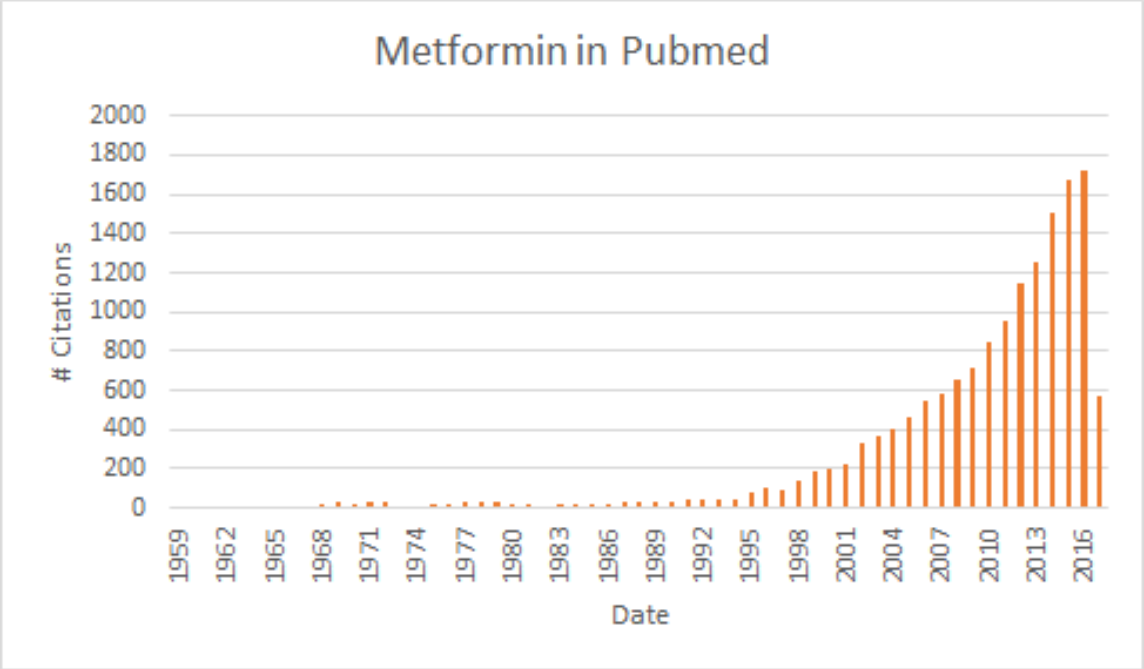

Figure 3 Metformin citations in Pubmed. Search conducted on 1st April, 2017.

Metformin's effects on lifespan

To provide an easy overview of metformin's effects on lifespan, I have summarized the data in three tables (see below). Table 1 summarizes data in simple organisms, Table 2 in rodents and Table 3 summarizes human data.

Simple organisms

In yeast chronological lifespan was extended in two studies (Borklu-Yucel *et al.*, 2015; Kazi *et al.*, 2017) but not in a third one (Choi *et al.*, 2013). In a recently-published paper Kazi *et al.* (2017) show that metformin extends the chronological lifespan of yeast. To the best of my knowledge three studies have been published examining the effect of metformin on lifespan in the roundworm *C. elegans* (Cabreiro *et al.*, 2013; De Haes *et al.*, 2014; Onken and Driscoll, 2010). A fourth paper has investigated the effect of buformin (Bakaev *et al.*, 2002). *C. elegans* is one of the most studied model organisms in aging research and the organism in which the first life extending mutations were discovered. Furthermore, the roundworm is also a popular model to study the effect of various pharmacological treatments on lifespan. In 2010 Onken and Driscoll demonstrated that exposure of *C. elegans* to 50 millimolar (mM)

metformin increased median lifespan by about 40%. However maximal lifespan was not increased. Metformin treatment also the slowed age-related decline in locomotory activity suggesting an improved healthspan. De Haes *et al.* (2014) found that the lifespan of *C. elegans* reached an optimum at 25 mM metformin (25% increase in mean lifespan). Concentrations of 50 mM or above resulted in a non-significant extension of mean lifespan. Surprisingly enough old worms also start to develop "wrinkles". This disorganization of the "skin" (called the cuticle) was prevented by metformin treatment. Finally, David Gems and colleagues conducted a large number of lifespan tests in *C. elegans* with metformin and phenformin. This group discovered a dose-dependent increase in lifespan with an optimal lifespan extension achieved at 50 mM (36% increase in mean lifespan). A higher dose had a significant smaller effect (only 3% increase in mean lifespan at 100 mM). The authors further demonstrated that phenformin similarly increased lifespan and that metformin administration from middle age onwards resulted in a modest increase in lifespan (8% at 25 mM). The surprising result from this study however was the finding that metformin reduced lifespan in the absence of living bacteria.

In the fruit fly *Drosophila melanogaster* no effect of metformin on lifespan was found at low to moderate dosages and at high dosages lifespan was decreased (Jafari *et al.*, 2007; Slack *et al.*, 2012; Shirazi *et al.*, 2014). However it did rescue the shortened lifespan of amyloid-β overexpressing flies (Niccoli *et al.*, 2016). Amyloid-beta is an aggregation-prone protein that forms the plaques found in the brain of Alzheimer's disease patients (see below). Furthermore, metformin reduced mortality in obese flies infected with a mold (*R. oryzae*). Metformin however did not improve survival in obese non-infected flies even though it did induce weight loss (Shirazi *et al.*, 2014). Finally, metformin significantly extended the mean and maximum lifespans of male and female crickets (Hans *et al.*, 2015).

Biguanide	Model	Concentration	Lifespan	Reference
Metformin	Baker's yeast	1 nM to 1mM	No effect on chronological lifespan	Choi *et al.*, 2013
Metformin	Baker's yeast	0.5 mM to 100 mM	Chronological lifespan was extended between 10 mM to 100 mM.	Borklu-Yucel *et al.*, 2015
Metformin	Baker's yeast	25 mM	Chronological lifespan was extended by 20-25%	Kazi *et al.*, 2017
Buformin	Roundworm *C. elegans*	0.00001 mg/ml to 1 mg/ml	Optimal lifespan extension at 0.1 mg/ml: 23% increase in mean lifespan and 26% increase in maximum lifespan.	Bakaev *et al.*, 2002
Metformin	Roundworm *C. elegans*	1 mM, 10 mM, and 50 mM	At 50 mM metformin median survival is increased by about 40%. No effect at 1 mM and 10 mM.	Onken and Driscoll, 2010

Metformin	Roundworm *C. elegans*	50 mM	Multiple independent lifespan studies. Mean lifespan extension between 13% and 57%.	De Haes *et al.*, 2014
Metformin	Roundworm *C. elegans*	25 mM to 100 mM	Mean lifespan was extended by 18% (25 mM), 36% (50 mM), and 3% (100 mM)	Cabreiro *et al.*, 2013
Metformin	Fruit flies	0.4 mg/mL, 0.8 mg/mL or 1.6 mg/mL in diet	No effect on mortality rate	Jafari *et al.*, 2007
Metformin	Fruit flies	1 mM to 100 mM	No effect for male flies between 1mM and 50 mM but a significant decrease upon 100 mM. No effect for female flies between 1mM and 10 mM but a significant decrease at higher concentrations.	Slack *et al.*, 2012
Metformin	Normal weight fruit flies	5 mM to 100 mM	There was a dose-dependent reduction in survival. However, the authors did not report a statistical analysis.	Shirazi *et al.*, 2014
Metformin	Obese fruit flies	5 mM to 100 mM	There was a dose-dependent reduction in survival. However, the authors did not report a statistical analysis.	Shirazi *et al.*, 2014
Metformin	Female cricket	1.78×10^{-3} g metformin/g food	Mean: +47% Max: +45%	Hans *et al.*, 2015
Metformin	Male cricket	1.78×10^{-3} g metformin/g food	Mean: +39% Max: +35%	Hans *et al.*, 2015

Table 1 Effect of biguanides on lifespan in simple organisms.

Rodents

The first study examining the effect of a biguanide on lifespan dates from 1980. Dilman and Anisimov (1980) studied the effect of phenformin on the lifespan of female C3H/Sn mice and found that mean lifespan was extended by 23%. In 2003 these authors published a review in which they re-analyzed all the lifespan data that their lab had produced on biguanides since the 1980s (Anisimov *et al.*, 2003). According to this new analysis phenformin extended the mean (21.1%) and the maximal (26%) lifespan of the female C3H/Sn mice. It also increased the mean lifespan of the oldest 10% of survivors by 28.4%. The reason for using the mean

lifespan of the last 10% survivors is because this particular measure of maximal lifespan is less susceptible to single outliers compared to just reporting the lifespan of the oldest individual in the study. Increased lifespan however does not mean that aging is slowed. It could for example be that the treatment reduces young mortality while having no effect on the age-related increase in mortality (= aging). Therefore the authors also studied several measures of population aging including the time needed for the mortality rate to double and the rate of aging. Phenformin treatment resulted in a decline in the aging rate of 31.2% while the mortality rate doubling time was increased 1.45-fold. Both of these measures indicate that metformin increased lifespan by slowing down aging. Phenformin had no effect on mean lifespan in outbred female LIO rats but it did increase the maximum lifespan (9.8%) and mean lifespan of the oldest 10% survivors (10.1%). In this study phenformin also failed to impact measures of the aging rate (Anisimov, 1982; Anisimov *et al.*, 2003).

Buformin was shown to increase mean lifespan (7.3%), maximum lifespan (5.5%), and mean lifespan of the oldest 10% survivors (12%). Buformin treatment was shown to decrease the rate of aging and to cause a 1.49-fold reduction in tumor incidence (Anisimov, 1980; Anisimov *et al.*, 2003).

Nine studies have been published examining the effect of metformin on lifespan in rodents (see table 2). As can be seen in figure 4 metformin largely had a positive effect on mean or median lifespans. Though one study found a significant decrease of 13.4% in male 129/Sv mice (Anisimov *et al.*, 2010). Maximum lifespan was also extended in several studies.

The effect of metformin on lifespan may be age-, gender-, strain-, and dose-dependent (see Table 2). The combination of metformin and rapamycin may extend lifespan more than either drug alone (Strong *et al.*, 2016). Interestingly, long-term rapamycin treatment causes some side effects (glucose intolerance and hyperlipidemia) which could be improved by metformin treatment (Bulterijs, 2011).

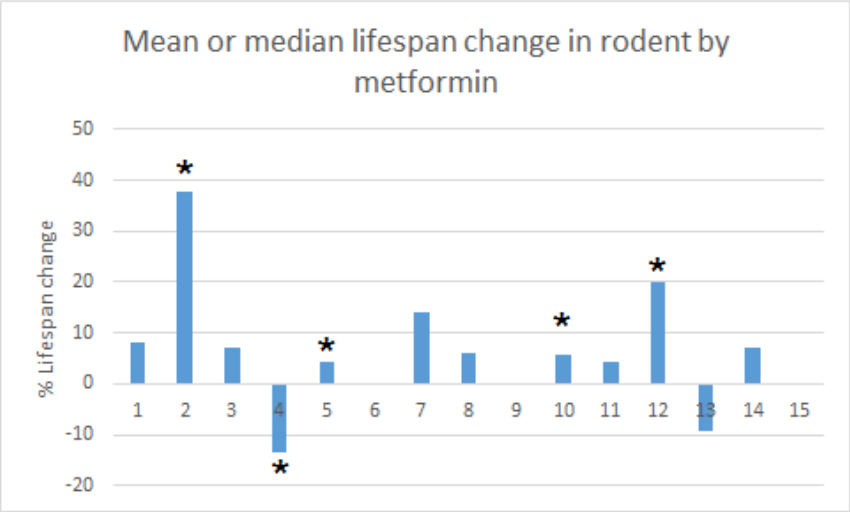

Figure 4 Mean or median changes in lifespan by metformin in rodents. The asterisks indicate significance by whatever criteria applied in the original paper. 1-15 refers to the order in which the metformin data have been tabled in table 2. One data point, a 14.4% decrease in lifespan in male C57BL/6 mice was censored because the dose used (1% of diet) was toxic while lifespan was extended in that same study by the low (0.1%) dose.

Biguanide	Model	Lifespan	Cancer incidence	Reference
Phenformin	Female C3H/Sn mice	Mean: + 21.1% Max: +26% Max (10%): +28.4%	3.8-fold decrease in mammary adenocarcinomas	Dilman and Anisimov, 1980
	Female outbred LIO rats	Mean: no effect Max: +9.8% Max (10%): +10.1%	No effect on spontaneous cancer incidence	Anisimov, 1982
Buformin	Female LIO rats	Mean: +7.3% Max: +5.5% Max (10%): +12%	1.49-fold decrease in tumor incidence	Anisimov, 1980
Metformin	FVB/N HER2/neu mice	Mean: +8% (NS) Max: +16.2% Max (10%): +13.1%	Metformin decreased the incidence and reduced tumor size of breast cancer	Anisimov *et al.*, 2005
	Female SHR mice	Mean: +37.8% Max: +10.3% Max (10%): +20.8%	No effect on spontaneous cancer incidence	Anisimov *et al.*, 2008
	Female FVB/N HER2/neu mice	Mean: +7% (NS) Max: -9.3% Max (10%): -11%	Metformin slows down the development of breast cancer	Anisimov *et al.*, 2010a
	Male 129/Sv mice	Mean: -13.4% Max: no effect Max (10%): no effect	No effect	Anisimov *et al.*, 2010b
	Female 129/Sv mice	Mean: +4.4% Max: no effect Max (10%): no effect	3.5-fold reduction in malignant tumors.	Anisimov *et al.*, 2010b
	Fischer-344 rats	Mean: no effect Max: no effect Max (10%): no effect	Not	Smith *et al.*, 2010
	Female SHR mice (started at 3 months of age)	Mean: +14.1% (NS) Max: +3% (NS) Max (10%): no effect	The first animal to die from cancer in the metformin group was 22% older than that from the control group.	Anisimov *et al.*, 2011
	Female SHR mice (started at 9 months of age)	Mean: +6.1% (NS) Max: -9% (NS) Max (10%): -8% (NS)	The first animal to die from cancer in the metformin group was 25% older than that from the control group.	Anisimov *et al.*, 2011

	Female SHR mice (started at 15 months of age)	Mean: no effect Max: no effect Max (10%): no effect	The first animal to die from cancer in the metformin group was 5% older than that from the control group.	Anisimov *et al.*, 2011
	Male C57BL/6 mice (0.1% metformin)	Mean: +5.8%	No data	Martin-Montalvo *et al.*, 2013
	Male C57BL/6 mice (1% metformin)	Mean: -14.4% (toxic dose)	No data	Martin-Montalvo *et al.*, 2013
	Male B6C3F1 mice	Mean: +4.15% (NS)	No data	Martin-Montalvo *et al.*, 2013
	Male 129/Sv (neonatal)	Mean: +20% Max: +3.5% (NS) Max (10%): +3.2%	No effect	Anisimov *et al.,* 2015
	Female 129/Sv (neonatal metformin)	Mean: -9.1% (NS) Max: -3.8% (NS) Max (10%): -3.3% (NS)	No effect	Anisimov *et al.,* 2015
	Male UM-HET3	Median: +7% (NS)	No data	Strong *et al.*, 2016
	Female UM-HET3	Median: no effect	No data	Strong *et al.*, 2016

Table 2 Effect of the different biguanides on lifespan and cancer incidence in rodents. The data for the older Anisimov studies have been derived from the 2003 re-analysis (Anisimov *et al.*, 2003). No effect signifies that the effect was less than the arbitrary chosen value of 3%. (NS) means not significant by whatever definition used in the original paper.

Human data

In the UK Prospective Diabetes Study (UKPDS) the patients treated with metformin had a 36% reduction in all-cause mortality compared to conventional therapy based on diet and exercise. In contrast patients in this trial who received sulfonylurea/insulin therapy only had an 8% reduction in all-cause mortality. Metformin was also superior in reducing diabetes-related death, myocardial infarction and stroke compared to sulphonylurea/insulin therapy (UK Prospective Diabetes Study Group, 1998).

Multiple trials show that metformin lowers mortality when compared to other interventions (table 3). Surprisingly enough, Bannister *et al.* (2014) found that mortality in type 2 diabetes patients treated with metformin was lower than in non-diabetic controls.

Metformin compared with	Study period	Outcome	Reference
Diet/Exercise	Average follow-up 10.7 years	36% reduction in all-cause mortality compared to conventional treatment	UK Prospective Diabetes Study Group, 1998
Sulfonylurea	Average follow-up 5.1 years	Odds ratio of 0.60 for metformin over sulfonylurea for all-cause mortality	Johnson *et al.*, 2002
Sulfonylurea (meta-analysis)	24 weeks to 10.7 years	No difference	Hemmingsen *et al.*, 2014
Conventional therapy	10 year follow-up of UKPDS study	27% reduction in all-cause mortality	Holman *et al.*, 2008
Non-diabetics	Average follow-up 2.8 years	Median survival time was 15% lower in non-diabetics	Bannister *et al.*, 2014
Non-use of metformin	Study period was just over 7 years	41% reduction in all-cause mortality	Hippisley-Cox, 2016
Insulin	Average follow-up 3.5 years	Hazard ratio of 0.60 for metformin + insulin over insulin monotherapy for all-cause mortality	Holden *et al.*, 2016

Table 3 Effect of metformin compared to other treatments on mortality in diabetes patients.

The Targeting Aging with Metformin (TAME) trial will enrol roughly 3,000 elderly (65-79 years old). This placebo-controlled, randomized clinical trial will investigate the effect of metformin on a composite outcome that includes cardiovascular events, cancer, dementia, and mortality (Barzilai *et al.*, 2016).

The Me.Me.Me trial is a phase III randomized controlled trial in which the effect of metformin-treatment on the risk for age-related non-communicable chronic diseases will be investigated in people who suffer from metabolic syndrome but are otherwise healthy (Pasanisi *et al.*, 2017).

Finally, the Metformin in Longevity Study (MILES) will test metformin in older adults with impaired glucose tolerance and investigate if the gene expression profile becomes more similar to that of young healthy subjects (ClinicalTrials.gov Identifier: NCT02432287).

Metformin as a CR mimetic

Calorie restriction (CR) is the most robust experimental method to increase lifespan. It has been demonstrated to increase lifespan in a wide variety of model organisms from yeast to monkeys (Fontana *et al.*, 2010). CR mimetics are drugs that mimic the beneficial effects of a calorie restricted diet without reducing calorie intake (Madeo *et al.*, 2014).

Stephen Spindler and colleagues tested the effect of metformin on gene expression in the livers of mice. Eight weeks of metformin treatment reproduced 75% of the gene expression changes observed in long term calorie restriction (CR). In comparison eight weeks of CR only reproduced 71% of gene expression changes observed in long term CR. These data support the idea that metformin works as a CR mimetic (Dhahbi *et al.*, 2005; Spindler, 2006). These data were later confirmed in the livers and muscles of animals treated with metformin for 30 months (Martin-Montalvo *et al.*, 2013).

Metformin and CR also share some other similarities. Both reduce oxidative stress (Sohal and Weindruch, 1996), lower insulin/IGF-1 signaling (Hursting *et al.*, 2013), increase SIRT1 activity (Chen *et al.*, 2008), activate autophagy (Wohlgemuth *et al.*, 2007), and reduces inflammation (González *et al.*, 2012). Furthermore, metformin and calorie restriction share a high similarity in the diseases improved by them. Both interventions decrease the risk for cardiovascular disease (Weiss and Fontana, 2011), cancer (Hursting *et al.*, 2013), type 2 diabetes (Prasannarong *et al.*, 2012), while increasing the risk for amyotrophic lateral sclerosis (ALS) in a mouse model (Pedersen and Mattson, 1999; Patel *et al.*, 2010). Only references for calorie restriction are given as the effect of metformin on these molecular and pathophysiological markers has been discussed in great detail in the sections below.
The metabolic effects of CR and metformin show major differences (Bulterijs, 2011). CR increases fatty acid synthesis (Weindruch *et al.*, 2001), cholesterol biosynthesis (Pedroso *et al.*, 2014), and gluconeogenesis (Weindruch *et al.*, 2001) while metformin has the opposite effect (see below).

Protective effects of metformin on diseases

Given that metformin is used for the treatment of type 2 diabetes it is unnecessary to discuss that here. It's sufficient to point out that metformin has not only been shown to be useful for the treatment of type 2 diabetes but is also effective in reducing the risk for the development of type 2 diabetes in high risk patients (such as those suffering from metabolic syndrome) (Hostalek *et al.*, 2015).

Cancer

Various animal studies have shown that metformin reduces spontaneous and induced (such as by exposure to carcinogens) cancers as well as reducing cancer incidence in animals that are genetically susceptible to cancer (Anisimov *et al.*, 2005a & b; Eikawa *et al.*, 2015; Zhang *et al.*, 2016). Metformin was shown to decrease the rate of proliferation and induce cell cycle arrest in ovarian cancer cells in culture. Furthermore, metformin pre-treatment reduced the number of tumor implants by 60% in mice that were injected with an ovarian cancer cell line (Lengyel *et al.*, 2014). Surprisingly, metformin treatment reduced several stemness markers

as well as spheroid body formation in breast and ovarian cancer cells. This suggests that metformin may reprogram cancer cells into non-cancer cells (Hu *et al.*, 2014). Metformin has also shown promise as an adjuvant to other cancer treatments. For example, metformin sensitizes non-small lung cancer cells (Storozhuk *et al.*, 2013), liver cancer cells (Liu *et al.*, 2012), pancreatic cancer (Wang *et al.*, 2015), breast cancer, connective tissue cancer cells, and cancer stem cells (Song *et al.*, 2012) to ionizing radiation. Similarly, metformin has also been shown to increase the effectiveness of chemotherapy (Hirsch *et al.*, 2009; Iliopoulos *et al.*, 2011; Rocha *et al.*, 2011; Dong *et al.*, 2012; Lin *et al.*, 2013). However, metformin reduced cisplatin-mediated apoptosis of multiple cancer cell lines with the sole exception of a mouse melanoma cell line in which metformin stimulated cisplatin-induced cell death (Janjetovic *et al.*, 2011). These data may suggest that metformin's effect on cancer is dependent on the cancer type. Also the activation of AMPK by metformin could possibly improve survival of cancer cells in established tumors by protecting these cells from metabolic stress (Pryor and Cabreiro, 2015).

Metformin treatment appear to protect healthy tissues from chemotherapy-induced damage. For example, metformin prevented chemotherapy-induced cognitive impairment in mice (Zhou *et al.*, 2016a). Furthermore, metformin protected mice from chemotherapy-induced peripheral neuropathy (Mao-Ying *et al.*, 2014). Finally, metformin was shown to reduce the toxic effect of the chemotherapy drug doxorubicin on the heart (Kelleni *et al.*, 2015).

Zhang *et al.* (2016) have synthesized approximately 140 biguanides and screened them for high affinity for OCT1 and OCT3 (uptake in cells) but low affinity for OCT2 (urine excretion). This resulted in the identification of NT1014 which showed much higher potency compared to metformin for growth inhibition of ovarian cancer cells. Another novel biguanide, N1-hexyl-N5-benzyl-biguanide mesylate (HBB), showed much higher potency compared to metformin against breast cancer cells (Guo *et al.*, 2014). Metformin derivatives in which the methyl side chain is replaced by a longer alkyl chain terminated by a mitochondrial-targeted cation (PPh_3^+) have a much higher potency against cancer (Kalyanaraman *et al.*, 2017). For example, Mito-Met_{10} was 100-fold more potent than phenformin in pancreatic cancer cells (Cheng *et al.*, 2016).

Multiple epidemiologic studies demonstrate that type 2 diabetes patients treated with metformin have a lower risk for cancer compared to those on other anti-glycemic treatments (Evans *et al.*, 2005). In a systematic review of 41 observational studies it was found that metformin use is associated with a lower risk of cancer death, incidence of any cancer, as well as multiple specific cancer locations (liver, colorectal, pancreas, stomach, esophagus) but not in others (breast, lung, ovarian, uterus, prostate, bladder, kidney, and melanoma) (Franciosi *et al.*, 2013). Gandini *et al.* (2014) conducted a meta-review on cancer risk and mortality in people treated with metformin. Overall cancer risk was reduced by 31% and overall cancer mortality by 34% but between study heterogeneity was large. Thakkar *et al.* (2013) equally found a 30% reduction in cancer risk in a meta-analysis of cohort studies. However, when case-control studies were analysed the benefit dropped to 10% and no effect was found when randomized, placebo-controlled trials were analysed. Two other meta-reviews of randomized controlled trials equally found no effect of metformin on cancer (Stevens *et al.*, 2012; Franciosi *et al.*, 2013). In another meta-analysis that combined cohort, case-control, and randomized controlled trials it was found that metformin was associated with a lower cancer risk (Wu *et al.*, 2015a). A subgroup analysis looking at metformin

monotherapy did find a 16% lower cancer risk but this did not reach significance (Stevens *et al.*, 2012). A major limitation of these systematic reviews on randomized controlled trials is that the average follow-up is very short. The average follow-up of studies included in the Stevens *et al.* (2012) meta-analysis was just 4.1 years and for mortality it was even only 2.8 years. Another limitation of trials is that they cannot make the distinction between a reduced cancer risk from metformin versus an increased cancer risk from other anti-glycemic treatments.

Metformin users had a significant better survival and reduced the risk of cancer recurrence in hepatocellular carcinoma after liver resection (Seo et al., 2016; Chan *et al.*, 2017). In contrast, Bhat *et al.* (2014) found no improvement in survival by metformin in newly diagnosed patients with hepatocellular carcinoma. Metformin failed to improve survival in patients with advanced pancreatic cancer in a double-blind, randomized, placebo controlled phase 2 trial (Kordes *et al.*, 2015).

Lewis Cantley, director of the Cancer Center at Beth Israel Deaconess Medical Center, told Gary Taubes in an interview that "Metformin may have already saved more people from cancer deaths than any drug in history" (Taubes, 2012). However, the data from randomized placebo-controlled trials to support a metformin-induced reduction in cancer risk and/or mortality remains low. There's also a clear lack of studies in non-diabetic patients. Multiple clinical studies are currently ongoing that will hopefully provide better evidence about metformin's effectiveness in treating cancer (Chae *et al.*, 2016).

Cardiovascular disease

Metformin treatment improved several classical measures of cardiovascular health including reductions in total cholesterol, low-density lipoprotein (LDL) cholesterol, lipoprotein(a), and Apo B levels in women suffering from PCOS syndrome (Kilicdag *et al.*, 2005). However in the same study it was also found that metformin treatment lead to a decrease in high-density lipoprotein (HDL) cholesterol, which is generally considered to be protective against atherosclerosis. Metformin decreased total cholesterol, triglycerides, LDL, and VLDL in patients with metabolic syndrome (Paul *et al.*, 2016). Metformin therapy initiated after myocardial infarction in patients without known diabetes resulted in a slight decrease of LDL levels and a decrease in LDL particle size (Eppinga *et al.*, 2016). Six months of metformin therapy had no effect on LDL and HDL concentrations in normoinsulinemic PCOS patients (Romualdi *et al.*, 2008). The effect of metformin may be race dependent as a one-year treatment with metformin lead to an increase in HDL cholesterol in Caucasian and African-American but not in Hispanic pre-diabetics (Zhang *et al.*, 2015a). Other studies have also found that metformin increases HDL cholesterol levels (Wu *et al.*, 1990). Metformin treatment has been shown to increase homocysteine levels (see below). In another study in patients suffering from a genetic abnormality in cholesterol metabolism (type II B hyperlipidemia) that leads to high blood levels of triglycerides and LDL cholesterol it was found that metformin therapy resulted in a decrease in total cholesterol and LDL cholesterol levels (Pentikäinen *et al.*, 1990). In a systematic review of 41 studies, with a combined number of over 3,000 patients, it was found that metformin treatment resulted in a decrease in plasma triglycerides, total cholesterol, and LDL cholesterol. However the decrease in triglycerides could be attributed to the metformin-induced decrease in blood glucose levels and hence metformin had no direct effect on triglyceride metabolism. Metformin had no

effect on blood pressure nor on HDL cholesterol (Wulffelé *et al.*, 2004). Metformin decreased serum lipoprotein(a) levels in some (Velazquez *et al.*, 1997; Bell and Ovalle, 1998; Kilicdag *et al.*, 2005) but not all studies (Landin *et al.*, 1994; Testa *et al.*, 1996).

Metformin inhibits the insulin-stimulated synthesis of plasminogen activator inhibitor-1 (PAI-1) (Anfosso *et al.*, 1993). Metformin also reduces PAI-1 levels in HIV patients (Hadigan *et al.,* 2001) and in women suffering from PCOS syndrome (Tan *et al.*, 2009). In cultured endothelial cells, metformin treatment protected against oxidized LDL-induced increases in oxidative stress, apoptotic cell death, and restored eNOS activity (Hung *et al.*, 2016). Metformin also reduced palmitic acid-induced lipid accumulation in cultured macrophages (Song *et al.*, 2010). Metformin also inhibits the monocyte to macrophage differentiation which contributes to the inflammatory environment inside atherosclerotic plaque. Furthermore, metformin reduced plaque formation in an Ang-II-induced atherogenesis $ApoE^{-/-}$ mice model (Vasamsetti *et al.*, 2015). Metformin treatment significantly reduced the progression of aortic atherosclerosis in a rabbit model (Li *et al.*, 2009). AMPK activators, such as metformin, have also been found to shift the polarization of macrophages from the proinflammatory M1 to the anti-inflammatory M2 state (Hattori *et al.*, 2015). Indeed, metformin has been shown to reduce the secretion of various proinflammatory cytokines from macrophages (see below). Furthermore, C-reactive protein (CRP) levels are reduced by metformin treatment (see below). Metformin significantly increased the flow-mediated dilatation of the brachial artery in normoinsulinemic PCOS patients indicating an improvement in endothelial function (Romualdi *et al.*, 2008).

Metformin-treatment leads to a decrease in vitamin B_{12} and folic acid levels (see below). Vitamin B_{12} and folic acid are involved in the biochemical conversion of homocysteine to methionine (Selhub, 1999). Hence a metformin-induced B_{12} and folic acid deficiency could impair this conversion and lead to an increase in homocysteine levels. Homocysteine has emerged as a new risk factor for cardiovascular disease (Refsum *et al.*, 1998; Humphrey *et al.*, 2008) and dementia (Seshadri *et al.*, 2002; Wald *et al.*, 2011). Multiple studies have confirmed higher homocysteine levels in patients using metformin (Hoogeveen *et al.*, 1997; Wile and Toth, 2010). Furthermore, intervention studies have also found that metformin increases serum homocysteine levels (Carlsen *et al.*, 1997; Kilicdag *et al.*, 2005; Sahin *et al.*, 2007; Wulffelé *et al.*, 2003). However, some studies have failed to observe an effect of metformin on homocysteine levels (Pongchaidecha *et al.*, 2004; Yilmaz *et al.*, 2005). Furthermore, several intervention studies have failed to observe a decrease in cardiovascular events or mortality by vitamin B_{12} and folic acid supplementation despite significant reductions in homocysteine levels (Toole *et al.*, 2004; Bonaa *et al.*, 2006; Lonn *et al.*, 2006; Martí-Carvajal *et al.*, 2013).

In the UK Prospective Diabetes Study (UKPDS) metformin treatment reduced the risk of myocardial infarction by 39% over a 10 year period compared to conventional treatment. When mortality from all macrovascular causes (myocardial infarction, sudden death, angina, stroke, and peripheral disease) was combined then the metformin group had a 30% lower risk compared to the conventional treatment. However, it should be pointed out that macrovascular mortality in the metformin group did not differ from the other intensive therapy groups hence it could be that metformin's effect on macrovascular disease risk reduction is solely due to a reduction in blood glucose levels and not to a specific benefit of metformin itself (UK Prospective Diabetes Study Group, 1998). Metformin does not reduce high blood

sugar levels in normoglycemic individuals hence it remains unclear if metformin could have a beneficial effect on cardiovascular disease in people without diabetes.

A ten-year follow-up of the UKPDS study confirmed that those on metformin therapy still had a lower risk of heart disease (33% reduced compared to the conventional treatment group) (Holman *et al.*, 2008). Metformin did not associated with an improvement in an aggregate of micro- and macrovascular morbidity and mortality in a randomized placebo-controlled trial with a follow up of 4.3 years. However, metformin did reduce the risk for macrovascular disease (Kooy *et al.*, 2009). Metformin monotherapy was associated with a lower risk of cardiovascular morbidity and mortality in a retrospective cohort analysis of new users of oral antiglycemic drugs when compared to sulphonylurea monotherapy (Johnson *et al.*, 2005). Metformin had no effect on cardiovascular events when compared to other antiglycemic drugs while it did cause a significant reduction when compared to placebo or no therapy in a meta-analysis. By using a meta-regression the authors found that metformin appears to be more effective in trials of longer duration and in trials with lower minimum and maximum age (i.e. in younger patients) (Lamanna *et al.*, 2011).

Patients who suffered from ischaemic cardiomyopathy were given either metformin or a placebo for three years. Most but not all patients in the trial had type 2 diabetes or impaired glucose tolerance. The incidence of reinfarction, occurrence of angina pectoris, acute cardiovascular events, and deaths were lower in the metformin group. The largest reduction was found in reinfections (8.9% in the control group versus only 1.6% in the metformin group) (Sgambato *et al.*, 1980). Metformin increases survival of worms exposed to long-term anoxia (LaRue and Padilla, 2011).

Twelve weeks of metformin treatment also reduced infarct size and improved the preservation of left ventricular ejection fraction in rats after an experimentally-induced myocardial infarction (Yin *et al.*, 2011). Others have also observed that metformin improved cardiac function after an experimental-induced heart failure in rats (Wang *et al.*, 2011a), mice (Gundewar *et al.*, 2009; Sun and Yang, 2017) and dogs (Sasaki *et al.*, 2009). Interestingly, Gundewar *et al.* (2009) reported that metformin therapy started at the time of ischemia induction and continued for 4 weeks improved survival by 47%. Metformin started 1 week after the experimental-induction of ventricular hypertrophy and continued for 8 weeks resulted in an improvement of haemodynamic function and a significant reduction in ventricular hypertrophy (Zhang *et al.*, 2011). The AMPK activator AICAR similarly prevented cardiac hypertrophy (Li *et al.*, 2007; Meng *et al.*, 2011).

Aneurysms

Aneurysms are weakened spots in the vascular wall that give rise to blood-filled bulges. The rupture of an aneurysm can be fatal depending on its location in the body. Especially deadly are aneurysms in the Circle of Willis in the brain, in the aorta, and in the abdominal aorta. Hsu *et al.* (2016) analysed data extracted from the Taiwanese National Health Insurance Research Database and found that metformin use was associated with a lower risk of aortic aneurysms. Fujimura *et al.* (2016) found that metformin use by diabetic patients was associated with a decreased enlargement of aneurysms in the abdominal aorta. Furthermore, the association was dose-dependent. Metformin also reduced the incidence of aortic aneurysms in an Ang-II-induced ApoE$^{-/-}$ mice model (Vasamsetti *et al.*, 2015).

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Stroke

Metformin (50 mg/kg/day) administered to newly born rats protected against the damaging effects of experimentally-induced ischemia on the brain. More specifically metformin attenuated the behavioral deficits, improved spatial learning, increased oligodendrocyte progenitor cell proliferation and promoted the recovery of normal myelin sheet architecture (Qi *et al.*, 2016). A single dose of metformin 24h before the induction of experimental stroke significantly reduced infarct volume by 29% as well as neurological deficits (Jiang *et al.*, 2014a). Metformin treatment started 24 h after stroke promoted functional recovery, pushed the microglia/macrophages to an anti-inflammatory M2 phenotype, increased angiogenesis and neurogenesis (Jin *et al.*, 2014). Metformin administration for three weeks starting 24 h after the experimental induction of a stroke in mice improved stroke-induced behavioral deficits and enhanced the formation of new blood vessels in the damaged tissue (Venna *et al.*, 2014). In another study metformin therapy was started at the time of reperfusion (ie: directly after the experimental stroke). Here it was found that metformin therapy significantly reduced the ischemia-induced brain atrophy volume. Furthermore, metformin also induced angiogenesis and neurogenesis (Liu *et al.*, 2014a). Metformin reduced infarct size, improved neurobehavioral outcomes, and decreased blood-brain barrier permeability in an experimental stroke model when given for 14 days after stroke (Liu *et al.*, 2014b).

Cheng et al. (2014) used data from the Taiwan National Health Research Institute database to investigate the effect of metformin use on stroke risk in diabetic patients. After a 4-year follow up, metformin use was associated with a significant decrease in the risk for stroke (Hazard Ratio: 0.468). Mima *et al.* (2016) found that neurological severity of stroke was lower in type 2 diabetes patients treated with metformin compared to those on other treatments.

Autoimmune diseases

Autoimmune diseases are diseases in which the immune system attacks self-antigens leading to tissue damage. Common autoimmune diseases include type 1 diabetes mellitus, rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease.

Rheumatoid arthritis (RA) is a disease characterized by inflammation of the joint followed by its destruction. Inflammation is a key part of the pathophysiology of RA and treatment consists of various anti-inflammatory drugs. Metformin has been shown to have anti-inflammatory properties (see below) and hence may be useful in the treatment of RA. Indeed, metformin significantly reduced the severity of arthritis in a mice model of RA (Son *et al.*, 2014).

Anecdotally, I would like to mention the case of a friend of mine whose grandmother had suffered from RA for 38 years. She had swollen and painful joints despite being on anti-inflammatory drugs. At the advice of her grandson in June 2014 she implemented dietary changes and started taking various supplements (including metformin). Her condition improved in the months after the start of the treatment and she is currently off the anti-inflammatory medications and nearly free of pain. While she used to struggle with walking the stairs in her home she now regularly walks several kilometres without problems.

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Metformin delayed the onset and attenuated the disease progression in a mouse model of multiple sclerosis (Nath *et al.*, 2009). Metformin ameliorated experimental autoimmune encephalomyelitis, an experimental model of autoimmune-induced brain inflammation believed to mimic human diseases such as multiple sclerosis (Sun *et al.*, 2016). Metformin treatment also ameliorated inflammatory bowel disease (IBD) in a DSS-induced (Lee *et al.*, 2015) and a genetic-induced (Xue *et al.*, 2016) IBD mouse model. Metformin suppressed the induction of the autoimmune phenotype and reduced autoantibody production in a mouse model of lupus (Lee *et al.*, 2017). The combination of metformin and the inhibitor of glucose metabolism, 2-deoxy-D-glucose, significantly reduced the enlargement of the spleen, inhibited the autoantibody production, and reversed T-cell activation in the TC mouse model of lupus (Yin *et al.*, 2015). Furthermore, metformin reduced clinical flares of lupus in a proof-of-concept clinical trial (Wang *et al.*, 2015a).

Several clinical trials with metformin for autoimmune diseases are currently ongoing: Graves disease (ClinicalTrials.gov Identifier: NCT02535975), Lupus (ClinicalTrials.gov Identifier: NCT02741960).

Other diseases

Positive effects

Epidemiological research indicates that metformin usage is associated with a lower risk of open-angle glaucoma in diabetic patients (Richards *et al.*, 2015). A current ongoing clinical trial is testing metformin on age-related macular degeneration (ClinicalTrials.gov identifier: NCT02684578). Metformin reduces lens opacity in mice and may hence help to prevent cataracts (Martin-Montalvo *et al.*, 2013).

Metformin protected against allergic eosinophilic inflammation in mice (Park *et al.* 2012; Calixto *et al.*, 2013). Metformin users had a lower risk of asthma-related hospitalization and asthma exacerbation compared to non-users (Li *et al.*, 2016). Frequent metformin use was associated with a lower risk for psoriasis in a cohort study based on Taiwan's National Health Insurance claim database (Wu *et al.*, 2015b). Using the same database, Chen *et al.* (2017a) found that metformin use was associated with a lower risk of asthma.

Metformin has anti-fibrotic properties in a mouse model of lung fibrosis (Choi *et al.*, 2016; Sato *et al.*, 2016), renal fibrosis (Cavaglieri *et al.*, 2015; Shen *et al.*, 2016), and inhibits liver fibrosis in a mouse model of type 2 diabetes (Qiang *et al.*, 2010). A trial is underway to evaluate the effect of metformin on liver fibrosis in hepatitis C and hepatitis C + HIV infected patients (ClinicalTrials.gov identifier: NCT02306070). Metformin attenuated connective tissue deposition in the heart of MSG-induced obese rats (Burlá *et al.*, 2013). Metformin inhibits pressure overload-induced cardiac fibrosis by downregulating the TGF-β pathways (Xiao *et al.*, 2010). Indeed, Sasaki *et al.* (2009) found that TGF-β mRNA levels were decreased by metformin in the heart of dogs. Furthermore, metformin inhibits the Ang II-induced differentiation of cardiac fibroblasts into myofibroblasts, a critical event in the progression of cardiac fibrosis (Bai *et al.*, 2013). Indeed, metformin treatment successfully inhibited Ang II-induced TGF-β production and cardiac fibrosis in mice (Chen *et al.*, 2017b).

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The case study discussed in the autoimmune disease section (see above) also suffered from non-alcoholic fatty liver disease, possibly as a side effect of the decades long use of the anti-arthritis drug methotrexate, was also reversed after the dietary, dietary supplement and metformin intervention. Some studies indicate that metformin may improve non-alcoholic fatty liver disease (Garinis *et al.*, 2010). Indeed, metformin also improves fatty liver in mice (Lin *et al.*, 2000; Kim *et al.*, 2010; Woo *et al.*, 2014; Kim *et al.*, 2016; Karise *et al.*, 2017). Six months of metformin therapy resulted in 16% of enrolled patients who no longer had evidence of non-alcoholic fatty liver disease. Though, metformin was not superior to lifestyle therapy (Nar and Gedik, 2009). However a meta-analysis failed to find a benefit of metformin on histological response, but liver function tests (ALT and AST) showed an improvement (Li *et al.*, 2013).

Metformin exhibited antimicrobial activity in an *in vitro* assay (Dash *et al.*, 2011). Metformin increased neutrophil chemotaxis, phagocytosis of bacteria, and bacterial killing (Park *et al.*, 2013). Metformin reduces the growth of tuberculosis bacteria including that of drug resistant strains (Singhal *et al.*, 2014). Metformin may also reduce the risk of *Clostridium difficile* infection in diabetic patients (Eliakim-Raz *et al*., 2015). Metformin use was associated with a lower risk of hospital-treated infections in diabetic patients compared to other glucose-lowering drugs (Mor *et al.*, 2016). Based on an analysis of the Taiwan's National Health Insurance Research Database it was found that metformin use was associated with a lower risk of sepsis (Shih *et al.*, 2015).

Metformin protected against experimentally-induced intervertebral disc degeneration in a rat model (Chen *et al.*, 2016). Metformin use was associated with less pain in patients suffering from lumbar radiculopathy pain, which can be a consequence of intervertebral disc degeneration (Taylor *et al.*, 2013).

Metformin also reduced serum deprivation-induced toxicity and cell death in stratial cells expressing mutant huntingtin (Jin *et al.*, 2016; Vázquez-Manrique *et al.*, 2016). Metformin enhanced touch response in worms expressing mutant huntingtin (Vázquez-Manrique *et al.*, 2016). Metformin significantly increased lifespan (+20.1%) in mice expressing a mutant form of human huntingtin, a model for Huntington's disease (Ma *et al.*, 2007). Chronic metformin therapy resulted in a modest improvement in behavioural and locomotory function in a mice model of Huntington. However, metformin therapy did not reduce striatal atrophy nor did it improve mitochondrial function (Adhihetty *et al.*, 2010). In contrast, Jin *et al.* (2016) did find an improvement in mitochondrial function in metformin-treated cultured cells expressing mutant huntingtin.

Metformin increases muscle strength in old *OXYS* rats (Kolosova *et al.*, 2016) and protects muscle against cardiotoxin injury (Langone *et al.*, 2014). In addition metformin has been found to improve the formation of postsynaptic sites with a "youthful architecture" on myotubes in cell culture. However, metformin had no effect on neuromuscular junctions in mice (Stockinger *et al.*, 2017). Elderly metformin users had a better muscle strength, as measured by a handheld dynamometer, compared to nonusers (Sumantri *et al.*, 2014).

Metformin may also improve symptoms in women suffering from cyclic edema (Valensi *et al.*, 1995; Soudet *et al.*, 2017). Metformin also decreased mortality in experimentally-induced acute seizures in mice without changing the severity of the seizures. Metformin did however

facilitate the termination of the acute seizures and also shortened the duration of chronic seizures in another mouse model (Yang *et al*., 2017).

Negative effects

Metformin had a negative effect on disease onset and progression in an amyotrophic lateral sclerosis (ALS) mouse model (Kaneb *et al.*, 2011). Metformin treatment decreased exercise capacity in newly diagnosed patients with metabolic syndrome (Paul *et al.*, 2017) or with insulin-resistance (Cadeddu *et al.*, 2012). Similarly, Braun *et al.* (2008) observed a decrease in exercise capacity in healthy males and females with a normal body weight given metformin. Metformin also decreased exercise capacity after a single dose in mice (Rouquet *et al.*, 2014). However, a single dose of metformin (1000 mg) did not affect exercise capacity in humans (Johnson *et al.*, 2008).

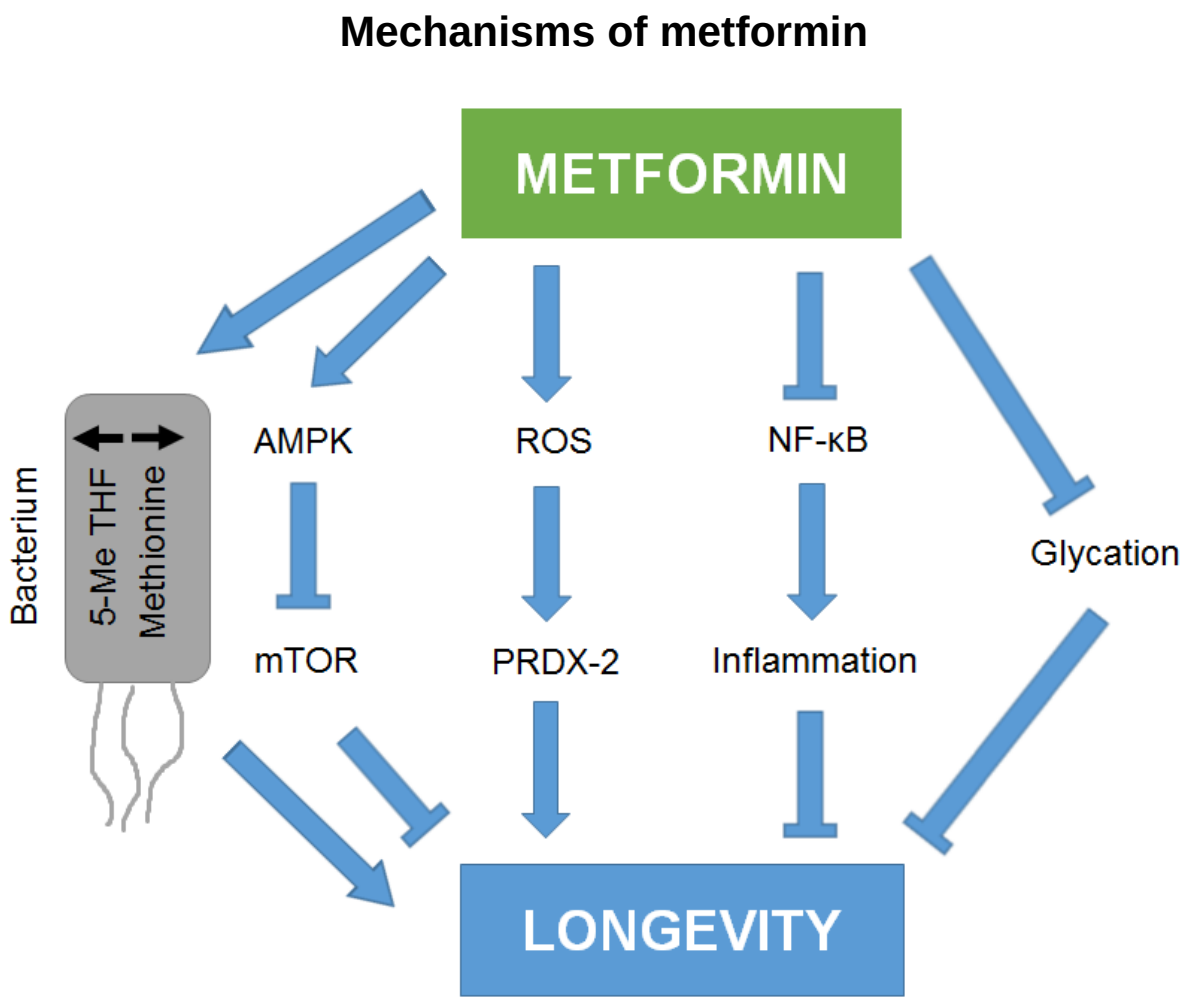

Figure 5 Some of the mechanisms through which metformin could promote longevity. The blunt-ended arrows indicate inhibition.

Despite over half a century since its discovery, we still haven't obtained a full picture about the mechanisms by which metformin exerts its beneficial effects. For example, recent studies continue to uncover new mechanisms through which metformin could possibly reduce blood sugar levels (Miller *et al.*, 2013; Madiraju *et al.*, 2014). Here we present some

of the known mechanisms but due to space limitations it is impossible to discuss the entire scientific literature on this subject. We solely focus on mechanisms that provide a possible mechanistic explanation for the life extending effects of metformin (Figure 5).

Signalling cascades

AMPK is an important energy sensor that acts as a master regulator of metabolism. AMPK is activated in low cellular energy conditions (Long and Zierath, 2006). Metformin activates AMPK in virtually all cell types ever tested (Bulterijs, 2011). The inhibition of complex I of the electron transport chain by metformin is a possible mechanism for this (Owen *et al.*, 2000). However, Vytla and Ochs (2013) unexpectedly found that metformin stimulated ATP production. Given that genetic overexpression of the catalytic subunit of AMPK extends lifespan in worms (Apfeld *et al.*, 2004; Mair *et al.*, 2011) and fruit flies (Ulgherait *et al.*, 2014) it's plausible that AMPK activation contributes to the life extending effects of metformin.

A second mechanism is the direct and indirect inhibition of the metabolic regulator mTOR by metformin (Bulterijs, 2011). Inhibition of mTOR by genetic or pharmacological (e.g. rapamycin) means is well known to extend lifespan in several model organisms including roundworms and rodents (Harrison *et al.*, 2009; Lamming *et al.*, 2013). mTOR is one of the biggest hubs in the cell to regulate growth and energy state. mTOR integrates signals from amino acid abundances, cellular energy state and growth factors (Saxton and Sabatini, 2017). Multiple studies show that metformin decreases mTOR signaling (Kalender *et al.*, 2010; Kickstein *et al.*, 2010; Ben Sahra *et al.*, 2011; Nair *et al.*, 2014; Howell *et al.*, 2017). However, a recent study failed to observe a decrease in mTOR activity in the skeletal muscle of aged mice treated with metformin (Dungan *et al.*, 2016). This result is in line with a previous study that found that a pharmacological activator of AMPK (which is an inhibitor of mTOR) failed to activate AMPK in the muscles of old mice, only having an effect in the muscles of young mice (Reznick *et al.*, 2007). It might thus be possible that metformin fails to exert its geroprotective effects when administered to elderly animals. So far only one rodent study has tested the effect of metformin on lifespan when started at different ages. The results demonstrated that the effect of metformin on lifespan decreases with the age at which the treatment was started (Anisimov *et al.*, 2011).

The insulin/IGF-1 signalling (IIS) pathway has been linked to aging in worms, flies, and rodents. For example, knocking out a single copy of the IGF receptor (IGF-1R) in mice extends lifespan by 26% (Holzenberger *et al.*, 2003). Metformin treatment has been shown to increase insulin-like growth factor binding protein-1 (IGFBP-1) levels (De Leo *et al.*, 2000; Pawelczyk *et al.*, 2004). In healthy males short-term (15 days) of metformin therapy resulted in a significant reduction in serum IGF-1 and insulin levels (Fruehwald-Schultes *et al.*, 2002). Oral administration of metformin in mice decreased insulin and IGF-1 levels by about 20% and 35%, respectively (Memmott *et al.*, 2010). In addition metformin has also been found to reduce the signalling through IGF-1R as well as the expression levels of IGF-1R in various cancer cell lines (Karnevi *et al.*, 2013; Sarfstein *et al.*, 2013; Zhang *et al.*, 2015b).

The histone deacetylase SIRT1 is a well-known "anti-aging" protein. However, recent evidence has questioned these effects (Burnett *et al.*, 2011). We have previously discussed Sirt1 and refer the reader to that article for more background (http://www.longecity.org/forum/blog/201/entry-3579-nicotinamide-riboside/). Metformin increases SIRT1 protein levels in white adipose tissue (WAT) of obese db/db mice (Caton *et*

al., 2011), in liver cells of ob/ob mice (Song *et al.*, 2015) and in human endothelial cells (Hung *et al.*, 2016). Metformin increased SIRT1 protein levels in mouse microvascular endothelial cells maintained in high glucose but not in normal glucose (Arunachalam *et al.*, 2014). Metformin significantly increased SIRT1 deacetylase activity in human umbilical vascular endothelial cells (HUVECs) cultured for 3 days in high glucose followed by 3 days of normal glucose (Zhang *et al.*, 2015c). Zheng et al. (2012) found a dose-dependent increase in SIRT1 activity in endothelial cells exposed to one week of high glucose followed by two weeks of normal glucose + metformin. Furthermore, when hyperglycemia was induced in rats for two weeks followed by four weeks of normoglycemia + metformin, SIRT1 levels were significantly increased in the retinas compared to the control. Metformin also increased SIRT1 activity but not protein levels in skeletal muscle cells (Bogachus and Turcotte, 2010).

SIRT1 activates the forkhead box O (FOXO) transcription factors by deacetylation (Daitoku *et al.*, 2011). As discussed in the last paragraph, metformin activates SIRT1 leading to the hypothesis that metformin may also lead to the activation of FoxO transcription factors. Indeed, metformin treatment reduced acetylated FoxO1 levels in mouse microvascular endothelial cells maintained in high glucose but not in normal glucose (Arunachalam *et al.*, 2014). Metformin also upregulated and increased nuclear translocation of FOXO1 in adipocytes (Barbato *et al.*, 2013). In contrast, metformin significantly decreased FOXO1 levels and reduced its nuclear localization in human and mouse aortic endothelial cells grown in high glucose (Li *et al.*, 2015a). Metformin activated FOXO3a in a breast cancer cell line (Fonseca *et al.*, 2012; Queiroz *et al.*, 2014). Metformin treatment extended lifespan of the *daf-16* null mutant *C. elegans* (Onken and Driscoll, 2010). DAF-16 is the worm's homolog of FOXO transcription factors leading to the conclusion that, at least in worms, metformin extends lifespan through DAF-16/FOXO independent pathways.

Inflammation

Metformin has also been reported to inhibit NF-κB, the master regulator of inflammation (Isoda *et al.*, 2006; Huang *et al.*, 2009; Tan *et al.*, 2009; Kim *et al.*, 2011; Martin-Montalvo *et al.*, 2013). Metformin prevented the secretion of the proinflammatory cytokine TNF-α from macrophages (Hyun *et al.*, 2013). Inhibition of NF-κB by metformin is able to prevent the senescence-associated secretory phenotype (SASP, see below) (Moiseeva *et al.*, 2013). Furthermore, metformin therapy has been shown to reduce serum levels of C-reactive protein (CRP) in women suffering from PCOS (Morin-Papunen *et al.*, 2003; Velija-Asimi, 2007), in those at risk for developing diabetes (Goldberg *et al.*, 2014) and in type 2 diabetes patients (Chakraborty *et al.*, 2011; Esteghamati *et al.*, 2013). Metformin (2 μM) prevented the lipopolysaccharide (LPS)-induced secretion of proinflammatory cytokines (IL-1 and TNF-α) by macrophages. However, metformin in the absence of LPS had no effect on the secretion of proinflammatory cytokines (Bułdak *et al.*, 2014). In another study metformin was found to reduce the pro-inflammatory cytokine IL-1β but not TNF-α while increasing the anti-inflammatory cytokine IL-10 in macrophages stimulated by LPS (Kelly *et al.*, 2015). Metformin was found to significantly decrease TNF production while having no effect on NF-κB activation in isolated human monocytes stimulated with LPS or oxidized LDL (Arai *et al.*, 2010). Metformin significantly reduced the NF-κB subunit p65 and the phosphorylation of IκB in aortic vessel wall and decreased serum hs-CRP levels in an atherosclerotic rabbit model (Li *et al.*, 2009). Phosphorylation of IκB leads to its proteasomal degradation and thereby

relieving its inhibitory effect on NF-κB. Hence the decrease in phosphorylation of IκB will result in lower nuclear localization of NF-κB. Treatment with metformin significantly reduced the mRNA levels of the proinflammatory cytokines IFN-γ, TNF-α, IL-17, IL-1β, and IL-6 in the brain of mice suffering from experimental autoimmune encephalomyelitis (Nath *et al.*, 2009). However, 12 weeks of metformin treatment actually increased TNF-α levels in non-diabetic men with coronary heart disease (Carlsen *et al.*, 1998). Given the central role of inflammation in aging (Chung *et al.*, 2009), it seems reasonable to suggest that metformin may extend lifespan by decreasing inflammation.

Cell senescence

In the 1960s Hayflick and Moorhead observed that primary human cells could only undergo a finite number of cell divisions in culture before irreversibly ceasing to divide (Hayflick and Moorhead, 1961). Cells that remain alive but have irreversibly stopped dividing are called senescent cells. A variety of stressors can induce cell senescence such as oxidative stress and oncogene activation. In this way cell senescence can act as an anti-tumor mechanism by permanently arresting the division of cells with high levels of DNA damage. However, cell senescence is a double-edged sword as senescent cells can stimulate the growth of nearby tumor cells.

Senescent cells accumulate with age (Dimri *et al.*, 1995; Jeyapalan *et al.*, 2007). This is either caused as a result of age-related decline in immune-mediated clearance of senescent cells or because senescent cells are generated in elderly individuals at a rate faster than their removal (Rodier and Campisi, 2011). Removing senescent cells in progeroid (Baker *et al.*, 2011) or normal (Baker *et al.*, 2016) mice through an artificial genetic construct has been found to increase lifespan and prevent several age-related phenotypes. senescence-associated secretory phenotype (SASP).

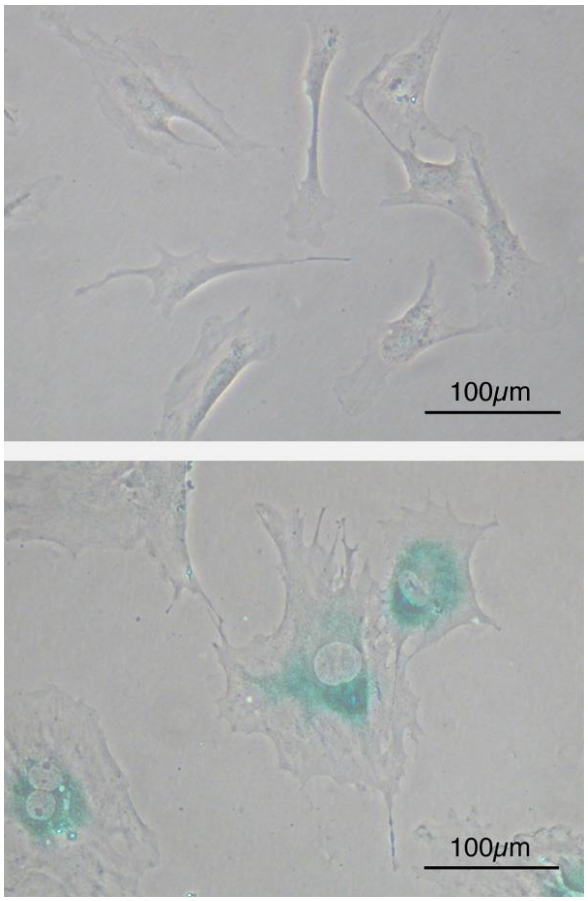

Figure 6 Normal mouse embryonic fibroblasts (MEFs, top) and senescent MEFs (bottom). The blue-green color in the senescent cells is caused by a senescent cell-specific staining technique (sa-β-Gal). Credit: Y tambe (https://commons.wikimedia.org/w/index).

As reported above, metformin is able to inhibit the senescence-associated secretory phenotype (Moiseeva *et al.*, 2013). Oxidative-stress induced cell senescence (as determined by the senescence marker p16INK4a) was reduced in a dose-dependent manner by metformin treatment (Chen *et al.*, 2016). Metformin reduced cell senescence (as measured by the sa-β-Gal assay) in mouse microvascular endothelial cells maintained in high glucose

but no effect was found in normal glucose (Arunachalam *et al.*, 2014). Furthermore, metformin significantly reduced sa-β-Gal staining in HUVECs cultured for three days in high glucose followed by three days in normal glucose (Zhang *et al.*, 2015c). Metformin also reduced sa-β-Gal staining in myoblasts exposed to ceramide (Jadhav *et al.*, 2013).

In contrast, multiple studies show that metformin is able to induce senescence in cancer cell lines (Yi *et al.*, 2013; Li *et al.*, 2015b). The induction of senescence by metformin in cancer cells could potentially explain the anti-cancer effects of metformin (see above). Indeed, metformin significantly increased the number of mouse embryonic fibroblasts that become senescent after exposure to the chemotherapy drug doxorubicin. Metformin also induced premature senescence in two human diploid fibroblast cell lines (Cufí *et al.*, 2012). Doxorubicin interferes with topoisomerase-II-mediated DNA repair and increases the generation of free radicals that can damage cellular components including the DNA (Thorn *et al.*, 2011). The study by Cufí *et al.* (2012) indicates that metformin may lower the threshold for stress-induced senescence. Hence cells that experience oncogenic-like stimuli may sooner go into senescence thereby reducing the risk for further oncogenic transformation.

Mitohormisis

We often think that the relationship between a stress (such as exposure to radiation) and a negative health outcome (such as cancer risk or mortality) is a linear dose-response curve. However, lots of research has shown that small levels of stress often exert protective effects and this phenomenon has been termed 'hormesis'. The specific hormetic response to low levels of reactive oxygen species (ROS) produced by mitochondria has been termed 'mitohormesis' (Ristow and Schmeisser, 2014). Metformin treatment lead to an increase in mitochondrial ROS production and this was essential for the life extending effect of metformin. Addition of antioxidants reduced the ROS levels and abolished metformin's effect on lifespan. The authors further showed that the mitohormesis signaling happens through the ROS-induced activation of a protein known as peroxiredoxin 2 (PRDX-2) (De Haes *et al.*, 2014). This research was presented at the second Eurosymposium on Healthy Ageing (https://youtu.be/dy6c6qY6BRw).

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Decrease in progerin levels

Hutchinson-Gilford Progeria Syndrome (HGPS) is a rare inherited disorder that is often described as "premature aging". Patients suffering from HGPS often die around their 13th birthday. HGPS is characterized by an error in the splicing of lamin A, which is involved in the "skeleton" of the cell nucleus, leading to the formation of a truncated protein known as progerin. Progerin accumulates in the nucleus of people suffering from HGPS leading to abnormalities in gene expression and a characteristic abnormal shape of the cell nucleus (known as "nuclear blebbing", see figure 7). Interestingly, in recent years it was found that low levels of progerin accumulate in the nucleus during "normal" aging (Scaffidi and Misteli, 2006). Metformin treatment decreased the expression of progerin and improved the morphology of the cell nucleus in cells from progeria patients and in cells engineered to express progerin (Egesipe *et al.*, 2016; Park and Shin, 2017).

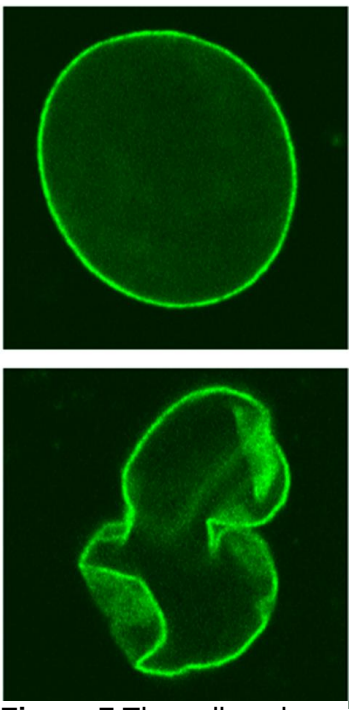

Figure 7 The cell nucleus of a healthy individual (top) and from a progeria patient (bottom). Credit: Scaffidi *et al.* (2005).

Metabolic effects

The classical hypothesis for the mechanism by which metformin activates AMPK is the inhibition of mitochondrial respiration at complex I leading to a decrease in cellular ATP levels and a concomitant increase in AMP. AMP is an allosteric activator of AMPK (Bulterijs, 2011). If this hypothesis were correct, metformin would cause a decrease in cellular ATP levels which would result in large shifts in metabolic fluxes. However, most studies have been conducted in isolated mitochondria or in permeabilized cells. An alternative mechanism for the metformin-induced increase in AMP levels without decreasing ATP concentration has been proposed (Ouyang *et al.*, 2011).

Cells build an energy reserve in the form of phosphocreatine by transferring a phosphate group from ATP to creatine. When energy is needed phosphocreatine can donate the phosphate group back to ADP generating ATP (Persky and Brazeau, 2001). Metformin (10-20 mM) improved phosphocreatine recovery after ATP depletion (by either dinitrophenol or azide) in cultured muscle cells (Vytla and Ochs, 2013). This result suggest that metformin actually stimulates energy production rather than decreasing it. Though it should be pointed out that at higher metformin concentrations (40-80 mM) phosphocreatine recovery was decreased.

Metformin has a whole host of metabolic effects. Some of them are clearly involved in its antidiabetic action such as the inhibition of gluconeogenesis in the liver (Hundal *et al.*, 2000). Metformin also reduces plasma free fatty acid concentrations in diabetic individuals (Perriello

et al., 1994) and this may help to reduce lipotoxicity. Metformin stimulates fatty acid oxidation in muscle and liver (Zhou *et al.*, 2001; Collier *et al.*, 2006; Wang *et al.*, 2014). Interestingly, overexpression of fatty acid oxidation genes extends lifespan in fruit flies (Lee *et al.*, 2012). Metformin also reduces cholesterol and lipid biosynthesis (Scott and Tomkin, 1983; Zhou *et al.*, 2001). The latter also suggests that inhibition of the electron transport chain is an unlikely explanation for the mechanism of metformin, as fatty acid oxidation requires electron transport chain activity.

In 2013 David Gems and colleagues showed that metformin extended the lifespan of *C. elegans* through influencing the metabolism of the bacteria on which the worms grew. Metformin changed microbial folate and methionine metabolism. When worms were grown on a bacteria-free medium or on dead bacteria metformin administration actually reduced lifespan. This suggests that in worms metformin has toxic effects on the host but these are overshadowed by the beneficial effects from changes in the microbiome metabolism (Cabreiro *et al.*, 2013). Another study had already demonstrated that interfering with folate synthesis in bacteria extended lifespan in *C. elegans* (Virk *et al.*, 2012). A more recent study further confirmed that folate had no direct effect on *C. elegans* lifespan strengthening the conclusion that the effect is solely related to changes in the bacteria (Virk *et al.*, 2016) Metformin has been shown to induce changes in the gut microbiome composition and metabolism in mice (Lee and Ko, 2014) and humans (Forslund *et al.*, 2015). Cabreiro *et al.* (2013) also speculated that the lack of lifespan extension seen in fruit flies by metformin might be caused by a metformin-resistant microbiome in flies.

Decreases in dietary methionine intake have been shown to extend the lifespan in yeast (Lee *et al.*, 2014; Ruckenstuhl *et al.*, 2014), fruit flies (Troen *et al.*, 2007; Lee *et al.*, 2014), and rodents (Orentreich *et al.*, 1993; Richie *et al.*, 1994; Miller *et al.*, 2005; Lopez-Torres and Barja, 2008). In addition methionine restriction extended replicative lifespan of human cells in culture (Kozieł *et al.*, 2014). Methionine restriction lowers mitochondrial ROS production resulting in lower oxidative protein damage (Sanz *et al.*, 2006; Caro *et al.*, 2008; Caro *et al.*, 2009; Sanchez-Roman *et al.*, 2011).

Oxidative stress

A radiolysis experiment suggests that metformin is a weak scavenger for the $OH^{\bullet}$ radical. In the same study polymorphonuclear cells were extracted from the blood of healthy volunteers and the researchers tested if metformin could decrease the ROS production in these cells after stimulation. Metformin showed an 8% inhibition of ROS after PMA and a 28% decrease in ROS after fMLP-stimulation but neither reached significance (Bonnefont-Rousselot *et al.*, 2003). Khouri *et al.* (2004) confirmed the finding that metformin is a scavenger for $OH^{\bullet}$ but not for superoxide radicals *in vitro*. Metformin was not found to have antioxidant effects in a cell free assay ferric reducing antioxidant power assay (Othman *et al.*, 2016).

H_2O_2 levels in whole head homogenate of crickets were decreased by metformin treatment (Hans *et al.*, 2015). ROS levels were decreased in white blood cells isolated from type 2 diabetes patients treated with metformin (Chakraborty *et al.*, 2011). Superoxide production after stimulation in platelets extracted from type 2 diabetes patients treated with metformin was similar to that of healthy controls and lower than in glibenclamide or diet-treated patients (Gargiulo *et al.*, 2002). Metformin reduced mitochondrial ROS production and

malondialdehyde levels in rats fed a high fat diet (Pintana *et al.*, 2012). Metformin reduced ROS production and lipid peroxidation in rat pancreatic β-cells stimulated with free fatty acids (Piro *et al.*, 2012). Metformin significantly reduced palmitic acid-induced ROS production in human aortic endothelial cells (Hou *et al.*, 2010). Metformin also reduced ROS production in unstimulated or stimulated (by either angiotensin II, PMA or high glucose) bovine aortic endothelial cells (Ouslimani *et al.*, 2005; Mahrouf *et al.*, 2006). Treating skin cells from progeria patients with metformin resulted in a significant decrease in reactive oxygen species (ROS) production (Park and Shin, 2017). Metformin decreased ROS production in mouse microvascular endothelial cells maintained in high glucose but not in normal glucose (Arunachalam *et al.*, 2014). Metformin prevented the lipopolysaccharide (LPS)-induced increase in ROS production and malondialdehyde levels (Bułdak *et al.*, 2014; Kelly *et al.*, 2015). However, other studies have found that metformin increases ROS production in adipocytes (Anedda *et al.*, 2008) and preadipocytes (Jaganjac *et al.*, 2017).

When old mice were supplemented with metformin they had an increase in the expression levels of the antioxidant enzyme superoxide dismutase 2 (SOD2) (Park and Shin, 2017). Metformin increased catalase and SOD2 levels in LPS-stimulated and in unstimulated macrophages while SOD1 was only increased in unstimulated cells (Bułdak *et al.*, 2014). Metformin increased SOD2 (+47%), catalase (+134%), glutathione reductase (+187%), and glutathione peroxidase (+146%) levels and increased catalase enzymatic activity (+59%) as well as glutathione peroxidase enzymatic activity (+106%) in fructose-fed mice. Furthermore, in mice fed the control diet (without fructose) catalase enzymatic activity was increased by 151% in the metformin group (Karise *et al.*, 2017). Metformin ameliorated the MPTP-induced decrease in SOD and catalase activity, glutathione levels and the increased level of lipid peroxidation in a mouse model of Parkinson's disease (Patil *et al.*, 2014). Metformin also attenuated the haloperidol-induced increase in malondialdehyde and the reduction in glutathione and catalase (Adedeji *et al.*, 2014). Metformin also increases SOD2 and catalase levels in normal fibroblasts and in fibroblasts from fibromyalgia patients (Alcocer-Gómez *et al.*, 2015). Metformin treatment increased glutathione levels in blood and liver but not in heart of diabetic and normal mice (Ewis *et al.*, 1995). Metformin decreased ROS levels and increased protein levels of SOD2 and catalase in endothelial cells cultured for one week in high glucose followed by 2 weeks in normal glucose + metformin (Zheng *et al.*, 2012). Hung *et al.* (2016) found lower ROS levels and an increased activity of SOD in human endothelial cells pre-treated with metformin before oxidized LDL exposure. Similarly, Martin-Montalvo *et al.* (2013) also found higher levels of SOD2 in metformin treated mice. Furthermore, they also found that metformin lowers the mitochondrial production of superoxide and reduces the levels of 8-iso-PGF2α indicating a decrease in lipid peroxidation. Metformin activates Nrf2 in a liver cell line (Martin-Montalvo *et al.*, 2013), in mice brain (Prasad *et al.*, 2017), as well as the Nrf2-homolog SKN-1 in worms (Onken and Driscoll, 2010). Metformin increased glutathione levels, improved antioxidant potential, and lowered malondialdehyde, protein carbonyl, and ROS levels in brain tissue of rats (Geetika *et al.*, 2017). Metformin also decreased malondialdehyde in fructose treated mice (Karise *et al.*, 2017).

Metformin treatment prevented the hydrogen-peroxide-induced apoptosis in skin cells from fibromyalgia patients (Alcocer-Gómez *et al.*, 2015). Metformin also ameliorated the pentylenetetrazole-induced decrease in glutathione levels and the increase in malondialdehyde levels (Zhao *et al.*, 2014). Twelve weeks of metformin treatment in newly diagnosed type 2 diabetes patients significantly increased the ferritin reducing ability of

plasma, a measure of the antioxidative capacity of blood plasma. Furthermore, the advanced oxidation protein product levels decreased and the levels of the antioxidant enzyme paraoxonase were increased by metformin treatment (Esteghamati *et al.*, 2013; Mirmiranpour *et al.*, 2013). Chakraborty *et al.* (2011) also observed a decrease in advanced oxidation protein product levels after 24 weeks of metformin therapy.

Glycation inhibitor

Glycation is the non-enzymatic reaction between reducing sugars or reactive carbonyl compounds (such as glyoxal and methylglyoxal) with proteins (figure 8). Glycation has been recognized as one of the drivers of biological aging (Sjöberg and Bulterijs, 2009). Metformin has a strong structural similarity to the well known inhibitor of glycation aminoguanidine (Bulterijs, 2011). Aminoguanidine is the gold standard glycation inhibitor that is used as a positive control in research on glycation (Richardson *et al.*, 2015). There are several possible mechanistic explanations for how metformin could protect against glycation. Firstly, metformin is used to lower blood glucose levels in diabetic patients and hence the rate of glycation would be decreased. However, metformin does not lower blood glucose levels in people with normal blood glucose levels (normoglycemic). Secondly, metformin may decrease oxidative stress resulting in a lower production of reactive carbonyl compounds (see above). Finally, metformin may act as a scavenger reacting with reactive intermediates of glycation and thereby decreasing their concentration (Harding and Ganea, 2006).

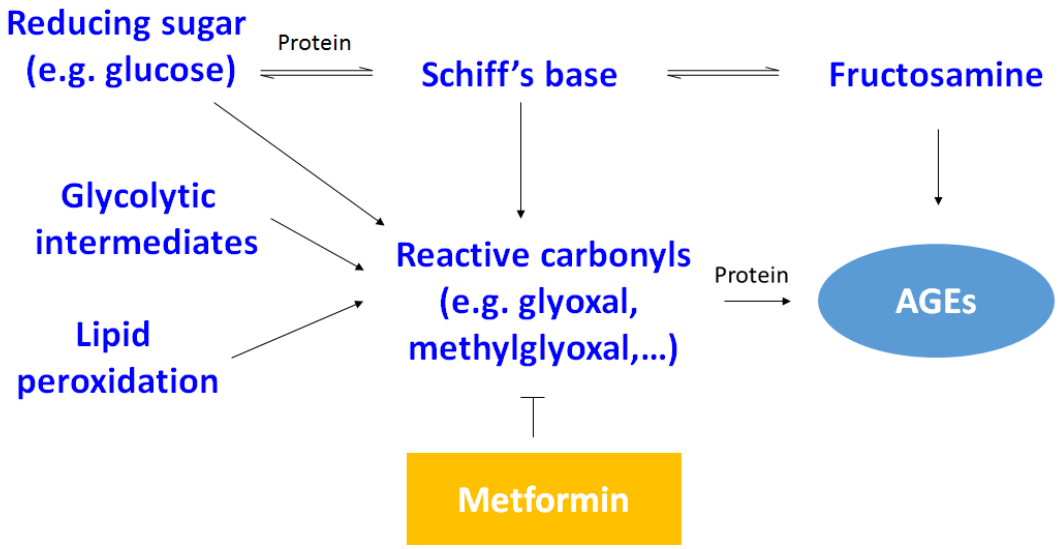

Figure 8 Formation pathways of advanced glycation end products (AGEs).

When metformin is incubated with methylglyoxal at 37 °C for three hours the free methylglyoxal concentration decreases by 92.1% (Huang *et al.*, 2016). But does metformin also scavenge methylglyoxal in the presence of proteins? This question has been answered by multiple studies. For example, Ruggiero-Lopez *et al.* (1999) incubated bovine serum albumin (BSA) with either glyoxal or methylglyoxal for 6 days at 37 °C to induce glycation. The inhibitory effect of metformin was investigated and compared to aminoguanidine. The addition of metformin reduced glyoxal-induced glycation (as measured by fluorescence) by 37% and methylglyoxal-induced glycation by 45%. In contrast, aminoguanidine decreased glyoxal-induced glycation by 85% and methylglyoxal-induced glycation by 58%. The efficacy

of metformin in reducing methylglyoxal-induced (Kiho *et al.*, 2005; Ahmad *et al.*, 2013) and glucose-induced (Tanaka *et al.*, 1997; Rahbar *et al.*, 2000) protein glycation has been confirmed by other studies.

Metformin administration to type 2 diabetes patients (Beisswenger *et al.*, 1999) or women suffering from PCOS (Diamanti-Kandarakis *et al.*, 2007) has been found to lower serum levels of methylglyoxal. The addition product produced by the scavenging reaction between methylglyoxal and metformin was demonstrated to be present in the urine of type 2 diabetic patients treated with metformin (Kinsky *et al.*, 2016). Twelve weeks of metformin treatment in type 2 diabetes patients lead to a significant decrease in serum AGEs in patients newly diagnosed (Esteghamati *et al.*, 2013). Twenty-four weeks of metformin therapy decreased plasma pentosidine levels in type 2 diabetes patients (Chakraborty *et al.*, 2011). Furthermore, 6-month metformin treatment in women with PCOS led to lower serum advanced glycation end product (AGE) levels (Christakou *et al.*, 2014).

Human neuronal stem cells exposed to AGEs had a reduced cell viability and increased levels of proinflammatory cytokines. Metformin treatment protected against AGE-induced cellular toxicity (Chung *et al.*, 2015, 2017). Schurman *et al.* (2008) treated cells with AGE-modified protein and observed oxidative stress and cell death. However, these alterations could be prevented by metformin treatment. The authors showed that AGE-modified protein lead to an upregulation of the receptor for advanced glycation end products (RAGE) on the cell surface while this upregulation was prevented by metformin. RAGE has a positive feedback cycle so that upon binding of AGE-protein to a RAGE receptor the cell expresses more RAGE protein leading to an even stronger AGE-RAGE signal. Metformin apparently reduces this positive feedback cycle and hence reduces AGE-modified protein toxicity (Schurman *et al.*, 2008; Ishibashi *et al.*, 2012a&b; Zhou *et al.*, 2016b). Metformin also reduced AGE-induced increases in the expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) while increasing the expression of the anti-inflammatory cytokine IL-10. Furthermore, metformin reduced NF-κB nuclear translocation and shifted the polarization of macrophages to the anti-inflammatory M1 phenotype (Zhou *et al.*, 2016b).

DNA damage

In the genetic disease Fanconi anemia, patients experience high levels of chromosome breaks. Exposing fibroblasts from a Fanconi anemia patient to metformin for 48 h significantly reduced the level of these chromosome breaks. Furthermore, it was demonstrated that aminoguanidine (see above) equally reduced chromosome damage (Zhang *et al.*, 2016). Metformin reduced the number of DNA adducts and the amount of oxidative DNA damage in cigarette smoke-exposed mice (Izzotti *et al.*, 2014). Metformin-treated mice have also been shown to harbor lower levels of the DNA strand break marker γ-H2AX foci (Arkadieva *et al.*, 2011). Skin cells from progeria patients treated with metformin also show a significant decrease in γ-H2AX foci (Park and Shin, 2017). Similarly in fruit flies γH2avD (the fly equivalent of γ-H2AX foci) were reduced by 36% in intestinal stem cells of metformin treated flies (Na *et al.*, 2013). Metformin was shown to reduce paraquat-induced increases in ROS and DNA mutations. Furthermore, metformin also decreased oncogenic Ras-induced increases in ROS and DNA damage (Algire *et al.*, 2012). Metformin treatment ameliorated insulin-induced increases in DNA damage in cell culture and in rats (Othman *et al.*, 2016).

However, metformin was found to induce DNA damage in Chinese hamster ovary (CHO-K1) cells as detected by the comet assay. In contrast, the authors failed to find chromosome aberrations in the CHO-K1 cells and micronucleus assays in mice also failed to find DNA damage (Amador *et al.*, 2012). Metformin was also found to enhance the formation of oxidative DNA damage in DNA exposed to H_2O_2 and Cu^{2+} *in vitro*. However, no increase in oxidative DNA damage was found in the absence of H_2O_2 and Cu^{2+} (Ohnishi *et al.*, 2016). As discussed above metformin lowers ROS levels in the cell and this might compensate for the metformin-induced enhancement of ROS damage.

Metformin may induce DNA damage in cancer cell lines possibly by increasing ROS production (Marinello *et al.*, 2015). Furthermore, metformin has been found to radiosensitize cancer cells through inhibition of DNA repair proteins (Jeong *et al.*, 2015; Wang *et al.*, 2015b). However, this might be beneficial as it could contribute to metformin's anti-cancer effects (see above). As discussed above, metformin seems to lower ROS production in normal cells.

Cell death

Metformin reduced mitochondrial permeability transition pore opening in β-cells (Lablanche *et al.*, 2011) and in the ischemic heart (Bhamra *et al.*, 2008). The opening of the mitochondrial permeability transition pore is one of the key events in mitochondrial-induced apoptosis. Metformin reduced the induction of endothelial cell death by exposure to oxidized LDL (Valente *et al.*, 2014). Metformin protected against H_2O_2-induced cardiomyocyte apoptosis (Sasaki *et al.*, 2009). Metformin reduced AGE-induced apoptotic cell death in kidney cells (Ishibashi *et al.*, 2012b). Metformin also reduced serum deprivation-induced cell death in stratial cells expressing mutant huntingtin (Vázquez-Manrique *et al.*, 2016). Metformin administration increased the urinary excretion of cell-free DNA and mtDNA in young but even more in old rats (Gaziev *et al.*, 2016). Cell-free DNA and mtDNA in the urine is a marker of cell death *in vivo*. The authors interpret this finding as that metformin may potentially increase the cell death of cells containing structural or functional abnormalities, which may be beneficial. The effect of metformin of β-cell death will be discussed below.

Autophagy

Metformin induced autophagy in the brain of rats leading to protection from experimentally induced stroke. Treatment with an autophagy inhibitor completely abolished the metformin-induced neuroprotection (Jiang *et al.*, 2014a). Metformin protected nucleus pulposus cells from exogenous oxidant-induced apoptosis and cell senescence by activating autophagy (Chen *et al.*, 2016). Metformin also increased autophagy induction and flux in liver cells from ob/ob mice (Song *et al.*, 2015). Metformin has been found to increase autophagy in cancer cells (Tomic *et al.*, 2011; Feng *et al.*, 2014; Takahashi *et al.*, 2014; Sessen *et al.*, 2015; Nazim *et al.*, 2016). Few direct data exist in non-cancerous cells on the link between metformin and autophagy but based on our mechanistic understanding metformin should activate autophagy. Metformin is an inhibitor of mTOR (see above) and mTOR suppresses autophagy hence we expect that metformin would stimulate autophagy. Metformin stimulated the acidification of the lysosomal/endosomal compartments (Labuzek *et al.*, 2010).

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Weight loss

Body weight was also lower in metformin-treated adult C57BL/6 mice but metformin attenuated the age-related weight loss. Body weight in old mice treated with metformin surpassed that of control mice (Martin-Montalvo *et al.*, 2013). Age-related weight loss is a predictor of mortality (Wedick *et al.*, 2002; Knudtson *et al.*, 2005; Alley *et al.*, 2010). However in other studies the body mass of old metformin-treated mice was lower than that of control mice (Anisimov *et al.*, 2008; Anisimov *et al.*, 2010b). Yet other studies found no difference in body weight over the whole lifespan (Anisimov *et al.*, 2010a). It is also possible that the effect of metformin on body mass may be sex dependent. Indeed, in one study it was found that metformin-supplemented male mice were lighter while female mice were heavier compared to controls (Anisimov *et al.*, 2015).

Short-term (15 days) of metformin therapy had no effect on body weight or body fat in healthy males with a normal body weight (Fruehwald-Schultes *et al.*, 2002). Twenty-four weeks of metformin treatment reduced BMI levels from 27 to 23 kg/m² in a double-blind, placebo-controlled, randomized trial of type 2 diabetes patients in India (Chakraborty *et al.*, 2011). Six weeks of metformin treatment lead to a significant decrease in body weight and BMI in patients with type 2 diabetes (Sahin *et al.*, 2007). Metformin reduced weight gain by 3 kilo after a 4.3 year follow-up in diabetes patients (Kooy *et al.*, 2009). A Cochrane review found that metformin decreased body weight and BMI in obese children and adolescents (Mead *et al.*, 2016). A meta-analysis of randomized placebo-controlled trials found that lifestyle + metformin lead to a greater decrease in BMI (by 0.7 kg/m²) than lifestyle + placebo (Naderpoor *et al.*, 2015). Another meta-review indicated that metformin is effective in reducing antipsychotics-induced weight gain (Zheng *et al.*, 2015). Finally, in a meta-analysis that included all randomized placebo-controlled trials for which data on weight change were available found that metformin was associated with a decrease in body weight of 1.1 kg (Domecq *et al.*, 2015). In conclusion we can say that there's convincing evidence that metformin use in humans leads to a decrease in body weight and BMI but we should bear in mind that this decrease is very small compared to what is normally obtained by diet and lifestyle interventions. For example, one short term (8 weeks) diet study found an approximate weight loss of 7 kg (Lopez-Legarrea *et al.*, 2013). The exception to this is the Indian trial in which 24 weeks of metformin-treatment produced an impressive decline in BMI of 4 units. In the placebo group BMI was decreased by just 0.5 units (Chakraborty *et al.*, 2011).

Safety of metformin treatment

Minor side effects

Metformin has several non-serious side effects such as gastrointestinal problems (diarrhea, flatulence, vomiting, upset stomach, abdominal bloating, anorexia, and nausea), taste disturbances including a metallic taste in the mouth, and dermatological problems (erythema, pruritus, and urticaria) (Product monograph: Glucophage®, 2009).

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Lactic acidosis

The most serious concern with biguanide therapy is the development of lactic acidosis. Lactic acidosis has a mortality rate of about 30-50% (DeFronzo *et al.*, 2016). The two other biguanides (phenformin and buformin) have been withdrawn because of the risk for lactic acidosis. Certain conditions increase the risk for lactic acidosis and hence metformin is contraindicated for people suffering from these conditions. Contraindications for metformin use include renal or hepatic insufficiency, circulatory dysfunction such as congestive heart failure, during stress situations (such as severe infections, trauma or surgery), severe dehydration, during radiological investigations with iodinated contrast materials, unstable or insulin-dependent diabetes, people who suffer from a metabolic acidosis, people who have an excessive intake of alcohol, people with a history of lactic acidosis, and in very elderly people (Product monograph: Glucophage®, 2009; DeFronzo *et al*., 2016). It should also not be used in people suffering from a known hypersensitivity or allergy to metformin nor during pregnancy or breastfeeding (Product monograph: Glucophage®, 2009).

Wang *et al.* (2003) found an approximate 5-fold increase in blood lactate levels in mice injected with metformin compared to saline. The concentration of the drug needed to achieve half of the maximal effect is called the EC_{50} value. The lower the EC_{50} the higher the potency of the drug. Wang *et al.* (2003) also compared the EC_{50} values for all three biguanides for their ability to increase blood lactate levels. The EC_{50} for metformin was 734 µM compared to 119 µM for buformin and only 4.97 µM for phenformin. These data illustrate that metformin is much less potent than buformin and phenformin in increasing blood lactate levels. Human studies typically show a small increase in blood lactate concentrations (DeFronzo *et al.*, 2016).

Salpeter *et al.* (2010) conducted a large meta-review of 347 studies and found no increased risk for lactic acidosis in metformin-treated patients compared to those on other diabetes medications. Furthermore blood lactate levels were not significantly different between both groups. The incidence of lactic acidosis is estimated at 5-9 cases per 100,000 patient years of metformin use (DeFronzo *et al.*, 2016). The lack of lactic acidosis cases in published trials probably reflects the fact that trials typically exclude patients at risk for lactic acidosis and that trial participants receive standard of care (DeFronzo *et al.*, 2016). Lactic acidosis is a risk especially in patients with contraindications for the use of metformin or in people who take an overdose (Yamada *et al.*, 2016).

Hypoglycemia

Hypoglycemia (overly low blood sugar level) is a common adverse effect of many medications used in type 2 diabetes. After all the goal of these medications is to lower blood sugar levels. However, when metformin is used as monotherapy (in the absence of other blood sugar lowering drugs) this risk of hypoglycemia is negligible (Cheng and Fantus, 2005).

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Decrease in vitamin B_{12} levels

Berchtold *et al.* (1969) reported that short-term (2-3 months) metformin treatment caused malabsorption of vitamin B_{12}. In a recent randomized placebo controlled trial it was found that treatment of type 2 diabetes patients with 850 mg three times a day for 4.3 years resulted in a 19% decrease of serum B_{12} levels (de Jager *et al.*, 2010). Prevalence of B_{12} deficiency in metformin-treated patients has been reported to range from 5.8% to as high as 28% (Pflipsen *et al.*, 2009; Reinstatler *et al.*, 2012; Ko *et al.*, 2014; Ahmed *et al.*, 2016; Damião *et al.*, 2016; Kancherla *et al.*, 2017). Reinstatler *et al.* (2012) found that 5.8% of metformin-treated diabetes patients had B_{12} deficiency compared to only 2.4% in those not using metformin. In a meta-analysis of 29 studies with a total of over 8,000 patients it was demonstrated that metformin use was associated with lower serum vitamin B_{12} levels and a higher incidence of B_{12} deficiency (Niafar *et al.*, 2015).

One surprising study revealed that an increased intake of calcium reversed the malabsorption of vitamin B_{12} by metformin (Bauman *et al.*, 2000). However this finding is explained by the presence of calcium ions in the receptor that is needed for vitamin B_{12} absorption in the small intestine (Birn *et al.*, 1997).

Vitamin B_{12} deficiency can cause peripheral neuropathy, impaired memory, delirium, megaloblastic anemias, pancytopenia, and hyperhomocysteinemia (Kibirige and Mwebaze, 2013). Multiple case reports have been published that link metformin use to one of the B_{12} deficiency diseases (Mahajan and Gupta, 2010). While the decrease in vitamin B_{12} levels can occur quickly after initiation of metformin therapy, it can take up to 5-10 years before overt clinical manifestations of vitamin B_{12} deficiency become evident due to the large body stores of this vitamin, primarily in the liver, that are not quickly depleted (Kibirige and Mwebaze, 2013).

Sadly many long-term users are never tested for vitamin B_{12} status. In fact current clinical guidelines do not make any recommendations on vitamin B_{12} testing or prevention in metformin users (Fogelman *et al.*, 2016). In a recent study it was found that only 37% of long-term metformin users had their vitamin B_{12} status tested (Kancherla *et al.*, 2017).

Decrease in folate levels

In the metabolism section we discussed how metformin extended lifespan of *C. elegans* by interfering with folate metabolism. However, we consider a decrease in folate levels in the host to be a negative side effect of metformin treatment as Virk *et al.* (2016) demonstrated that host folate had no effect on lifespan.

A deficiency in folate can lead to anemia, neurological disorders, birth defects, occlusive vascular disease, and colonic polyposis (Haslam and Probert, 1998).

Short term (6 weeks) of metformin treatment resulted in a 10% decrease of serum folate levels (Sahin *et al.*, 2007). Serum folate levels were decreased by 7% after 16 weeks of metformin treatment in a placebo-controlled, randomized trial (Wulffelé *et al.*, 2003). Similarly, 40 weeks of metformin treatment lead to a 8% decrease in non-diabetic male patients with cardiovascular disease (Carlsen *et al.*, 1997). However, some studies failed to

find a decrease in serum folate levels in diabetic patients treated with metformin (Carpentier *et al.*, 1976; Pongchaidecha *et al.*, 2004; de Jager *et al.*, 2010).

Decrease in testosterone levels

It is well known that testosterone levels decline with age in males (Yeap, 2009). Several prospective studies have demonstrated an association between low testosterone levels and increased mortality in middle-aged and older men (Shores *et al.*, 2006; Laughlin *et al.*, 2008; Tivesten *et al.*, 2009). However such studies cannot demonstrate if this association is causal. It could for example be that men who are sicker tend to have lower testosterone levels. Indeed, testosterone has been found to be decreased by acute and chronic illnesses (Shores, 2014). To the best of my knowledge no intervention study has tested the effect of metformin supplementation on lifespan. Though, one interesting result comes from the Interventions Testing Program. 17-α-estradiol extended lifespan of male but not female mice (Strong *et al.*, 2016). 17-α-estradiol inhibits the activity of the enzyme 5α-reductase which converts testosterone in its more active form dihydrotestosterone.

Treatment with 1500 mg/d metformin decreased serum testosterone levels by 23% in women with breast cancer (Campagnoli *et al.*, 2012, 2013). In women suffering from PCOS metformin treatment (500 mg three times per day) for 30-32 days led to an approximate 19% reduction in plasma free testosterone levels (De Leo *et al.*, 2000). Similarly, three months and six months of metformin therapy significantly reduced testosterone levels in normoinsulinemic women with PCOS syndrome (Romualdi *et al.*, 2008). A meta-review of randomized placebo-controlled trials with metformin for the treatment of PCOS found a decrease in testosterone levels (Naderpoor *et al.*, 2015). Pawelczyk *et al.* (2004) found a 37% decrease in testosterone levels and a 16% increase in sex hormone-binding globulin (SHBG) in obese PCOS patients treated with 500 mg metformin three times a day. SHBG binds to testosterone and thereby decreases the level of "free" testosterone. Only free testosterone can penetrate cells to activate the signalling pathways. Thus higher SHBG levels result in less testosterone signalling. Tan *et al.* (2009) found an approximate 28% decrease in testosterone levels after 6 months of metformin treatment compared to before metformin treatment in women suffering from PCOS.

There's a surprising lack of studies that investigate the effect of metformin on testosterone levels in males. Two weeks of metformin treatment in normal men lead to a significant reduction in total testosterone and free testosterone with a concurrent increase in sex hormone binding globulin levels (Shegem *et al.*, 2002). In addition three months of metformin treatment plus a hypocaloric diet lead to a significant decrease in total testosterone levels in diabetic men as well as in free testosterone levels in the non-diabetic men (Ozata *et al.*, 2001).

The decrease in testosterone levels by metformin is yet another interesting parallel between metformin and calorie restriction (Cangemi *et al.*, 2010).

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Cognitive decline

The hallmarks of Alzheimer's disease (AD) are the accumulation of amyloid-β in plaques and the formation of neurofibrillary tangles made of aggregated hyperphosphorylated tau protein (Bloom, 2014). In addition some have described AD as type 3 diabetes because insulin resistance in the brain is a common pathophysiologic finding in the brain of AD patients (de la Monte and Wands, 2008; Kandimalla *et al.*, 2016). This lead to the hypothesis that metformin could potentially be a treatment for AD.

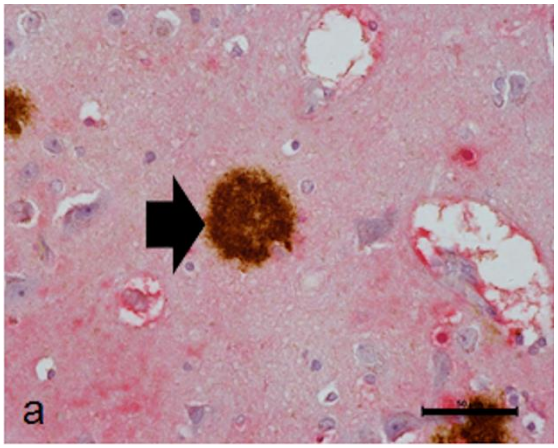

Figure 9 A diffuse plaque in the brain of an aged human. Credit: (Mathur *et al.*, 2015).

Chen *et al.* (2009) have shown that metformin (2 mM) stimulates the accumulation of amyloid-β in neurons *in vitro*. An observation confirmed by a more recent study by Son *et al.* (2016). However insulin prevented the production of amyloid-β. Metformin could even enhance the ability of insulin to decrease amyloid-β production (Chen *et al.*, 2009). In another paper the authors exposed primary neurons to metformin (2.5 mM) in the presence of insulin and observed a decrease in the levels of the enzyme BACE1 which is involved in the production of amyloid-β (Hettich *et al.*, 2014). Metformin increased amyloid-β protein levels in *C. elegans* but decreased the oligomerization (Ahmad and Ebert, 2016). Picone *et al.* (2015) reported that metformin increased amyloid precursor protein (APP) expression as well as induced the formation of amyloid-β aggregates in cell culture. However, the lowest concentration of metformin used in this experiment was 12.5 mM. To put this in contrast, the highest concentration in the brain of mouse fed 50 mg/kg metformin was only 17µM (Wilcock and Bailey, 1994). Picone *et al.* (2015) confirmed that insulin treatment reduced metformin-induced amyloid-β production. Hence, even though several cell culture studies suggest that metformin increases amyloid-β levels these studies have as limitation that they all use supraphysiological metformin concentrations and that the increase in amyloid-β is attenuated by the presence of insulin. *In vivo* insulin is obviously present and hence the *in vivo* state may more reflect the experiments conducted in the presence of insulin. Picone *et al.* (2015) also reported that metformin treatment increased ROS production and decreased antioxidant gene expression in a neuronal cell line. A final contradiction of this paper with the literature is the observed activation of NF-κB by metformin (see above). This is equally in contradiction with the general observed trend for lower ROS levels and higher expression of antioxidant genes in metformin treated cells (see above). As already reported above, the lab of Linda Partridge demonstrated that metformin rescued the shortened lifespan of amyloid-β overexpressing fruit flies by increasing the uptake of glucose in the neurons (Niccoli *et al.*, 2016). Metformin delayed the amyloid-β-induced paralysis, improved neurotransmitter function, reduced amyloid-β oligomerization in *C. elegans* (Ahmad and Ebert, 2016). Metformin also ameliorated amyloid-β-induced mitochondrial dysfunction in human neural stem cells (Chiang *et al.*, 2016).

Tau needs to be hyperphosphorylated before it can aggregate. Phosphatase enzymes can remove phosphate groups from tau and hence protect against the formation of neurofibrillary

tangles. The phosphatase PP2A is activated by metformin, and metformin treatment reduced phosphorylation of tau in mouse neurons. Furthermore the authors administered metformin (5 mg/ml) in the drinking water of mice for 16-24 days and found a decrease in phosphorylation of tau in the brain (Kickstein *et al.*, 2010). Metformin injections also decreased total tau and phosphorylated tau levels in the brain of diabetic mice (Li *et al.*, 2012). Barini *et al.* (2016) confirmed that metformin reduces the phosphorylation of tau but paradoxically find that tau aggregation was increased. When metformin was given to a human tau transgenic mouse the number of tau inclusions in the brain was increased. Recently it was found that metformin treatment also decreases phosphorylation of α-synuclein, a protein found in inclusions in the brain of Parkinson's disease patients (Pérez-Revuelta *et al.*, 2014).

As we already discussed above, metformin increases homocysteine levels and high homocysteine levels have been linked to a higher risk for dementia (Seshadri *et al.*, 2002; Wald *et al.*, 2011). However, lowering plasma homocysteine levels did not reduce cognitive decline in elderly patients with Alzheimer's disease or dementia (Clarke *et al.*, 2014; Zhang *et al.*, 2017).

Metformin injections (200 mg/kg/d) failed to improve spatial learning and memory in diabetic mice (Li *et al.*, 2012). While metformin did improve cell proliferation and neuroblast differentiation in the dentate gyrus, a part of the brain believed to be important in the formation of new memories, in a rat model of diabetes (Hwang *et al.*, 2010). Furthermore, metformin was found to promote neurogenesis and improve spatial memory in normal (non-diabetic) mice (Wang *et al.*, 2012).

The risk for dementia was decreased in diabetic people treated with either metformin or sulfonylureas (Hsu *et al.*, 2011). In the Singapore Longitudinal Aging Study the long-term (>6 years) use of metformin was associated with a reduced risk for cognitive decline (Ng *et al.*, 2014). Moore *et al.* (2013) found that patients with type 2 diabetes that used metformin had worse cognitive performance than those managing their diabetes with other treatments. In an abstract presented at the AD/PD 2017 conference in Vienna researchers using data from the Taiwan's National Health Insurance research database with a 12-year follow up showed that metformin use in diabetic patients was linked to a higher risk for Parkinson's disease, Alzheimer's disease, vascular dementia, as well as all-cause dementia. In addition the risk for dementia and Parkinson's disease showed a dose- and duration-dependent effect (Kuan and Huang, 2017). However, metformin treatment was neuroprotective in a mice model of Parkinson's disease (Patil *et al.*, 2014). The human studies have all been conducted in diabetic people. So it remains possible that metformin negatively affects the risk for Alzheimer's disease by increasing amyloid-β levels but that in diabetes patients this is overshadowed by a larger reduction in the risk for Alzheimer's disease from the improvement in insulin sensitivity.

Alzheimer's disease has sometimes been called diabetes type 3 because of the finding that neurons in Alzheimer's disease show insulin resistance and that experimental induction of insulin resistance mimics multiple features of Alzheimer's disease (de la Monte and Wands, 2008; Kandimalla *et al.*, 2016). Metformin improved insulin sensitivity in a cell culture model of neuronal insulin resistance. Furthermore, this resulted in decreased tau hyperphosphorylation and reduced amyloid-β levels (Gupta *et al.*, 2011).

One hypothetical explanation for the increased risk of Alzheimer's disease in type 2 diabetics treated with metformin is that type 2 diabetes patients may also develop neuronal insulin resistance. As discussed above, metformin increases amyloid-β production in cultured neurons but this is abolished by the addition of insulin. Insulin resistant neurons no longer respond to insulin and hence metformin increases amyloid-β production. If this hypothesis proves right then metformin could potentially increase Alzheimer's disease risk in insulin resistant patients while lower it in normal people. Sadly no data exist on the incidence of Alzheimer's disease in non-diabetic metformin users.

As already discussed above, Zhou *et al.* (2016a) demonstrated that metformin treatment in mice protects against chemotherapy-induced cognitive impairment. Metformin also ameliorated the haloperidol-induced memory deficit (Adedeji *et al.*, 2014). Metformin improved learning behavior and memory in rats fed a high fat diet (Pintana *et al.*, 2012). However, in a study by McNeilly *et al.* (2012) metformin was ineffective in preventing a high fat diet-induced cognitive deficit in rats.

Beta-cell function and apoptosis

Exposure of β-cells to metformin for 24 h leads to a decrease in insulin production and secretion in response to a high glucose stimulus. Metformin in concentrations as low as 40 μM could induce β-cell apoptosis after long exposure. The number of apoptotic cells is time- and dose-dependent (Kefas *et al.*, 2004). These results are confirmed by Wang *et al.* (2011b) who observed that half of the cells cultured for 72 hours in metformin were unviable. Metformin was found to decrease the proliferation and promote apoptosis in MIN6 cells (Jiang *et al.*, 2014b). Hinke *et al.* (2007) found a concentration-dependent reduction in cell viability in metformin-exposed MIN6 cells. MIN6 are typically thought of as a pancreatic β-cell line but analysis shows that MIN6 cells also have features of other pancreatic endocrine cells (Nakashima *et al.*, 2009). The finding that metformin might induce β-cell death is strengthened by the finding that other AMPK activators (Kefas *et al.*, 2003; Cai *et al.*, 2007; Kim *et al.*, 2007) or genetic AMPK overexpression (Riboulet-Chavey *et al.*, 2008) also induce apoptosis in β-cells. However, Cai *et al.* (2007) and Kim *et al.* (2007) reported that AICAR-induced activation of AMPK caused apoptosis in part through an increase in ROS. While as was discussed above metformin seems to decrease ROS production.

In contrast, metformin protected against dysfunction and cell death in ER-stress induced apoptosis in a mouse β-cell line (Jung *et al.*, 2012). Metformin inhibited the opening of the permeability transition pore, a key event in mitochondrial-induced apoptosis, in permeabilized and intact rat pancreatic β-cell line (INS-1). Furthermore, metformin increased viability in INS-1 cells exposed to high glucose or fructose concentrations (Lablanche *et al.*, 2011). Metformin suppressed palmitic acid-induced apoptosis in MIN6 cells (Jiang *et al.*, 2014b) and in rat insulinoma cells (Simon-Szabó *et al.*, 2014). The protective effect of metformin against palmitic acid-induced apoptosis is strengthened by other studies that find that metformin restores insulin secretion in β-cells exposed to free fatty acids or high glucose (Patanè *et al.*, 2000; Piro *et al.*, 2012). Metformin also reduced ROS production in pancreatic β-cells stimulated by free fatty acids (Piro *et al.*, 2012). Also as discussed before metformin had protective effects, such as decreased ROS production (Hou *et al.*, 2010) and reduced lipid accumulation (Song *et al.*, 2010), in other cell types exposed to palmitic acid.

Finally, Chang *et al.* (2016) found no effect from metformin on cell death in rat insulinoma cells. Metformin also failed to protect against methylglyoxal-induced cell death.

Previous LongeCity article on metformin

I've previously published a metformin review on LongeCity:
http://www.longecity.org/forum/page/index.html/_/articles/metformin-a-life-extension-drug-r32

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