Characterization of metformin–microbiome interaction in type 2 diabetes

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Introduction

Diabetes mellitus (DM), commonly referred to as diabetes, is a group of complex metabolic disorders characterised mainly by hyperglycaemia. It affects millions of people worldwide and its prevalence is on the rise. Diabetes and its complications have a great health and economic impact, as this disorder increases the risk of blindness, infection, limb amputation, cardiovascular disease, cancer, among others. While the mechanisms behind the onset and progression of diabetes are not fully understood, it is known that both genetic and environmental factors are involved[1,2].

The two most common forms of this disorder are type 1 (T1DM) and type 2 (T2DM) diabetes. T1DM is an autoimmune disorder involving the destruction of the insulin-producing β-cells in the pancreas, with onset most commonly in younger age groups. In contrast, T2DM is typically developed later in life, and the pathophysiology of the disease in each patient has a variable contribution of β-cell dysfunction and tissue insulin resistance[2].

Multiple lifestyle aspects are known to be risk factors for T2DM - such as obesity and sedentary behaviour, as the accumulation of fat in the liver and muscles is known to impair cell uptake of glucose[2]. Intestinal dysbiosis is also associated with various components of T2DM. The composition and functionality of the gut microbiome influences the host on a systemic level by triggering immune and endocrine responses, through the production of metabolites which promote the chronic inflammation typical of DM or which regulate insulin secretion, such as short-chain fatty acids like butyrate[1]. Understanding the part of the gut microbiome in DM is crucial to choosing and developing adequate treatments for DM patients. In fact, it is known that metformin, a first-line treatment for T2DM, has a significant effect on the microbiome[3]. On the other hand, McCreight et al.[4] described the passage of metformin through the gastrointestinal tract and supported the hypothesis that a large accumulation of metformin in the intestine could contribute to the glucose-lowering effect of the drug, perhaps directly through the change in microbiome composition[5].

Metformin's mode of action is not completely understood; however, it is known that it decreases hepatic gluconeogenesis and promotes muscular glucose uptake[6]. It has an oral bioavailability of 50-60% and incomplete gastrointestinal absorption, and it is excreted in urine without being metabolised. Metformin is generally well-tolerated: common side effects include gastrointestinal complaints, usually in the beginning of treatment[7]. It has been shown to reduce the risk of cardiovascular disease and colon cancer[6,7].

Studies in animals and humans have been carried out in order to understand the effect of metformin in bacterial growth. Specific taxa were found to be highly regulated by metformin in European women, such as Enterobacteriaceae (including Escherichia, Shigella, Klebsiella and Salmonella), whose levels increased, Clostridium and Eubacterium, which decreased[8]. Higher levels of Enterobacteriaceae were also found at baseline in Nordic T2DM patients treated with metformin versus untreated patients[9].

In a Colombian adult population diagnosed with T2DM, sequencing of the 16S rRNA gene revealed no differences in the number of operational taxonomic units (OTUs) observed between groups (metformin-treated versus untreated group). However, the diversity was significantly reduced by treatment, suggesting that metformin alters the bacterial community structure in the gut microbiota of T2DM patients. In this Colombian T2DM population, metformin significantly increased the OTUs belonging to the *Megasphaera* and *Prevotella* genera, while it reduced the OTUs of *Clostridiaceae 02d06* and *Barnesiellaceae*, as well as the *Oscillospira* genus[10].

Significant changes in the abundance of *Escherichia* and *Intestinibacter* were also observed in metformin-treated groups, a finding that is in agreement with results reported in a cross-sectional study comparing groups of T2DM-treated and untreated people[3]. However, the growth of *Escherichia coli in vitro* was not affected by metformin, which demonstrates the limitations of *in vitro* studies. In rat models, metformin had no impact on gut microbiome diversity of rats fed a standard diet, suggesting its effect is diet-dependent[11]. Metformin increased the abundance of the beneficial bacteria *Lactobacillus* and *A. muciniphila* in high fat diet-fed rats which had previously developed insulin resistance[12].

Akkermansia muciniphila and *Bifidobacterium bifidum* are two species found to be increased in T2DM patients treated with metformin. Earlier studies demonstrated they are both mucin-degrading bacteria. Mucin is a glycoprotein component of the mucus gel coating of the human gastrointestinal tract epithelium. Increased production of mucin increases the thickness of the mucus layer covering the mucosa and, thus, strengthens epithelial barrier function. Metformin-related increase of these bacteria in the gut has been linked to improved glucose metabolism[5,13].

In addition, a transcriptome analysis of metformin's effects on two distinctly-related bacterial species in a gut simulator showed that metformin regulates genes which encode for metalloproteins or metal transporters[14]. Nonetheless, further studies are required to fully map the interaction mechanisms between metformin and the microbiome.

With this in mind, we propose an experimental design making use of *A.muciniphila* and *B.bifidum* strains and faecal samples from a group of healthy individuals and T2DM patients. First, we will culture each bacterial strain in Petri dishes with and without metformin and perform proteomic and metabolomic analyses. The findings will be the basis for a gut simulator experiment in order to validate the culture results and whether the effects of metformin on the two species hold up in the context of the whole microbiome, considering the known effects of metformin in other bacteria.

Experimental Design

In vitro bacteria culture

Two species of gut bacteria – *Akkermansia muciniphilla* and *Bifidobacterium bifidum* – will be cultured as described in Yoshihara *et al*.[15]. We will cultivate each bacterial species for 30 days, in five replicates of minimal medium with no metformin, to act as control, and medium supplemented with three concentrations of metformin - a minimal dose, a clinical dose, and an excessive dose. We will measure the optical density of the media every 24 hours, in order to estimate culture growth. Subsequently, in order to understand how metformin affects each species, protein extraction and purification as well as mass spectrometry will be performed for proteomics and metabolomics analysis, as described in Chen & Gerszten[16]. The results will be crossed with protein databases to gather information on known functions.

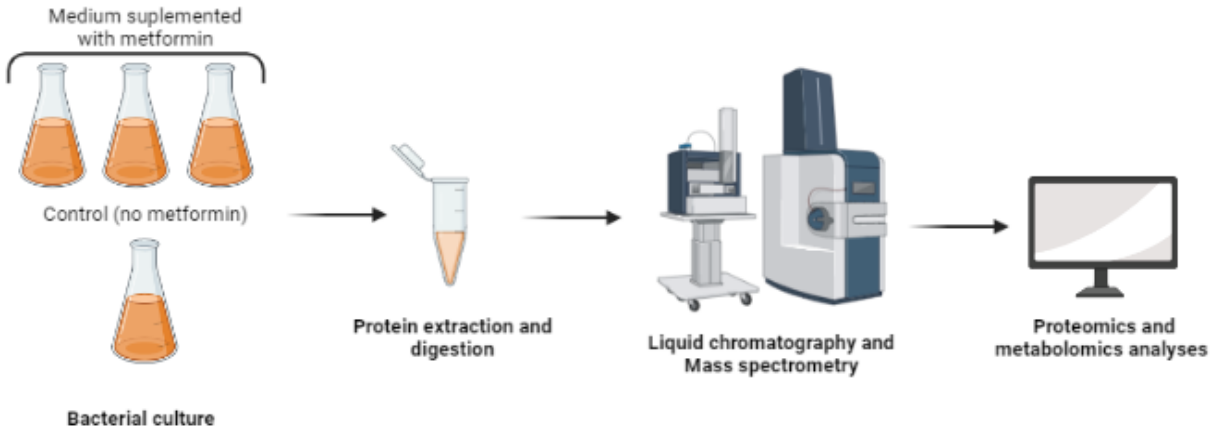

Figure 1 - General overview of the in vitro culture experiment. *A.muciniphila* and *B. bifidum* will be cultivated for 30 days in a minimal medium with no metformin (control) and in a medium supplemented with different concentrations of metformin. Five replicates will be prepared for each strain and metformin concentration. Proteomics and metabolomics analyses will be carried out with the use of LC-MS/MS.

Sample collection

We will gather faecal samples from 30 healthy individuals and 30 type 2 diabetics aged 18-65, to account for microbiome diversity and variability. We will exclude people who are pregnant, who have ever been treated with metformin, or who are being treated with any other medication.

A partnership with Portuguese public hospitals will facilitate contact with patients willing to participate. All participants will be required to give informed consent. We will certify that all applicable institutional regulations concerning the ethical use of information and samples from human volunteers will be followed.

***In vitro* gut simulator**

In order to explore the effect of metformin on a gut microbial community *in vitro*, samples from participants will be divided in four groups: healthy; type 2 diabetic (control groups); healthy with a clinical dose of metformin; and type 2 diabetic with a clinical dose (treatment groups).

Faecal samples will be prepared as described in Wu *et al*.[14] and an aliquot will be inoculated in an in vitro gut simulator such as described in Wiele. T *et al*.[17]. After a time to allow for simulator stabilisation, metformin will be added to both treatment groups and the simulator will run for another 30 days.

An aliquot will be collected from the gut simulator after the established period, followed by DNA extraction and qPCR to evaluate the population growth of *A.muciniphila* and *B.bifidum* in control and treated samples as described in Collado *et al*.[18].

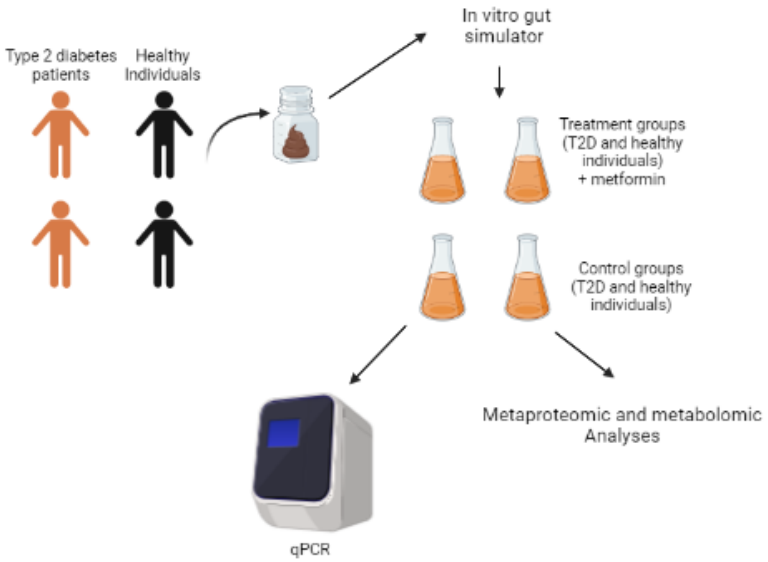

Figure 2 - General overview of in vitro gut simulator experiment. Faecal samples obtained from healthy individuals and type 2 diabetes patients will be inoculated in the in vitro gut simulator. Metformin will be added after a period of stabilisation (treatment group). DNA and protein will be extracted from the four groups of samples: healthy; type 2 diabetic (control groups); healthy with a clinical dose of metformin; and type 2 diabetic with a clinical dose (treatment groups) to perform qPCR, metaproteomics and metabolomics analyses, respectively.

Similar as in the pure cultures of *A.muciniphila* and *B.bifidum*, proteomics and metabolomics analysis will be conducted to study the interaction between the bacteria and metformin, now in the midst of a gut microbiome-like community. Computational tools will be used to filter the large output datasets of these analyses, in order to select results concerning our two species of interest and to compare differences between control VS. treatment groups and microbiome from healthy VS. diabetic individuals.

Expected results and concluding remarks

Omics-based analyses will allow us to understand what mechanisms are involved in the interaction of bacteria-metformin and associate them with known functions of *A.muciniphila* and *B.bifidum*. We hypothesise that enzymes, membrane transporters, and transcription factors, as well as signalling pathway elements and metabolites may be some of the molecules affected by treatment with metformin.

Further studies should focus on broadening the knowledge on how the microbiome as a whole reacts to metformin treatment, particularly *in vivo* models, as well as functional validation of proteins found in this study. Subsequently, researchers should direct their efforts to understand how the changes in microbiome affect the host metabolism and the management of diabetes.

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