Review

Rethinking the Paradoxical Increase in Endogenous Glucose Production in Response to Metformin: Should We Retain the Glucagon Antagonistic Effect of the Drug?

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Abstract: Metformin, the treatment of first choice in type 2 diabetes (T2D), is known to mainly act by decreasing endogenous glucose production (EGP) in the liver. Paradoxically, in the last decade several reports documented increased EGP after metformin treatment. This increase, was often attributed to pronounced rises in glucagon, consistent with counter-regulatory response to the glucose lowering effect of metformin. However, considering that hyperglucagonemia, but not hypoinsulinemia, is a main driver of EGP in T2D, increased EGP should have been a common finding. This observation, together with the finding that metformin antagonizes glucagon effects on energy expenditure and protein synthesis, concurrently to its effects on EGP and the emerging evidences demonstrating increased branched chain and gluconeogenic amino acids in response to metformin treatment may points to a liver alpha-cell skeletal muscle cross talk similar to that observed in the liver-alpha cell axis. Here, we provide a mechanistic perspective to this latter possibility, based on mechanistic studies of metformin's transcriptional targets; retaining thus its glucagon antagonistic and presumably anti-gluconeogenic effects on liver and attributing the increase in EGP to renal gluconeogenesis. We finally discuss how increased EGP might reflect an adverse response to metformin treatment, providing support from clinical and epidemiological data.

Abbreviations:

T2D; Type 2 diabetes
EGP; Endogenous glucose production
AMPK; AMP-activated protein kinase
AAs; Amino acids
BCAAs; Branched chain amino acids
CREB; cAMP-responsive element-binding protein regulated transcription coactivator 2 CRTC2; CREB-regulated transcription coactivator 2

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Introduction

Metformin is a biguanide derivative that was initially synthesized from galegine; an isoprenyl guanidine derived from the plant French lilac (Galega officinalis) [1]. Years later, metformin and other biguanides were first synthesized, but their glucose lowering effects were overlooked until the seminal work by Sterne [2] who introduced metformin for the first time to treat T2D individuals. At that time, although little was known about the mechanisms of its action, metformin was deemed as an inhibitor of endogenous glucose production (EGP) based mainly on studies of other biguanides, particularly phenformin, and on the recurrent observation of increased gluconeogenic precursors (lactate, alanine and glycerol) which suggested inhibition of gluconeogenesis [3]. Later studies confirmed these premises with the introduction of clamp-based approaches and showed that metformin, indeed, inhibits EGP; thus, improving hepatic insulin resistance, and increases peripheral and whole-body glucose uptake [4, 5].

Another landmark in metformin's mechanisms of action was the establishment that the activation of AMP-activated protein kinase (AMPK) underlies its inhibitory effect on EGP [6]. This finding led to a proliferation of studies giving the liver a central role in the glucose lowering effects of metformin [7-9]. Although other mechanisms have been proposed for metformin since its early days, the inhibition of EGP received most of the interest.

However, recent studies challenged this paradigm by demonstrating that metformin, in contrast, may increase EGP in T2D patients [10]. In fact, the latter study is not the first study showing such findings. Christensen, Højlund [11] previously showed that metformin potentiates EGP in healthy individuals after 42 hours of fasting. Konopka, Esponda [12] also showed that metformin may increase EGP in prediabetic individuals. In all of these studies the increase in EGP was accompanied by an increase in glucagon levels.

Considering that increased EGP and hyperglucagonemia are key features of T2D, the abovementioned findings could be interpreted as adverse responses to metformin treatment or alternatively could be considered as a counter-regulatory response. Although most of these studies opted for the counter-regulatory response, there is evidence to suggest that metformin still counteract glucagon effects [12]. In fact, proofs that metformin may counteract glucagon effects came from the study that demonstrated concomitantly an increase in EGP [12]. Surprisingly, this occurred in concomitance to increased gluconeogenic amino acids (AAs) levels, which may suggest a liver-alpha cell crosstalk [12, 13].

How an antihyperglycaemic drug that is supposed to curtail liver glucose production may, in contrast, result in the opposite and why this occurs in concomitance of a differential effect on fasting blood glucose in healthy versus well controlled T2D individuals? How metformin can counteract some effects of glucagon and potentiate others? In this perspective we offer a critical appraisal of the literature of metformin's effects on EGP. We provide, in light of the emerging evidences of metformin on protein metabolism, a hypothetical scenario to the paradoxical metformin-induced increase in EGP and its glucagon antagonistic effects. We finally discuss how this increase in EGP might reflect an adverse response.

The Metformin-EGP paradox: an adverse effect to metformin or a counterregulatory adjustment?

Until recently, the liver was central to the glucose lowering effects of metformin. Specifically, metformin was mostly known as an inhibitor of liver glucose production or as is commonly called EGP [14]. Zhou, Myers [6] were the first to establish that AMPK underlies metformin's inhibitory effects on EGP. However, these findings were challenged by Foretz's group, who demonstrated that in liver specific AMPK knockout mice, metformin still reduce EGP; where a strong correlation was found between decreased gluconeogenesis and [AMP]:[ATP] ratio, suggesting an AMPK-independent role for the decrease in

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cellular energy status seen in this process [15]. Subsequent studies confirmed the AMPK-independent mechanisms and established that metformin, through AMP-related mechanisms, could inhibit adenylate cyclase resulting in a marked reduction in cyclic AMP and thus could interfere with glucagon signaling and gluconeogenesis [16, 17]. One other study by the group of shulman suggested that metformin may alter EGP by inhibiting the mitochondrial glycerol-3-phosphate dehydrogenase, thus interfering with cytosolic redox state and resulting in augmented cytosolic NADH/NAD+ ratio and impaired gluconeogenesis from redox-dependent substrates (i.e. lactate and glycerol) in rats [18, 19]. Altogether, these findings indicate that metformin could lower EGP by independent or combined mechanisms that involve alterations of key gluconeogenic enzymes, glucagon antagonism and/or alteration of gluconeogenesis from redox-related substrates.

However, in the last decade a number of studies have challenged this paradigm. Christensen, Højlund [11] were the first to demonstrate that metformin could increase EGP in healthy humans. In this study, investigators wanted to test the extent to which metformin could block EGP under extreme counter-regulatory condition. To do so, they subjected healthy individuals to a 60-h glycogen depleting fasting and noticed that metformin under such condition potentiates EGP. Of note, although this study is the first to pay attention to the fact that metformin could increase EGP in humans, it is important to highlight that such increase can be traced back to 1989 when Penicaud, Hitier [20] showed in fa/fa insulin resistant rats that metformin could increase EGP. However, these findings did not receive much attention given that decreased EGP by biguanides prevailed at that time. The second study that paid attention to the fact that metformin could increase EGP was the study of Konopka, Esponda [12]. The authors extended their findings to postabsorptive state. They first showed that EGP levels were unaltered after a 2-week period of metformin treatment in prediabetics. However, it was only when subjects were individually inspected that authors noticed a heterogeneous pattern of response in a subgroup of 3 subjects. In this subgroup, EGP was increased in concomitance to increased glucagon levels and this accounted for the absence of overall change in EGP. The authors followed by a somatostatin clamp-based approach to investigate interaction between hyperglucagonemia and metformin and found that metformin potentiates glucagon-induced stimulation of EGP [12].

Gormsen, Sondergaard [10] further extended his findings to healthy and well controlled T2D individuals, where they showed that metformin increased EGP and that this increase is accompanied by 18-30 % increase in glucagon levels. This is surprising, because as mentioned earlier one of the mechanisms underlying metformin's ability to decrease EGP is glucagon antagonism [16]. Considering that increased EGP levels and hyperglucagonemia are key features of T2D [21], these findings could be interpreted as adverse responses to metformin treatment and thus one would expect glycemic control to worsen.

This, however, was not the case; where the elegant design of Gormsen, Sondergaard [10] enabled the dissociation of increased EGP and glucagon levels from worsened glycemic control. In this study, paradoxically, fasting blood glucose decreased in T2D patients whereas it was unaltered in healthy individuals [10]. In response to gormsen's article, McCreight, Mari [22] published a research letter confirming increased EGP in healthy individuals without any notable effects on glucose and insulin levels. Importantly, in both these studies there was a concurrent corresponding increase in non-insulin mediated glucose uptake in the basal state. Gormsen, Sondergaard [10] also reported an increase in insulin mediated glucose uptake as evidenced by increased glucose disposal under hyperinsulinemic euglycemic clamp.

Blood glucose levels has been well defined in normal subjects and generally show very narrow range of variation [23]. The maintenance of this steady state is dependent upon net balance between EGP and glucose removal from systemic circulation. This implies that any change in glucose delivery will be compensated by a reciprocal change in glucose removal and vice versa so that plasma glucose stays relatively stable [23]. Therefore, under relatively normal glycemic levels in the above-mentioned studies, it tempting

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to speculate that the increase in glucose uptake would be compensated by a comparable counter-regulatory increase in EGP to prevent hypoglycemia. However, this cannot be firmly assumed taking into accounts limitations inherent to whole-body measurement of glucose kinetics that may fail to adequately describe tissue-specific glucose dynamics [24].

Tracing glucose kinetics from the available substrate flux studies: connecting the dots toward an integrative understanding of metformin's effects on EGP

Identification of the exact pathway of glucose utilization after metformin treatment is critical for integrative understanding of its mechanisms of action. As mentioned earlier, the first report of an increase in EGP following metformin treatment dates back to 1989 [20]. This study, although too old, it provides interesting insights on how metformin may increase EGP. The authors of this study reported in genetically modified *fa/fa* rats that metformin increases EGP and that this increase was secondary to increased glucose cycling [20]. Three years later, Bailey, Wilcock [25] reported that metformin decreases basal glucose and increases lactate concentrations in hepatic portal vein but not in hepatic vein, a finding consistent with increased intestinal glucose utilization and increased lactate extraction by the liver. Intriguingly, in the postprandial state, lactate concentrations in hepatic vein exceeded those in hepatic portal vein, which suggested at that time that hepatic lactate extraction and consequently glucose cycling were blunted in postprandial state. These findings were partially reproduced in a more recent study; where authors subjected mice to a high fat diet and found that addition of metformin to high fat diet blunted or at least attenuated the deleterious effect of this diet on glycemic and weight control [26]. The authors also reported increased lactate concentrations and reduced pH levels in portal vein but not in peripheral veins suggesting increased intestinal glycolytic glucose utilization and increased glucose cycling [26]. Similar to Penicaud, Hitier [20], authors followed by a stable isotope investigation to trace glucose kinetics; where they reported increased doubly labeled glucose-1,6-^{13}C after oral ingestion of mono-labeled glucose-1-^{13}C. Importantly, these findings suggest breakdown of mono-labeled glucose-1-^{13}C to lactate and its conversion back to glucose-1,6-^{13}C in the aldolase reaction, consistent with increased glucose cycling. However, the findings from this study do not appear to support blunted postprandial glucose cycling [26].

An important observation from the two latter studies is that animal models were non diabetic, which similar to the above-reported human studies, may suggest that increased EGP could reflect a counterregulatory response to metformin's glucose lowering effects [10]. Moreover, similar Gormsen, Sondergaard [10] and McCreight, Mari [22], Penicaud, Hitier [20] also reported that metformin increases non-insulin mediated glucose uptake. It is important to note in this regard that increased non-insulin-mediated glucose uptake may reflect increased intestinal extraction and utilization of glucose from the systemic pool. Although some studies including the study of Gormsen, Sondergaard [10] reported increased insulin mediated glucose uptake during hyperinsulinemic euglycemic clamp, a non-insulin contribution to insulin-mediated glucose uptake could not be excluded [27]. In agreement with this view, Penicaud, Hitier [20] in *fa/fa* rats demonstrated that glucose uptake was not affected in insulin dependent tissues and was even decreased in skeletal muscle tissue under euglycemic levels, consistent with an increased uptake of glucose by non-insulin sensitive tissues. The only notable significant increase in glucose uptake was seen in intestines. Similarly, intestinal glucose uptake accounted for most of glucose uptake under hyperglycemic conditions in this study [20].

At first glance, one may outweigh the view that the increased EGP could reflect a counter-regulatory response to the glucose lowering effects of metformin and that the hepato-centric and anti-gluconeogenic effects of metformin could be overridden by the hypoglycemic stress. However, hyperglucagonemia is often observed in T2D and prediabetic patients and glucagon levels as high as those observed in the study of Gormsen, Sondergaard [10] and Konopka, Esponda [12] have been reported in T2D and prediabetics [28-30]. Further, hyperglucagonemia, but not hypoinsulinemia, was shown to be the main

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driver of EGP [31]. Therefore, if hyperglucagonemia could override metformin's anti-gluconeogenic and glucagon antagonistic effects, why would not the increase in EGP be a common finding in metformin studies? Quite intriguing, also, is the finding that metformin concurrently to its promoting effects on glucagon-induced increase in EGP, antagonized glucagon-induced increase in energy expenditure and decrease in protein synthesis. With the two latter effects being most probably exerted in the liver [32]. Finally, the decrease in basal glucose uptake in Penicaud, Hitier [20] study raises the question as to whether such a decrease may reflect increased insulin resistance and thus could be responsible for increased branched chain amino acids (BCAAs) observed after metformin treatment in some studies [33]. Understanding how metformin could concurrently promote some of glucagon's effects and antagonize others and if this may involve tissue-specific adjustments in insulin sensitivity is of extreme importance because it may not only reveal other drug targets but also may help in early identification of responders to treatment.

Reinterpreting increased EGP in light of emerging metformin's effects on protein metabolism: Should we hold the counter-regulatory premise?

A key puzzling observation from Konopka, Esponda [12] is that metformin antagonized some of the catabolic effects of glucagon including glucagon-induced increase in energy expenditure and decrease in protein synthesis. Although, measured at whole-body level, these attenuating effects of metformin may reflect its effects on the liver. This is because skeletal muscle lacks glucagon receptor and glucagon-induced decrease in protein synthesis has been attributed to substrate limitations due to increased liver AAs consumption [31]. Moreover, the fact that under metformin treatment, increased EGP coincided with increased glucagon and gluconeogenic AAs levels including the glucagontropic AA precursor, alanine, raises several questions and may open the door to new interpretations. hyperalaninemia has also been reported elsewhere after metformin treatment [34]. If, as suggested by Konopka, Esponda [12]; metformin increases EGP by increasing AAs incorporation into gluconeogenic pathways, would not be more realistic to observe a decrease in gluconeogenic AAs concentrations? Of course, one may argue that this increase might have exceeded liver capacity to extract alanine. But considering that liver glucose production from alanine reaches saturation at concentrations 20-30 times that of physiological level which is far beyond gluconeogenesis from any other AA makes this possibility unlikely [35]. Consequently, one might not completely rule out a possible antigluconeogenic or glucagon antagonistic effects of metformin on glucagon signaling. Although increased EGP would suggest the inverse, there are some instances during which EGP increases under glucagon antagonism/resistance. These instances are commonly referred to as the concept of liver alpha-cell axis.

BCAAs are elevated in T2D [36]. Metabolomic profiling studies revealed a signature of altered BCAAs catabolism in obese individuals that is strongly associated with insulin resistance [37]. Similar to alanine, metformin has been reported to increase BCAAs particularly leucine and isoleucine [33]. Although it did not appear to have an effect on total BCAAs concentration in the study of Konopka, Esponda [12], metformin significantly increased isoleucine and numerically increased leucine. The latter being increased to a similar extent to the increase seen Walford, Davis [33] study, consistent with increased type II error. Other studies, reported no effects of metformin and pioglilitazone bi-therapy on BCAAs [38]. The lack of effect in the last study is suggestive of a blunting effect of metformin on BCAAs [38], given that pioglitazone has previously been shown to decrease BCAAs concentrations in patients with nonalcoholic steatohepatitis [39]. These findings are somehow surprising and unexpected, given that metformin is an insulin sensitizer and metformin induced-improvement in insulin sensitivity was expected to translate to decreased BCAAs levels. However, despite increased whole-body insulin sensitivity, an increase in BCAAs was observed in these studies. It is important to highlight in this regard

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that improvement in whole-body insulin sensitivity may fail to reflect tissue-specific insulin sensitivity [24]. Further, as stressed earlier, whole-body insulin sensitivity as measured during hyperinsulinemic euglycemic clamp or surrogate markers may be confounded by non-insulin stimulated glucose uptake that could contribute to insulin stimulated glucose uptake [27, 40, 41]. Whether this increase occur in insulin sensitive tissues or in non-insulin sensitive tissues remains unexplored. The study of Penicaud, Hitier [20] gave a glimpse into what tissues might contribute to this increase in non-insulin mediated glucose uptake by demonstrating in *fa/fa* rats a decrease in basal skeletal muscle glucose uptake and a 200% increase of intestinal glucose uptake under hyperglycemic conditions, but this needs further confirmation in humans. Specifically, whether the decrease in basal skeletal muscle glucose uptake is the result of increased insulin resistance or to the decrease in fasting glycemia and insulinemia, consistent with a mass-action effect worth further investigation. In relation to protein metabolism, both insulin resistance and insulin deficiency could translate at the skeletal muscle level to increased BCAAs catabolism and thus elevated plasma concentrations of BCAAs [42, 43]. Glucagon also may profoundly increase BCAAs concentrations [43]. Whether these effects of metformin on protein metabolism are isolated insignificant observations or adverse responses to the treatment or alternatively represent a specific metabolic signature of metformin worth further investigations.

A scenario whereby metformin may affect protein metabolism through a liver alpha-cell cross talk

According to the concept of the liver alpha-cell axis, lipid or pharmacological-induced glucagon antagonism may in a feedback loop stimulate glucagon secretion [13]. Because glucagon stimulates AA-induced urea formation, its antagonism may decrease urea formation from AAs and thus increasing AAs concentrations in the blood [13]. AAs in turn in a feedback loop may increase glucagon secretion to compensate for the degree of glucagon antagonism/resistance. Of course, we are aware that in the study of Konopka a decrease in AAs other than gluconeogenic AAs occurred, but in the absence of a direct evaluation of gluconeogenic AAs flux, it is difficult to distinguish whether this decrease was the result of increased incorporation of AAs into gluconeogenic pathways or as suggested by the liver-alpha cell axis concept was the result of a compensatory increase in glucagon [13].

Quantitively, alanine alone accounts for more than 50 % of liver AA uptake [35]. Therefore, if the decrease seen in certain AAs resulted from their incorporation into gluconeogenic pathways, alanine would be the most expected AA to fall. Also of importance, it seems that alanine is the most important driver of the liver-alpha cell axis and was even integrated together with glucagon into an index to predict abnormalities affecting this axis [13, 44]. This, together with the growing body of evidences showing that metformin may increase glucagon-stimulated EGP [10, 12, 22] and that this effect may depend on AAs [12] may suggest that metformin mediates a liver alpha-cell cross talk. Accordingly, we propose that metformin may antagonize glucagon action by a mechanism analogous to that observed in the liver-alpha-cell axis (Fig.1). If our premise is correct, then there should be some mechanistic support for this premise.

Metformin may promote a liver alpha-cell cycle – Transcriptional support

cAMP-responsive element-binding protein (CREB)-regulated transcription coactivator 2 (CRTC2) is an important glucagon-inducible gluconeogenic transcription factor. Metformin may antagonize glucagon signaling through AMPK-mediated disassembly of CRTC2 transcriptional complex [45]. In a similar fashion to metformin's differential effects on glucose control in healthy versus T2D individuals, Erion, Kotas [46] showed that knock down of CRTC2, resulted in a differential effect on EGP and glycemic control in a rat model of extreme hyperglycemia and another one of mild hyperglycemia – profoundly

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improving EGP and glucose levels in the former but not in the latter. Surprisingly, the less profound effects on EGP and glucose tolerance were the result of a compensatory increase in glucagon, a pattern mirroring that observed in the study of Konopka, Esponda [12] and that seen in the liver-alpha cell axis. Erion, Kotas [46] wanted to test the relative contribution of CRTC2 to the regulation of hepatic gluconeogenesis given the prevailing idea that it is a critical component of the gluconeogenic transcriptional response characterizing fasting and T2D. Specifically, CRTC2 dephosphorylation in response to glucagon and chronic hyperglycemia was thought to underlie the increase in EGP by modulating CREB targeting of prototypical gluconeogenenic genes including glucose-6-phosphatase (*G6pase*) and phosphenolpyruvate carboxikinase *(PEPCK)* [46]. Intriguingly, while the expression of these genes was substantially reduced in the extreme hyperglycemia rat model of CRTC2 knock down, it was unaltered in normal and presumably in the mild hyperglycemia rat model of CRTC2 knock down. The authors found rather a reduced expression of genes that promote AA shuttling into catabolic pathways, joining the clinical studies pointing to an effect of metformin on protein metabolism [12, 33, 38]. Together with the observed increase in glucagon, these findings may point to the feedback loop of AAs-stimulated glucagon secretion suggested by liver-alpha cell axis. To address the hypothesis – that increased glucagon may be blunting the improvement of EGP and thus glucose tolerance, authors treated rats with somatostatin or a glucagon-neutralizing antibody and they showed in both cases that EGP and glucose tolerance was ameliorated, thus providing direct evidence that it is, indeed, the compensatory increase in glucagon that was responsible for the blunted glycemic ameliorations. Although was not dedicated to investigate the liver-alpha cell axis, this study points to a possible liver-alpha cell crosstalk similar to that described by the liver alpha-cell axis concept.

What remains problematic in this study is the paradoxical improvement in EGP and glycemic control despite increased glucagon levels in the extreme hyperglycemia rat model of CRTC2 Knock down. This finding overlaps with the current state of knowledge with regard to metformin's action– that metformin may increase EGP as it can do the inverse depending on the degree of glycemic control, especially that metformin has been reported to promote AMPK-mediated disassembly of CRTC2 transcriptional complex [45, 47]. What still to be determined, though, is whether the differential expression profiles of G6Pase and PEPCK mediates the differential effect of CRTC2 knock down and presumably to metformin on EGP and glucose tolerance in the extreme and mild glycemia rat models. With regard to metformin, there is data showing that metformin continues to reduce EGP even when G6Pase and PEPCK are forced to express [15]. Further, the glycemic control was unaltered despite decreased G6pase and PEPCK gene expression in CRTC2 knockout mice models [48]. Similarly, expression profiles of G6pase and PEPCK correlated poorly with increased rates of EGP in T2D humans and mice livers [49], even though inhibition of CRTC2 could correct the defect [50]. Combined with findings from Erion, Kotas [46] these findings may suggest the contribution of other gene targets, and particularly genes involved in the AAs metabolism, to the increased EGP levels in T2D and to metformin response. The increased alanine levels in response to metformin treatment in the study of Konopka, Esponda [12] perfectly matches the decreased expression profile of alanine aminotransferase in the study of Erion, Kotas [46] and thus not only suggest a role of CRTC2 in the mechanism of action of metformin but also implicate AAs in this process (Fig.1).

It should be noted, however, that physiological relevance of metformin's transcriptional repression of gluconeogenic gene expression; particularly metformin's-induced phosphorylation and nuclear exclusion of CRTC2 through the LKB-1-AMPK pathway has been challenged by the study of Foretz, Hebrard [15], who demonstrated that metformin still suppress glucose production in AMPK knockout mice and AMPK and LKB1 knock out primary hepatocytes and this occurs in the absence of CRTC2 phosphorylation. However, the acute nature (glucose challenge 30 minutes after metformin administration) and the mode of administration (oral vs intraperitoneal injection) may account for the

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discrepancies between the study of Foretz, Hebrard [15], Shaw, Lamia [45] and He, Sabet [47].

An extrahepatic contribution to metformin's-induced increase in EGP

The compensatory increase in glucagon after CRTC2 Knock down in both rat models in light of the differential effect on blood glucose could also point to a superior glucagon resistance in the extreme hyperglycemia rat model that may have conditioned the decrease in EGP and glucose levels in this model but not in the model of mild hyperglycemia, although the authors suggested that increased glucagon is relatively unimportant in the setting of hypercorticosteronemia and very low insulinemia characterizing diabetic zucker rat model. But if metformin acts by antagonizing glucagon action, how would then EGP increase? Although studies investigating the liver-alpha cell axis dissociated glucagon effects on AAs metabolism from its effects on EGP; indicating that the effects of glucagon on hepatic glucose production persist and are independent from its effect on AAs, it is important to note that EGP as measured during classical hyperinsulinemic euglycemic clamp studies is assumed to reflect liver glucose delivery to the systemic circulation, yet non-hepatic tissues, such renal tissue, may also contribute to EGP [23, 51].

A Renal contribution to metformin's-induced increase in EGP

Renal gluconeogenesis, classically considered insignificant due to technical limitations, has gained momentum in T2D [51, 52]. Recent studies demonstrated that there is a hepatorenal reciprocity according to which a decrease in glucose release by one organ is compensated by an increased release from the other organ [51, 52]. Several examples of renal compensation were documented in the literature among which animal models of liver failure and patients undergoing liver transplantation in whom hypoglycemia is generally uncommon thanks to a compensatory increase in renal glucose production. Therefore, although EGP seems to be not affected under glucagon resistance or antagonism one may not firmly assume that glucagon-mediated hepatic glucose production is preserved in the absence of direct investigations of the relative contribution of renal gluconeogenesis to increased EGP under such conditions.

An extrahepatic contribution to EGP was also suggested by the study of Le Lay, Tuteja [48]. In this study the authors generated a liver specific CRTC2 knock out mouse model to study the contribution of CRTC2 to the early fasting response. Surprisingly, while glucose metabolism was not affected in vivo, isolation of CRTC2$^{-/-}$ hepatocytes and their culture revealed decreased glucose production compared to control hepatocytes upon stimulation by glucagon. Although, other hormonal cues may come at play and stimulate liver glucose production at the whole-body level, these findings could also suggest compensatory mechanisms by other organs, mainly the kidneys.

This postulation may, however, contradict with the findings of Konopka, in that glucagon is the main driver of the increase seen in EGP, and consequently liver is the main player in such increase given that glucagon does not appear to have an effect on kidneys. However, isolation of glucagon effect by somatostatin infusion may affect other glucoregulatory hormones including insulin. Thus, a caveat toward the generalization of conclusions of this study is that the observed effects of metformin on EGP may not be solely explained by the increase in glucagon. Indeed, evidences of increased renal compensation exists in conditions characterized by insulinopenia like prolonged fasting and in mice with liver-specific knock-down of pyruvate carboxylase [53]. In the latter study, similar to Le Lay, Tuteja [48] study mice exhibited a normal in vivo EGP and glucose profiles at the whole-body level. However, when liver was isolated and perfused hepatic glucose production appeared to be subject of substantial decrease that was consistent with a decreased expression of *G6pase* and *PEPCK* [53]. Fasting glucagon/insulin ratio was also elevated in this study. While the study of Le Lay, Tuteja [48] did not examine renal gluconeogenesis, Cappel, Deja [53] established that the relatively normal EGP in the setting of

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hepatic gluconeogenesis suppression was compensated for by renal gluconeogenesis as evidenced by elevated M3 enrichment in most tricarboxylic acid cycle intermediates, suggesting increased utilization of the anaplerotic pathway for gluconeogenesis in the post-absorptive state [53].

Could hyperglucagonemia underly an adverse response to metformin treatment?

Although at this point it is too early to talk about an adverse response, it is important to highlight that the response to metformin is heritable up till 34% [54] . Moreover, although the increase in EGP coincided with improvements in both fasting and postprandial glycemic profiles in some studies [10, 12], there is evidence to suggest that metformin could adversely increase postprandial blood glucose by 40% [55]. In this latter study, although the increase in glucose was not accompanied by a significant rise in glucagon levels, authors did not completely exclude this possibility because glucagon levels were subject to high interindividual variability [56], just like the study of Konopka, Esponda [12]. Similarly, Pilmark showed that metformin, indeed, may increase basal EGP and that its combination with exercise seems also to increase postprandial EGP. Importantly, this increase in postprandial EGP in response to metformin and exercise seems to limit the magnitude of the decrease in postprandial glucose. Moreover, in a recent study metformin was also demonstrated to result in a non-significant 47 % increase in postprandial glucagon levels in T2D individuals, but this coincided with improved glucose concentration compared to when placebo was administrated [57]. In this study, the glucose lowering effects of metformin seemed to be mediated by GLP-1 because when GLP-1 signaling was antagonized glucose levels rose and this coincided with a very important rise in glucagon, suggesting that there might be a certain threshold for glucagon to be able to stimulate EGP just as suggested by Konopka, Esponda [12]. Therefore, it seems that the increase seen in GLP-1 modulates the effect of metformin on glucagon secretion and consequently on EGP. It is important in this context to highlight that Liang, Xu [58] et al showed that adjustment for basal GLP-1 levels removed the statistical significance of the association between the rs2289669 of the SLC47A1 gene; encoding for the multidrug and toxin extrusion 1, a transporter involved in the hepatic and renal excretion of metformin, and enhanced glycemic response. Altogether these findings may implicate GLP-1 and glucagon in interindividual response variation to metformin.

Conclusion

The observation that metformin antagonizes glucagon effects on protein synthesis and energy expenditure concurrently to increased gluconeogenic AAs strongly questions the growing number of reports indicating that pronounced counter-regulatory response may overcome metformin's antigluconeogenic and glucagon antagonistic effects on the liver and thus may increase EGP. Mechanistic studies of one of metformin's gluconeogenic transcriptional targets, CRTC2, strongly implicates protein metabolism in the mechanism of action of metformin in the liver, joining thus clinical studies in humans. All together these observations in addition to the emerging evidences indicating that metformin may increase BCAAs concentrations, may suggest a liver alpha-cell cross talk similar to that observed in the liver alpha-cell axis in which the increase in glucagon could compensate for the degree of glucagon antagonism in the liver. Although studies investigating the liver-alpha cell axis hold the view that the gluconeogenic pathway is not influenced in this axis, the absence of studies assessing renal gluconeogenesis strongly limit these claims, which may suggest that the increase in EGP could arise from renal gluconeogenesis. Whether these effects of metformin on protein metabolism are isolated insignificant observations or adverse responses to the treatment or alternatively represent a specific metabolic signature of metformin worth further investigations. Future studies should consider an integrative design involving the study of regional and whole-body protein and gluconeogenic substrate dynamics using multiple tracer approaches to comprehensively

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enlighten the relative contribution of different gluconeogenic substrate to gluconeogenesis.

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Author contribution statement

J.M proposed the topic, drafted the manuscript, T.A, A.E.H, I.L and M.Z helped in drafting and manuscript revision. A.B, A.O and E.B contributed to the discussion and revised all the aspect of the manuscript. All authors read and approved this final version of manuscript.

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Figure caption

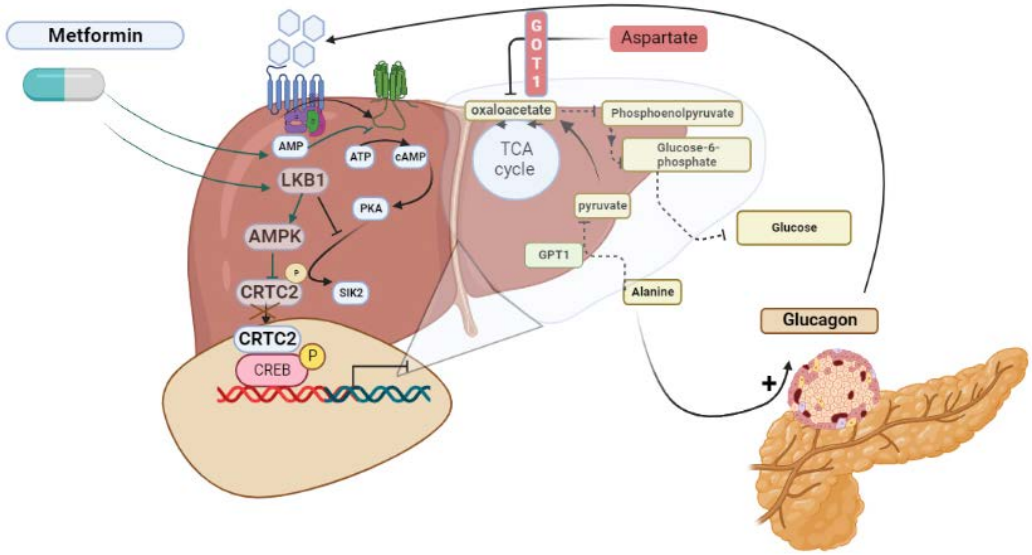

Figure 1. Glucagon antagonizing effects of metformin may drive a liver alpha-cell cross talk. Glucagon binds to glucagon receptor and promotes production of cAMP. cAMP activates PKA, which phosphorylates and thus inhibits the salt inducible kinase 2 (SIK2) and dephosphorylates and thus promotes the nuclear translocation of CRTC2. CRTC2, then forms a complex with CREB and promotes gluconeogenic gene transcription. Metformin can antagonize glucagon action both allosterically; through increased level of AMP which allosterically inhibits Adenylyl cyclase, and transcriptionally by promoting phosphorylation and thus nuclear exclusion of CRTC2 through AMPK and LKB1. LKB1 may also promote CRTC2 phosphorylation by a SIK2 dependent mechanisms. Nuclear exclusion of CRTC2 will inhibit gluconeogenic gene expression including genes involved in protein catabolism (GOT1, GPT1, etc…) which may increase concentrations of gluconeogenic amino acids, including alanine. Alanine may drive a liver alpha-cell crosstalk by promoting glucagon secretion to compensate for the degree of glucagon antagonism.