

Of mice and men: is there a future for metformin in the treatment of hepatic steatosis?

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Abstract:

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver diseases, of which the first stage is steatosis. It is one of the most common liver diseases in developed countries and there is a clear association between type 2 diabetes (T2DM) and NAFLD. It is estimated that 70% of people with T2DM have NAFLD and yet there is currently no licenced pharmacological agent to treat NAFLD. Whilst lifestyle modification may ameliorate liver fat, it is often difficult to achieve or sustain, thus there is great interest in pharmacological treatments for NAFLD. Metformin is the first line medication in the management of T2DM and evidence from animal and human studies has suggested that it may be useful in reducing liver fat via inhibition of lipogenesis and increased fatty acid oxidation. Findings from the majority of studies undertaken in rodent models clearly suggest that metformin may be a powerful therapeutic agent to specifically reduce liver fat accumulation; data from human studies is less convincing. In this review we discuss the evidence for the specific effects of metformin treatment on liver fat accumulation in animal and human studies and the underlying proposed mechanisms to try and understand and reconcile the difference in findings from rodent and human work.

Introduction

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver diseases including hepatic steatosis, non-alcoholic steatohepatitis (NASH) and cirrhosis, in those who do not consume significant amounts of alcohol ^[1, 2]; it is one of the most common liver diseases in developed countries ^[3, 4]. There is an association between type 2 diabetes (T2DM) and NAFLD, with one being predictive of the other ^[5]. Prevalence of NAFLD in people with T2DM is reported to be ~70% ^[6-8] although due to sensitivity of NAFLD quantification measurements it is likely the prevalence is underestimated. There is currently no licenced pharmacological agent for the treatment of NAFLD, therefore lifestyle changes are encouraged. Weight loss (5-10% body weight) is the current recommended treatment and is, when achieved, very effective with degree of steatosis being significantly reduced ^[9] and in some situations completely resolved ^[10].

A first line medication in the management of T2DM is metformin, there are now in excess of 20 million prescriptions a year ^[11]. Data from the UK Prospective Diabetes Study (UKPDS) demonstrated that metformin improved glucose control, reduced diabetes-related endpoints and mortality ^[12]. Given the clear benefits of metformin in individuals with T2DM and the link between insulin resistance/T2DM and NAFLD there have been several studies investigating whether any liver specific benefit can be derived from its use. In this review we discuss the specific effects of metformin treatment on liver fat content from studies undertaken using rodent, human and in vitro cellular models. We suggest reasons why findings between animals and humans are not consistent and try to delineate discrepancies between human studies to ask the question - is there a future for metformin in the treatment of hepatic steatosis?

How metformin may have therapeutic value in NAFLD

Metformin is an insulin sensitizer that results in lower blood glucose levels in patients with T2DM and impaired glucose tolerance (IGT); the potential mechanisms of metformin action have been extensively reviewed elsewhere ^[13]. Here we focus on the hepatic mechanisms by which metformin mediates its therapeutic effects in T2DM and highlight how these may be beneficial for the treatment of NAFLD and more specifically hepatic steatosis.

Traditionally, the liver is thought to be the primary site of metformin action ^[14, 15] where it exerts its anti-hyperglycemic effect through inhibition of gluconeogenesis ^[13, 18]. Although the mode of action for metformin is not fully understood, the long-established view is that metformin inhibits complex 1 of the mitochondrial respiratory chain leading to increased AMP:ATP ratios ^[19] and subsequent AMPK activation. This switches off energy consuming pathways such as gluconeogenesis and lipogenesis and increases fatty acid oxidation (FAO) ^[20]. Therefore it is plausible that metformin could be a therapy for NAFLD as increased FAO and decreased lipogenesis in the liver would lead to net loss in intrahepatic triacylglycerol (IHTAG) content. However, it has been shown that metformin exerts effects in an AMPK-independent manner; in AMPK-deficient hepatocytes metformin suppressed gluconeogenesis, via reduced expression of glucose-6-phosphatase, and reduced intracellular ATP concentration, making it unavailable for glucose production ^[19]. Moreover, it has been shown that metformin induces a mild energy stress leading to increased AMP levels (not direct AMPK activation) ^[20, 21] and this is responsible for metformin's glucose-lowering effects ^[22-24]. AMP inhibits a rate controlling enzyme of gluconeogenesis, fructose-1,6-bisphosphatase (FBP1), in mice and when FBP1 is rendered insensitive to AMP metformin's ability to reduce hyperglycemia is reduced ^[23]. Whether or not AMPK is critical for metformin's action does not negate the potential role for metformin in NAFLD treatment; as the energy stress

mediated by metformin that leads to increased AMP levels would potentially be sufficient to promote FAO and reduce lipogenesis thus reducing IHTAG.

Metformin's effect on IHTAG

Metformin has been shown in humans, animals and in cells *in vitro* to both reduce and maintain IHTAG content. In this section we summarise the key studies that have specifically investigated the effects of metformin on IHTAG content.

Evidence that metformin reduces IHTAG content:

Animal studies

In the majority of animal studies metformin treatment has been reported to decrease IHTAG content (Table 1). Lin *et al* were the first to show that in obese (*ob/ob*) mice, low dose metformin (350 µg/kg/d) for 4 weeks significantly reduced IHTAG content (from moderately severe to almost undetectable measured by histology) compared to untreated animals ^[32]. Similar findings in *ob/ob* mice have also been reported ^[32]. In the Lin study, metformin treated mice had a greater decrease in epididymal adipose tissue mass compared to control animals, which could not be explained by reduced calorie intake ^[32]. Therefore the effects of metformin on IHTAG cannot be excluded as being secondary to reductions in fat mass in this study.

Genetically obese and diabetic mice spontaneously develop hepatic steatosis ^[32, 36] but the majority of rodent models induced IHTAG accumulation through diet. A consistent finding from these studies is that metformin reduced, to varying degrees, IHTAG content (Table 1). For example, mice on a methionine- and choline-deficient plus high fat (MCD+HF) diet treated for 8 weeks with metformin (37.5 mg/kg/d), had a reduction in IHTAG from ~75% to

50%, compared to MCD+HF diet only mice ^[37]. Maslak et al reported that mice fed a high fat diet (HFD) in combination with a high dose of metformin (616 mg/kg/d) had a reduction in IHTAG content (~17 versus 7%) compared to mice fed only the HFD ^[38]. In HFD mice, metformin (310 mg/kg/d) reduced liver fat (from 34% to 20%) however, the mice treated had a ~11% reduction in body weight ^[39] which may contribute to the decrease in IHTAG content when metformin is given. As well as decreasing IHTAG, it has also been shown that metformin alters intra-hepatocellular lipid composition reducing diacylglycerol (DAG) and ceramide content and increasing unsaturated fatty acids within the livers of metformin treated mice compared to untreated mice ^[38] and in HFD + metformin treated rats ^[40].

Human studies

A consistent finding is that patients treated with metformin have modest weight loss (the current first line treatment for NAFLD) ^[31] and improvement in hepatic insulin sensitivity; however, evidence that metformin reduces IHTAG content in humans is inconsistent. Approximately half of the human studies report that metformin reduces IHTAG content in some or all subjects (Table 2). For example, a clinical trial comparing the effects of metformin against vitamin E in NAFLD patients demonstrated that the relative amount (%) of IHTAG before and after metformin treatment was significantly reduced (~40% before vs 20% after) in those who had follow up biopsies (31% of subjects) ^[32]. The reduction in IHTAG was not accompanied by changes in plasma lipid profiles ^[50]. When metformin was given in combination with dietary advice IHTAG was reduced to a greater extent than dietary advice alone, despite similar weight loss in both groups ^[51]. In other studies (where metformin was given for either 6 or 11 months) 25-30% of patients showed histological improvement in steatosis post treatment ^[34, 35]. Although IHTAG content was not directly measured, changes in liver volume (measured by ultrasound) combined with decreased

plasma ALT levels were indicative of decreased IHTAG ^[36]. In a larger study (63 patients with NAFLD) metformin treatment improved liver enzyme profiles: a surrogate marker of reduced IHTAG content ^[37].

Evidence that metformin maintains IHTAG content

Animal studies

To our knowledge only one rodent study has not shown a significant effect of metformin on IHTAG content. Ford and colleagues reported that in HFD mice, very high dose metformin alone (2.5 g/kg/d for 5 weeks) had no effect on IHTAG content (remaining at ~40%) ^[38].

However, when given in combination with salicylate there was a trend towards a decrease in IHTAG (P= 0.066 HFD versus HFD plus metformin and salicylate); and salicylate alone did not have any effect on IHTAG in vivo ^[38]. The authors concluded that the synergistic activation of AMPK induced by combination therapy exerts the beneficial effect on IHTAG content.

Human studies

Almost half of the studies in humans that investigate the effect of metformin on IHTAG show that metformin maintains IHTAG content (Table 2). A meta-analysis of 11 randomised controlled trials investigating the effect of metformin on NAFLD, found that despite improving surrogate markers of cardiovascular disease and T2DM (e.g. weight, waist circumference, HbA1c, HOMA-IR, HDL-cholesterol), metformin failed to improve liver histology including steatosis, inflammation and hepatocyte ballooning ^[39]. A meta-analysis of studies in NASH patients reported that, based on histological scores, metformin had no significant effect on hepatic steatosis ^[56].

In biopsy defined NAFLD, no significant changes in histological defined steatosis were observed in patients following 6 months treatment with metformin compared to placebo [57, 58]. Similarly in children with biopsy confirmed NAFLD, Lavine et al found that treatment with metformin (1 g/d 96 weeks) had no effect on IHTAG content or serum lipid profiles [53]. A pilot study undertaken to assess the effect of diet and exercise in combination with either metformin or placebo in patients with NASH but without T2DM, found no difference in hepatic steatosis in the diet and exercise plus either metformin or plus placebo groups [43]. Therefore, it is likely that the observed improvement in steatosis were due to lifestyle intervention alone.

Tiikkainen et al found no change in IHTAG content (13% to 14% before and after treatment measured by MRI) in 10 drug-naive patients with T2DM treated with metformin for 16 weeks at maximal clinical dose (2 g/d) despite a modest decrease in body weight (84 +/- 4 kg before vs. 82 +/- 4 kg after, $P < 0.05$) [60]. In a pilot study assessing the use of metformin as a treatment for NASH, it was found that although 48 weeks of metformin treatment significantly improved some histological features there was no significant change in IHTAG content measured by MRI (14.9% vs 13% pre and post metformin) [45]. Furthermore, when the authors divided patients into those who responded (31% patients) and those who did not (in terms of predefined histological criteria for NASH) there was still no difference in IHTAG content (-0.8% vs -0.2% responders vs non-responders) [45].

In vitro studies support both cases

In vitro studies tend to support the findings that metformin reduces IHTAG content. Using a 3D perfusion system Kostrzewski and colleagues showed that when cryopreserved human hepatocytes were cultured with free fatty acids (600 μ M, 14 days) and metformin (100 μ M, 7

days) there was a reduction in intracellular TG compared to untreated control cells; the metformin treated cells utilised less free fatty acids from cell culture medium ^[62, 63] which may indicate that glucose is preferentially taken into the cells. It has been shown that cryopreserved human hepatocytes display alterations in mitochondrial machinery especially at the level of complex I ^[61, 64-66] resulting in reduced oxygen consumption and mitochondrial respiration compared to fresh cells. As metformin may exert some of its effects through complex I and given the fact that mitochondria are critical for FAO, findings in cryopreserved hepatocytes must be taken with caution. For example treatment with metformin alone had no effect on lipogenesis in mouse primary hepatocytes whereas it significantly suppressed lipogenesis in cryopreserved human hepatocytes ^[38]. Work in HepG2 cells has shown that metformin treatment high dose (2 mM) for 24 hours decreased intracellular TG levels in an AMPK dependent manner ^[48, 49] under high glucose conditions (> 25 mM).

Mechanism by which metformin may affect IHTAG content

Effect on hepatic lipogenesis

Evidence that metformin suppresses hepatic lipogenesis

Animal and cell models have shown that metformin induced reduction in IHTAG content may occur, at least in part through reduced lipogenesis. This effect is probably dependent on AMPK as metformin has been shown to robustly inhibit lipogenesis in murine primary hepatocytes only when AMPK is present ^[50]. Ford et al indirectly measured de novo lipogenesis (DNL) through respiratory exchange ratio (RER) and found the RER was lower in metformin treated mice despite comparable degrees of adiposity to controls ^[69]; thus suggesting decreased DNL in animals treated with metformin. Many studies have shown that metformin decreases expression of lipogenic genes ^[23, 27, 29, 51] (Table 1). For example,

metformin is reported to decrease SREBP-1c expression, one of the master regulators of the DNL pathway, in HFD fed mice ^[29]. Additionally, in fructose fed mice, metformin treatment decreased the expression of the transcriptional regulators of DNL, and IHTAG content ^[52]. ACC1 inhibition by AMPK seems to be critical to this effect; when ACC1 is rendered constitutively active (and cannot be inhibited by AMPK) ^[43] mice spontaneously develop steatosis which cannot be reversed by treatment with metformin ^[71]. In the same study similar findings were observed in hepatocytes isolated from wild type or constitutively active ACC1 mice; these mice did not show improved insulin sensitivity following metformin treatment suggesting ACC inactivation by metformin is important for both the insulin-sensitising and lipid lowering effects of metformin ^[72].

Evidence that metformin channels glucose into lipogenesis

Despite strong evidence that metformin inhibits lipogenesis in an AMPK dependent manner, it may also increase glucose uptake into insulin sensitive tissues including the liver ^[54]. Increased uptake of glucose into the liver could be stored as glycogen or channelled into the DNL pathway. If channelled into the DNL pathway this may contribute to maintained IHTAG content observed in some studies. However, to our knowledge this has not been shown in animal models and has not been investigated in humans. However, glycogen synthase (GS), a key enzyme in glycogen production, has been reported to be impaired in skeletal muscle of obese T2DM humans ^[55]; metformin treatment had no effect on human skeletal muscle GS activity despite increased glucose disposal ^[55]. Activity of GS has also been shown to be impaired in the livers of HFD rats ^[75], thus it could be hypothesised that when glucose is taken into the liver following treatment with metformin, it is channelled into lipogenic rather than glycogen synthesis pathways, due to impaired GS activity.

Effect on fatty acid oxidation

Evidence that metformin increases FAO

In animal models, two studies have directly measured FAO in response to metformin treatment and reported increased FAO and decreased IHTAG content ^[33]. In primary rat hepatocytes treatment with 0.5 mM metformin for 4 hours increased FAO (using ¹⁴C-oleate) and suppressed lipogenic gene expression via AMPK activation ^[58]. More recent work has shown that metformin's ability to increase FAO (and inhibit lipogenesis) is enhanced when small molecule AMPK specific activators are used in combination with metformin in both rat and human primary hepatocytes ^[59]. This findings suggest that either 1) metformin does not robustly activate AMPK, or 2) metformin increases FAO independently of AMPK. Other studies have reported elevated gene expression of markers of FAO ^[41] and increased mitochondrial biogenesis in mice fed a HFD and treated with metformin ^[78] which would potentially lead to greater oxidative capacity. Metformin has also been shown to prevent HFD induced loss of PPAR-alpha expression in mice, which may be indicative of enhanced FAO ^[29]. Tokubuchi and colleagues investigated the effects of 2 weeks treatment with metformin on energy metabolism in healthy and T2DM subjects and concluded that metformin reduced fasting RQ ^[60] suggesting a shift in the utilisation of fat as a fuel following metformin treatment ^[60] which may be indicative of enhanced FAO. However, metformin also increased postprandial RQ in both healthy and T2DM groups, suggesting enhanced aerobic glycolysis and does not support enhance FAO via AMPK ^[60]

Evidence that metformin supresses FAO

Some *in vitro* and in human studies have reported that metformin does not increase FAO. Metformin treatment (0.5mM for 18 hours) had no effect on FAO in murine primary hepatocytes from either WT or constitutively active ACC knock-in mice ^[53] nor in mouse

primary hepatocytes ^[38] . Additionally, an acute high dose metformin (1 hour at 5 or 50 mM) treatment in rat primary hepatocytes had no effect on oxidation rates or fatty acid esterification ^[61]. Here, low dose 0.05-0.5 mM metformin treatment of rat hepatocytes for 24 hours has been shown to decrease expression of genes involved in FAO and ketogenesis ^[61] suggesting suppression of oxidative capacity by metformin. In line with this Perriello and colleagues found that metformin treatment suppressed whole body fat oxidation, measured by indirect calorimetry, compared to placebo group ^[62] in both obese and non-obese T2DM patients. Liver specific FAO is difficult to measure, a recent study utilised [¹¹C]palmitate and [¹⁴C]VLDL-TG in humans, to explore if metformin treatment improved fatty acid metabolism through the modulation of intrahepatic fatty acid partitioning ^[63]. They found no change in fasting hepatic fatty acid metabolism (measured by [¹¹C]palmitate PET) or in whole body FAO (using [¹⁴C]VLDL-TG) post-metformin treatment ^[63] suggesting that metformin does not promote enhanced FAO in humans. Due to the discrepancies observed between human studies it is possible that acute treatment with metformin may lead to an initial increase in FAO however; it appears that with longer-term treatment this effect is lost.

Potential mechanisms by which metformin reduces or maintains IHL content are summarised in Figure 1 (rodents) and Figure 2 (humans).

Other mechanisms by which metformin may affect hepatic lipid content

In addition to metformin's effects on lipogenesis and FAO other potentially mechanisms which may influence hepatic lipid content have been described. A study reported that in mice on a HFD, treatment with metformin reduced lipid droplet content and size and increased FGF-21 expression in the liver ^[64]. The authors concluded that enhanced FGF-21 was responsible for the loss of hepatic fat as incubation of HepG2 cells with metformin in the

presence of FGF-21 inhibition led to a retention of intra-hepatocellular lipid ^[64]. Additionally, using *ob/ob* mice and primary murine hepatocytes it was shown that metformin reduced intra-hepatocellular lipid content and reduced the expression of adipose differentiation-related protein (ADRP) ^[25]; over expression of ADRP in hepatocytes perturbed metformin's ability to reduce TAG accumulation ^[25]. Both these findings suggest that metformin may reduce hepatic lipid content in an AMPK-independent mechanism in animals and in vitro.

Why does the literature not agree?

It is clear from the preclinical literature that metformin may have beneficial effects on IHL content via enhanced FAO and inhibited lipogenesis however, the reasons why some human studies do not find an effect of metformin on hepatic steatosis need to be better understood in order for metformin to be used clinically in NAFLD. In order to shed light on this, this section focuses on the differences between human and animal studies and importantly tries to delineate the reasons for differences within the human studies.

Is it a case of dose?

Taking the cell, animal and human data together, findings are inconsistent and discrepancies may, in part, be explained by difference in metformin dose. For example, in humans treated with metformin, mean plasma concentrations are 2.7 mg/L or 20 μ M (range 0 – 800 μ M) with 94% of subjects having plasma levels under 10 mg/L (75 μ M) ^[65]. Whereas the majority of cellular studies have utilised supra-physiological concentrations ^[66] (from 0.5 mM to 50 mM). In rodent studies metformin doses range from 0.35 mg/kg/d up to 2.5 g/kg/d (Table 1) with the majority giving in excess of 60 mg/kg/d which would equate to > 4.5 g/d in for a human; the maximal human dose is 2 g per day (~26 mg/kg based on a 75 kg human) ^[65]. However, a dose of 2.5 g/kg/d (almost 10 times maximal dose in humans), did not result in

any significant effect on hepatic fat content in HFD mice ^[39]. The study by Lin et al is the lowest dose of metformin investigated, and equates to the maximal human dose ^[31], and they reported hepatic fat was significantly reduced; thus dose of metformin cannot completely explain the discrepancy in findings. All human studies used clinically relevant doses (1.5 – 2 g/d or 0.5 g/d long acting drug or 1 g/d in adolescents) and are therefore comparable to each other (Table 2).

Pharmokinetics of metformin: species variation

Differences in pharmacokinetics between humans and animals should be taken into account and may explain why apparently higher doses are utilised in animal models. OCT1 (*SCL22A1*) is the main transporter of metformin into the liver and the expression of hepatic OCT1 varies between humans and animals, with higher hepatic expression in humans ^[67] and high expression in rodent kidneys compared to humans ^[68] meaning that metformin may have altered pharmacokinetics in different species ^[69]. Numerous genetic variants of OCT1 have been described, with carriers having higher metformin plasma concentrations and reduced therapeutic effect ^[69]. As there is a large inter-individual variation in human hepatic OCT1 expression, this may go some way in explaining variations between human studies. This variation in OCT1 subtypes explains why the plasma levels of metformin in humans is difficult to assess and quantify and ranges widely. MATE1 (*SCL47A1*) is thought to be involved with hepatic excretion of metformin and is similarly expressed in mice ^[67, 68] however, in rats MATE1 is only expressed in the kidney. These variations in transporter expression between species as well as potential substrate specificity differences may explain some of the discrepancies between human and animal findings in regards to metformin effect on liver fat.

Does timing play a role?

Duration of treatment does not appear to be a factor in the ability of metformin to reduce liver fat content in rodent models as treatment times varied from a single dose^[35] up to 14 weeks^[44, 81] with the majority treating for 4-5 weeks (Table 1). In the human studies, duration of treatment also varied (between 12 - 96 weeks, Table 2) but does not correlate to outcome; 48 weeks of treatment two studies report no change in liver fat content^[54, 58] whilst one study reported a reduction^[34] (Table 2).

It has been established that the time of day that drugs are taken can cause differences in their therapeutic value; this is true for metformin administration in mice where kinetics of metformin induced AMPK activation are influenced by time of day despite no changes in metformin uptake into hepatocytes^[53, 54]. Metformin leads to alterations in circadian phase in the liver compared to muscle of mice^[74]. This effect of timing on drug kinetics and pharmacology may explain some discrepancies between humans and animals as rodents are nocturnal and humans' diurnal. Species differences in clock components have been established^[75] but it is also important to note that animal studies are normally carried out under constant lighting conditions (darkness or light) to eliminate possible 'masking' influences of light, circumventing the clock mechanism. Whereas in human studies the time of day (usually twice daily) that metformin is taken is not reported or controlled. This may go a long way to explain discrepancies between animal and human and within human studies. The importance of the circadian clock in the action of metformin and its therapeutic potential needs confirming and therefore human studies where time of day of metformin treatment is controlled may give important insight into whether or not it is an effective therapy in NAFLD in humans.

Hepatic fat quantification method in humans

The method used to quantify hepatic fat content may in part explain the discrepancies in findings between human studies. Although biopsy is the gold standard for assessing IHL content in humans it has a number of limitations: lack of follow up, heterogeneity of liver fat content ^[76], subjective interpretation and it is invasive, therefore the use of non-invasive methods are becoming more common including ultrasonography, computed tomography (CT), MRI, and MRS (reviewed ^[77]). Of these imaging modalities ultrasound appears to be utilised most often ^[33, 78-80] and report a reduction in liver fat content in the majority of subjects after metformin however ultrasound has limited sensitivity in individuals with high body mass index (BMI) (≥ 40 kg/m²) ^[77] and the current grading system is simplistic and therefore may not be suitable for evaluating patients with NAFLD after therapeutic intervention such as metformin. In contrast MRI/MRS offer direct quantification of liver fat content with higher accuracy ^[81]; the only studies that utilised MRI reported there was no change in liver fat content with metformin treatment ^[44, 45].

Cohort of humans used in the study

The cohort of participants used in a particular study may play a role, with some studying individuals with T2DM whilst others have studied obese or insulin resistant individuals (Table 2). The effect of metformin on non-diabetics may be different to diabetics and due to the diversity in participant phenotype across studies, factors including: duration since diagnosis, assessment of steatosis and use of other therapeutics may explain some of the variation in findings. Hyperglycemia has been shown to inhibit AMPK activity in rats ^[82] and HepG2 cells ^[48]; this may partially explain why studies in humans, report inconsistent results as fasting glucose will vary depending on exclusion and inclusion criteria of the study and the individuals' glycaemic control. Therefore the blood glucose concentrations of subjects may

impact on AMPK activity and in part, explain discrepancies between study findings.

Additionally, metformin has been shown to increase GLP-1 concentrations in the human gut^[83]. This may be important as GLP-1 levels have been shown to be progressively reduced as T2DM progresses^[84]; GLP-1 has been shown to reduce hepatic DNL via AMPK activation in rats^[85] but it remains unclear if this effect also occurs in humans. Therefore in subjects with established T2DM or poorly controlled T2DM, metformin may not promote GLP-1 secretion from the gut and thus not be able to reduce DNL, this may add to the discrepancies in human findings.

Finally, genetic variation in humans may also be a confounding factor: for example, polymorphisms in the metformin transporter OCT1 have been described (as discussed earlier), with the most common being rs628031 (A>G) which reduces OCT1 expression levels and may affect uptake of metformin into tissues^[86]. Additionally, presence of the PNPLA3 gene variant rs738409 (C>G) is associated with increased liver fat content in humans^[87] and may also act as a confounding factor in how metformin impacts on hepatic fat content and metabolism, metformin may be unable to reverse hepatic steatosis in carriers due to the variant being strongly associated with steatosis^[87].

Future potential of metformin for NAFLD

Co-treatment with other AMPK/T2DM drugs

There is growing evidence that combination therapy significantly improves metformin's anti-steatotic potential; co-treatment of primary hepatocytes with metformin and salicylate has been shown to exert synergistic activation of AMPK and suppression of lipogenesis in a dose dependent manner^[38] suggesting co-treatment with metformin and other AMPK activating drugs may be beneficial in NAFLD. Rosiglitazone (given alone or in combination with

metformin) was shown to be superior in histological improvement in NAFLD patients compared to metformin alone ^[72, 88] and significantly reduced IHTAG content (15% vs 7% pre vs post rosiglitazone) whilst metformin alone did not (13% vs 14% pre vs post metformin) ^[44]. In a recent study in patients already prescribed metformin (for at least 3 months) who still had steatosis (~15% liver fat measured by MRI), treatment with an additional diabetic therapeutic (liraglutide or sitagliptin) significantly reduced intrahepatic lipid content (from ~ 15% to 12%) and body weight (by ~2.5 kg) ^[89]. Evidence from HFD murine models also supports this as treatment alone with metformin has been shown to be less effective than when given in combination with telmisartan (angiotensin II receptor antagonist) and sitagliptin (second line drug of choice in T2DM) which reduced IHTAG content to levels observed in chow fed mice ^[29]. Altogether this suggests that metformin may be beneficial in NAFLD in order to treat or prevent T2DM and a conjunction therapy would be beneficial to promote or synergistically increase a reduction in IHL content.

Metformin effects on extrahepatic tissues

Altering gut microbiome

It is now accepted that glucose lowering properties of metformin may also be mediated by gastrointestinal effects ^[90-92]. Work in rodent models has demonstrated that infusion of metformin directly into the duodenum activates AMPK ^[90] and results in suppressed hepatic glucose output through a duodenal AMPK-neuronal pathway. Loss of duodenal AMPK negates metformin induced suppression in hepatic glucose production ^[90]. Suggesting that at least some of the therapeutic effects of metformin are mediated through a gut-liver axis. Recently the gut microbiome has been suggested to have a causative effect in NAFLD AS. Hepatic steatosis in mice has been shown to be transmittable through fecal transplant ^[93]. Most recently, metformin has been shown to alter gut microbiota in T2DM patients;

metformin promotes a shift back to a non-diabetic microbiotic profile ^[92] with enhanced butyrate and propionate production (both of which are able to regulate intestinal gluconeogenesis) ^[94, 95]. Additionally, the transfer of human faecal samples from metformin-treated individuals to germ free mice improves glucose tolerance ^[91]. This effect of metformin on gut microbiota supports its potential role in the treatment of NAFLD; preclinical studies have suggested a causal role of gut microbiota in NAFLD (reviewed in ^[96]) and there is a clear association between dysbiosis and NAFLD in humans. Additionally, progression of NAFLD in humans is associated with overall decrease in microbial diversity in the gut ^[96]. Therefore metformin could potentially reduce hepatic steatosis through gut-liver axis and additionally prevent progression of NAFLD to more serious liver disease.

Effects on BAT

Metformin uptake into murine brown adipose tissue (BAT) has been shown to be similar to that in liver ^[88]. In obese mice metformin has been reported to elevate the expression of BAT markers and inhibit white adipose tissue (WAT) differentiation via enhanced FGF-21 from the liver ^[44]. It could be hypothesised that if metformin activates BAT, increased energy expenditure might be beneficial to reduce fat content in peripheral tissues; whether metformin is able to directly activate BAT remains unclear. In the 1990s it was shown that treatment of mice (250mg/kg/d) ^[98] or rats (320mg/kg/d) ^[99] with metformin did not activate BAT and more recently in a human BAT *in vitro* cell model, metformin decreased BAT oxygen consumption ^[88].

Conclusion

In this review we asked the question if there is a future for metformin in the treatment of hepatic steatosis. In support of this, pre-clinical data in rodents suggests that metformin may

be a powerful therapeutic agent to specifically reduce IHL content; however it remains unclear whether metformin treatment would reduce IHL in humans. Recent collaborative guidelines from the European Association for the Study of the Liver (EASL), European Association for the Study of Diabetes (EASD) and European Association for the Study of Obesity (EASO) suggest that data is insufficient for evidence-based recommendations for the use of metformin to treat NAFLD ^[100]. However, as metformin may mediate a multitude of effects that could be beneficial in the treatment of NAFLD, including restoration of gut microbiota and increased BAT activity, further investigations into why metformin may reduce hepatic steatosis in some, but not all human studies, warrants further investigation.

Future human studies should include control of medication timing to account for variations in therapeutic effect controlled by circadian clock, larger cohorts, description and separation of genotypes and MRI quantification of IHL content. Moreover, the potential for metformin to work synergistically with other drugs, such as salicylate, or lifestyle interventions should be further investigated. Ideally, to fully understand the effect of metformin on hepatic lipid metabolism researchers should employ detailed investigations to better appreciate how metformin affects hepatic lipid metabolism. For example, if metformin increases hepatic glucose uptake, this glucose may be channelled into hepatic DNL pathways, as excess carbohydrate is a precursor for hepatic DNL ^[101]. Increased DNL would negate any increases in FAO and result in maintained liver fat content. Stable isotope tracers could be utilised to assess DNL in humans pre- and post-metformin treatment; to our knowledge this has not been investigated.

It is currently unclear whether other benefits of metformin treatment negate the potential lack of change in liver fat content in some individuals. For example, the potential use of

metformin in cancer treatment, including hepatocellular carcinoma (HCC) is of growing interest ^[101, 102]. Further work is required to understand and clarify the variability in metformin's effect on IHL content; elucidating this may lead to a more personalised approach, with effective strategies and therapies for the treatment of NAFLD. Is there a future for metformin in the treatment of hepatic steatosis? Potentially yes, if it can be understood why metformin reduces hepatic liver fat in some individuals but not others. It is likely that the use of metformin with another AMPK activating drug e.g. salicylate, could be a powerful tool to treat diabetes and NAFLD synergistically.

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Table 1. Summary of animal studies that have investigated the effects of metformin on hepatic steatosis

Animal model	Diet	Metformin dose	Effect on steatosis	Other hepatic lipid observations	Ref
Ob/Ob mice (C57BL-6)	Chow	350 ug/kg/d for 4 wk	↓ steatosis	↔ serum TG ↓ SREBP-1 and FAS expression	[32]
Ob/Ob mice (C57BL-6)	Chow	75mg /kg/d for 4 wk	↓ steatosis	↓ hepatic TG content ↓ hepatic ADRP expression ↓ serum TG ↓ serum LDL	[34]
Ob/Ob mice (C57BL-6)	Chow	300 mg/kg/d for 4 wk	↓ steatosis	↑ SIRT1 ↓ serum total cholesterol	[33]
Ob/Ob mice (C57BL-6)	Chow	50 or 100mg/kg/d for 4 wk	↓ steatosis	↓ serum TG ↑ AMPK expression	[103]
db/db mice (C57BL-Ksj)	chow	1 dose; high dose 400mg/kg vs low dose 50mg/kg	N / A	Altered hepatic FA and carbohydrate metabolism gene expression	[35]
C57BL/6 mice	HFD for 5 wk	2.5 g/kg for 5 wk	↔ steatosis	↓ RER	[40]

				↓ hepatic TG content	
C57BL/6 mice	HFD 12 wk	50 mg/kg/d for 6 wk	↓ steatosis	↓ hepatic DAG content ↓ lipogenesis	[41]
C57BL/6 mice	MCD + HFD for 8 wk	37.5mg/kg/d for 8 wk	↓ steatosis, inflammation and fibrosis	↔ serum TG ↑ serum total cholesterol ↓ lipogenic genes	[37]
C57BL/6J mice	HFD for 12 wk	150 mg/kg/d for 4 wk	↓ steatosis	↑p-AMPK ↓ FAS and ACC1 expression	[104]
C57BL/6 mice	HFD	10mg/kg/d or 50mg/kg/d for 14 wk	↓ steatosis	↓ lipogenic gene expression ↓ LDL and total cholesterol ↓ FFA ↔ serum TG	[44]
C57BL/6 mice	HFD for 10 wk	310 mg/kg/d 6 wk	↓hepatic steatosis	↑ beta-oxidation ↓ SREBP-1 expression ↑ number of hepatic mitochondria	[39]

C57BL/6 mice	HFD for 15 wk	616mg/kg in drinking water for 5 wk	↓ steatosis	↑ AMPK expression ↑ mitochondrial biogenesis ↓ plasma TG and total cholesterol ↓ hepatic TG/ DAG, ceramide content	[38]
C57BL/6 mice	HFD for 5 m	50 or 200mg/kg/d for 15 d	↓ steatosis	↓ hepatic TG content ↓ SREBP-1, FAS and ACC1 expression	[105]
C57BL/6 mice	HFD for 12 wk	300mg/kg/d for 10 d	↓ steatosis	↑ FA oxidation ↑ p-AMPK	[42]
C57BL/6 mice	HFD for 14 wk	200mg/kg/d for 14 wk	↓ steatosis	↓ plasma TG ↓ FAS and SCD expression	[81]
C57BL/6 mice	F for 18 wk	250mg/kg/d 8 wk	↓ steatosis	↓ SREBP-1, PPAR γ , FAT/CD36 expression ↑ PPAR α , PGC1 expression ↑ p-AMPK	[52]

Wistar rats	HFD for 8 wk	300mg/kg/d for 3 wk	N / A	↓ plasma FFA	[106]
				↓ DAG and	
				↑ mitochondrial markers	
				↑ p-AMPK	
Wistar rats	HFD for 2 wk	120 mg/kg/d for 2 wk	N / A	↓ Hepatic glycogen	[46]
				↓ plasma FFA on chow and HFD	
Sprague-Dawley rats	Chow	2.5 mg/kg/d for 2 wk	N / A	↑ FA oxidation gene expression	[43]
				↑ p-AMPK/ACC	
Goto–Kakizaki rats	HFD for 2-6 m	60 mg/kg/d for 4 wk	No steatosis	↓ FFA	[107]
			observed		
			↔ hepatic TG		
			content		

Abbreviations: wk, weeks; m, months, d, day; h, hours; HFD, high fat diet; MCD, Methionine Choline Deficient; FA, fatty acid; FFA, free fatty acid; TG, triglyceride; LDL, low density lipoprotein cholesterol; DNL; de novo lipogenesis; RER, respiratory exchange ratio; DAG, diacylglycerol; SCD, stearoyl-CoA desaturase; FAS, fatty acid synthase; ACC1, acetyl CoA carboxylase ; F, fructose.

Table 2. Summary of human studies investigation the effect of metformin on hepatic steatosis and lipid metabolism

Total subjects (F/M)	T2DM	BMI (kg/m ²)	% weight change	Age (y)	Metformin Dose	Duration treatment	Measurement of hepatic steatosis	Findings	Re f
11 (4/7)	Y	30.6 ± 1*	- 2.9	46 ± 4*	1g/d	16 wk	MRI/S	↔ hepatic steatosis ↓ body weight	[53]
9 (1/8)	N	32.2 ± 1†	- 2.8 ^a	50.2 ± 9.1†	0.5g/d (long- acting)	12 m	Biopsy	↔ hepatic steatosis (compared to placebo group)	[43]
26 (13/13)	N	33.9 ± 6.8†	- 5.7	44 ± 13.7†	2g/d	48 wk	Biopsy and MRI/S	↔ hepatic steatosis ↔ TG ↓ body weight Improved ALT	[45]
20 (4/16)	Y	31.4 ± 3.9†	- 4.4	44.3 ± 9.0†	2.5-3g/d	6 m	Biopsy	↔ hepatic steatosis ↓ body weight	[55]

								↓ serum cholesterol and LDL	
21 (10/11)	Y	23.8 ± 0.5 vs 30.9 ± 1.3* (n=7 vs n=14)	N / A	49 ± 3 vs 50 ± 2*	single 500mg dose	N / A	N / A	↓ whole body FAO (more robust in non-obese) ↓ FFA ↓ 3OHB	[69]
57 (10/47)	N	34 ± 5†	+ 13.6	13.1 ± 2.4†	1g/d	96 wk	Biopsy	↔ hepatic steatosis ↔ liver histology	[56]
49 (27/21)	N	31.2 ± 3.6†	- 2.9	47.9 ± 8.3†	0.85g/d	48 wk	Biopsy	↔ hepatic steatosis ↓ body weight	[58]
55 (15/40)	N	28.7 ± 3.6†	- 7.3 ^a	42 ± 10†	2g/d	12 m	Biopsy	↓ hepatic steatosis ↓ severity of steatosis	[60]
15 (6/9)	N	25–44)‡	- 1.1	51 ± 12†	0.02g/kg/d	48 wk	Biopsy	↓ hepatic steatosis (in 30% of patients)	[34]

17 (6/11)	N	30.1 ± 3.4†	- 8.0 ^a	39.8 ± 10.6†	0.85g/d	6 m	Ultrasound	↓ hepatic steatosis ↓ severity of steatosis	[78]
20 (18/2)	N	36.5 ± 4.9†	- 7.8	40.8 ± 13†	1g/d	6 m	Ultrasound	↓ hepatic steatosis (25% patients) ↓ body weight	[61]
19 (15/4)	Y	31.0 ± 4.0*	- 7.1 ^a	49.4 ± 8.6*	1.7g/d	6 m	Biopsy	↓ severity of steatosis ↓LDL ↓ALT	[63]
32 (15/17)	Y	32.6 ± 5.8*	N / A	51.9 ± 10.9*	1.7g/d	4 m	N / A	↓ plasma TG ↑ HDL	[37]
12 (6/6)	Y	30.3 ± 5.7†	- 0.5	64 ± 5†	2g/d	3 m	N / A	↔ body weight ↔ FAO	[63]
29 (10/19)	Y	26.8 ± 0.7*	- 4.8	46.3 ± 2.3*	2g/d	24 wk	Ultrasound	↓ hepatic steatosis	[65]
87 (0/87)	Y	28.9 (25.4- 32.8)‡	N / A	57.5 (40- 69)‡			Liver/spleen ratio CT	↓ liver/spleen ratio	[108]]

21			- 7.6						
(0/21)	N	37.2 ± 3.4†		15-19\$	1g/d	6-12 m	Ultrasound	↓ hepatic steatosis	[80]

Data presented as: *mean±SEM; †mean±SD; ‡ median (range) \$range; ^aBMI % change.

Abbreviations: wk, weeks; m, months, d, day; n / A, not assessed; FAO, fatty acid oxidation; TG, triglyceride; LDL, plasma low density lipoprotein cholesterol; HDL, plasma high density lipoprotein cholesterol; ALP; plasma alanine aminotransferase.

Metformin: AMPK dependent mechanism

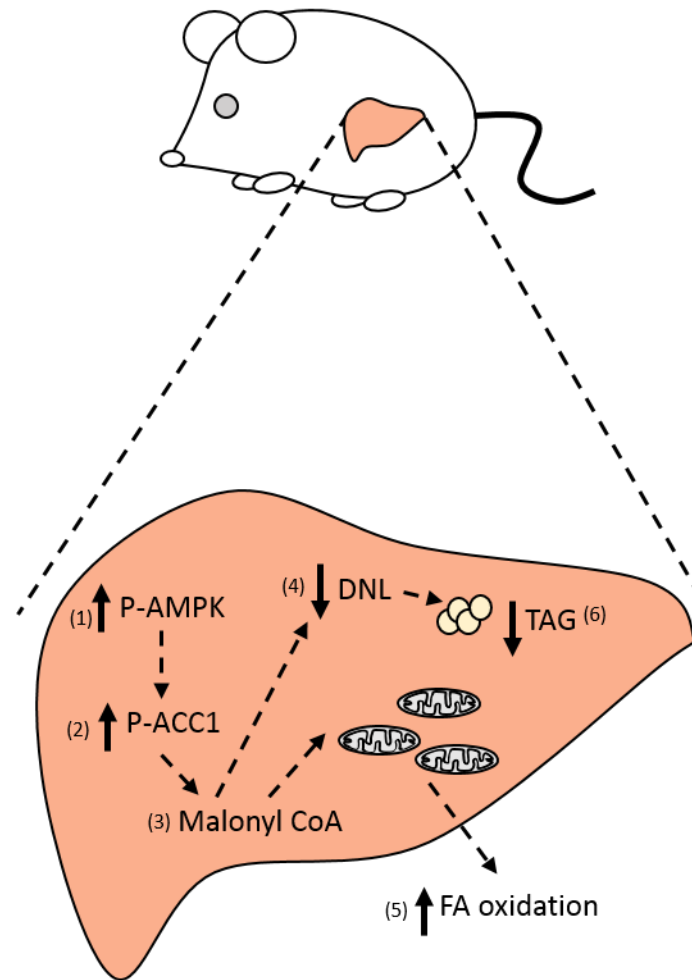


Figure 1: Overview summary of the most established mechanism by which metformin reduces liver fat content in rodents. Briefly, metformin treatment increases phosphorylation of AMPK (1) leading to increased phosphorylation of ACC1 (2) and subsequent decrease in malonyl-CoA (3). Decreased malonyl-CoA inhibits DNL (4) and increases fatty acid entry into the mitochondria via CPT1 and subsequent increased fatty acid oxidation (5). The overall effect is net loss in TAG content within the liver (6).

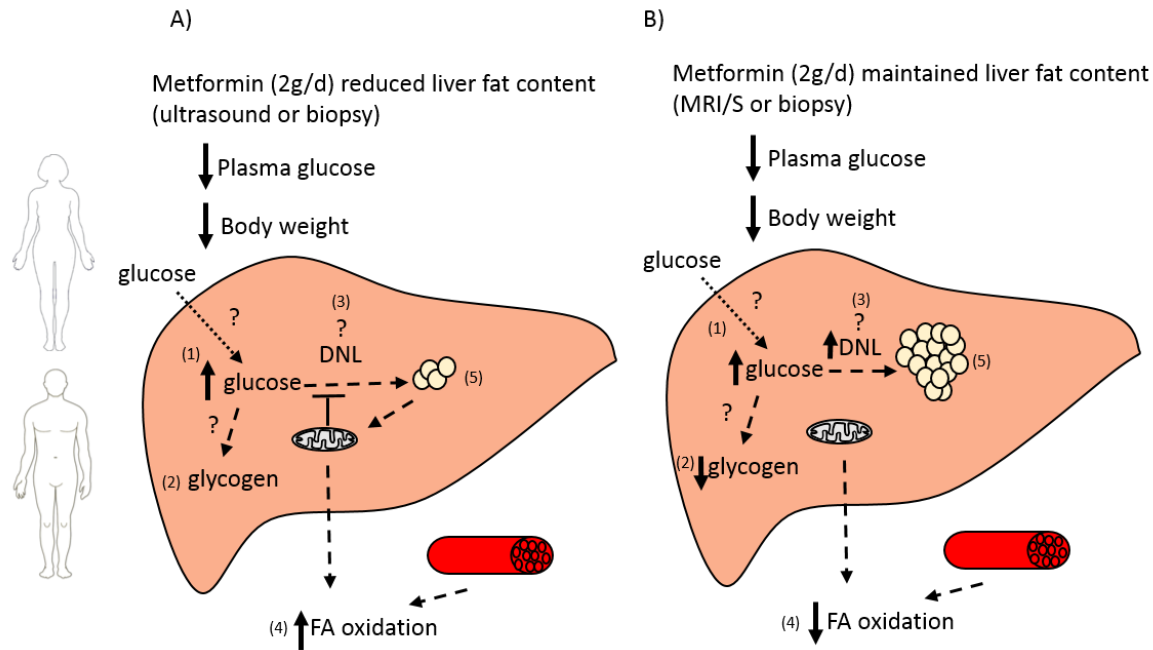


Figure 2: Overview summary of proposed potential mechanisms by which metformin A) reduces and/or B) maintains liver fat content in humans. A) Metformin treatment reduces plasma glucose and reduces body weight. Excess glucose may be taken up by the liver (1) and channelled into either glycogen (2) or DNL (3) however through increased whole body fatty acid oxidation (4) (potentially via AMPK activity) liver fat content is reduced (5). B) Metformin treatment reduces plasma glucose and reduces body weight. Excess glucose may be taken up by the liver (1) and channelled into either glycogen (2) or DNL (3) however whole body fatty acid oxidation is not increased (4). DNL is an energetically costly pathway and therefore the net effect is maintenance of hepatic lipid content as TAG rather than increased hepatic TAG (5).