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Review

Protection by metformin against severe Covid-19: An in-depth mechanistic analysis

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SUMMARY

Since the outbreak of Covid-19, several observational studies on diabetes and Covid-19 have reported a favourable association between metformin and Covid-19-related outcomes in patients with type 2 diabetes mellitus (T2DM). This is not surprising since metformin affects many of the pathophysiological mechanisms implicated in SARS-CoV-2 immune response, systemic spread and sequelae. A comparison of the multifactorial pathophysiological mechanisms of Covid-19 progression with metformin's well-known pleiotropic properties suggests that the treatment of patients with this drug might be particularly beneficial. Indeed, metformin could alleviate the cytokine storm, diminish virus entry into cells, protect against microvascular damage as well as prevent secondary fibrosis. Although our in-depth analysis covers many potential metformin mechanisms of action, we want to highlight more particularly its unique microcirculatory protective effects since worsening of Covid-19 disease clearly appears as largely due to severe defects in the structure and functioning of microvessels. Overall, these observations confirm that metformin is a unique, pleiotropic drug that targets many of Covid-19's pathophysiology processes in a diabetes-independent manner.

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Introduction

Since the start of the coronavirus disease 2019 (Covid-19) pandemic, the impact of various widely prescribed drugs (such as renin-angiotensin system blockers and statins [1]) on Covid-19-related outcomes has been closely scrutinized. The antidiabetic agent metformin (MET) deserves particular attention in this respect because type 2 diabetes mellitus (T2DM) is one of the main comorbidities associated with the severity of Covid-19 [2], and most current guidelines recommend MET as the first-line drug treatment for T2DM [3]. Indeed, MET is a pleiotropic drug with beneficial effects that go far beyond its impact on blood glucose homeostasis [4]. This pleiotropic profile makes MET a good drug candidate for attenuating the severity of Covid-19 [5]. Indeed, several observational studies of patients with diabetes have found an association between MET treatment and better COVID-related outcomes [6].

The "metformin hypothesis" is supported by the large body of literature data on MET's ability to reduce inflammation and infection as well as its unique favourable effects on the microcirculation [7]. Here,

we review a range of mechanisms that might explain how MET could improve outcomes for patients with Covid-19.

A rational set of protective mechanisms

The complexity of the pathophysiology of Covid-19 suggests that its treatment should be multifaceted. With this regard, it has been suggested that cationic drugs are more likely to be effective in treating Covid-19 [8]. As such, MET – probably the most pleiotropic drug known [9] – might therefore be a good candidate for treating Covid-19, especially given the current trend towards repurposing drugs [10]. Indeed, besides its antihyperglycaemic effects (first reported in the late 1920s), MET reappeared in 1949 as an anti-influenza agent named flumamine [11], before being launched in 1957 as a treatment for T2DM. Over the last few decades, observations of MET's worldwide use have progressively revealed the drug's numerous effects in a broad range of disease settings [12].

Pleiotropic protective effects of metformin

A growing body of preclinical and clinical data has highlighted MET benefits in nephropathy [13], cancer prevention and/or

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treatment [14], neurodegenerative diseases [15], ageing [16], infections, lung fibrosis, polycystic ovary syndrome (PCOS), and many more.

MET and infections

As mentioned earlier, MET was first marketed as a drug for influenza. In obese mice with influenza, MET increases the survival rate [17]. There are also reports of MET's activity against hepatitis B virus [18], hepatitis C virus [19], and Zika virus [20]. For dengue, MET showed good efficacy in patients with diabetes [21]. Effects against prions have also been suggested [22]. A recent publication described MET's inhibition of virus-host interactions in human papillomavirus-positive cancer cells [23]. In people with diabetes, MET use was associated with a significantly reduced risk of infections compared to insulin or sulphonylureas [24]. It was recently suggested that biguanides could be administered by inhalation in the treatment of influenza and possibly Covid-19 [25] and that MET might even be repositioned as an antimicrobial. Over the last decade, many studies have shown that MET influences the gut microbiota and suggested that this is one of the major mechanisms of action for its beneficial effect on "meta-inflammation" and associated metabolic disorders [26]. MET also shows anti-*Plasmodium* activity [27].

MET, the immune response, and inflammation

The initial immune response to infecting pathogens is characterised by the acute production of pro-inflammatory molecules by Th17 T cells, M1 macrophages, and neutrophils, possibly leading to what has been termed a "cytokine storm" [28]. This pro-inflammatory phase is followed by healing processes mediated by regulatory T cells (Tregs) and a switch in the macrophage phenotype from M1 to M2. This step is critical for recovery and might be modulated by MET.

Interference with the cytokine storm might be one mechanism by which MET alleviates the severity of Covid-19. During the past years MET effects on inflammation have been the subject of many reports. Indeed, MET decreases cytokine levels in vascular cells [29], experimental myocarditis [30], sepsis [31], gut inflammation [32], interleukin (IL)-10 deficient mice [33], lipopolysaccharide (LPS)-stimulated mouse colon cells [34], metabolic syndrome induced by fructose [35], PCOS [36], and animals or patients with diabetes [37]. Interestingly, these effects of MET are independent of its antihyperglycaemic action [38].

The NLRP3 inflammasome (which is overactivated in Covid-19) is largely responsible for the cytokine storm [39]. MET reduced the NLRP3 inflammasome's activity in various settings: subjects with obesity and T2DM [40], diabetic cardiomyopathy [41], and myocardial injury [42], as well as periodontitis and LPS-induced lung injury in rodents [43]. MET also blocked the NLRP3 inflammasome and IL-1 β secretion in keratinocytes suggesting possible protection against psoriasis [44]. MET also reduces CD4 T cell counts, increases CD8 T cell counts [45], and binds high mobility group box 1 (HMGB1) [46]. In rodents with LPS-induced sepsis, MET reduced infiltration by neutrophils and macrophages [47].

IL-6 is considered to be the main cytokine involved in Covid-19 pathogenesis, although this has been challenged: some studies found that the IL-6 levels in Covid-19 were similar to (or even lower than) those in other forms of acute respiratory distress syndrome (ARDS) [48]. A Covid-specific cytokine profile (with elevated levels of IL-1 receptor antagonist and IL-8) has been linked to higher mortality [49]. It is noteworthy that MET reduced the secretion of IL-6 and IL-1 β by macrophages primed with the Covid-19 spike protein [50].

Several reports indicate that MET reduces the number of pro-inflammatory Th17 cells and increases the number of Tregs. This effect has been reported in autoimmune insulinitis [51], liver submitted to ischaemia/reperfusion, liver transplantation [52], experimental arthritis [53], autoimmune encephalomyelitis [54], and experimental

multiple sclerosis [55]. MET also increased the number of Tregs in obese individuals with asthma [56].

Monocytes and macrophages (MPs) are part of the front line in immune defence and are strongly involved in combating infection by severe ARDS [57]. High MP counts are indeed found in the lung alveoli [58]. MPs can broadly be divided into M1 (pro-inflammatory) and M2 (anti-inflammatory) phenotypes. Oxidative phosphorylation is blunted in M1 MPs, which prevents the switch to M2 [59]. Many studies have shown that MET stimulates the M1 to M2 phenotype switch. In obese mice, MET reduced the number of M1 MPs and the level of monocyte chemoattractant protein-1 in adipose tissue and increased the M2:M1 ratio [60]. Expression of M2-like genes was promoted by MET in MPs from hyperlipidaemic rats [61]. In the context of ischaemia, MET increased M2 polarisation in microglia [62]. In the bone marrow, MET increased M2 count and reduced osteolysis [63]. In skin, topical treatment with MET favoured wound healing by reducing NLRP3 activity and increasing the MP2 count [64]. In another study, MET reduced tumour progression and angiogenesis by increasing the M2 switch [65]. These data evidence MET's ability to correct the balance between M1 and M2 MPs and thereby reduce the severity of inflammation.

Inflammation is accompanied by oxidative stress, which has an important role in Covid-19 [66]. There is a large body of evidence on MET's antioxidant effects in hyperglycaemia [67], advanced glycation end-product (AGE)-induced injury [68], the response to palmitic acid [69], and LPS-activated MPs in vitro [70] and in vivo; in the latter setting, MET was found to act at the mitochondrial level. A recent study demonstrated that MET activated the transcription factor fork head box O3 (FOXO3) and thus reduced the level of oxidative stress in immune cells [71]. MET inhibited the proliferation of pancreatic cancer cells by reducing the level of oxidative stress – notably by inhibiting NADPH oxidase 4 (NOX4) [72]. Another suspected source of oxidative stress in Covid-19 is serum iron load, due to interaction between the virus and haemoglobin; this leads to Fe³⁺ accumulation and hyperferritinaemia [73]. MET's ability to bind iron might provide an additional means of limiting oxidative stress in Covid-19 [74].

MET, virus entry, and virus fate

Unfortunately, it is not known whether or not MET modifies the viral load in patients with Covid-19. However, a MET-associated reduction in the severity or progression of the disease is suggested by the association between metformin treatment on admission and more favourable Covid-19 outcomes. The uptake of SARS-CoV-2 by cells occurs mainly through angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2). Studies of ACE2's organ distribution have highlighted the lung and gut as particularly receptor-rich areas [75]. Moreover, the vascular endothelium can be affected by SARS-CoV-2, leading to endothelialitis. This effect appears to be due directly to the viral spike protein per se [76].

It has been suggested that by activating AMP-activated protein kinase (AMPK), MET causes ACE2 to be phosphorylated, leading to a conformational change that might prevent SARS-CoV-2 from binding to the receptor [77]. In vitro, MET increases the mRNA expression of ACE2 and TMPRSS2 in human hepatocytes [78]. Lastly, the level of ACE2 activity is modulated by the local microbiome [79]. In view of MET's well-known effects on the composition of the microbiota, this might constitute another means of action for the drug [80].

A large proportion of Covid-19 patients presents gastrointestinal symptoms because ACE2 and TMPRSS3 are strongly expressed in the intestine, which is a potential site of virus replication [81]. Therefore, the intestinal microbiota might play a key role in Covid-19, and the persistence of dysbiosis in survivors is common [82]. It is well known that MET interacts with intestinal microbiota and reduces intestinal inflammation [83], as illustrated by its effects on the IL-18 level.

Lastly, MET might dampen a viral attack by acting on proteases. If the virus is to fuse with cell membrane and inject RNA into cells, the

SARS-CoV-2 spike protein must be cleaved into two subunits. The spike protein has several possible sites for cleavage by cysteinyl proteases or cathepsins [84] (particularly cathepsin L). Studies with MET and its complex with Zn have evidenced inhibitory effects on cysteinyl proteases and cathepsin [85].

MET, lung pathology and fibrosis

Several animal studies have shown that MET diminishes the lung injury induced by LPS administration. MET relieved ARDS [86] and reduced TLR4 signalling and permeability across the pulmonary endothelium [87]. In rabbits submitted to high-pressure ventilation, lung injury was reduced by MET [88]. In humans, MET had contrasting effects on mortality in patients with chronic obstructive pulmonary disease [89] and non-Covid-19 ARDS [90]. Moreover, Oh and Song published the results of two population-based cohort studies from South Korea: in their first study [91], pre-treatment with MET did not reduce hospital mortality in ARDS patients, but in the second study, decreased the risk of developing Covid-19 by 30% [92]. An important question is therefore whether or not MET's putative beneficial effects on lung injury are due to MET treatment prior to hospital admission and/or to MET maintenance during the hospital stay. Several lines of evidence suggest that MET pre-treatment has a critical role: *i*) MET reduces tuberculosis in patients with diabetes; *ii*) ongoing use of MET versus other antidiabetic agents was associated with a duration-dependant 15% reduction in the risk of severe COPD exacerbations [93]; *iii*) in a recent cohort study, long-term MET use was associated with a lower risk of pneumonia and pneumonia-related death [94]; and *iv*) it is noteworthy that MET use was linked to a lower computed tomography score in Covid-19 patients [95]. The relevance of these observations to Covid-19 should, however, be considered with caution because SARS-CoV-2 spreads throughout the body in a particularly rapid aggravation phase. Indeed, albeit in a rather short series, in patients with diabetes hospitalised for Covid-19 and categorised into "continuation of metformin throughout the hospital stay", "discontinuation of metformin on admission", and "no metformin" groups, the beneficial association between Covid-19 outcomes and metformin treatment was related to the drug's continuation during the hospital stay, rather than previous exposure [96].

With regard to the time course of MET administration, it is critically important to note that lung fibrosis can start as early as 2–3 weeks after the SARS-CoV-2 infection. This is a consequence of intensive inflammation [97] and is therefore not unexpected – particularly in severe cases. MET's antifibrotic effects have only been described recently but have been observed in various cell systems and tissues. In lung fibroblasts, MET reduced collagen synthesis through AMPK activation [98]. In lung tissue, MET reduced bleomycin-induced fibrosis [99]. In radiation-induced pneumonitis, MET decreased levels of fibrosis markers such as inflammatory cell infiltration, oedema, alveolar thickening, and collagen deposition [100]. With regard to the kidney, MET was found to reduce interstitial fibrosis in cyclosporin A-induced fibrosis [101]. Fibrosis in adipose tissue after doxorubicin treatment [102] was reduced by MET in insulin-resistant rodents and in obese animals [103]. These effects have recently been reviewed [104].

MET and the reduction in mortality

Many *in vitro* and animal studies have shown that MET protects against mortality or cell death in various disease situations (reviewed in [105] or following exposure to toxins [106]). An impressive example is the spectacular survival of animals submitted to haemorrhagic shock and treated with MET upon reperfusion [107]. In humans, MET has been associated with a decrease of all-cause mortality and major adverse cardiovascular events in people with pathologies or conditions as diverse as diabetes [108,109], patients in the ICU [110], perioperative patients [111], sepsis [112], stroke [113], heart failure

[114], acute coronary syndrome [115], and chronic kidney disease [116]. Importantly, MET's beneficial effect was independent of the degree of prevailing hyperlactataemia in critically ill patients [117].

Putative mechanisms underlying MET's protective effects

Effects on the microvasculature

In this chapter, we will consider successively aspects relative to COVID-infection then to MET's effects.

Clinical research has started to show that with increasing duration and severity, Covid-19 becomes a predominantly vascular disease with generalised endotheliopathy [118]. The microcirculation is an important but often unappreciated player in early pathological vascular processes [119]. Microvascular dysfunction is evident in reactivity tests and is linked to the cytokine storm [120].

A growing body of evidence reflects the unexpected extent and intensity of microvascular lesions throughout the body. Evidence for endothelialitis, hyperpermeability, and disturbed haemostasis is increasingly reported [121]. Evidently, the fact that subjects with diabetes, arterial hypertension or obesity are more prone to more severe Covid infection is not a coincidence since these diseases are well known to impair (micro)vascular function. Several experts have suggested that SARS-CoV-2 replicates in endothelial cells; this replication might be further promoted if the endothelium has already been damaged [122]. The virus spike protein's disorganising effects on the microvasculature were reviewed recently [123]. Interestingly, autopsy material indeed showed early lesions in capillaries and microthrombi in arterioles; as well as some damage to larger vessels [124]. In cardiac autopsy tissue, high levels of ACE2 and TMPRSS2 expression were found in capillaries but not in larger vessels [125]. This finding indicates that SARS-CoV-2 infection strongly affects processes that are specific to small vessels [126]. It is particularly noteworthy that one study observed the persistence of vascular dysfunction in patients convalescing from Covid-19 [127] – possibly due to the persistence of ACE2 activity in plasma [128] and a lasting reduction in capillary volume [129].

MET's effects: Over the past 30 years, the results of many animal and clinical studies have highlighted the exquisite effects of MET on microvessels. A particular feature of MET is that it has a greater effect on microvessels (arterioles and capillaries) than on large vessels [130]. Preservation of microvascular responsiveness, inhibition of capillary permeability and of leucocyte adhesion are amongst the hallmark properties of MET, which largely explain the drug's long-term effects on diabetic complications and other ischaemia-related diseases [7].

Microvascular blood flow and pericytes

Microflow distribution is regulated by precapillary "sphincters" and scattered capillary pericytes (corresponding to smooth muscle cells in large vessels) that generate a cyclic flow motion in capillary beds [131]. Intravital microscopy reveals so-called "vasomotion", which regulates the opening and closing of microvascular units so that the available blood is used as efficiently as possible, adapting supply to meet local metabolic needs. This phenomenon is highly specific to microvessels and has a key role in tissue health.

Pericytes (or their podocyte equivalents in kidneys) are contractile cells that regulate microvessel haemodynamics and are closely connected to endothelial cells. Pericyte or podocyte loss leads to uncontrolled endothelial damage and results in angiogenesis and retinopathy or nephropathy. The involvement of pericytes in viral pathologies is increasingly recognised: loss of pericytes is indeed observed in Covid-19 [132], and pericyte disruption by the virus has been shown in heart [133] and in neurological complications of Covid-19 [134]. Although the presence of pericytes in lung microvessels is subject to debate [135], it is important to note that the SARS-

CoV-2 receptor ACE2 is mainly located on pericytes [136] and pericytes are also subjected to fibrin deposition [137]. If the presence of pericytes is confirmed, it could mean that pericytes (rather than the endothelium) might be the virus's first point of attack in systemic deterioration – at least in the lungs. The putative sequence of the phenomena seems to be that of a primary infection of pericytes leading to endothelial dysfunction and increased permeability. This hypothesis is supported by the observation that pericytes regulate neutrophil extravasation during inflammation [138].

MET's effects: Under basal conditions, MET barely stimulates vasomotion. In the diabetic hamster cheek pouch and bat wing, MET restored post-ischaemic vasomotion [139]. In a skin flap model, MET increased capillary perfusion and reduced oedema [140]. These effects have been confirmed in humans, as seen for skin capillary responsiveness in prediabetes [141] and in first-degree relatives of patients with diabetes. A recent report showed that MET acutely increased functional capillary density during the postprandial period in obese diabetic patients [142]. These effects can easily explain MET's beneficial effects on insulin resistance, since the latter is closely related to poor microflow distribution [143]. Capillary microflow is also closely dependant on haemorheological factors, such as red cell flexibility; indeed, a recent report highlighted this type of abnormality in erythrocytes from patients with Covid-19 [144]. MET has been shown to improve red cell membrane flexibility/viscosity in several studies [145].

MET has been shown to protect retinal pericytes primed with AGEs and podocytes of diabetic rats from death, as also evidenced by a lower level of the podocyte marker podocalyxin [146].

Microvascular permeability

Microvascular permeability is regulated largely but not exclusively by the glycocalyx (GC) and the tight junctions between endothelial cells [147]; both of these structures are highly sensitive to inflammation. Abnormally high (disease-induced) permeability leads to oedema and damage to surrounding tissues – essentially due to the oxidative stress generated by adhering or extravasated leukocytes. Pre-existing activated inflammasomes and increased permeability in patients with diabetes may underlie the greater severity of Covid-19 in this context. The viral spike protein is known to disrupt the blood-brain barrier [148].

MET's effects: MET has prominent anti-oedematous effects in pathological situations. In the Syrian hamster model of diabetes, MET completely blocked microvascular leakage [149]. Reductions in oedema were also seen in non-diabetic situations, such as brain and peripheral ischaemia [150], middle cerebral artery occlusion [151], traumatic brain injury [152], glioma-related oedema [153], cultured pulmonary endothelial monolayers, LPS-induced lung injury, and carageenan-induced swelling [154]. Clinically, MET reduced cyclic oedema with a remarkable success rate and independently of glycaemic changes [155].

The endothelial barrier is also strongly regulated by the proteins constituting the tight junctions, whereby paracellular permeability is controlled. It has been suggested that bacterial translocation across the gut wall might modulate the immune response to SARS-CoV-2. MET attenuated the loss of tight junctions in the small intestine [156], in the ileum of IL-10 deficient mice and in experimental colitis, and thereby prevents the translocation of bacteria out of the intestine. MET also increased tight junction protein expression in the brain of septic rats and in retinal pigment cells challenged with glyoxal [157]. In airway epithelial cell cultures, MET increased the number of tight junctions in the presence of *Staphylococcus aureus* [158] or *Pseudomonas aeruginosa* [159]. As in the intestine, this effect was due to increased phosphorylation of occludin and zonulin-1 by AMPK. Indeed, the tightness of endothelial junctions is also controlled by AMPK – a key target for MET [160]. Furthermore, it has been shown that occludin regulates AMPK activity in pericytes [161].

Haemostasis

Atypical microthromboembolism is probably the most surprising and yet largely unexplained finding in Covid-19. It testifies to a state of thrombo-inflammation and is considered as a main if not the key cause of death in Covid-19. Patients with Covid-19 exhibit microvascular thrombosis that mimics classical disseminated intravascular coagulation. Haemorrhage is also observed [162].

The clinical importance of haemostatic abnormalities is highlighted by the reported associations between elevated D-dimer, fibrinogen, C-reactive protein (CRP), ferritin and cytokines levels on one hand and the severity and mortality of Covid-19 on the other [163]. Even though some reports are discordant, most experts agree that patients display both hyperfibrinolytic and hypercoagulant activities, and the clots strongly resist conventional anticoagulant therapies. Microthrombosis might also be due to microparticles that circulate after inflammation [164]. However, some findings about fibrinolysis are discordant because hyperfibrinolysis is reportedly characterised by both higher D-dimer levels and fibrinolytic shutdown [165]. According to a novel “ferric ion” hypothesis, the presence of D-dimers and the persistence of fibrin clots suggest that the clots' structure is modified by the elevated ferric ion levels produced by haemolytic anaemia [166]. This free iron might induce the formation of an insoluble fibrin structure called parafibrin [167].

Plasminogen activator inhibitor-1 (PAI-1) is a major regulator of fibrinolysis. Covid-19 was associated with an increase in PAI-1 [168], the reason for it is still debated [169]. Another study discriminated between non-severe and severe/critical Covid-19 on the basis of endothelial-derived factors, including PAI-1 [170]. Platelets are amongst the other important factors in thrombotic processes; unusual platelet hyperreactivity has been reported [171].

MET's effects: The fibrinolytic effects of biguanides have been known for decades [172]. MET's main action was found to be the inhibition of plasminogen activator inhibitor-1 (PAI-1) in patients with diabetes, thereby favouring fibrinolysis [173]. MET also decreased levels of PAI-1 antigen, factor VII, and CRP [174]. In patients with diabetes and patients with PCOS, MET reduced D-dimer levels [175]. In view of the obvious complexity of fibrinolysis in Covid-19, it is difficult to know whether MET's effect on PAI-1 could have clinical consequences. If the above-described “ferric iron hypothesis” turns out to be true, MET might exert a key action through its metal-binding ability. It should be remembered that MET also reduces levels of factors favouring PAI-1 activation, such as cytokines. MET also has beneficial effects on levels of von Willebrand factor (vWF), vascular cell adhesion protein-1 (VCAM-1) and tissue factor [176] and on factor XIII and fibrin fibre thickness, resulting in changes in the structure of fibrin. MET reduced in vivo platelet activation in diabetic rats by decreasing mitochondrial DNA release [177]. A lower level of platelet activation was also observed in patients with diabetes [178], independently of changes in metabolic parameters. In rats, MET-inhibited electrical stimulation-induced thrombus formation in situ in carotid arteries [179] and mesenteric arteries [180]. It is noteworthy that this effect was as large as with aspirin.

The glycocalyx: the cornerstone of worsening Covid-19's pathophysiology?

A better understanding of the structure and physiology of the GC is probably the most recent novel contribution to vascular physiology, notably that of small vessels. Although the GC covering endothelial cells in the vasculature is only 10 to 100 nm thick, it is a key “gate keeper” for vascular homeostasis. GC acts as a protective coating by preventing clotting at the endothelium, limiting interaction with blood cells, regulating vessel permeability, and transmitting signals for endothelial dynamics via shear stress-induced mechanotransduction. The GC has a hair-like appearance, constituted by a matrix of glycosaminoglycans bound to proteoglycans and encased in hyaluronan. It thereby acts as a sieve by excluding molecules heavier than

70 kDa. Due to its direct contact with blood, the GC is stimulated permanently by many circulating factors and therefore constantly requires repair by resynthesis. This resynthesis notably concerns heparin sulphate (HS), the GC's main constituent.

Low shear stress induces GC degradation by lowering AMPK levels, while increasing AMPK levels blocks VCAM-1 and ICAM-1 and thus reduces macrophage recruitment in vivo [181]. Thus, loss of laminar flow in small vessels is a potential cause of endothelialitis and highlights the importance of controlled flow in microvascular units. It should be noted that GC thickness decreases with advancing age, which renders the endothelium more vulnerable.

An abnormal GC has been linked to kidney injury, respiratory failure, hepatic dysfunction, and fibrosis. Many circulating factors can attack the GC, reduce its thickness, and release GC components into peripheral blood. Inflammatory factors associated with sepsis (such as CRP, TNF α , and the interleukins) are amongst the factors that induce GC shedding. Moreover, GC synthesis is related to plasma protein levels and so might be impaired by hypoalbuminaemia.

Recent studies have highlighted the GC's key pathophysiological role in Covid-19 [182]. Observations of the sublingual microcirculation in patients infected with SARS-CoV-2 revealed strong heterogeneity in capillary flow, a lower proportion of perfused capillaries, and a lower erythrocyte velocity [183]. These findings were confirmed by a clinical report on Covid-19 patients, showing a loss of perfused capillary density of up to 90% and a great reduction in GC thickness, particularly in mechanically ventilated patients. These changes were accompanied by the systemic elevation of levels of indicators of endothelial dysfunction (such as vWF-cleaving protease and VEGF-A), which increased with the length of hospital stay [184]. Many observations also report elevated levels of various products shed from the GC (such as chondroitin sulphate and syndecan-1), which makes the endothelial surface thrombotic [185]. Circulating syndecan-1 levels and their temporal change are predictive of patient outcome [186], making syndecan-1 a relevant indicator of disease severity and evolution. Circulating endothelial GC components are distributed throughout the body and hyaluronan fragments induce more generalised endothelial dysfunction [187]. The shedding can be partly corrected by the administration of low-molecular weight heparin, which is a heparanase inhibitor [188]. Heparanase activity increases in sepsis and it is known that the lower GC thickness in sepsis is correlated with the mortality rate [189]. Similarly, the GC is thinner in cases of post-influenza ARDS. These observations have prompted some researchers to suggest that the GC has an essential role in the pathophysiology of Covid-19 [190]. HS is by far the most prominent component of the GC, and was found to bind SARS-CoV-2 [191]. Under normal circumstances, the GC shields the ACE2 receptor from interacting with the SARS-CoV-2 spike protein [192]; however, HS degradation induces severe endothelial dysfunction and further favours the spreading of endothelial damage throughout the organism via the release of HS by-products [193]. Levels of HS are controlled by commensal bacteria, the number of which decrease with age; this might explain why older adults are more susceptible to Covid-19. Recently, several clinical investigations confirmed the presence of microcirculatory lesions in Covid-19 patients. Accordingly, therapies aimed at increasing HS levels might be of value. Moreover, as observed in ARDS, heparanase activity was elevated in Covid-19 patients and was correlated with disease severity [194]. Interestingly, sulphated polysaccharides block SARS-CoV-2 infections in vitro [195].

MET's effects: Although few data are available for MET, the drug's established pharmacological effects fit with putative protection against GC damage. Indeed, in diabetic rats, MET maintained the GC's thickness despite the lack of an effect on hyperglycaemia - indicating a direct protective effect [196]. In animals fed a high-fat diet, MET protected the myocardial perfusion reaction to an adenosine challenge (a marker of GC integrity) [197]. In rats with chronic diabetes,

MET decreased the adhesion of cancer cells to endothelium by increasing the GC's barrier activity. In human diabetics, MET treatment led to decreased urinary glycosaminoglycan excretion, suggesting protection against endothelial GC shedding [198].

MET protects cells

In both diabetic and non-diabetic situations, many reports have shown the beneficial effects of MET on cell survival in vitro or on organ/function preservation. It is noteworthy that this protection has been observed in several studies of ischaemia or ischaemia/reperfusion in the heart, brain, and peripheral organs [199]. For example, MET was associated with a reduction in myocardial infarct size [200], an almost 100% survival rate in hamsters submitted to haemorrhagic shock, and good outcomes in patients with diabetes after a stroke [201]. Interestingly, MET protects against ischaemia injury at doses that are much lower than those used to treat diabetes.

MET and membrane homeostasis

Hormone signalling is strictly dependant on membrane receptor binding and subsequent post-receptor signalling. These key processes require an optimal membrane composition and structure, which depends on the positions of interactions between membrane lipids and proteins and on links with the cell cytoskeleton. For example, the insertion of glucose transporters and the latter's intrinsic activity are closely related to membrane structure. In turn, the membrane's structure defines its fluidity (viscosity). Many factors reduce membrane fluidity, including hyperinsulinaemia, hyperglycaemia, and a lipid-rich diet.

It has long been known that biguanides are membrane-active compounds, due to their structure and cationic nature. Several in vitro and ex vivo experiments have evidenced a bell-shaped dose-effect curve for MET in lipid membranes, erythrocytes submitted to hyperglycaemia [202], and erythrocytes from MET-treated patients with diabetes [203]. Interestingly, MET's major pharmacological effects are mirrored by its effect on membrane fluidity [204]. Hence, membrane fluidity might be an important contributor to the efficacy of MET.

MET and cell death

Cell death is a major consequence of infection by SARS-CoV-2. The cells can be killed through various mechanisms (including necrosis, apoptosis, pyroptosis, and ferroptosis), depending on the initial cause of cell stress and the stimulatory pathway(s).

Apoptosis results from the loss of cellular energy due to hypoxia or ischaemia. As mentioned above, are strongly affected by severe, virus-associated inflammation. Microvascular function depends closely on endothelial function, which is under the control of pericytes or podocytes. Given that endothelial cells and pericytes/podocytes interact, pericyte loss leads to severe diseases such as retinopathy, collapse of the blood-brain barrier, and kidney dysfunction. Pericyte/podocyte apoptosis is typically induced by cytokines or oxidative stress in the context of inflammation or hyperglycaemia [205]. Both elevated levels of angiotensin II (potentially a key factor in SARS-CoV-2 infection) and hyperglycaemia reduce AMPK activity in podocytes [206]. The same factors can induce the apoptosis of endothelial cells. MET inhibits apoptosis in retinal pigment epithelium cells [207], hyperglycaemia-cultured endothelial cells and insulin islets [208], and many more.

Pyroptosis (cell death induced by inflammation) was inhibited by MET after myocardial infarction via an action on AMPK and the NLRP3 inflammasome [209]. MET also inhibited pyroptosis in intestinal ischaemia/reperfusion and diabetic periodontitis.

Ferroptosis is a consequence of uncontrolled lipid peroxidation. It is associated with oxidative stress and exposure to labile/free iron. Ferroptosis is downregulated by AMPK, one of MET's main targets

[210]. In Covid-19, haeme is attacked on its 1-beta chain, which dissociates the iron from the porphyrin ring. Hyperferritinaemia, high levels of toxic free ferric iron, and anaemia due to haemolysis have been reported in Covid-19. Ferritin is known to have major roles in some diseases and has been linked to the severity of SARS-CoV-2 infections [211]. Fe^{3+} is highly toxic, and iron overload is associated with inflammation, hypercoagulation, and immune dysfunction. In a study in patients with Covid-19, MET reduced circulating ferritin levels [95] – an effect previously reported in patients with diabetes and in lean and overweight women with PCOS. The ability of biguanides to bind metals (including ferric iron) contributes to MET's beneficial effect in Covid-19 infection.

The mechanisms of MET's mitochondrial effects are subject to much debate. This is due to both the use of supratherapeutic drug concentrations and the mitochondria's particular features when studied in vitro. To justify the use of mM MET concentrations in vitro (when the plasma level is about 10 μ M in clinical practice), some researchers have claimed that mitochondria are able to concentrate the drug. Considering these critical limitations, one key mechanism whereby MET could avoid cell death is inhibition of the mitochondrial transition pore (MTP), a key mechanism in cell survival/death. MET inhibited MTP opening and the release of cytochrome c in neurons [212], hyperglycaemia-cultured endothelial cells [213], pancreatic INS1-beta-cells, and cardiac cells submitted to hypoxia/reoxygenation [214]. In isolated, perfused hearts from normal or diabetic rats, MET reduced the infarct size after post-ischaemic reperfusion; this effect was partly due to inhibition of MTP opening [215]. Similar results for heart ischaemia were obtained in vitro and in vivo, with the inhibition of mitochondrial complex 1 and MTP. This non-exhaustive list of MET's effects on cell death clearly demonstrates the drug's potential for limiting the severity and spread of disease.

Cellular and molecular mechanisms of MET, as applied to the pathophysiology of Covid-19

MET and hypoxia

Most hospitalised patients with Covid-19 are admitted because of respiratory complications and need oxygen or mechanical ventilation. This state of hypoxia is caused by lung damage and (in some cases) haemolytic anaemia, which reduces oxygen transport by erythrocytes. Under normal conditions, hypoxia is countered by the activation of hypoxia-inducible factor 1 (HIF-1). However, viral infection leads to supranormal levels of HIF-1. In order to replicate, SARS-CoV-2 requires glycolytic conditions, such as those found in patients with diabetes. The generation of oxidative stress by SARS-CoV-2 means that monocytes from infected patients strongly express HIF-1, with detrimental effects on the immune system and T cell dysfunction, IL-1 secretion, and epithelial cell death [216]. Elevated HIF-1 activity might explain (at least in part) the higher mortality in obese or diabetic patients infected with SARS-CoV-2. Furthermore, HIF-1 favours fibrosis and might be a valuable therapeutic target in the vicious circle formed by lung hypoxia and HIF-1 induction in Covid-19 patients.

MET was shown to lower HIF-1 expression/activity in T cells [217], hyperinsulinaemia [218], hypoxic myeloma cells [219], oral squamous carcinoma cells [220], and cancer fibroblasts [221].

MET and hydrogen sulphide (H_2S)

The well-known physiological role played by thiol-containing molecules is an important factor in the severity of Covid-19 [222]. Hydrogen sulphide is produced through the metabolism of sulphur-containing molecules by several enzymes. Accordingly, the gut microbiota is one of the major sources of H_2S in the body. H_2S was notably found to be a gasotransmitter capable of acting

synergistically and can even replace NO in the vascular endothelium. Aside from its prominent involvement in vascular physiology and its beneficial effects on endothelial dysfunction, H_2S reduces the activity of the NLRP3 inflammasome, participates in regulation of the immune system, has anti-inflammatory and antiviral properties, decreases neutrophil transmigration, inflammation and ferroptosis in septic lung injury, inhibits autophagy and endoplasmic reticulum stress, alleviates ventilator-induced lung injury, promotes macrophage M2 polarisation, and maintains the integrity of the tissue barrier. These properties fit well with the pathological characteristics and pathways of SARS-CoV-2 infection, making H_2S an exciting therapeutic target. H_2S levels are reduced in Covid-19 [223] and linked to disease severity and death rates [224]. However, these findings must be interpreted with caution because the dose range for H_2S is narrow and the gas typically acts in a hormetic manner. It is also interesting to note that H_2S was shown to protect the GC [225] and decrease fibrosis [226].

The similarity between the effects of H_2S and those of MET is striking and raises the question of whether MET acts through this gas. Indeed, it has been found that MET increases H_2S levels [227]. In vivo, exposure to MET and the H_2S donor NaHS decreases renal damage. Although it remains to be determined how MET leads to an elevation of H_2S , in vitro MET exposure led to greater expression of cystathionine γ -lyase, one of the enzymes involved in H_2S synthesis [228]. The major natural contribution of the intestinal microbiota to H_2S production through the metabolism of various thiols in food provides another potential source of H_2S generation related to MET. Interestingly, it has been suggested that sulphur donors constitute an adjuvant therapy for Covid-19 [229]. For example, some components of garlic bind to viral structures [230] and so might be of therapeutic value in Covid-19.

MET, ER stress, and the UPR

The endoplasmic reticulum (ER) responds to various nociceptive stimuli by developing so-called ER stress (ERS), which is notably characterised by the unfolded protein response (UPR). ERS has a constant, key role in maintaining cellular homeostasis by eliminating cellular debris from organelles and from viruses. Intracellular structures are in close contact with each other, and the activity and direction of these interconnected processes depend on the nature and duration of the stimulus. Basal and short-term ERS and the UPR are beneficial for cell life; for example, the UPR helps to maintain the lung's vascular barrier function. However, prolonged activation can override these pathways' fine control mechanisms; ERS and UPR become toxic and ultimately lead to cell dysfunction and death. Intensive ERS leads to foam cell formation, cytokine production, and pulmonary fibrosis. ERS is closely linked to the production of inflammatory factors and can lead to obesity and cardiac dysfunction. A reduction of ERS is associated with a lower level of inflammasome formation [231].

MET was shown to reduce ERS in various pathological situations: angiotensin II-induced hypertension [232], palmitate-stimulated B cells [233] and cardiac cells, thapsigargin-induced cardiac dysfunction [234], hyperglycaemia [235], and renal fibrosis [236]. In the nervous system, MET regulated the UPR in a galactose-ageing model of neurodegeneration [237]. In cerebral ischaemia-reperfusion, MET reduced levels of ERS-related proteins and increased neurological scores and survival [238].

MET, lysosomes, and autophagy

Autophagy is a highly conserved physiological process through which cells maintain homeostasis and energy sparing. It is considered to be an important processing mechanism for virus endocytosis and is therefore a potentially valuable therapeutic target. Many

factors (such as ERS and caloric restriction, for example) can stimulate autophagy. This process starts with the formation of autophagosomes around the xenofactor; the cargo is then transported via early and late endosomes to lysosomes, where it is destroyed. Cells can thereby eliminate infections, provided that the system is not overwhelmed. Autophagy in Covid-19 is currently a hot topic, since some viruses can circumvent and reduce autophagy pathways and use them for replication by blocking the fusion with lysosomes [239]. It has therefore been suggested that limiting autophagy would be an effective strategy soon after infection by SARS-CoV-2 [240]. Like most other pathways involved in these processes, autophagy is a double-edged sword. More generally, activating autophagy is considered to be an effective means of increasing lung function in pathological situations [241]. The stimulation of autophagy is considered to be one of MET's main mechanisms of action in metabolic, ischaemic or inflammatory situations (for a review, see [242]).

Some researchers have suggested that targeting lysosomes might be a better strategy than trying to block the virus or its entry into cells [243]. A key factor in the autophagy/lysosomal process is the organelles' pH because viruses need an acidic milieu in which to replicate. Weak or strong bases can induce deacidification, and so small alkalinising molecules might therefore block viral activity [244]. Due to its guanidine structure, MET is a weak base capable of reaching lysosomes and increasing the pH. Some very elegant research on MET and AMPK showed that MET acted at the endosomal/lysosomal level by mimicking a starving cellular state that activated v-ATPase in lysosomes [245]. This promoted displacement of AXIN/LKB1 and stimulated AMPK (for more details, see [246]). This energy-linked stimulation of AMPK also explains MET's prolongation of the life span in *C. elegans* [247]. On this basis, it was recently suggested that MET can be used to treat Covid-19 [248]. Furthermore, lysosomes can be damaged by infectious agents. Galectins recognise these endomembrane lesions so that the cells respond by activating AMPK and autophagy. A study of specific modifications in galectin markers showed that MET also induced these modifications in a macrophage cell line [249].

Throughout this review it is seen that stimulation of AMPK could represent at least one common denominator of these various mechanisms. Indeed AMPK is known to be a hub of intracellular signalling. Due to the great amount of evidence for AMPK stimulation by MET, and even though not all MET effects are mediated by this pathway, the reader is referred to the dedicated literature [250].

Fig. 1 summarises the many aspects of AMPK's involvement in physiological and biochemical processes related to multifactorial diseases like Covid-19. This involvement ranges from effects on viral attack (at the top of the Figure) to the development of lung and/or vascular fibrosis (at the bottom) and thus follows the main steps in disease progression.

Conclusion

Severe Covid-19 is characterised by an initial infection in the upper airways, which then spreads throughout the body and appears as a generalised microvascular disease with atypical haemostatic defects (microthromboses) in capillaries and, ultimately, fibrosis. A deep look into the vast body of pharmacological data on MET reveals activities that are clearly suited to counter the main pathological events in Covid-19. MET's unique but often unappreciated microvascular effects appear to target Covid-19's switch from an infectious state to a vascular disease. The drug's preservation of small vessel integrity in terms of motricity, microflow regulation, permeability and probably haemostasis notably – but not exclusively – provides a realistic explanation of the impressive clinical benefit related to this compound. Lastly, it is important to remember that all the various pharmacological effects of MET described here can occur in a diabetes-independent manner.

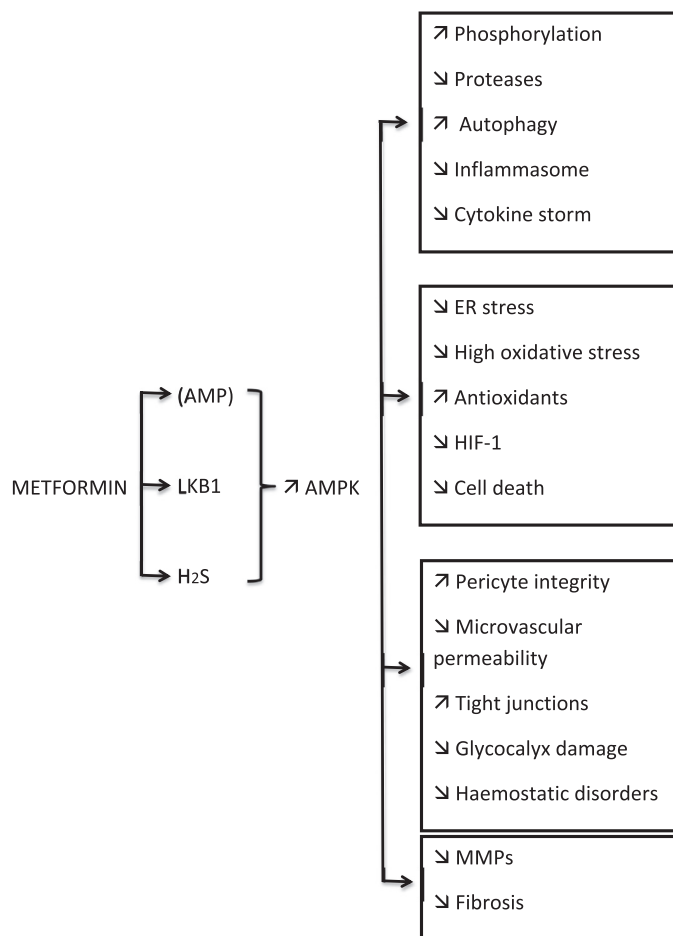


Fig. 1. AMPK's multifaceted involvement as a major mechanism in the physiological and biochemical processes of multifactorial diseases (e.g. Covid-19).

AMP, adenosine monophosphate; AMPK, 5'-AMP-activated protein kinase; LKB1: serine–threonine liver kinase B1; H₂S, hydrogen sulphide; ER, endoplasmic reticulum; HIF-1, hypoxia-inducible factor 1; MMP, matrix metalloproteinase.

Contribution statement

Conception of the research, and drafting: NW, AAS and JDL; critical revision of the manuscript: all co-authors.

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