UNIVERSITY OF LATVIA

Faculty of Biology

Ilze Elbere

DOCTORAL THESIS

METFORMIN EFFECTS ON GUT MICROBIOME AND EPIGENETICS IN TYPE 2 DIABETES PATIENTS AND HEALTHY INDIVIDUALS

Promotion to the degree of Doctor of Biology

Molecular Biology

Supervisor: Dr. Biol., Prof. Jānis Kloviņš

Riga, 2021

The doctoral thesis was carried out in Latvian Biomedical Research and Study Centre, at Human Genetics and Molecular Medicine department, and Faculty of Biology, Department of Molecular Biology, University of Latvia from 2017 to 2021.

The research was supported by ERDF grant 1.1.1.1/16/A/091 and author received Ltd "MikroTik" scholarship administrated by Foundation of University of Latvia.

Form of the thesis: collection of research papers in biology, subfield – molecular biology.

Supervisor: Prof., Dr. biol. Jānis Kloviņš

Reviewers:

1) *Asoc. prof., Dr. biol. Aija Linē, Latvian Biomedical Research and Study Centre;*
2) 2) *Prof., PhD, Susanne Brix Pedersen, Technical University of Denmark;*
3) *Prof., Dr. med. Mārcis Leja, University of Latvia*.

The thesis will be defended at the public session of the Doctoral Committee of Biology, University of Latvia, at 11:00 on July 1st, 2021 at Latvian Biomedical Research and Study Centre, Ratsupites Str. k-1.

The thesis is available at the Library of the University of Latvia, Kalpaka blvd. 4.

This thesis is accepted for the commencement of the degree of Doctor of Biology on _____ ___ 20___ (*norāda mēnesi, dienu un gadu*) by the Doctoral Committee of Biology, University of Latvia.

Chairman of the Doctoral Committee _____/ *vārds, uzvārds/*
(paraksts)

Secretary of the Doctoral Committee _____/ *vārds, uzvārds/*
(paraksts)

ABSTRACT

Metformin is an antidiabetic agent widely used as a first-line treatment for type 2 diabetes (T2D). It has various advantages as well as variable therapeutic effects, contraindications, and side effects. The pharmacodynamic effects of metformin have been widely studied, interaction with gut microbiome or epigenetic regulation of the host along with other therapeutic target sites have been highlighted, yet details of these mechanisms remain obscure. Moreover, metformin's pleiotropic effects have shown significant results in the treatment of many other diseases outside T2D. The high subject-specific variance and dynamic nature of both the gut microbiome and DNA methylation profile makes them a significant target for precision medicine biomarker discovery.

The aim of this study was to identify taxonomic and functional gut microbiome biomarkers as well as epigenetic signatures of the host for metformin pharmacodynamics, therapy efficacy and tolerance. Using massive parallel sequencing based approaches for gut microbiome profiling we observed significant and immediate reduction of inner diversity in the healthy cohort and changes in the taxonomic profile caused by metformin therapy in both newly diagnosed T2D patients and healthy individuals. Employing shotgun metagenomics, we presented the possibility to use baseline sample composition as a prediction tool for metformin therapy efficacy and tolerance. In addition, our study on global DNA methylation profile and its changes during metformin use presented the first and currently the only study evaluating longitudinal effects in peripheral blood cells of healthy human individuals. The results depicted that the genes representing the top-ranked differentially methylated probes corresponded to the main functional groups associated with previously described targets of metformin therapy: regulatory processes of energy homeostasis, inflammatory responses, tumorigenesis, and neurodegeneration. These results altogether bring novel data that could be used in future studies and in development of microbiome modulation approaches as well as precision medicine based treatment algorithms.

KOPSAVILKUMS

Metformīns ir plaši izmantots pirmās izvēles antidiabētiskais medikaments 2. tipa cukura diabēta (T2D) ārstēšanai. Tam ir vairākas priekšrocības, tomēr raksturīga arī mainīga terapeitiskā iedarbība, kontrindikācijas un blakusparādības. Metformīna farmakodinamiskā iedarbība ir plaši pētīta, tiek uzsvērta tā mijiedarbība ar zarnu mikrobiomu, kā arī saimniekorganisma epiģenētisko regulāciju un citiem terapeitiskajiem mērķiem, tomēr informācija par šiem mehānismiem joprojām ir neskaidra. Papildus tam metformīna plejotropā iedarbība ir parādījusi būtiskus rezultātus daudzu citu slimību ārstēšanā ārpus T2D. Gan zarnu mikrobiomam, gan DNS metilācijas profilam raksturīgās indivīd-specifiskas iezīmes un dinamika padara tos par nozīmīgu mērķi precīzijas medicīnas biomarķieru atklāšanai.

Šī pētījuma mērķis bija identificēt taksonomiskos un funkcionālos zarnu mikrobioma biomarķierus, kā arī saimniekorganisma epiģenētiskās iezīmes metformīna farmakodinamikai, terapijas efektivitātei un tolerancei. Izmantojot lielapjoma paralēlas sekvenēšanas pieejas zarnu mikrobioma profilēšanai, mēs novērojām metformīna terapijas izraisītu būtisku un tūlītēju iekšējās daudzveidības samazināšanos veselo indivīdu kohortā, kā arī taksonomiskā profila izmaiņas gan jaundiagnosticētiem T2D pacientiem, gan veseliem indivīdiem. Izmantojot visa metagenoma sekvenēšanu, mēs prezentējām iespēju izmantot pirms terapijas uzsākšanas iegūtā mikrobioma parauga taksonomisko sastāvu kā rīku metformīna terapijas efektivitātes un tolerances prognozēšanai. Papildus tam mūsu publikācija par globālo DNS metilēšanas profilu perifērajās asins šūnās un tā izmaiņām metformīna lietošanas laikā parādīja pirmo un pašlaik vienīgo pētījumu, kurā tika analizēta ietekme longitudinālā griezumā uz veseliem indivīdiem. Rezultāti parādīja to, ka gēni, kas atbilst top diferencēti metilētajām zondēm, reprezentē galvenās funkcionālās grupas, kas saistītas ar iepriekš aprakstītajiem metformīna terapijas mērķiem: enerģijas homeostāzi regulējošie procesi, iekaisuma reakcijas, tumoriģenēze un neirodeģenerācija. Šie rezultāti kopumā sniedz jaunas zināšanas, kurus kalpos par pamato turpmākiem pētījumiem un mikrobiomu modulācijas pieeju, kā arī precīzijas medicīnā balstītu ārstēšanas algoritmu izstrādē.

TABLE OF CONTENTS

ABBREVIATIONS

16S rRNA	16S ribosomal RNA
18S rRNA	18S ribosomal RNA
5hmC	5-hydroxymethylcytosine
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
AXIN	scaffold protein AXIN
BFT	*B. fragilis* toxin
BMI	body mass index
bp	base pairs
CBP	CREB binding protein
CEC	colon epithelial cell
CGI	CpG islands
CPM	counts per million
CRC	colorectal cancer
CREB	cyclic AMP response element binding protein
DMP	differentially methylated probe
DMR	differentially methylated region
DNMT	methyltransferase
DNA	deoxyribonucleic acid
DPP-4	dipeptidyl peptidase 4
ERK	extracellular signal-regulated kinase
ETBF	Enterotoxigenic *Bacteroides fragilis*
FBP1	fructose 1,6 bisphosphatase-1
FMT	faecal microbiota transplantation
FXR	farnesoid X receptor
GI	gastrointestinal
GLP-1	glucagon-like peptide 1
GPCR	G protein-coupled receptor
GUDCA	glycoursodeoxycholic acid
H19	long noncoding RNA H19
HTS	high-throughput sequencing
ITS	internal transcribed spacers
LEfSe	Linear discriminant analysis Effect Size
LKB1	liver kinase B1
lncRNA	long non-coding RNA
LPS	lipopolysaccharides
M0	samples collected before metformin treatment
M10h	samples collected 10 hours after the first metformin intake
M24h	samples collected 24 hours after the first metformin dose
M7d	samples collected 7 days after starting the therapy
mGDP	mitochondrial glycerophosphate dehydrogenase
miRNA	micro RNA
MODY	maturity onset diabetes of the young
mRNA	messenger RNA
MRCC1	mitochondrial respiratory chain Complex I
mTORC1	mammalian target of rapamycin complex I
ncRNA	non-coding RNA
NMDS	non-metric multidimensional scaling
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PERMANOVA	permutational multivariate analysis of variance
PGM	Personal Genome Machine
piRNA	Piwi-interacting RNA
RCP	Regression on Correlated Probes
RNA	ribonucleic acid
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosyl-L-methionine
SCFAs	short-chain fatty acids
SGLT-2	sodium-glucose co-transporter-2
SHP	small heterodimer partner
siRNA	short interfering/ silencing RNA
SLC22A1	Solute Carrier Family 22 Member 1
SLC22A3	Solute Carrier Family 22 Member 3
SLC47A1	Solute Carrier Family 47 Member 1
SNP	single nucleotide polymorphism
sPLS-DA	sparse Partial least squares discriminant analysis
T1D	type 1 diabetes
T2D	type 2 diabetes
TET2	Ten-Eleven Translocation-2 protein
TGF	transforming growth factor
TLR4	Toll-Like Receptor 4
TMAO	trimethylamine-N-oxide
TSS	transcription start site
VIP	variable importance in projection

INTRODUCTION

Diabetes mellitus is one of the most prevalent diseases worldwide, with T2D constituting more than 90% of cases. It has been recognized as a global burden for healthcare and economics. Therefore, timely identification of individuals at risk, improved detection and monitoring of T2D patients, effective treatment with reduced incidence of comorbidities, and improved awareness are the key elements for decreasing the future burden of this disease. Metformin is a first-line antidiabetic agent widely used for treatment of T2D. It has various advantages as well as variable therapeutic effects, contraindications, and side effects. The pharmacodynamic effects of metformin have been widely studied, interaction with gut microbiome or epigenetic regulation of the host along with other therapeutic target sites have been highlighted, yet details of these mechanisms remain obscure.

Importance of this work: Early prediction of efficacy and tolerance for antidiabetic therapy is a significant way to develop precision medicine based approaches, therefore, improving quality of life for the patients. New knowledge about metformin pharmacodynamics is important to the development of efficient treatment algorithms for T2D patients and creation of new treatment strategies for metformin therapeutic targets outside T2D.

Aims of the study: To identify taxonomic and functional gut microbiome biomarkers as well as epigenetic signatures of the host for metformin pharmacodynamics, therapy efficacy and tolerance.

Tasks to reach the aims:

1) Analyse the metformin effects on taxonomic profile of healthy human gut microbiome.
2) Investigate the short-term metformin effects on peripheral blood DNA methylation profile in healthy individuals.
3) Evaluate the similarities and population specific features of metformin induced taxonomic and functional changes in gut microbiome both in healthy and in newly diagnosed T2D patients.
4) Determine possible biomarkers for metformin therapy efficacy and tolerance

1. LITERATURE REVIEW

1.1. Human microbiome

1.1.1. Characterization and development

The human microbiome is defined as the collection of microorganisms (bacteria, archaea, viruses, eukaryotes) in a specific body site – habitat – together with their genomes and the surrounding environmental conditions, e.g. biochemical products (Marchesi and Ravel 2015). Moreover, the most recent discussions and amendments put additional emphasis on the temporal and spatial dynamics of the microbiome, as well as the integration, interaction, and coevolution with the host as part of the microbiome definition (Berg *et al.* 2020). The main human microbiome niches are skin, oral, respiratory, urogenital, and intestinal microbiomes, with most of them having several smaller subpopulations, dependent on physiological and environmental differences within the human body. Microbiome composition and functional capacity have been strongly proven to be associated with maintaining the health of the host as well as with the pathogenesis of various diseases (Figure 1). Unfavourable shifts in the human microbiome are defined as dysbiosis and have been studied and characterized in the context of inflammatory bowel disease, obesity, type 1 and type 2 diabetes, allergies, multiple sclerosis, autism, cancer, and several other diseases (Lloyd-Price *et al.* 2016, Paun and Danska 2016, Vivarelli *et al.* 2019, Chandra *et al.* 2020).

Currently, it is mostly acknowledged that the colonization with the first microbial communities starts during birth, however, in recent years some studies have highlighted the possibility of microbiome subpopulations in the placenta, amniotic fluid, and meconium, therefore supporting *in utero* colonization hypothesis (Perez-Munoz *et al.* 2017). Nevertheless, the health of the mother and her microbiome, mode of delivery, feeding type (breastfeeding versus formula), early antibiotic use, and even pet keeping in the household have been established as the most important factors affecting the microbiome of an infant, its development in the first years of life, and effects on health in adulthood (Tanaka and Nakayama 2017, Kim *et al.* 2019, Moore and Townsend 2019).

During life, the human microbiome is still highly dynamic, however, mostly maintaining its person-specific features. There has been characterized a wide range of factors impacting the microbiome throughout life, such as host genetics, diet, age, lifestyle (sleep, exercise, stress levels, smoking), hormonal shifts, use of antibiotics and other medications, infections, traveling, and other interactions with the environment (Rojo *et al.* 2017).

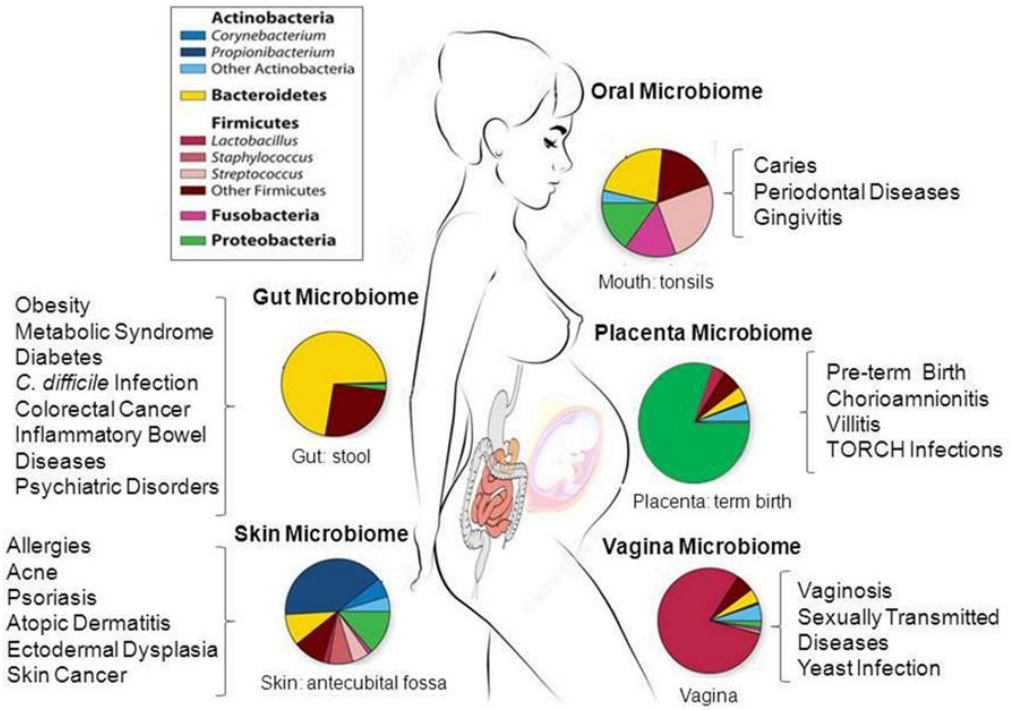


Figure 1. Taxonomic composition of various human microbiome subpopulations characterizing a healthy individual, as defined by data from previous large-scale human microbiome projects, and examples of various diseases associated with dysbiosis of their subpopulations. Adapted from (Belizario and Napolitano 2015).

The definition of a healthy human microbiome is still unclear as there are several factors continuously affecting the microbiome and high inter-individual and intra-individual variety exists. At the moment determination of core microbiome or sets of specific features common to healthy microbiomes, such as prevalent organisms or molecular pathways, as well as dynamic modelling of compositional and functional fluctuations throughout life have been used to distinguish healthy or dysbiotic microbiomes (Lloyd-Price *et al.* 2016, Aguirre de Carcer 2018).

1.1.2. Gut microbiome

The most diverse and most studied human microbiome subpopulation is specifically the human gut microbiome. It has been characterized as one of the most densely populated microbial populations on Earth, composed of more than 1000 species (Rinninella *et al.* 2019), with the dominant phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia*, where the first two composes approximately 90% of the gut microbiota (Qin

et al. 2010). Gut microbiome continuously interacts with the host and other microbiome subpopulations, therefore, ensuring a number of evolutionary developed vital functions:

(1) Extraction, absorption, and synthesis of various metabolites and nutrients, such as short-chain fatty acids (SCFAs), bile acids, and vitamins, therefore, enhancing the metabolic capacity and interacting with metabolic regulation of the host (Brestoff and Artis 2013, Kho and Lal 2018);
(2) Protection from infections induced by opportunistic pathogens (colonization resistance), mostly through competition processes - antimicrobial peptide secretions, pH modification, nutrient metabolism, and effects on cell signalling pathways (Kim *et al.* 2017, Ducarmon *et al.* 2019);
(3) Strengthening the integrity of the gut and shaping the intestinal epithelium, thus, ensuring a mechanic barrier (Natividad and Verdu 2013);
(4) Regulation of development, homeostasis, and function of both innate and adaptive immune systems of the host (Maynard *et al.* 2012).

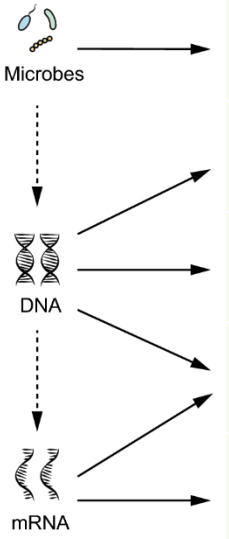
In addition to these functions, studies have shown the gut microbiome as a key player in the regulation of several physiological processes and systems of the host. Gut-brain axis, gut-liver axis, gut-muscle axis, and gut-skin axis are some of the most studied examples of these systemic interactions mediated by the gut microbial communities (O'Neill *et al.* 2016, Tripathi *et al.* 2018, Osadchiy *et al.* 2019, Przewlocka *et al.* 2020).

The gut microbiome is highly variable in the context of biogeography throughout the gastrointestinal (GI) tract. Host physiology, nutrient availability, competition, pH, and oxygen levels are only some of the factors behind this variability (Mark Welch *et al.* 2017). In addition, it is important to note that a vast majority of the studies are analysing specifically the faecal microbiome, which is significantly different from various subpopulations associated with the GI tissue. However, the data obtained from faecal microbiome can serve as non-invasive biomarkers often depicting similar shifts in microbial communities compared to biopsy samples (Tang *et al.* 2015, Engevik and Versalovic 2019).

1.1.3. Microbiome analysis

For many years, microbiome studies were done using culture-dependent methods, which still have significant limitations when it comes to studying the human gut microbiome, and even now, it is estimated that approximately half of the prokaryotic diversity found in the mammalian gut microbiome cannot be grown in any known culture (Lagkouvardos *et al.* 2017).

During the previous decade, the development of high-throughput sequencing (HTS) approaches has fostered a rapid advancement in microbiome studies. HTS methods can be classified into three categories: microbiome, DNA, and mRNA level analyses. (1) Culturome, in nowadays comprehension, is a recent method where HTS and other molecular methods have been combined with innovative culture techniques, therefore, renewing the interest in applying the culture-based approaches for microbiome studies (Lagier *et al.* 2018, Zou *et al.* 2019). (2) Amplicon based approach is estimated to be the most used HTS method for microbiome studies. Amplicon studies comprise various types of marker genes used for taxonomical profile determination of microbiome: 16S ribosomal RNA (*16S rRNA*) gene for prokaryotes; 18S ribosomal RNA (*18S rRNA*) gene for eukaryotes; internal transcribed spacers (ITS) for fungi (Woese and Fox 1977, Schoch *et al.* 2012). (3) The shotgun metagenome approach includes sequencing and analysis of all present DNA, therefore offering insight into the microbiome's functional potential as well (Sharpton 2014). (4) Metatranscriptomic approach profile the mRNA levels of the microbial community, characterizing the current functional activity (Bashiardes *et al.* 2016). However, the new approaches have brought new challenges, therefore, independently of the methods used their advantages and limitations should be taken into account (Figure 2).



Method	Advantages	Limitations
Culturome	• High-throughput • Targeted selection • Provides microbial isolates	• Expensive • Laborious • Influenced by media and the environment
Amplicon (16S/18S/ITS)	• Quick analysis • Low-biomass requirement • Applicable to samples contaminated by host DNA	• PCR and primer biases • Resolution limited to genus level • False positive in low-biomass samples
Metagenome	• Taxonomic resolution to species or strain level • Functional potential • Uncultured microbial genome	• Expensive • Time-consuming in analysis • Host-derived contamination
Virome	• Can identify RNA and DNA viruses • Quick diagnosis	• Most expensive • Difficult to analysis • Severe host-derived contamination
Metatranscriptome	• Can identify live microbes • Can evaluate microbial activity • Transcript-level responses	• Complex sample collection and analysis • Expensive and complex in sequencing • Host mRNA and rRNA contamination

Figure 2. Advantages and limitations of current high-throughput sequencing-based approaches used in microbiome research. Adapted from (Liu *et al.* 2020).

Factors, such as sample type, amount of biomass, and the scientific question are used for the selection of the most suitable approaches. Most importantly, it is advisable to integrate different methods, therefore obtaining insight into both the taxonomic and functional profiles of the analysed microbiome. Such an approach is defined as multi-omics and includes not only HTS based methods but proteomics and metabolomics analyses, as well as it may include integration with data from the host (Wang *et al.* 2019, Liu *et al.* 2020).

1.2. Epigenetic regulation

1.2.1. Main mechanisms of the epigenetic regulation

The term "epigenetics" was introduced in 1942 by Conrad Waddington, thereby stating – "an epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Waddington 1942). Based on our current understanding, epigenetic mechanisms are defined as processes that regulate gene expression through the alteration of chromatin structure without changing the nucleotide base sequences. These epigenetic influences can be further inherited both in transgenerational and in a mitotic way. The three most characterized mechanisms of epigenetic regulation are histone modifications, DNA methylation, and non-coding RNAs (ncRNAs). Importantly, most of the epigenetic modifications are reversible, therefore, this field offers a considerable promise for new targeted and precision medicine-based therapies (Allis and Jenuwein 2016, Kronfol *et al.* 2017, Cavalli and Heard 2019).

The DNA in each eukaryotic cell is organized in tightly regulated structures – approximately 145-147 base pairs (bp) of DNA are wrapped around an octameric and globular protein complex, thus, forming the "nucleosomal core particle". Each octamer is formed by two dimers containing H2A and H2B core histones, and one tetramer containing two H3 and two H4 core histones. Also, the linker histone H1 is needed to form a full nucleosome and stabilise higher-order chromatin structures (Allan *et al.* 1980, Luger *et al.* 1997). In addition to the physical regulation of DNA accessibility determined by the nucleosomes, histones may carry various posttranslational modifications, which as well have a significant impact on chromatin accessibility and therefore the gene activity. The most studied histone modifications include acetylation, methylation, phosphorylation, sumoylation, ubiquitinylation, ADP ribosylation, and deamination (Kouzarides 2007). More recently, some new types of modifications have been characterized, such as propionylation and butyrylation (Kebede *et al.* 2015). Most of the best-studied histone modifications are located on the N-terminal tail regions, nevertheless, other

regions of histones can be modified, such as the central globular domains. All these modifications establish the "histone code" and function through disrupting chromatin contacts or by affecting the recruitment of other non-histone proteins to chromatin. Interestingly, histone modifications can affect each other, as well as they can interact with DNA methylation (Rothbart and Strahl 2014).

DNA methylation is a common epigenetic modification of DNA in mammals. It is a mechanism involving a covalent transfer of a methyl group (CH_3) from the universal donor S-adenosyl-L-methionine (SAM) to the C-5 position of the cytosine ring by DNA methyltransferases (DNMTs) (Borchiellini *et al.* 2019). In mammals, DNA methylation can occur at cytosines in any context in the genome, however, more than 98% of this modification occurs specifically on cytosines that precede guanine – CpG sites (Lister *et al.* 2009). CpG-rich genome regions are called CpG islands (CGI), usually located in the proximity of transcription start sites (TSSs) of the ~70% of human protein-coding genes (Saxonov *et al.* 2006). The addition of the CH_3 group mediates conformational changes in the major groove of DNA, in that way altering the DNA-protein binding and further the gene expression (Liebl and Zacharias 2019). Previously, the majority of studies have been focused on the methylation effects within CGIs located in the promoter or close to TSSs of protein-coding genes. However, more recently the significance of methylation in other genomic regions have been acknowledged, such as CG shores (up to 2 kb from CGI), shelves (2-4 kb from CGI), and open sea (the rest of the genome) (Visone *et al.* 2019). The effect of DNA methylation is proved to be mostly dependent on the genomic localization. For example, methylation of TSS-associated CGIs negatively correlates with gene expression, whereas, methylation in gene-body has shown a positive correlation with gene expression (Teissandier and Bourc'his 2017).

Non-coding RNAs are defined as functional molecules that do not have the protein-coding ability. Currently, all ncRNAs can be classified as housekeeping ncRNAs and regulatory ncRNAs. The latter are further arbitrarily divided into two groups based on their size – small/ short-chain (≤200 nt) and long (>200 nt) –, and there are several subtypes within both of these groups. Most often, the following three subtypes of short ncRNAs are studied – short interfering or silencing RNA (siRNA), micro RNA (miRNA), and Piwi-interacting RNA (piRNA). Long non-coding RNAs (lncRNAs) as well can be divided into subclasses, but often these classes are not specified. It has been described that ncRNAs can interact with genes, therefore, up- or down-regulating their expression, interact with chromatin organizing proteins, guide methylation, etc. (Peschansky and Wahlestedt 2014). More recently, diverse chemical modifications of cellular RNAs have been described and termed as "RNA

epigenetics" or "epitranscriptome", covering more than 100 known types of post-transcriptional modifications RNA epigenetics (Liu and Pan 2015).

A significant characteristic of epigenetic regulation is that it is dynamic, can be modified, and is strongly affected by various environmental and behavioural factors throughout life. Such factors as environmental pollutants, physical activity levels, sleep pattern, stress levels, diet, medication use, smoking, and gut microbiome profile fluctuations have a significant effect on epigenetic patterns and their dynamics (Alegria-Torres *et al.* 2011). As most of the aforementioned factors are controllable, new lifestyle-based primary care, and preventive medicine recommendations and approaches have been developed and are definitely an emerging field at the moment (Lee *et al.* 2020). In addition, it is important to emphasize that the mechanisms of epigenetic regulation altogether cannot be viewed only individually, but as a complex regulatory system with various levels of interaction.

1.2.2. Interaction between epigenetic regulation and human gut microbiome

The current understanding of various molecular mechanisms how regulatory interactions between the host and its microbiome are implemented mostly remains elusive (Carbonero 2017). Nevertheless, studies have shown that changes in taxonomic and functional profiles of the gut microbiome correlate with epigenetic changes, moreover, various metabolites produced by microbiota, such as SCFAs, biotin, folates, and trimethylamine-N-oxide (TMAO), can regulate the epigenetic modifications of the host (Nicholson *et al.* 2012, Devaux and Raoult 2018). In mice, microbiota modulates the expression of numerous lncRNAs in various metabolic and other organs (Dempsey *et al.* 2018), as well as its modulated changes of *miR-181* expression levels in adipose tissue have been demonstrated (Virtue *et al.* 2019). In regards of DNA methylation, the action of DNMTs can be affected by metabolic activities of the microbiome, especially those involving the synthesis of metabolites participating in one-carbon metabolism, as these metabolites can further serve as methyl group donors for SAM (Mischke and Plosch 2013). In addition, communities in the gut can metabolize choline into various metabolites, including trimethylamine, which further can be metabolized into TMAO – known to be responsible for reduced methylation levels amongst other effects (Romano *et al.* 2015, Sun *et al.* 2016, Romano *et al.* 2017). As another mechanism, SCFAs, for example, butyrate, induce phosphorylation of ERK (extracellular signal-regulated kinase), which results in downregulation of DNMT1 and further demethylation of specific genes (Sarkar *et al.* 2011). The gut-derived SCFAs can also regulate the action of histone acetyltransferases and histone deacetylases, therefore, contributing to the regulation of histone modifications and chromatin

remodelling (Krautkramer *et al.* 2016, Yuille *et al.* 2018). As the epigenetic modifications are central mechanisms taking part in directing the transcriptional response to the environmental cues, they have been proposed to be a potential interface for the microbiota to implement dynamic interaction with the host genome and metabolism (Woo and Alenghat 2017).

In addition to the described systemic interactions, more insights of microbiome effects on host epigenetics have been gained in the context of colon epithelial cells (Allen and Sears 2019) and this specific case can be used as an example characterizing the complexity and significance of microbiome-epigenome interactions (Figure 3).

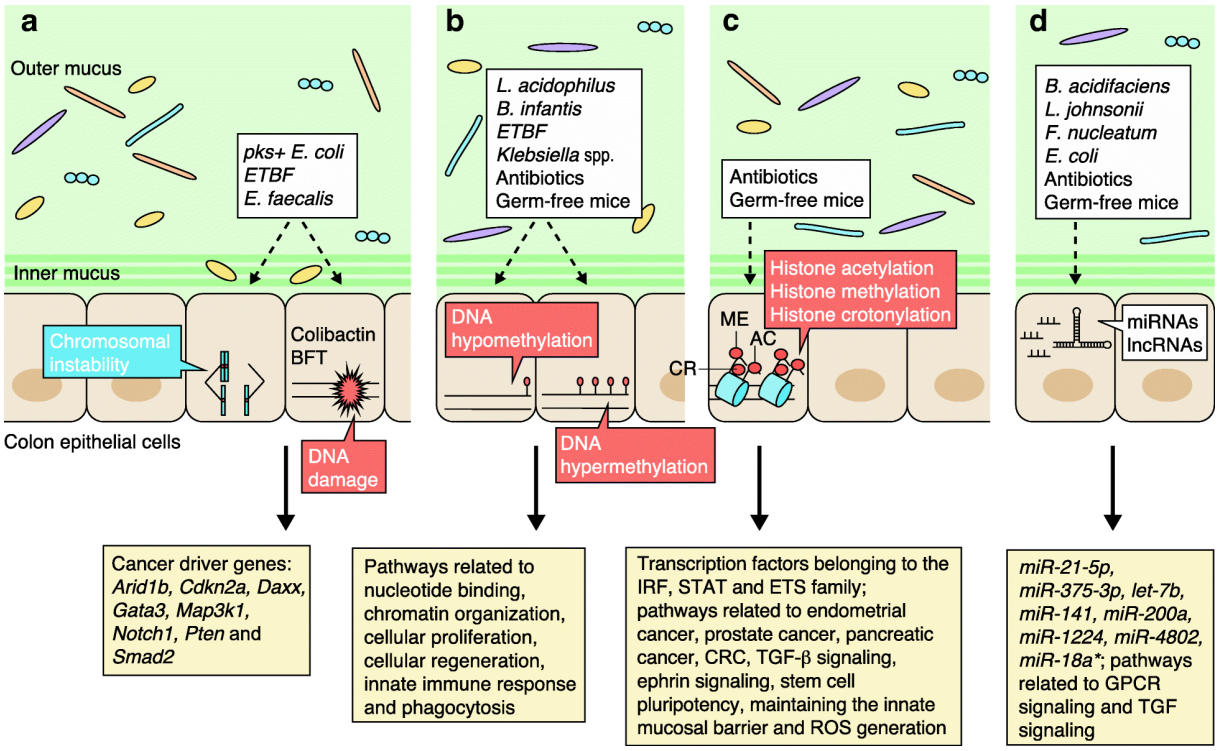


Figure 3. Effect of the gut microbiome on the colon epithelial cell (CEC) genome and epigenome. **A.** Enterotoxigenic *Bacteroides fragilis* (ETBF) and *pks* + *Escherichia coli* cause DNA damage in CECs that is mediated by *B. fragilis* toxin (BFT) and colibactin, respectively. *Enterococcus faecalis*, through the impact on macrophages, induces chromosomal instability and tumor-inducing DNA mutations in cancer driver genes. **B.** Antibiotics, germ-free mice, and specific microbes induce both the hypermethylation and the hypomethylation of genes belonging to pathways that are dysregulated in colorectal cancer (CRC). **C.** Studies with antibiotics and germ-free mice have shown – gut microbes do not generally affect global chromatin structure in CECs, but do cause changes in the accessibility of transcription factor binding sites, in histone modifications, and in the location of those modified histones. **D.** Antibiotics, germ-free mice, and specific microbes have been used to show that gut microbes

alter the expression of onco-miRNAs and anti-onco-miRNAs in CECs. They also alter the expression of long non-coding RNAs (lncRNAs) that are involved in G protein-coupled receptor (GPCR) and transforming growth factor (TGF) signalling. Abbreviations: *ETS* e26 transformation-specific, *IRF* interferon regulatory factor, *miRNA* microRNA, *ROS* reactive oxygen species, *STAT* signal transducer, and activator of transcription. Adapted from (Allen and Sears 2019).

1.3. Type 2 diabetes mellitus

1.3.1. Characterization and pathogenesis mechanisms

Diabetes Mellitus is a heterogeneous and complex group of metabolic disorders characterized by elevated blood glucose levels secondary to defects in insulin secretion, insulin action, or both. Currently, more than 460 million adults (age 20-79) worldwide have diabetes, and the prevalence of diabetes is estimated to continue to rise rapidly, as it will likely reach up to 700 million by 2045 (Kharroubi and Darwish 2015, International Diabetes Federation 2019). Approximately 5% of all cases are type 1 diabetes (T1D) – induced by autoimmune destruction of β cells of the pancreas. T1D usually develops in childhood or the teenage years (Saberzadeh-Ardestani *et al.* 2018). Also, other diabetes types of diabetes have been characterized, such as gestational diabetes (hyperglycaemia during pregnancy) (Plows *et al.* 2018) and the monogenic maturity onset diabetes of the young (MODY) (Hoffman and Jialal 2020). However, more than 90% is specifically type 2 diabetes (T2D), characterized by the presence of insulin resistance (inability of insulin-sensitive tissues to respond to insulin) with an inadequate compensatory increase in insulin secretion. The onset of T2D is usually later in life, however, the increasing obesity in adolescents has led to an increase of T2D in younger populations (Zheng *et al.* 2018). Most importantly, it is estimated that a large portion of T2D patients are still undiagnosed or the length of diagnosis is different than the true duration from the onset of T2D (International Diabetes Federation 2019).

Currently, it is known that T2D risk factors and pathogenesis mechanisms include a complex combination of genetic, metabolic, and environmental factors. Previous genome-wide association studies have shown the polygenic nature of T2D, with the discovered loci predicted to impact intermediate mechanisms of T2D pathophysiology: insulin resistance, lowering insulin secretion with normal fasting glycemia, reducing insulin secretion with fasting hyperglycemia, altering insulin processing (Dimas *et al.* 2014). A sedentary lifestyle, the energy-dense Western-style diet, and consequential obesity are the major modifiable risk

factors associated with increased insulin resistance, inflammation, and development of T2D (Bao *et al.* 2014, Bellou *et al.* 2018).

Moreover, the chronic hyperglycaemia of diabetes is associated with long-term damage and even failure of various organ systems, therefore, various diabetes complications are common to both T1D and T2D patients. Diabetes complications are mostly divided into two main groups – microvascular and macrovascular. Microvascular complications are the most common and include nephropathy, neuropathy, and retinopathy. Macrovascular complications contain cardiovascular disease, stroke, and peripheral artery disease. Other complications that cannot be classified into these two groups are occurring as well – reduced resistance to infections, dental disease, impaired wound healing, etc. (Papatheodorou *et al.* 2018).

1.3.2. T2D and gut microbiome

The human gut microbiome has been associated with the pathophysiology of most chronic diseases, including T2D. As a modifiable factor and a key element in T2D development, the gut microbiota has received significant attention in a high number of studies focusing on early T2D prediction, management, and treatment. Because of the high inter-individual and regional variability characteristic to the microbiome, as well the effects of various treatment strategies, it has been challenging to obtain consistent results across different study cohorts analysing associations between specific taxa and T2D, like many other diseases (Forslund *et al.* 2015, He *et al.* 2018). However, a recent review summarized 42 previous human microbiome studies to highlight common associations at the genus level, as a result, *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Akkermansia*, and *Roseburia* were shown to be negatively associated with T2D, while *Ruminococcus*, *Fusobacterium*, and *Blautia* were described to be positively associated with T2D. Interestingly, the widely used diversity indexes or the *Bacteroidetes/Firmicutes* ratio did not show consistent results in relation to T2D (Gurung *et al.* 2020). As the methods for gut microbiome detection have developed rapidly and costs have been reduced, the increased number of studies employing shotgun metagenomics has highlighted the need to focus on species and even strain-specific effects (Yan *et al.* 2020).

Such processes as gut permeability, inflammation, glucose and lipid metabolism, as well as energy homeostasis of the host - widely known to be regulated by gut microbiota inhabitants – have been proposed as the main molecular mechanisms of microbiome effects on T2D (Gurung *et al.* 2020). Nevertheless, numerous therapeutic applications based on microbiome modulation to improve metabolic profile of the host are continuously being created and tested. The simplest

of them being the dietary intervention, often adding prebiotics (e.g. dietary fibre) to diet, therefore, supporting growth of beneficial bacteria, such as species producing SCFAs. Usually, lifestyle modifications, which include dietary interventions, are the first step for T2D treatment. However, the response to such dietary interventions usually have variable results due to interindividual differences of microbiome composition (Marin-Penalver *et al.* 2016, Houghton *et al.* 2018). Another type of microbiome targeted T2D therapy is administration of probiotics – "live microorganisms that confer health benefits on the host when administered in proper amounts". Animal studies show positive effects on improving such parameters as insulin resistance or glucose-insulin homeostasis, nevertheless, results from human studies does not offer consensual results yet (Sun and Buys 2016, Tiderencel *et al.* 2020). As the third approach, faecal microbiota transplantation (FMT) should be mentioned. Current results show that FMT could improve insulin sensitivity, however, further conclusions are limited mostly due to small study sizes. Moreover, similarly to others the efficacy of this approach is affected by baseline microbiome composition of the recipient (Kootte *et al.* 2017). In addition to the abovementioned directly targeted microbiome modulation with aim to improve the health of the host, various antidiabetic agents have presented direct or secondary impact on the microbiome composition, which results in both beneficial and adverse effects (Kyriachenko *et al.* 2019). Overall, development of microbiome modulation approaches for T2D treatment as well as for improvement of pharmacodynamic effects for antidiabetic agents currently represent an emerging field of research and innovation (Brunkwall and Orho-Melander 2017, Gurung *et al.* 2020, Quigley and Gajula 2020).

1.4. Type 2 diabetes therapy and metformin pharmacodynamics

1.4.1. T2D therapy

A number of international guidelines have been created for the management of T2D (Aschner 2017, Doyle-Delgado *et al.* 2020). With the first choice of action being lifestyle-related modifications (improved diet, physical activities, reducing smoking), drug therapy options and algorithms have rapidly evolved. The recommended starting pharmacological treatment is metformin, following combination therapy or other alternatives in cases of metformin inefficiency, intolerance, or specific patient-related factors. Other commonly used antidiabetic medications include insulin, sulfonylureas, sodium-glucose co-transporter-2 (SGLT-2) inhibitors, glucagon-like peptide 1 (GLP-1) receptor agonists, dipeptidyl peptidase 4 (DPP-4) inhibitors, and thiazolidinediones, with each of them having specific efficacy levels, effects on cardiovascular system, weight, and risks for side effects (Quattrocchi *et al.* 2020).

1.4.2. Metformin

Metformin (1,1-dimethylbiguanide) is the first choice antidiabetic agent for the treatment of T2D, characterized by low costs and high safety. It has been widely used in clinical practice for more than 60 years and has clear benefits to not only glucose metabolism but diabetes complications as well. However, despite the long history of use and the high research interest, the mechanisms of metformin's action are still not fully understood (Rena *et al.* 2017, Lv and Guo 2020).

More interestingly, a number of studies have given insight into other therapeutic targets of metformin outside T2D therapy, such as obesity, polycystic ovary syndrome (PCOS), cancer, neurodegenerative, liver, cardiovascular, renal diseases, and even aging (Lv and Guo 2020). The main currently known underlying mechanisms for these diverse targets have been summarized in Figure 4.

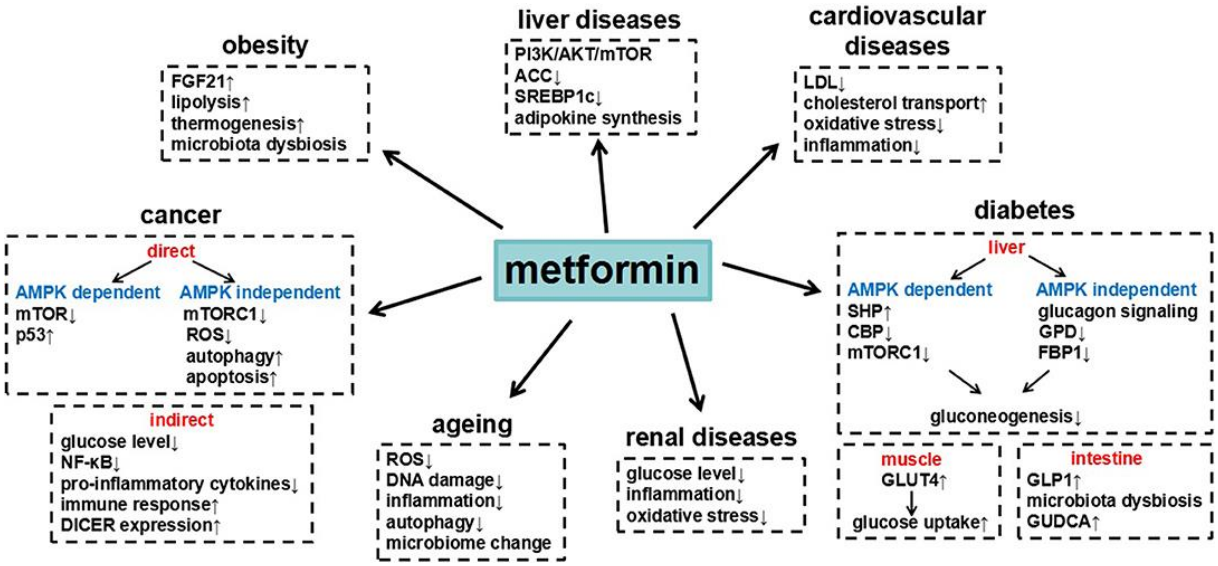


Figure 4. Summary of the major underlying mechanisms for metformin action in various diseases. FGF21 - fibroblast growth factor 21; PI3K – phosphatidylinositol-3-kinase; AKT - protein kinase B; mTOR - mammalian target of rapamycin; ACC - acetyl-CoA carboxylase; SREBP1c - sterol-regulatory-element-binding protein 1c; LDL - low-density lipoprotein; AMPK - adenosine 5'- monophosphate - activated protein kinase; SHP - small heterodimer partner; CBP - CREB binding protein; mTORC1 - mammalian target of rapamycin complex I; GDP - glycerol-3-phosphate dehydrogenase; FBP1 - fructose-1, 6-bisphosphatase-1; GLP1 - glucagon-like-peptide-1; GUDCA - glycoursodexoycholic acid; GLUT4 - glucose transporter 4; ROS - reactive oxygen species; NF-κB - nuclear factor-κB; p53 - tumor protein p53. Adapted from (Lv and Guo 2020).

The primary antidiabetic action of metformin is through the suppression of hepatic glucose production by 25-40% (Hundal *et al.* 2000) and this effect is accompanied by insulin-stimulated systemic glucose disposal (predominantly in skeletal muscle, mediated through glucose transporter 4 (GLUT4)). Moreover, metformin increases peripheral glucose utilization by the intestine, and, interestingly, it has been shown that the concentration of metformin in the jejunum reaches 30 – 300 times higher values than in the plasma (Bailey *et al.* 2008). Other effects of metformin include an increase in insulin signalling, a decrease in fatty acid and triglyceride synthesis, and an increase in fatty acid β-oxidation (Gong *et al.* 2012). It is important to note that metformin is not metabolized and, therefore, is excreted unchanged in faeces and urine (Graham *et al.* 2011).

Metformin's anti-hyperglycaemic effects are mostly exerted through AMP-activated protein kinase (AMPK)-dependent or AMPK-independent pathways. Mitochondria is characterized as one of the main molecular targets for metformin's action. Specifically, the inhibition of the mitochondrial respiratory chain Complex I (MRCC1) is widely studied. This results in suppressed ATP production and increased cellular AMP:ATP and ADP:ATP ratios, which further activate the cellular energy sensor AMPK (Vial *et al.* 2019). In addition, recently it has been shown that metformin could activate AMPK via the lysosomal pathway – the AXIN/LKB1-v-ATPase-Ragulator pathway (Zhang *et al.* 2016). AMPK activation further leads to activation of small heterodimer partner (SHP), inhibition of phosphorylation of CREB (cyclic AMP response element binding protein) binding protein (CBP), thus suppressing the expression of gluconeogenic genes (Kim *et al.* 2008, He *et al.* 2009). Moreover, AMPK-dependent inhibition of the mammalian target of rapamycin complex I (mTORC1) as well results in suppression of gluconeogenesis (Howell *et al.* 2017).

As for AMPK-independent metformin effects in T2D therapy, during the previous years, the inhibition of mitochondrial glycerophosphate dehydrogenase (mGDP) has been shown to be a significant contributor to metformin's glucose-lowering effects (Madiraju *et al.* 2014). However, the relative contributions of inhibition of MRCC1 and mGDP in metformin effects need to be clarified (Rena *et al.* 2017). Another recent discovery is the metformin's ability to directly target fructose-1,6-bisphosphatase-1 (FBP1), thus inhibiting hepatic glucose production (Hunter *et al.* 2018).

In addition to a number of effects in the liver and muscles, metformin stimulates GLP-1 release in the intestine (both fasting and postprandial) by the enteroendocrine L cells, thus enhancing insulin secretion (Mannucci *et al.* 2004, Holst 2007, Bahne *et al.* 2018). Moreover, the gut microbiota has been characterized and is continuously being studied as one of the central targets

and mediators for metformin effects in T2D and other diseases, as well as a significant contributor to both efficacy and tolerance of metformin (McCreight *et al.* 2016).

1.4.3. Metformin pharmacodynamics using multi-omics

Pharmacodynamics is the study of a drug's molecular, biochemical, and physiologic effects or actions (Marino and Zito 2020). The advances in technological opportunities have supported the rapid development of medication pharmacodynamics studies with a recent focus on the application of various –omics based approaches. Therefore, a new direction – pharmaco-omics – has been emerged and it offers new promises for the development of optimized and individualized treatment strategies (Weinshilboum and Wang 2017). In addition, since 2010 a more specific field focusing on interactions between drugs and the microbiome has been defined as "pharmacomicrobiomics", which investigates the multiple levels of variation represented by the microbiome components and its complexity that may have effects on drug response and disposition (Rizkallah *et al.* 2010, Doestzada *et al.* 2018).

A number of *in vitro* and animal experiments on metformin pharmacodynamics have been implemented (Luizon *et al.* 2016, Udhane *et al.* 2017, Meng *et al.* 2020), moreover, the data in human studies have accumulated significantly as well. Metformin effects on the human body have been widely studied with a focus on such –omics as genomics (Zhou *et al.* 2016), metabolomics (Safai *et al.* 2018), transcriptomics (Ustinova *et al.* 2019, Ustinova *et al.* 2020), epigenomics (Garcia-Calzon *et al.* 2017, Zhong *et al.* 2017, Garcia-Calzon *et al.* 2020), microbiomics (Forslund *et al.* 2015, Wu *et al.* 2017, Vallianou *et al.* 2019), and with a combination of these approaches.

Human gut microbiome studies regarding the metformin effects present a wide range of results, which are often different from animal studies and even present inconsistencies across the human study populations, however, some common effects have been clarified (Zhang and Hu 2020). It has been shown that metformin therapy reverses many of the bacterial changes occurring during T2D. The most common change in microbiota reported is the metformin-induced increase in abundance of *Akkermansia muciniphila* and other SCFA-producing bacteria such as *Bifidobacterium, Lactobacillus, Blautia* (de la Cuesta-Zuluaga *et al.* 2017, Wu *et al.* 2017). The increased ability of microbiota to produce SCFAs has been stated as one of the central mechanisms behind metformin's beneficial effects in the gut as well as at a systemic level. Moreover, the probiotic effects of *Akkermansia muciniphila* includes maintaining the integrity of the intestinal mucosa and regulation of host metabolism and immune responses, therefore,

the abundance of this species has been negatively correlated with obesity, diabetes, cardiovascular and immune diseases (Cani and de Vos 2017, Zhang *et al.* 2019). In addition, several studies in obese individuals or T2D patients, as well as healthy individuals have shown significant increase in abundance of *Escherichia* spp. (Forslund *et al.* 2015, Wu *et al.* 2017, Bryrup *et al.* 2019, Ejtahed *et al.* 2019), reduction in abundance of *Intestinibacter* spp. (Forslund *et al.* 2015, Wu *et al.* 2017, Bryrup *et al.* 2019), and changes in proportions of various *Bacteroides* species during metformin therapy (Paley *et al.* 2017, Sun *et al.* 2018).

Regarding the mechanisms behind the interaction between metformin and the habitants of microbiota, *in vitro* studies have demonstrated direct metformin effects on promoting the growth of such bacteria as *Akkermansia muciniphila* and *Bifidobacterium adolescentis*, and other interaction mechanisms have been proposed to be mediated through changes in bacteria-to-bacteria interactions or physiological/environmental conditions (Wu et al. 2017). As another significant mechanism of interaction the metformin's ability to impact the bile acid pool through changing the microbiome composition has been demonstrated. A recent study in T2D patients showed that metformin reduces the abundance of *Bacteroides fragilis* and its bile salt hydrolase activity, therefore, increasing levels of the bile acid glycoursodeoxycholic acid (GUDCA). In such way metformin acts through *B. fragilis*–GUDCA–intestinal FXR (farnesoid X receptor) axis which results in AMPK-independent improvement of glucose intolerance and insulin resistance (Sun *et al.* 2018).

Studies about metformin interaction with DNA methylation profile have been implemented only recently. It has been shown that metformin induce both hypomethylation and hypermethylation at the promoters of different genes. The results suggest that metformin can modify DNA methylation, possibly via regulation of the H19/SAHH axis (Zhong *et al.* 2017) or by AMPK-mediated inhibition of DNMT1 (Marin *et al.* 2017). Also, AMPK mediated effects on the global levels of 5-hydroxymethylcytosine (5hmC) has been proven, as metformin has a significant impact on the glucose-AMPK-TET2-5hmC axis, therefore, increasing the global 5hmC levels and proposing one of the key mechanisms for metformin's cancer preventing actions (Wu *et al.* 2018). Interestingly, reduced DNA methylation levels of transporter genes SLC22A1, SLC22A3, and SLC47A1 was observed in liver tissue of T2D patients on metformin therapy compared to subjects without antidiabetic treatment (Garcia-Calzon *et al.* 2017). However, the specific mechanisms and functional consequences of metformin's effects on DNA methylation and further on gene expression in still not fuly understood (Bridgeman *et al.* 2018).

2. MATERIALS AND METHODS

2.1. Study design and sample collection

2.1.1. Summary of all three publications

The study design describing the analysed cohorts of healthy individuals and newly-diagnosed T2D patients is summarized in figure 5.

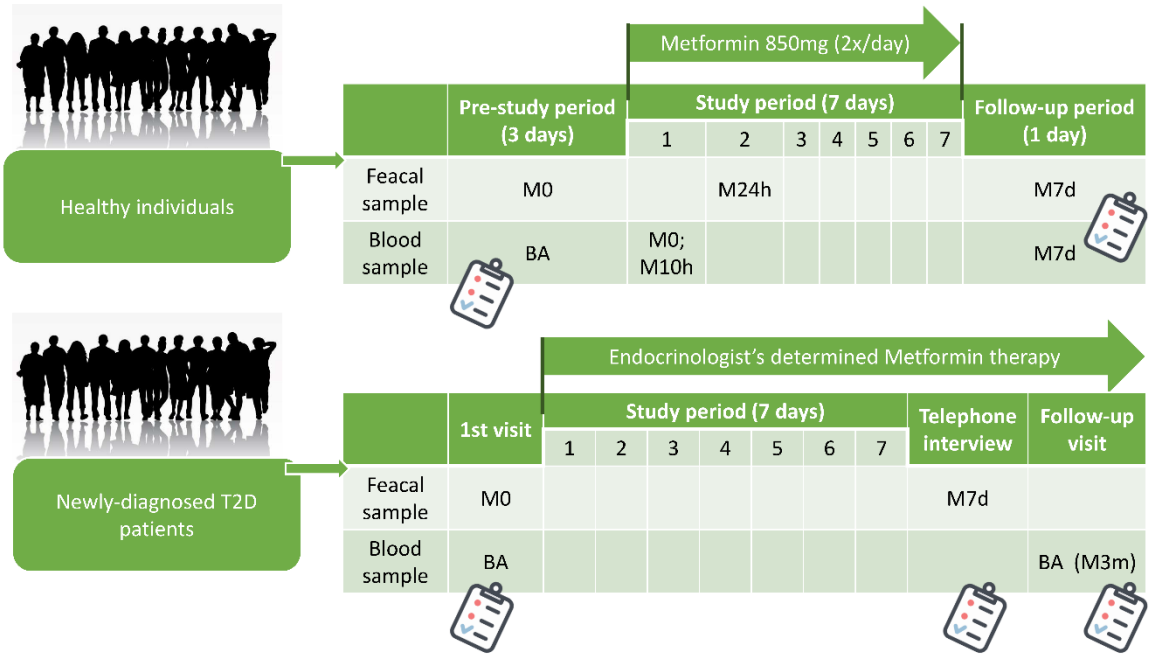


Figure 5. Visual representation of the study design summarizing cohorts analysed in three publications. Samples and visits were coded as follows: M0 – before starting metformin therapy; M10h – 10 hours after first metformin dose (but before the second dose); M24h – 24 hours after starting metformin therapy; M7d – after 7 days long metformin therapy; M3m – after three months of metformin therapy; BA – blood sample for biochemical/haematological analysis.

Study participants were recruited through the Genome Database of Latvian Population (Rovite *et al.* 2018). All samples and data from healthy individuals were obtained in the framework of a clinical trial (registration number: 2016-001092-74 (www.clinicaltrialsregister.eu)). The newly-diagnosed T2D patients were recruited within the framework or OPTIMED study. Informed consent was obtained from all participants at the beginning of the study. Inclusion/exclusion criteria for both cohorts are summarized in Apendix 1. The study was carried out in accordance with the principles of the Declaration of Helsinki, and approved by the Central Medical Ethics Committee (1/19-10-22) and State Agency of Medicines of the Republic of Latvia (17–1723).

Blood samples for hematological and biochemical analyses were collected in the fasting state 1–3 days before starting metformin administration. Data were used to evaluate significant health indicators for kidney and liver function, as well as other criteria characterizing the suitability of individuals for medicament therapy. All hematological and biochemical analyses were conducted in the same certified clinical laboratory. For the patient cohort, a repeated biochemical/hematological analysis was performed three months later (follow-up coded as a time point M3m).

2.1.2. Publication I: Association of metformin administration with gut microbiome dysbiosis in healthy volunteers

Eighteen healthy volunteers of Caucasian origin were included in this study. Participants took metformin (850 mg tablets; Berlin-Chemie AG, Germany) twice daily during meals with a glass of water for a period of 7 days. Diet, physical activities, and side effects were registered daily in specific questionnaires during the whole study period. Dietary data were registered using a 7-day food record during the week of metformin use, and an additional 2-day food record was filled before starting the use of metformin.

Stool samples in two aliquots were collected at three time points: before starting metformin treatment (M0) and 24 hours (M24h) and 7 days (M7d) after the first intake of metformin. After collection, faecal samples were stored at room temperature until delivery to the laboratory, and frozen at −80˚C as soon as possible but not later than within 24 hours of collection. Sample collection, storage, and handling were done by following the developed standard operating procedures with the aim to minimize unnecessary freezing and thawing cycles and to reduce the possibility of artefacts caused by temporary storage at room temperature.

2.1.3. Publication II: Significantly altered peripheral blood cell DNA methylation profile as a result of immediate effect of metformin use in healthy individuals

The study group involved 12 healthy metformin-naïve voluntary individuals. The research subjects received an 850-mg metformin tablet (Berlin-Chemie AG) twice a day for a week. Whole blood samples for methylation analysis were collected by certified medical personnel at three time points: (1) before starting metformin therapy (morning, fasting state) — M0, (2) 10h after first metformin intake, before the second tablet (evening) — M10h, and (3) after 7 days of metformin administration (morning, fasting state) — M7d.

2.1.4. Publication III: Baseline gut microbiome composition predicts metformin therapy short term efficacy in newly diagnosed type 2 diabetes patients

The study involved two longitudinal cohorts of participants: OPTIMED cohort of newly-diagnosed T2D patients (N=50) and a cohort of healthy individuals (N=35). Healthy individuals received 850mg metformin twice a day for 7 days, while T2D patients were treated with metformin monotherapy according to therapy prescribed by an endocrinologist (individual dosage, titration, etc.).

Stool samples were collected in two aliquots at pre-determined time points during the study, depending on the design for each study cohort. Samples were coded as follows: M0 – before metformin treatment, M24h – 24 hours after the first metformin dose (only in the study group of healthy individuals), and M7d – 7 days after starting the therapy. All samples were collected by participants at home, using sterile collection tubes without buffer (collection date and time were marked). Within 24 hours participants delivered samples to the closest clinical or research laboratory where samples were frozen at −80°C (delivery time was registered).

The information on anthropometric measurements, dietary habits, and biochemical/hematological analyses was obtained before starting metformin administration. Healthy volunteers registered their diet during the metformin administration, as well as any observed SE in special questionnaires. Patients of OPTIMED cohort were interviewed via phone by their endocrinologists after the first week of metformin therapy to register possible metformin-induced SE.

For the analysis of gut microbiome mediated metformin's therapy efficacy patients were divided into two subgroups based on the observed reduction of HbA_{1c} during three months long metformin therapy. Patients were defined as Responders if their HbA_{1c} levels had decreased by ≥12.6 mmol/mol (1%), or Non-responders if their HbA_{1c} levels had decreased by <12.6 mmol/mol (1%). This threshold has been previously established within a systematic review comparing three months long metformin therapy with placebo and used in other studies as well (Sherifali *et al.* 2010, Kashi *et al.* 2016).

2.2. Isolation of DNA

2.2.1. Microbial DNA

Microbial DNA was extracted from frozen stool samples using FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) and FastPrep Instrument according to the instructions of the manufacturer.

2.2.2. Human DNA

DNA isolation from whole blood samples collected from healthy participants was performed using the phenol-chloroform extraction method by Genome Database of Latvian Population as described before (Rovite *et al.* 2018).

2.3. Gut microbiome analysis

2.3.1. 16S rRNA amplicon library preparation and massive parallel sequencing

For each microbial DNA sample, the V3 region of the 16S rRNA gene was amplified using the Probio_Uni/Probio_Rev primer set (Milani *et al.* 2013). The amplified PCR products were purified using NucleoMag magnetic beads (Macherey-Nagel, Du¨ren, Germany), and their quantity and quality were evaluated with the Agilent 2100 Bioanalyzer DNA High Sensitivity chip (Agilent Technologies, Santa Clara, CA, USA). Sequencing of the amplicon libraries was performed with Ion Torrent Personal Genome Machine (PGM) System (Thermo Fisher Scientific; Ion 318 Chip Kit v2, Ion PGM Hi-Q Sequencing Kit, minimal sequencing depth per sample – 250 000 reads) according to the instructions of the manufacturer.

2.3.2. Shotgun metagenome library preparation and massive parallel sequencing

Shotgun metagenomic library preparation was done by fragmenting the microbial DNA at 300 bp (Covaris) and following the manual of the Ion Plus Fragment Library kit (ThermoFisher Scientific, USA). That included the following sample processing steps: (1) end repair after the physical fragmentation and clean-up with NucleoMag magnetic beads (Macherey-Nagel, Düren, Germany), (2) adaptor ligation, nick repair, and clean-up, (3) size selection in the range 360 – 440 bp, performed with BluePippin DNA 2% Dye-Free Agarose gel cassette with V1 Marker, and clean-up, and (4) amplification and clean-up. Samples were sequenced using Ion Proton sequencer with Ion PI Chip Kit v3 (>3 000 000 reads/sample).

2.4. DNA methylation analysis and RNA expression validation

The extracted human DNA samples were quantified with Qubit® 2.0 Fluorometer using Qubit dsDNA HS Assay Kit (TherfmoFisher Scientific, USA). For the bisulfite conversion, the EZ DNA Methylation-Gold TM kit (Zymo research, USA) was used according to the manufacturer's instructions. DNA methylation was determined by the Illumina Infinium

HumanMethylation450 BeadChip Array (Illumina, USA), using 500 ng of each bisulfite-treated DNA sample.

Total RNA for validation analysis was isolated from whole blood samples using PerfectPure RNA Blood Kit (5Prime GmbH, Ham- burg, Germany). Ribosomal RNS depletion was done with Low Input RiboMinus ™ Eukaryote System v2 (Thermo Fisher Scientific, USA). For cDNA library preparation, we used Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific, USA), and sequencing was performed on the Ion Proton ™ System and Ion PI ™ Chip (Thermo Fisher Scientific, USA).

2.5. Bioinformatical and statistical analyses

2.5.1. Publication I: Association of metformin administration with gut microbiome dysbiosis in healthy volunteers

Raw sequence data were processed using mothur software v.1.39.1 (Schloss *et al.* 2009). Analyses were done using a modified version of the publicly accessible MiSeq SOP. In the sequence filtering, step reads were removed if they were 75 bp or shorter, or contained ambiguous bases or homopolymers longer than eight bases. A representative sequence from each cluster was chosen and used to identify taxonomic groups from the SILVA database v.123 (Quast *et al.* 2013); the flip parameter was set as true. Chimeric sequences and sequences containing potential sequencing errors were removed using UCHIME (Edgar *et al.* 2011) or pre-clustering (threshold = 2), respectively. Operational taxonomic units were defined at ≥99 % sequence identity, using the OptiClust algorithm. Reads were classified using the naïve Bayesian classifier (Wang *et al.* 2007).

The correlation between gut microbiome taxa and the defined food groups was evaluated with Spearman's correlation analysis and the results were adjusted for multiple testing using the Benjamini–Hochberg method.

Statistical analyses were performed on taxonomic units found in at least 50% of samples with R program v.3.2.2 packages edgeR, limma, phyloseq, DESeq, vegan (adjustment for multiple testing by Benjamini–Hochberg method), and graphics were created with package ggplot2. Sample normalization was done as implemented in edgeR (calcNormFactors function) or the relative abundances were used if necessary. Additional analysis to detect differential abundance was performed using the Linear discriminant analysis Effect Size (LEfSe) method (Segata *et al.* 2011) integrated in the Galaxy framework. In particular, the non-parametric Kruskal–Wallis sum-rank test was used to detect differentially abundant taxa, and Linear Discriminant Analysis (LDA) was used to estimate the effect size. The genus level alpha diversity of each sample was

calculated by the Shannon index (Shannon 1948), beta diversity across samples was evaluated with non-metric multidimensional scaling (NMDS) using Bray–Curtis distances. Permutational multivariate analysis of variance (PERMANOVA) was used (permutations = 9999) for comparing the analyzed groups of ordinations. Statistical significance for changes of Shannon index and for taxonomic units between specific sample groups was evaluated by Wilcoxon signed-rank test.

2.5.2. Publication II: Significantly altered peripheral blood cell DNA methylation profile as a result of immediate effect of metformin use in healthy individuals

For methylation data analysis, IDAT files were imported using R package minfi (Aryee *et al.* 2014). Cell counts were estimated from methylation data using Houseman algorithm (Houseman *et al.* 2012) implemented in minfi.

Data preprocessing and normalization was done using Enmix (Xu *et al.* 2016). Briefly, probes with detection p-value >0.05 and probes with a multimodal distribution were filtered out. Background correction was performed with the function preprocessENmix using unused color channels as a background parameter estimate. Probe intensities were normalized using a quantile normalization method and probe type bias was adjusted using the Regression on Correlated Probes (RCP) method (Niu *et al.* 2016). Probes having a SNP or single base extension annotation in CpG site were excluded. Due to interrupted use of metformin by one of the study subjects, the sample taken after 1 week of metformin administration for that particular subject was discarded.

Batch effect was removed from data using slide and subsequently subjects as covariates as they showed the strongest influence on the probe methylation variability. Batch effect was removed using ComBat (Johnson *et al.* 2007) wrapped in the Enmix package. Differentially methylated probes between time points were identified using limma (Ritchie *et al.* 2015) on ComBat preprocessed data, adjusting for the following cell types estimated by minfi: CD8T, CD4T, NK and Gran. Inflation factor of p-value distribution was estimated using R package GenABEL (Aulchenko *et al.* 2007). All analyses were performed using R (3.3.3).

Statistically significant differentially methylated regions (DMRs) were identified with DMRcate software (Peters *et al.* 2015), FDR <0.05. Threshold for minimum number of probes within the region was set to three. DMRs were estimated from methylation M-values using the individual CpG site significance threshold at FDR <0.05. The interval between individual significant CpG sites had to be less than 1000 bp in the regions. The bandwidth scaling factor

was set as suggested in the manual (C=2). Regulatory information from Ensembl 91 regulation resources was added to identified differentially methylated probes (DMPs) and DMRs using Ensembl Regulation API (Zerbino *et al.* 2016).

Pathway enrichment analysis was performed with the IPA tool (Kramer *et al.* 2014). Information about enriched canonical pathways and networks was obtained performing the core analysis on all significant DMPs with FDR <0.05.

For RNA-seq data analysis, reads were mapped against human reference genome GRCh38 and read quantification was performed using STAR (2.5.3a) (Dobin *et al.* 2013). Obtained per-gene read counts were normalized using trimmed mean normalization and counts per million (CPM) values were calculated with edgeR (Robinson *et al.* 2010). ComBat (Johnson *et al.* 2007) implemented in R package sva (Leek *et al.* 2012) was used to adjust CPM values for subject specific effects and the Spearman correlation was estimated for the adjusted CPM values and the beta values for 11 selected CpG sites with SciPy (Olivier *et al.* 2002).

2.5.3. Publication III: Baseline gut microbiome composition predicts metformin therapy

Raw data from the sequencer were processed as follows: adapters were removed with cutadapt 1.16, sequences were trimmed with Trimmomatic v0.38 (5bp window, quality threshold = 20, average quality = 20, minimal length = 75), mapping was performed with bowtie2-2.3.5.1 using Homo sapiens genome Ensembl GRCh38 release-90 reference to remove host DNA sequences.

Composition and functionality from the remaining sequences of gut microbiome samples were analyzed using the HUMAnN2 pipeline (Franzosa *et al.* 2018), and taxonomic data were obtained with MetaPhlAn2 (Truong *et al.* 2015), analyses were performed with default parameters. Species level alpha diversity was calculated as the exponential of the Shannon index resulting in the effective number of species, and beta diversity was analyzed with NMDS using Bray-Curtis distances. Results of beta diversity were compared between subgroups with PERMANOVA. To explain the effects of environmental variables, adonis function (vegan package) was used to test the significance of individual variables, and complemented with Canonical Correspondence Analysis and visualized with biplot using R software (version 3.6.0) (Torondel *et al.* 2016). Evaluation of variables of interest was performed in two cases: (1) for all samples – both groups, baseline and follow up – to evaluate the contribution of age, gender and BMI; (2) only for T2D patient samples – to evaluate possible effect of the different prescribed metformin doses. Changes during metformin therapy and differences between study

subgroups within the taxonomic and functional profiles were evaluated by R package *limma* using *voom* transformation with sample-specific quality weights (further referred as limma+voom). All tests were adjusted by age, gender, and BMI, false discovery rate (FDR) adjusted values were used. T2D group data were adjusted by baseline HbA_{1c} levels. Only taxa present in ≥10% of samples were included. To compare metformin therapy response groups, the corrected data matrix was used for sparse Partial least squares discriminant analysis (sPLS-DA), a supervised model to reveal microbiota variation between groups. Key taxonomic groups responsible for the differential microbiota structure were detected using the "splsda" function in the R package "mix Omics" (Le Cao *et al.* 2011), tuning of sPLS-DA parameters was performed to determine the main taxonomic groups that enable discrimination of the subgroups with the lowest possible error rate. Taxonomic groups with variable importance in projection (VIP) > 1.5 were considered to be important contributors to the model. Additional cellular function enrichment analysis and visualization of functional profile data were performed using the Omics Dashboard integrated into MetaCyc (Paley *et al.* 2017). Statistical significance for changes/differences of the Shannon index and other analyzed parameters was evaluated by the Wilcoxon signed-rank test. Data normalizations were performed as integrated into the used tools, paired comparisons were used when appropriate.

3. RESULTS

I Association of metformin administration with gut microbiome dysbiosis in healthy volunteers

Highlights:

1. The metformin-induced reduction of inner diversity was observed at the markedly short time-period of 24 hours.
2. Individuals with side-effects had higher abundance of the opportunistic pathogen *Escherichia-Shigella* spp. before starting the metformin, and the inner diversity in the M7d sample compared to M24h sample increased only in the groups with side effects together with the abundance of *Escherichia-Shigella* spp.
3. Metformin administration induced reduction in abundance of the family *Peptostreptococcaceae* and three genera within it.
4. We did not observe a significant increase in abundance of *Akkermansia* spp. after correction.

PLOS ONE

RESEARCH ARTICLE

Association of metformin administration with gut microbiome dysbiosis in healthy volunteers

Ilze Elbere[1], Ineta Kalnina[1], Ivars Silamikelis[1], Ilze Konrade[2], Linda Zaharenko[1], Kristine Sekace[1], Ilze Radovica-Spalvina[1], Davids Fridmanis[1], Dita Gudra[1], Valdis Pirags[1,3], Janis Klovins[1,4]*

1 Latvian Biomedical Research and Study Centre, Riga, Latvia, **2** Riga East Clinical University Hospital, Riga, Latvia, **3** Department of Endocrinology, Pauls Stradins Clinical University Hospital, Riga, Latvia, **4** University of Latvia, Faculty of Biology, Riga, Latvia

* klovins@biomed.lu.lv

OPEN ACCESS

Citation: Elbere I, Kalnina I, Silamikelis I, Konrade I, Zaharenko L, Sekace K, et al. (2018) Association of metformin administration with gut microbiome dysbiosis in healthy volunteers. PLoS ONE 13(9): e0204317. https://doi.org/10.1371/journal.pone.0204317

Editor: Alan Landay, Rush University, UNITED STATES

Received: January 25, 2018

Accepted: August 25, 2018

Published: September 27, 2018

Data Availability Statement: All 16S rRNA sequence files are available from the "EBI Metagenomics" database (https://www.ebi.ac.uk/metagenomics/) under the study accession number PRJEB24497.

Funding: The study was funded by The National Research programme „Biomedicine for Public Health" (BIOMEDICINE). Project No 2 „Molecular Mechanisms, Pharmacogenetics and New Medicines for Treatment of Diabetes and Cardiovascular Complications". The funders had no

Abstract

Background

Metformin is a widely used first-line drug for treatment of type 2 diabetes. Despite its advantages, metformin has variable therapeutic effects, contraindications, and side effects. Here, for the very first time, we investigate the short-term effect of metformin on the composition of healthy human gut microbiota.

Methods

We used an exploratory longitudinal study design in which the first sample from an individual was the control for further samples. Eighteen healthy individuals were treated with metformin (2 × 850 mg) for 7 days. Stool samples were collected at three time points: prior to administration, 24 hours and 7 days after metformin administration. Taxonomic composition of the gut microbiome was analyzed by massive parallel sequencing of *16S rRNA* gene (V3 region).

Results

There was a significant reduction of inner diversity of gut microbiota observed already 24 hours after metformin administration. We observed an association between the severity of gastrointestinal side effects and the increase in relative abundance of common gut opportunistic pathogen *Escherichia-Shigella* spp. One week long treatment with metformin was associated with a significant decrease in the families *Peptostreptococcaceae* and *Clostridiaceae*_1 and four genera within these families.

Conclusions

Our results are in line with previous findings on the capability of metformin to influence gut microbiota. However, for the first time we provide evidence that metformin has an immediate effect on the gut microbiome in humans. It is likely that this effect results from the increase in

role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

abundance of opportunistic pathogens and further triggers the occurrence of side effects associated with the observed dysbiosis. An additional randomized controlled trial would be required in order to reach definitive conclusions, as this is an exploratory study without a placebo control arm. Our findings may be further used to create approaches that improve the tolerability of metformin.

Introduction

Metformin is a biguanide agent that is widely used as a first-line treatment of type 2 diabetes (T2D) [1]. Metformin has several advantages, including high safety indicators, high efficacy, neutral or lowering effect on body mass, and cardioprotective effects [2–4], resulting in broad indications for use over the 60 years it has been on the market. Nevertheless, metformin also has variable therapeutic effects, contraindications, and side effects which indicate the urgent need for a personalized approach when choosing treatment strategies [5].

It has been shown that intravenously administered metformin is less effective than its orally administered form [6]. Furthermore, metformin reaches a 30–300 times higher concentration in mucosa of small intestine compared to plasma, and up to 30% of the drug is eliminated through the feces [7, 8]. In addition, a delayed-release formulation of metformin improves glycemic control to the same extent as the immediate-release form despite lower systemic exposure [9]. These findings have led to the hypothesis that the effects of metformin are partially explained by its interaction with the gut microbiome. The connection between the effects of metformin and the gut microbiome has been supported by several recent studies [10–17]. These studies suggest that the gut microbiome is involved in both the therapeutic and side effects of the drug, yet details of this interaction remain obscure.

Current knowledge regarding the interaction between metformin and the gut microbiome highlights that metformin reduces inner diversity of the gut microbiome in mice fed a high-fat diet [13] and its administration increases relative abundance of *Akkermansia muciniphila* [10–14]. There is also evidence that metformin increases the abundance of some other mucin degrading and short-chain fatty acids producing genera [10], as well as opportunistic pathogens such as *Escherichia* spp. [11, 12]. Modulation of the gut microbiome is also hypothesized to be responsible for the anti-obesity action of metformin, not only in T2D patients but in prediabetic populations as well [18].

However, as pointed out previously, many of the earlier studies of the gut microbiome did not control for treatment regimens in T2D patients, subsequently leading to divergent conclusions [11]. It appears plausible that some of the potential clinical effects, e.g., metabolic control of longevity [19], anticancer properties [20], and testosterone lowering in patients with polycystic ovary syndrome [21] occur through alterations in the microbiome. Therefore, in this exploratory longitudinal study we evaluated the short-term effect of oral metformin administration on the human gut microbiome composition and diversity in healthy individuals, and the possible connection between these changes and metformin-related gastrointestinal (GI) side effects.

Materials and methods

Study design

Eighteen healthy volunteers of Caucasian origin were included in this exploratory study through the Genome Database of Latvian Population [22] as a part of an ongoing clinical trial

(50 individuals to be included in total), by assessing the 25 individuals available at the time. Baseline characteristics and registered clinical parameters are shown in Table 1. Major exclusion criteria were as follows: (1) use (during the past two months) of antibiotics, immunosuppressive drugs, corticosteroids, proton pump inhibitors, or pharmaceutical-grade probiotics; (2) oncological, autoimmune, or chronic gastrointestinal tract diseases, or T2D; (3) diarrhea in the past week; and (4) use of any other medications that are not compatible with metformin. A full list of inclusion/exclusion criteria can be found in the S1 Text. All participants, after full explanation of the purpose and nature of all procedures used, gave signed informed consent containing detailed information on the project (Fig 1). The study was carried out in accordance with the Declaration of Helsinki, and approved by the Central Medical Ethics Committee (1/16-05-12) and State Agency of Medicines of the Republic of Latvia (17–1723), clinical trial registration number: 2016-001092-74 (www.clinicaltrialsregister.eu).

Participants took metformin (850 mg tablets; Berlin-Chemie AG, Germany) twice daily during meals with a glass of water for a period of 7 days. Diet, physical activities, and side effects were registered daily in special questionnaires during the whole study period. Dietary data were registered using a 7-day food record during the week of metformin use, and an additional 2-day food record was filled before starting the use of metformin. We consulted a certified nutritionist and data from the dietary registry were divided into 11 food groups and labeled as follows: (1) milk and dairy products, (2) vegetables, (3) fruits, (4) meat and its products, (5) fish, (6) croppers, (7) nuts and seeds, (8) fat, (9) snacks, (10) sweetened drinks, and (11) alcohol. The cumulative summary characterizing the 7-day food records for each food group was expressed as a percentage from the combined amount of food consumption during the metformin treatment (S2 Table).

The primary endpoint of this study was the detection of significant changes in taxonomical composition of the gut microbiome. The secondary endpoint was the possible correlation between specific taxonomic units and the development of GI side effects. Compliance with the study was ensured by thorough explanation and detailed written instructions of the study protocol. Unused tablets were returned to the principal investigator.

All individuals were concurrently involved in an ongoing methylation profile analysis in leukocytes from whole blood samples taken at three specific time points during the study (unpublished data).

Sample collection

Blood samples for hematological and biochemical analyses were collected in the fasting state 1–3 days before starting metformin administration. Data were used to evaluate significant health indicators for kidney and liver function, as well as other criteria characterizing the

Table 1. Characteristics of the study group.

Characteristic	Value
Females/Males, n (%)	11 (61.1%)/ 7 (38.9%)
Age (years), median [IQR]	25.5 [7.5]
BMI, median [IQR]	24.2 [3.5]
ALAT (U/l), median [IQR]	20.5 [10.8]
Creatinine (μmol/l), median [IQR]	71.5 [13.5]
Fasting plasma glucose (mmol/l), median [IQR]	5.1 [0.5]

ALAT–alanine aminotransferase, BMI–body mass index, IQR–interquartile range

https://doi.org/10.1371/journal.pone.0204317.t001

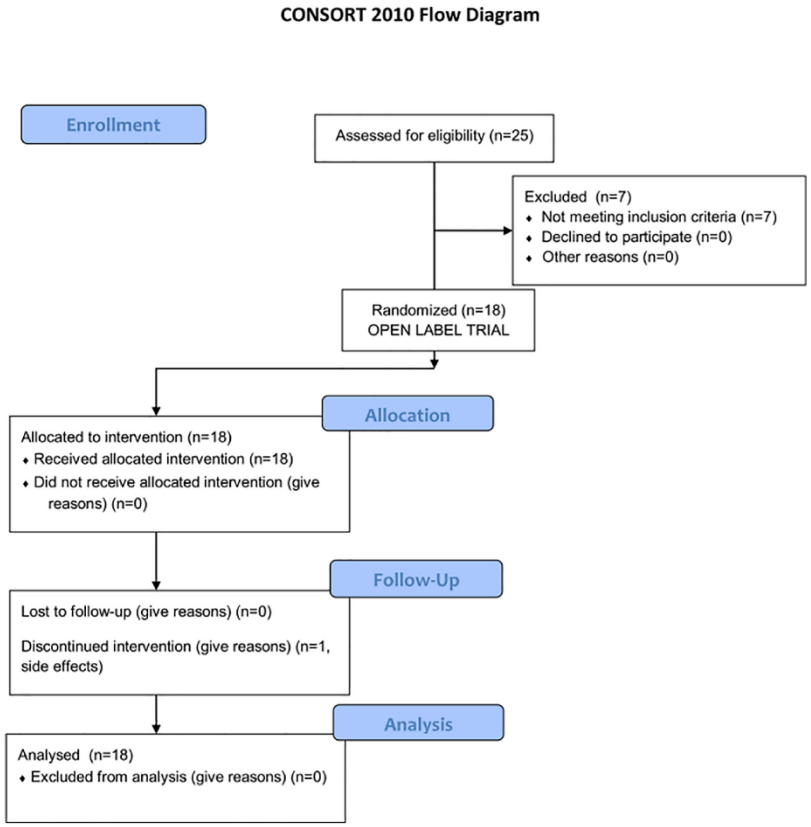


Fig 1. CONSORT flowchart of the open-label trial.

https://doi.org/10.1371/journal.pone.0204317.g001

suitability of individuals for medicament therapy. All hematological and biochemical analyses were conducted in the same certified clinical laboratory.

Stool samples in two aliquots were collected at three time points: before starting metformin treatment (M0) and 24 hours (M24h) and 7 days (M7d) after the first intake of metformin. After collection, fecal samples were stored at room temperature until delivery to the laboratory, and frozen at −80˚C as soon as possible but not later than within 24 hours of collection [23, 24]. Sample collection, storage and handling were done by following our developed standard operation procedures with the aim to minimize unnecessary freezing and thawing cycles and to reduce the possibility of artefacts caused by temporary storage at room temperature.

Bacterial DNA preparation and sequencing analysis

Microbial DNA was extracted from frozen stool samples using FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) and FastPrep Instrument according to the instructions of the manufacturer. DNA concentrations of the extracted samples were evaluated using Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity of the extracted microbial DNA was validated by agarose gel electrophoresis.

For each sample, the V3 region of the *16S rRNA* gene was amplified using the Probio_Uni/Probio_Rev primer set [25]. Each primer contained IonXpress adapter sequence and a unique barcode sequence. The amplified PCR products were purified using NucleoMag magnetic beads (Macherey-Nagel, Düren, Germany), and their quantity and quality were evaluated with the Agilent 2100 Bioanalyzer DNA High Sensitivity chip (Agilent Technologies, Santa Clara, CA, USA). Sequencing of the amplicon libraries was performed with Ion Torrent Personal Genome Machine (PGM) System (Thermo Fisher Scientific; Ion 318 Chip Kit v2, Ion PGM Hi-Q Sequencing Kit, minimal sequencing depth per sample– 250 000 reads) according to the instructions of the manufacturer.

Preprocessing and statistical methods

Raw sequence data were processed using mothur software v.1.39.1 [26]. Analyses were done using a modified version of the publicly accessible MiSeq SOP. In the sequence filtering, step reads were removed if they were 75 bp or shorter, or contained ambiguous bases or homopolymers longer than eight bases. A representative sequence from each cluster was chosen and used to identify taxonomic groups from the SILVA database v.123 [27]; the flip parameter was set as true. Chimeric sequences and sequences containing potential sequencing errors were removed using UCHIME [28] or pre-clustering (threshold = 2), respectively. Operational taxonomic units were defined at ≥99% sequence identity, using the OptiClust algorithm. Reads were classified using the naïve Bayesian classifier [29].

The correlation between gut microbiome taxa and the defined food groups was evaluated with Spearman's correlation analysis and the results were adjusted for multiple testing using the Benjamini–Hochberg method.

Statistical analyses were performed on taxonomic units found in at least 50% of samples with R program v.3.2.2 packages edgeR, limma, phyloseq, DESeq, vegan (adjustment for multiple testing by Benjamini–Hochberg method), and graphics were created with package ggplot2. Sample normalization was done as implemented in edgeR (calcNormFactors function) or the relative abundances were used if necessary. Additional analysis to detect differential abundance was performed using the Linear discriminant analysis Effect Size (LEfSe) method [30] integrated in the Galaxy framework. In particular, the non-parametric Kruskal–Wallis sum-rank test was used to detect differentially abundant taxa, and Linear Discriminant Analysis (LDA) was used to estimate the effect size. The genus level alpha diversity of each sample was calculated by the Shannon index [31], beta diversity across samples was evaluated with non-metric multidimensional scaling (NMDS) using Bray–Curtis distances. Permutational multivariate analysis of variance (PERMANOVA) was used (permutations = 9999) for comparing the analyzed groups of ordinations. Statistical significance for changes of Shannon index and for taxonomic units between specific sample groups was evaluated by Wilcoxon signed-rank test.

Results

Main characteristics of the samples

In total 53 stool samples were obtained from 18 healthy individuals. All characteristics depicted in Table 1, except for age and ALAT, corresponded to the Gaussian distribution. One participant withdrew from the trial at the fifth day of metformin administration due to severe GI side effects. The stool sample from this individual was collected after five days long metformin administration, and during the analysis it showed high similarity to all other M7d samples, so it was further analyzed together with this group.

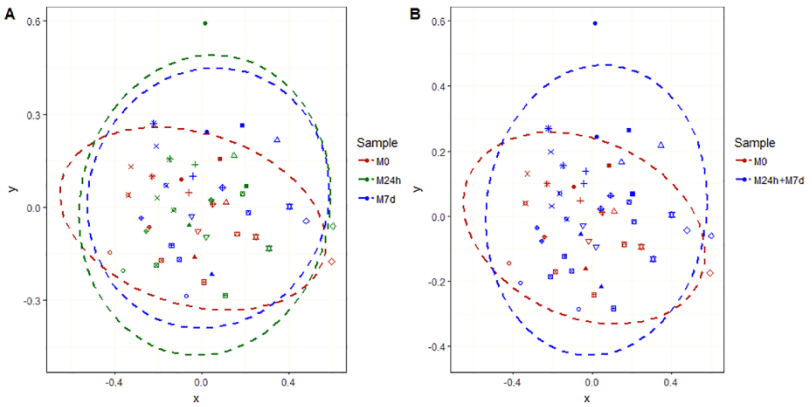


Fig 2. NMDS plots representing diversity between samples at genus level based on Bray–Curtis distances. (A) Comparison between all sample groups. (B) Comparison between M0 sample and samples during metformin administration (M24h + M7d). Ellipses represent the 95% confidence interval surrounding each group of samples. Different symbols represent participants of the study.

https://doi.org/10.1371/journal.pone.0204317.g002

After evaluation of registered side effects, we divided individuals into three groups according to the severity of GI side effects observed during the metformin administration: (1) no side effects (n = 3); (2) mild side effects defined by meteorism, stomach ache, nausea, and loss of appetite (n = 6); and (3) severe side effects defined by loose stools 1–3 times a day, diarrhea, and vomiting (n = 9). Only four individuals had loose stools (1–2 times per day) on day 1 of the study. The average time of occurrence for severe side effects was the day 3 of treatment. Full description on the registered adverse events can be found in S1 Table.

To evaluate the general differences in gut microbiota between the control sample and the samples taken after metformin administration we performed ordination analysis (Fig 2A and 2B) based on Bray–Curtis distances. As expected, gut microbiome communities were specific to each individual (PERMANOVA: $R^2 = 0.74$, $p = 0.001$) (S1 Fig). Thus, for further comparison of ordinations we used each individual as a nested factor. The analysis did not show any significant difference between the three groups of samples as defined by time points (M0, M24h, and M7d) (PERMANOVA: $R^2 = 0.028$, $p = 0.078$). Merging together both of the sample groups collected during and after metformin administration (M24h and M7d) and comparison with the control sample (M0) revealed a significant difference (PERMANOVA: $R^2 = 0.019$, $p = 0.036$).

Metformin reduces inner diversity of the gut microbiome

Comparing the Shannon index between the groups (Fig 3) we found that metformin therapy significantly reduces inner diversity of the gut microbiome immediately after the first two or three doses of metformin. After 7 days of metformin administration the inner diversity of the gut microbiome in study participants slightly increased, but was still significantly lower than before the use of metformin.

Changes in abundance of opportunistic pathogens in groups with different severity of GI side effects

To determine if the reduced inner diversity of the microbiome was associated with further gut microbiome dysbiosis, we analyzed changes in the abundance of common gut opportunistic

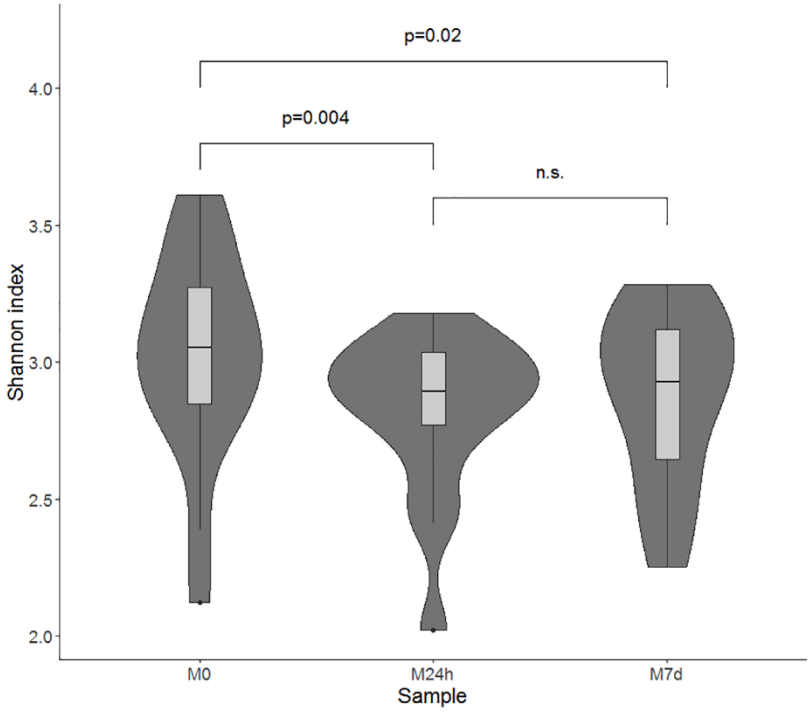


Fig 3. Alpha diversity changes during metformin therapy, evaluated at different time points. Samples marked as follows: M0—before starting metformin treatment; M24h - 24 hours after first intake of metformin; M7d - after 7 days treatment with metformin. Violin plot characterizing Shannon indexes combines boxplots, representing the median value and interquartile ranges, with kernel density plots.

https://doi.org/10.1371/journal.pone.0204317.g003

pathogen *Escherichia-Shigella* spp. We used the Wilcoxon-rank test for targeted analysis of possible changes in the relative abundance of *Escherichia-Shigella* genus comparing the three time points. There was no significant changes observed between the M0 (MED = 0.03%; IQR = 0.37%) and M24h ((MED = 0.05%; IQR = 0.14%) or M7d (MED = 0.46%; IQR = 1.04%). The relative abundance of these opportunistic pathogens was increased in the M7d sample when compared to M24h sample.

In order to test the possible relation of these changes with observed side effects we compared the changes in relative abundance of *Escherichia-Shigella* spp., as well as overall alpha diversity in different GI side effect categories (Fig 4). The inner diversity in the M7d sample compared to M24h sample increased only in groups with side effects. Thus in the group with mild side effects the median Shannon index for M7d sample was 3.03 (IQR = 0.21) compared to 2.97 (IQR = 0.15) in M24h sample, while in the group with severe side effects median was 2.88 (IQR = 0.66) for M7d sample compared to 2.72 (IQR = 0.42) for the M24h sample. We also observed increased presence of *Escherichia-Shigella* spp. in the samples taken before metformin administration from the participants later experiencing mild or severe side effects with the following median values of 0.21% (IQR = 1.57%) and 0.13% (IQR = 0.33%) respectively. The presence of *Escherichia-Shigella* spp. in the group with no side effects was beyond detectable limits.

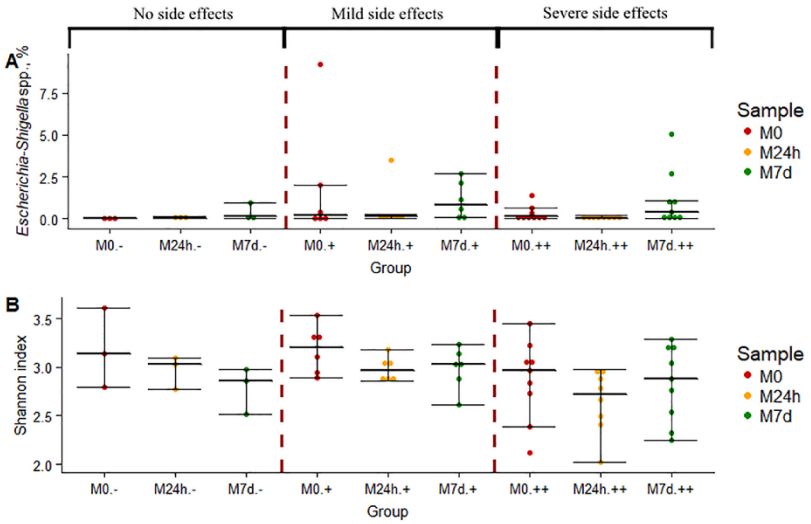


Fig 4. Changes in gut microbiome alpha diversity and abundance of opportunistic pathogen *Escherichia-Shigella* spp. at different time points within groups defined according to severity of GI side effects. (A) Changes in the relative abundance of *Escherichia-Shigella* spp. (B) Inner diversity changes, characterized by Shannon index. Samples marked as follows: M0—before starting metformin treatment; M24h - 24 hours after first intake of metformin; M7d - after 7 days treatment with metformin. Groups defined by observed side effects: "–"no side effects (n = 3), "+" mild side effects (n = 6), "++" severe side effects (n = 9). Dot plots depict median, 25th percentile and 75th percentile of data in each group. Dots beyond the bounds of the whiskers represent outliers.

https://doi.org/10.1371/journal.pone.0204317.g004

Differential abundance of taxonomic groups

To observe in-depth changes in the composition of the gut microbiome we used edgeR and evaluated the statistical significance of differential abundance of taxonomic groups between time points at every taxonomical level (phylum, class, order, family and genus). In total, 220 taxonomic groups presented in at least 50% of samples were tested. The main results are summarized in Table 2. There were no significant changes in representation of taxonomic groups at the phylum level at any of the contrasts between the M0, M24h, and M7d samples. One week treatment with metformin was associated with significant decreases in the families *Peptostreptococcaceae* and *Clostridiaceae_1* and four genera within these families: *Peptostreptococcaceae*_unclassified (family *Peptostreptococcaceae*), *Clostridiaceae_1*_unclassified (family *Clostridiaceae_1*), *Asaccharospora* (family *Peptostreptococcaceae*), and *Romboutsia* (family *Peptostreptococcaceae*). Comparison of the M24h and M7d samples showed significantly increased abundance of the order *Enterobacteriales*, including the only family in this order–*Enterobacteriaceae* with the genus, *Escherichia-Shigella*.

In addition, for graphic representation of differentially abundant taxa as well as their effect sizes and phylogenetic relationship, the LEfSe method was performed (Fig 5). This method detected 17 differentially abundant taxonomic clades, which mainly matched with those found with edgeR analysis.

In order to verify the findings from previous publications reporting that metformin increased abundance of *Akkermansia* spp., we performed a targeted Wilcoxon–rank test. Comparisons between two pairs were significant: M0 vs. M7d ($p = 0.03$) and M24 vs. M7d ($p = 0.01$) but the significance disappeared after performing the correction for multiple testing.

Table 2. Main significant changes in taxonomic units at all taxonomic levels.

Taxonomic level	Taxonomic group	Average abundance in sample groups,%			P–value [FDR*]		
		M0	M24h	M7d	M0 vs. M24h	M24h vs. M7d	M0 vs. 7d
Class	*Proteobacteria* unclassified	0.019	0.008	0.02	0.03 [0.62]		
	Gammaproteobacteria	1.16	0.50	1.71		0.002 [0.05]	0.008 [0.13]
	Verrucomicrobiae	0.45	0.30	1.14		0.03 [0.20]	
	Bacilli	1.02	0.83	1.31		0.03 [0.20]	0.04 [0.17]
	Epsilonproteobacteria	0.003	0.007	0.01			0.01 [0.13]
	Negativicutes	2.38	1.90	1.34			0.02 [0.15]
	*Proteobacteria*_unclassified	0.02	0.008	0.02	0.02 [0.68]		
	Enterobacteriales	0.99	0.41	1.55		**0.002 [0.04]**	0.005 [0.12]
Order	*Verrucomicrobiales*	0.45	0.30	1.14		0.03 [0.26]	
	Lactobacillales	1.00	0.81	1.29		0.03 [0.26]	0.04 [0.36]
	Selenomonadales	2.38	1.90	1.34			0.02 [0.26]
	Peptostreptococcaceae	1.17	0.93	0.23		**0.001 [0.02]**	**4.24E-06 [0.0002]**
	Clostridiaceae_1	0.70	0.51	0.13		**0.008 [0.12]**	**3.41E-05 [0.0007]**
Family	*Enterobacteriaceae*	0.99	0.41	1.55		**0.001 [0.02]**	0.004 [0.05]
	Streptococcaceae	0.58	0.41	0.68		0.01 [0.14]	
	Verrucomicrobiaceae	0.45	0.30	1.13		0.03 [0.21]	
	*Peptostreptococcaceae*_unclassified	0.91	0.72	0.18	0.04 [0.97]	**0.0006 [0.04]**	**1.86E-06 [0.0002]**
	*Clostridiaceae_1*_unclassified	0.63	0.49	0.10		0.032 [0.08]	**8.40E-06 [0.0005]**
	Asaccharospora	0.17	0.15	0.03		0.003 [0.08]	**1.64E-05 [0.0006]**
	Romboutsia	0.09	0.06	0.02		0.002 [0.07]	**2.92E-05 [0.0009]**
	Escherichia-Shigella	0.80	0.27	1.00		**0.0006 [0.04]**	0.008 [0.14]
Genus	*Streptococcus*	0.45	0.35	0.61		0.007 [0.16]	0.02 [0.31]
	*Enterobacteriaceae*_unclassified	0.19	0.13	0.48		0.01 [0.19]	0.004 [0.11]
	Ruminiclostridium_6	0.45	0.35	0.08		0.03 [0.45]	0.006 [0.13]
	Akkermansia	0.44	0.30	1.13		0.03 [0.48]	
	*Ruminococcaceae*_UCG-008	0.02	0.03	0.04			0.01 [0.16]
	Blautia	1.45	2.04	2.02			0.04 [0.52]

* Tendencies that maintained significance after false discovery rate (FDR) correction are marked in bold.

https://doi.org/10.1371/journal.pone.0204317.t002

This genus was present in 37 of 53 samples, but the tendency and direction of changes in abundance were not consistent in all individuals for this taxa.

In conclusion, to evaluate the possible confounding effect of diet, Spearman correlation analysis was carried out. We did not find any significant association between the changed taxa and our defined food groups after performing the correction for multiple testing.

Discussion

In order to observe unbiased short-term effects of metformin on the gut microbiome we used an exploratory longitudinal study design and included healthy individuals. We believe that this design should have minimized false associations and conclusions arising from unaccounted treatment status by metformin or other medications in T2D patients, including the unknown true duration of T2D before diagnosis and the high interindividual variation of the gut microbiome. It has been recognized that, in similar time series studies, individuals can be treated as their own controls before and during treatment [32]. In addition, the strong effect size in previously described metformin studies [12] allowed us to consider the longitudinal study design

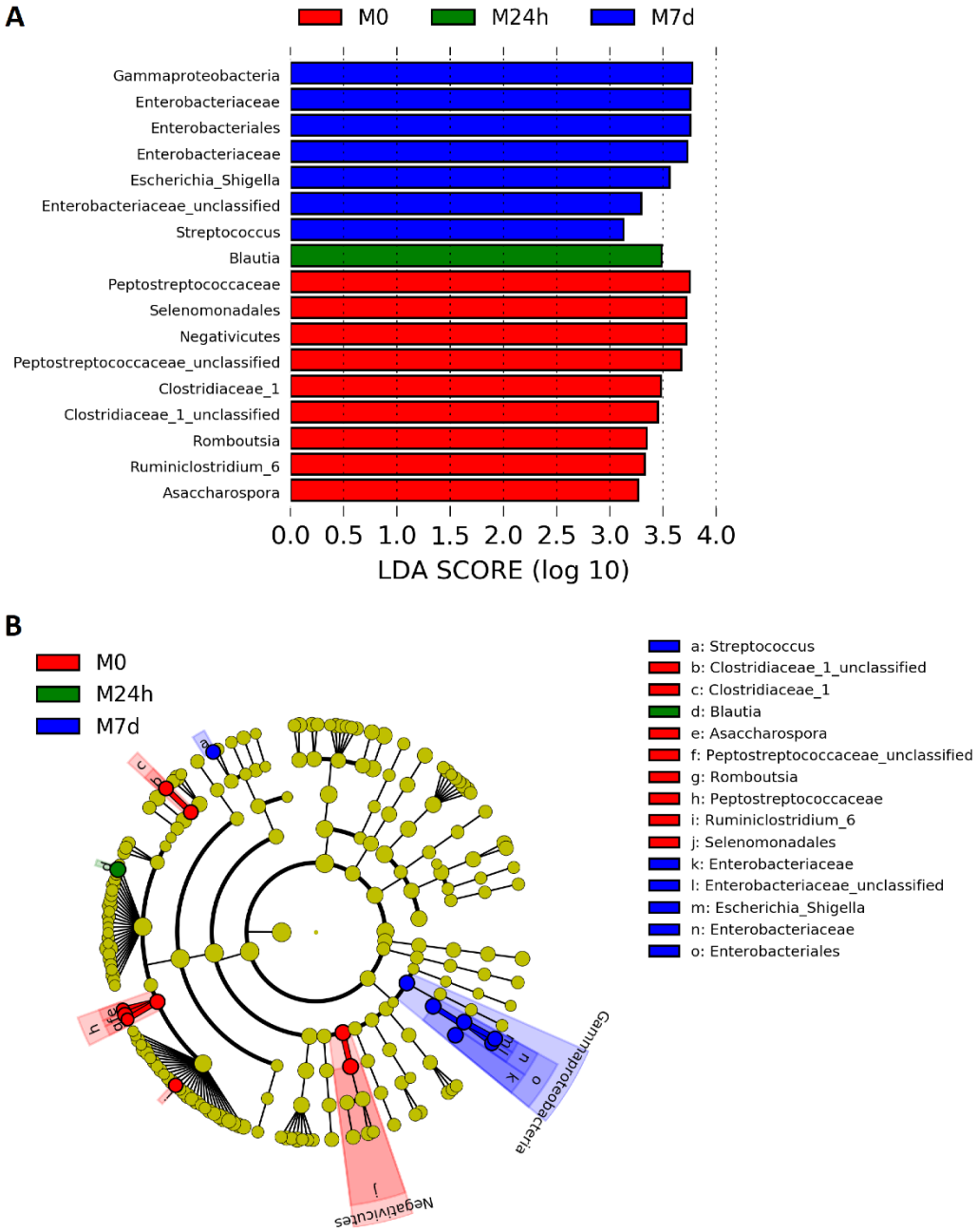


Fig 5. Comparison of LDA effect size of the significantly differentiating microbial taxa deduced using LefSe analysis. (A) Differences in abundance of taxonomic groups among all three sample. LDA cutoff = 2. Differentiating feature analysis was carried out with Kruskal–Wallis test raw p-value cutoff = 0.05. (B) Cladogram illustrating the phylogenetic relationship among the significantly differentiating gut microbiome taxonomic groups among the M0, M24h, and M7d samples.

https://doi.org/10.1371/journal.pone.0204317.g005

as sufficiently powerful to achieve the goal of our study. Taking in account our results, this study design has as well prevented any confounding effects induced by the known high inter-individual variety of diet [33], as we did not find any significant association between the changed taxonomic composition and data from the 7-day food record. Furthermore, there has been an increase in the use of metformin beyond diabetes, so this research may give additional insights into general features of the interaction between metformin and the gut microbiome, which may be further applicable to its use across a broad range of diseases, such as Alzheimer's disease [34], polycystic ovary syndrome [21], various types of cancers [20], and prevention of diabetes in individuals with prediabetic symptoms [35]. We also chose to include the first sampling point 24 hours after metformin administration in order to observe the effects as soon as possible and to avoid the potentially strong influence of diarrhea and other side effects known to occur after metformin administration. The generally accepted incidence of metformin-induced GI side effects is 20 – 30% [36, 37]. However, our data agreed with recent reports [38, 39], as we observed a high rate of side effects in our study (50% of study group experienced strong and 33% experienced mild side effects). This could be explained by the rather high initial dose of metformin, or the possibility that the design of the recent studies was more feasible for patients, which ensured higher treatment adherence and higher rate of reporting side effects.

Our findings that show the reduction in inner diversity of the gut microbiome during metformin treatment was in line with the previously observed effects of metformin effects in mice and rat models [13, 17]. In addition, a recent study using metagenome sequencing showed that metformin improves microbial gene richness among T2D patients, while metformin users generally have lower gene richness than healthy controls have [11]. It should be noted that, in our case, the reduction of diversity was observed at the markedly short time period of 24 hours, in the absence of diarrhea (only four participants experienced loose stools on the day 1). The small increase in inner diversity when comparing the M24h and M7d samples indicates the tendency of the gut microbiome to regain its ecological equilibrium even in participants experiencing diarrhea, as seen in the group of participants with severe side effects, in which nine people experienced loose stools or diarrhea. Likewise, this explains the growth of opportunistic pathogens including members from genus *Escherichia-Shigella* spp., which in previous studies has been associated with metformin treatment in T2D patients [11, 12]. Although, we cannot attribute the rapid increase of this genus between 24-hour and 7-day time points as a direct effect of metformin, this effect could be ensured by the trait of persistence of this genus [40] and high abilities to adapt [41]. Therefore, it can occupy the space open due to unfavorable conditions created by yet fully unknown effect of metformin. In other words, the reduced diversity in the gut presents *Escherichia-Shigella* spp. the free niche needed to emerge in larger numbers compared to the concurrent bacterial species. The connection between reduced alpha diversity and the further increase in the representation of opportunistic pathogens has been described before in the context of antibiotic treatment, various diseases and aging [11, 42–45].

The characteristic GI side effects in most cases manifest at the beginning of metformin therapy and usually disappear after several weeks [46, 47]. Several species from *Escherichia-Shigella* spp. have been identified as pathogens [48]. Assuming that the reason for adverse effects may be an increase of such opportunistic pathogens from *Escherichia-Shigella* spp., later reduction of adverse reactions could be associated with specific characteristics of these taxonomic groups. *Escherichia* and *Shigella* are two closely related genera that share bioenergetic mechanisms that allow them to fill a specific niche in the gut microbiome ecosystem [49]. Despite a competitive advantage as a facultative anaerobe, the population of *Escherichia coli* is known to be dependent on substrates provided by polysaccharide-degrading anaerobes [50]. Thus, the rapid initial growth might be terminated by the lack of mono- and disaccharides caused by

reduced abundance of anaerobic mucus-associated taxonomic groups and increased competition for the limited amount of energy substrates within the taxa. Also, T2D therapy accompanying a specific diet with reduced amount of simple carbohydrates [51] may play a role in limiting the amount of substrate. That could lead to further stabilization of the microbial ecosystem and recovery of metformin tolerance. Nevertheless, the initial side effects are the main reason for metformin discontinuation in 5% of patients [37]. Our results show an increased initial presence of *Escherichia-Shigella* spp. in the samples taken before metformin administration from the participants later experiencing side effects versus those without side effects (*Escherichia-Shigella* spp. below detectable limits). Development and implementation of a test for the presence of pathogens prior to metformin administration may allow stratification of treatment strategies (e.g. dose reduction or use of slow release forms) in high-risk patients.

A limitation of the present approach is the fact that analysis of *16S rRNA* sequencing results merge together various *Escherichia-Shigella* spp. species and strains with a wide spectrum of functions, effects, and ways of interaction [52]. Therefore, further metagenomic analysis in a longitudinal study providing information on gene richness, composition, and metabolic pathways could give deeper taxonomic and functional insight into the specificity of metformin-induced changes.

In addition, the sample collection procedure that involved temporary storage at room temperature prior to freezing can be seen as a possible limitation of the study. However, it has been shown in various studies that such approach does not significantly alter the microbiome composition if the storage is up to 24 hours [23, 24].

Despite the fact that it is still hard to distinguish whether dysbiosis of the gut microbiome is the cause or consequence of T2D and a specter of various other diseases, many therapeutic effects of gut microbiome modulation have been proven already [14, 53, 54]. It has been suggested that, despite induction of GI associated side effects, metformin may also exert its positive effects through its capability to modulate the gut microbiome. The strongest observable and specific effect of metformin in our study was the reduction in abundance of the family *Peptostreptococcaceae* and three genera within it. Members of this family, in principle, have been associated with compromised health–one of the most convincing examples being *Clostridium difficile*. Increased abundance of *Peptostreptococcaceae* has also been associated with such conditions as non-alcoholic fatty liver disease [55], ulcerative colitis [56], and colorectal cancer [57], as well as with reduced lifespan [58]. In addition, reduced abundance of this family has been found in mice fed with a low-fat diet [59] or with calorie restrictions [58]. Interestingly, both families, significantly decreased by metformin, have been described to show similar response tendencies in various studies. Both *Peptostreptococcaceae* and *Clostridiaceae_1* possibly mediate the effect of eugenol treatment on mucus production in mice [60] and may be associated with dietary protein restriction induced improvement of ileal barrier function in pigs [61].

In the context of T2D or metformin therapy, the family *Peptostreptococcaceae* in general has not been described before, but previous studies have found significantly reduced abundance of one genus within it–*Intestinibacter* spp.–associated with metformin treatment [11, 12]. The functional role of this genus is still unclear, as it has been defined only recently [62]. We did not observe any statistically significant changes in the abundance of this genus that might be explained by analysis of healthy individuals in our study group.

The possibly controversial role of these taxa could be explained by potential differences in genera and species composition within these families between human and animal gut microbiomes. Overall, these changes in taxonomic units show that metformin may have beneficial effects through modification of possibly unfavorable human gut microbiome composition.

Unlike previous studies, we did not observe a significant increase in abundance of *Akkermansia* spp. after correction. One of the reasons may be the low prevalence of this genus in our

study group that can be explained by population, age, or disease status based differences when comparing to other studies.

Another intriguing question is the mechanism of how metformin modifies the gut microbiome. Recently, it has been shown that metformin has a direct effect on some, but not all of the gut microbiome bacteria, that was demonstrated by decreased growth in the presence of metformin *in vitro* [12]. It is not yet clear whether these direct effects of metformin are sufficient to explain the broad range of taxa affected in gut. Alternatively, the microbiome changes at least in part can be the result of systemic effects of metformin on the host (e.g. altered enterohepatic circulation of bile acids and salts) as suggested in McCreight et al. (2016) [7]. Our data, however, show rapid metformin-induced effects, and thus are in favor of the direct action of metformin, although this has to be proven using additional *in vitro* studies.

In conclusion, we were able to present direct evidence of effects of metformin on the gut microbiome in humans using prospective study, and associate these changes with metformin side effects. As this is an exploratory study without a placebo control arm, it would require additional randomized controlled trial in order to reach definitive conclusions. Nevertheless, our results indicate the possibility of developing a personalized approach in metformin therapy by pre-screening gut microbiota for abundance of opportunistic pathogens, followed by adjusted therapeutic strategies in patients with higher risk of developing side effects.

Supporting information

S1 Text. List of inclusion/exclusion criteria.
(DOCX)

S2 Text. CONSORT checklist.
(DOCX)

S3 Text. Clinical trial information.
(PDF)

S1 Table. Summary of the registered side effects during the metformin use.
(DOCX)

S2 Table. Summary of 7-day food record during the metformin treatment.
(DOCX)

S1 Fig. Taxonomic composition in all samples at family level. Plot visualizes the high inter-individual diversity represented by most abundant taxonomic groups at family level. Each individual is marked with a personal identification code.
(PDF)

Acknowledgments

We acknowledge the Genome Database of Latvian Population and Latvian Biomedical Research and Study Centre for organization of participants' recruitment and bio-sample processing and data storage. The authors thank all the volunteers who participated in the clinical trial.

Author Contributions

Conceptualization: Ilze Elbere, Ineta Kalnina, Ilze Konrade, Linda Zaharenko, Ilze Radovica-Spalvina, Davids Fridmanis, Dita Gudra, Valdis Pirags, Janis Klovins.

Data curation: Ilze Elbere, Ivars Silamikelis.

Formal analysis: Ilze Elbere, Kristine Sekace.

Funding acquisition: Valdis Pirags, Janis Klovins.

Investigation: Ilze Elbere, Ineta Kalnina, Ilze Radovica-Spalvina, Dita Gudra.

Methodology: Ilze Elbere, Ineta Kalnina, Ivars Silamikelis, Kristine Sekace.

Project administration: Ineta Kalnina, Janis Klovins.

Resources: Davids Fridmanis.

Software: Ivars Silamikelis.

Supervision: Ineta Kalnina, Ilze Konrade, Valdis Pirags, Janis Klovins.

Validation: Ineta Kalnina, Ilze Radovica-Spalvina, Davids Fridmanis, Dita Gudra.

Visualization: Ilze Elbere.

Writing – original draft: Ilze Elbere.

Writing – review & editing: Ineta Kalnina, Ilze Konrade, Linda Zaharenko, Valdis Pirags, Janis Klovins.

References

1. American Diabetes Association. Standards of medical care in diabetes -2010. Diabetes Care. 2010; 33 Suppl 1:S11–61.
2. Bolen S, Feldman L, Vassy J, Wilson L, Yeh HC, Marinopoulos S, et al. Systematic review: comparative effectiveness and safety of oral medications for type 2 diabetes mellitus. Ann Intern Med. 2007; 147 (6):386–99. PMID: 17638715
3. Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M, et al. Management of hyperglycemia in type 2 diabetes, 2015: a patient-centered approach: update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care. 2015; 38(1):140–9. https://doi.org/10.2337/dc14-2441 PMID: 25538310
4. Fung CS, Wan EY, Wong CK, Jiao F, Chan AK. Effect of metformin monotherapy on cardiovascular diseases and mortality: a retrospective cohort study on Chinese type 2 diabetes mellitus patients. Cardiovasc Diabetol. 2015; 14:137. https://doi.org/10.1186/s12933-015-0304-2 PMID: 26453464
5. Nasri H, Rafieian-Kopaei M. Metformin: Current knowledge. J Res Med Sci. 2014; 19(7):658–64. PMID: 25364368
6. Bonora E, Cigolini M, Bosello O, Zancanaro C, Capretti L, Zavaroni I, et al. Lack of effect of intravenous metformin on plasma concentrations of glucose, insulin, C-peptide, glucagon and growth hormone in non-diabetic subjects. Curr Med Res Opin. 1984; 9(1):47–51. https://doi.org/10.1185/03007998409109558 PMID: 6373159
7. McCreight LJ, Bailey CJ, Pearson ER. Metformin and the gastrointestinal tract. Diabetologia. 2016; 59 (3):426–35. https://doi.org/10.1007/s00125-015-3844-9 PMID: 26780750
8. Graham GG, Punt J, Arora M, Day RO, Doogue MP, Duong JK, et al. Clinical pharmacokinetics of metformin. Clin Pharmacokinet. 2011; 50(2):81–98. https://doi.org/10.2165/11534750-000000000-00000 PMID: 21241070
9. Buse JB, DeFronzo RA, Rosenstock J, Kim T, Burns C, Skare S, et al. The Primary Glucose-Lowering Effect of Metformin Resides in the Gut, Not the Circulation: Results From Short-term Pharmacokinetic and 12-Week Dose-Ranging Studies. Diabetes Care. 2016; 39(2):198–205. https://doi.org/10.2337/dc15-0488 PMID: 26285584
10. de la Cuesta-Zuluaga J, Mueller NT, Corrales-Agudelo V, Velásquez-Mejía EP, Carmona JA, Abad JM, et al. Metformin Is Associated With Higher Relative Abundance of Mucin-Degrading Akkermansia muciniphila and Several Short-Chain Fatty Acid–Producing Microbiota in the Gut. Diabetes Care. 2016.
11. Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature. 2015; 528 (7581):262–6. https://doi.org/10.1038/nature15766 PMID: 26633628

12. Wu H, Esteve E, Tremaroli V, Khan MT, Caesar R, Manneras-Holm L, et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. Nat Med. 2017.
13. Lee H, Ko G. Effect of metformin on metabolic improvement and gut microbiota. Appl Environ Microbiol. 2014; 80(19):5935–43. https://doi.org/10.1128/AEM.01357-14 PMID: 25038099
14. Shin NR, Lee JC, Lee HY, Kim MS, Whon TW, Lee MS, et al. An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. Gut. 2014; 63(5):727–35. https://doi.org/10.1136/gutjnl-2012-303839 PMID: 23804561
15. Napolitano A, Miller S, Nicholls AW, Baker D, Van Horn S, Thomas E, et al. Novel gut-based pharmacology of metformin in patients with type 2 diabetes mellitus. PLoS One. 2014; 9(7):e100778. https://doi.org/10.1371/journal.pone.0100778 PMID: 24988476
16. Burton JH, Johnson M, Johnson J, Hsia DS, Greenway FL, Heiman ML. Addition of a Gastrointestinal Microbiome Modulator to Metformin Improves Metformin Tolerance and Fasting Glucose Levels. J Diabetes Sci Technol. 2015.
17. Zhang X, Zhao Y, Xu J, Xue Z, Zhang M, Pang X, et al. Modulation of gut microbiota by berberine and metformin during the treatment of high-fat diet-induced obesity in rats. Sci Rep. 2015; 5:14405. https://doi.org/10.1038/srep14405 PMID: 26396057
18. Maniar K, Moideen A, Bhattacharyya R, Banerjee D. Metformin exerts anti-obesity effect via gut microbiome modulation in prediabetics: A hypothesis. Med Hypotheses. 2017; 104:117–20. https://doi.org/10.1016/j.mehy.2017.06.001 PMID: 28673568
19. Barzilai N, Crandall JP, Kritchevsky SB, Espeland MA. Metformin as a Tool to Target Aging. Cell Metab. 2016; 23(6):1060–5. https://doi.org/10.1016/j.cmet.2016.05.011 PMID: 27304507
20. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. BMJ. 2005; 330(7503):1304–5. https://doi.org/10.1136/bmj.38415.708634.F7 PMID: 15849206
21. Moghetti P, Castello R, Negri C, Tosi F, Perrone F, Caputo M, et al. Metformin effects on clinical features, endocrine and metabolic profiles, and insulin sensitivity in polycystic ovary syndrome: a randomized, double-blind, placebo-controlled 6-month trial, followed by open, long-term clinical evaluation. J Clin Endocrinol Metab. 2000; 85(1):139–46. https://doi.org/10.1210/jcem.85.1.6293 PMID: 10634377
22. Rovite V, Wolff-Sagi Y, Zaharenko L, Nikitina-Zake L, Grens E, Klovins J. Genome Database of the Latvian Population (LGDB): Design, Goals, and Primary Results. J Epidemiol. 2018.
23. Cardona S, Eck A, Cassellas M, Gallart M, Alastrue C, Dore J, et al. Storage conditions of intestinal microbiota matter in metagenomic analysis. BMC Microbiol. 2012; 12:158. https://doi.org/10.1186/1471-2180-12-158 PMID: 22846661
24. Tedjo DI, Jonkers DM, Savelkoul PH, Masclee AA, van Best N, Pierik MJ, et al. The effect of sampling and storage on the fecal microbiota composition in healthy and diseased subjects. PLoS One. 2015; 10(5):e0126685. https://doi.org/10.1371/journal.pone.0126685 PMID: 26024217
25. Milani C, Hevia A, Foroni E, Duranti S, Turroni F, Lugli GA, et al. Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. PLoS One. 2013; 8(7):e68739. https://doi.org/10.1371/journal.pone.0068739 PMID: 23869230
26. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009; 75(23):7537–41. https://doi.org/10.1128/AEM.01541-09 PMID: 19801464
27. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 2013; 41(Database issue):D590–6. https://doi.org/10.1093/nar/gks1219 PMID: 23193283
28. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011; 27(16):2194–200. https://doi.org/10.1093/bioinformatics/btr381 PMID: 21700674
29. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007; 73(16):5261–7. https://doi.org/10.1128/AEM.00062-07 PMID: 17586664
30. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011; 12(6):R60. https://doi.org/10.1186/gb-2011-12-6-r60 PMID: 21702898
31. Shannon CE. A Mathematical Theory of Communication. Bell System Technical Journal. 1948; 27(3):379–423.

32. Goodrich JK, Di Rienzi SC, Poole AC, Koren O, Walters WA, Caporaso JG, et al. Conducting a microbiome study. Cell. 2014; 158(2):250–62. https://doi.org/10.1016/j.cell.2014.06.037 PMID: 25036628

33. Duvallet C, Gibbons SM, Gurry T, Irizarry RA, Alm EJ. Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. Nat Commun. 2017; 8(1):1784. https://doi.org/10.1038/s41467-017-01973-8 PMID: 29209090

34. Gupta A, Bisht B, Dey CS. Peripheral insulin-sensitizer drug metformin ameliorates neuronal insulin resistance and Alzheimer's-like changes. Neuropharmacology. 2011; 60(6):910–20. https://doi.org/10.1016/j.neuropharm.2011.01.033 PMID: 21277873

35. Lily M, Godwin M. Treating prediabetes with metformin: systematic review and meta-analysis. Can Fam Physician. 2009; 55(4):363–9. PMID: 19366942

36. Hoffmann IS, Roa M, Torrico F, Cubeddu LX. Ondansetron and metformin-induced gastrointestinal side effects. Am J Ther. 2003; 10(6):447–51. PMID: 14624284

37. Kirpichnikov D, McFarlane SI, Sowers JR. Metformin: an update. Ann Intern Med. 2002; 137(1):25–33. PMID: 12093242

38. Dujic T, Causevic A, Bego T, Malenica M, Velija-Asimi Z, Pearson ER, et al. Organic cation transporter 1 variants and gastrointestinal side effects of metformin in patients with Type 2 diabetes. Diabet Med. 2016; 33(4):511–4. https://doi.org/10.1111/dme.13040 PMID: 26605869

39. Florez H, Luo J, Castillo-Florez S, Mitsi G, Hanna J, Tamariz L, et al. Impact of metformin-induced gastrointestinal symptoms on quality of life and adherence in patients with type 2 diabetes. Postgrad Med. 2010; 122(2):112–20. https://doi.org/10.3810/pgm.2010.03.2128 PMID: 20203462

40. Amato SM, Orman MA, Brynildsen MP. Metabolic control of persister formation in Escherichia coli. Mol Cell. 2013; 50(4):475–87. https://doi.org/10.1016/j.molcel.2013.04.002 PMID: 23665232

41. Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P, et al. Organised genome dynamics in the Escherichia coli species results in highly diverse adaptive paths. PLoS Genet. 2009; 5(1):e1000344. https://doi.org/10.1371/journal.pgen.1000344 PMID: 19165319

42. Ticinesi A, Milani C, Lauretani F, Nouvenne A, Mancabelli L, Lugli GA, et al. Gut microbiota composition is associated with polypharmacy in elderly hospitalized patients. Sci Rep. 2017; 7(1):11102. https://doi.org/10.1038/s41598-017-10734-y PMID: 28894183

43. Francino MP. Antibiotics and the Human Gut Microbiome: Dysbioses and Accumulation of Resistances. Front Microbiol. 2015; 6:1543. https://doi.org/10.3389/fmicb.2015.01543 PMID: 26793178

44. Milani C, Ticinesi A, Gerritsen J, Nouvenne A, Lugli GA, Mancabelli L, et al. Gut microbiota composition and Clostridium difficile infection in hospitalized elderly individuals: a metagenomic study. Sci Rep. 2016; 6:25945. https://doi.org/10.1038/srep25945 PMID: 27166072

45. Morton ER, Lynch J, Froment A, Lafosse S, Heyer E, Przeworski M, et al. Variation in Rural African Gut Microbiota Is Strongly Correlated with Colonization by Entamoeba and Subsistence. PLoS Genet. 2015; 11(11):e1005658. https://doi.org/10.1371/journal.pgen.1005658 PMID: 26619199

46. Haupt E, Knick B, Koschinsky T, Liebermeister H, Schneider J, Hirche H. Oral antidiabetic combination therapy with sulphonylureas and metformin. Diabete Metab. 1991; 17(1 Pt 2):224–31.

47. Howlett HC, Bailey CJ. A risk-benefit assessment of metformin in type 2 diabetes mellitus. Drug Saf. 1999; 20(6):489–503. PMID: 10392666

48. Sims GE, Kim SH. Whole-genome phylogeny of Escherichia coli/Shigella group by feature frequency profiles (FFPs). Proc Natl Acad Sci U S A. 2011; 108(20):8329–34. https://doi.org/10.1073/pnas.1105168108 PMID: 21536867

49. Johnson JR. Shigella and Escherichia coli at the crossroads: machiavellian masqueraders or taxonomic treachery? J Med Microbiol. 2000; 49(7):583–5. https://doi.org/10.1099/0022-1317-49-7-583 PMID: 10882082

50. Jones SA, Chowdhury FZ, Fabich AJ, Anderson A, Schreiner DM, House AL, et al. Respiration of Escherichia coli in the mouse intestine. Infect Immun. 2007; 75(10):4891–9. https://doi.org/10.1128/IAI.00484-07 PMID: 17698572

51. Asif M. The prevention and control the type-2 diabetes by changing lifestyle and dietary pattern. J Educ Health Promot. 2014; 3:1. https://doi.org/10.4103/2277-9531.127541 PMID: 24741641

52. Gao YD, Zhao Y, Huang J. Metabolic modeling of common Escherichia coli strains in human gut microbiome. Biomed Res Int. 2014; 2014:694967. https://doi.org/10.1155/2014/694967 PMID: 25126572

53. Mattila E, Uusitalo-Seppala R, Wuorela M, Lehtola L, Nurmi H, Ristikankare M, et al. Fecal transplantation, through colonoscopy, is effective therapy for recurrent Clostridium difficile infection. Gastroenterology. 2012; 142(3):490–6. https://doi.org/10.1053/j.gastro.2011.11.037 PMID: 22155369

54. Rossi O, van Berkel LA, Chain F, Tanweer Khan M, Taverne N, Sokol H, et al. Faecalibacterium prausnitzii A2-165 has a high capacity to induce IL-10 in human and murine dendritic cells and modulates T cell responses. Sci Rep. 2016; 6:18507. https://doi.org/10.1038/srep18507 PMID: 26725514

55. Jiang W, Wu N, Wang X, Chi Y, Zhang Y, Qiu X, et al. Dysbiosis gut microbiota associated with inflammation and impaired mucosal immune function in intestine of humans with non-alcoholic fatty liver disease. Sci Rep. 2015; 5:8096. https://doi.org/10.1038/srep08096 PMID: 25644696

56. Lavelle A, Lennon G, O'Sullivan O, Docherty N, Balfe A, Maguire A, et al. Spatial variation of the colonic microbiota in patients with ulcerative colitis and control volunteers. Gut. 2015; 64(10):1553–61. https://doi.org/10.1136/gutjnl-2014-307873 PMID: 25596182

57. Chen W, Liu F, Ling Z, Tong X, Xiang C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. PLoS One. 2012; 7(6):e39743. https://doi.org/10.1371/journal.pone.0039743 PMID: 22761885

58. Zhang C, Li S, Yang L, Huang P, Li W, Wang S, et al. Structural modulation of gut microbiota in life-long calorie-restricted mice. Nat Commun. 2013; 4:2163. https://doi.org/10.1038/ncomms3163 PMID: 23860099

59. Cox LM, Cho I, Young SA, Anderson WH, Waters BJ, Hung SC, et al. The nonfermentable dietary fiber hydroxypropyl methylcellulose modulates intestinal microbiota. FASEB J. 2013; 27(2):692–702. https://doi.org/10.1096/fj.12-219477 PMID: 23154883

60. Wlodarska M, Willing BP, Bravo DM, Finlay BB. Phytonutrient diet supplementation promotes beneficial Clostridia species and intestinal mucus secretion resulting in protection against enteric infection. Sci Rep. 2015; 5:9253. https://doi.org/10.1038/srep09253 PMID: 25787310

61. Fan P, Liu P, Song P, Chen X, Ma X. Moderate dietary protein restriction alters the composition of gut microbiota and improves ileal barrier function in adult pig model. Sci Rep. 2017; 7:43412. https://doi.org/10.1038/srep43412 PMID: 28252026

62. Gerritsen J, Fuentes S, Grievink W, van Niftrik L, Tindall BJ, Timmerman HM, et al. Characterization of Romboutsia ilealis gen. nov., sp. nov., isolated from the gastro-intestinal tract of a rat, and proposal for the reclassification of five closely related members of the genus Clostridium into the genera Romboutsia gen. nov., Intestinibacter gen. nov., Terrisporobacter gen. nov. and Asaccharospora gen. nov. Int J Syst Evol Microbiol. 2014; 64(Pt 5):1600–16. https://doi.org/10.1099/ijs.0.059543-0 PMID: 24480908

II Significantly altered peripheral blood cell DNA methylation profile as a result of immediate effect of metformin use in healthy individuals

Highlights:

1. In total, 125 significantly modified sites were discovered, and 11 differentially methylated CpGs with the largest and most consistent changes in beta values at different contrasts were prioritized: *POFUT2*, *CAMKK1*, *EML3*, *KIAA1614*, *UPF1*, *MUC4*, *LOC727982*, *SIX3*, *ADAM8*, *SNORD12B*, and *VPS8*.
2. Genes corresponding to the top-ranked DMPs represent the main functional groups associated with previously described targets of metformin therapy: regulatory processes of energy homeostasis, inflammatory responses, tumorigenesis, and neurodegeneration.
3. A significant correlation between the expression levels and methylation changes of the corresponding CpG sites were found for three genes: *UPF1*, *MUC4*, *KIAA1614*.
4. The pathway enrichment analysis revealed metformin's association with various pathways some of which already has been described in connection with metformin action but not in the context of epigenetic regulation.

Elbere et al. Clinical Epigenetics (2018) 10:156
https://doi.org/10.1186/s13148-018-0593-x

Clinical Epigenetics

RESEARCH Open Access

Significantly altered peripheral blood cell DNA methylation profile as a result of immediate effect of metformin use in healthy individuals

Ilze Elbere[1†], Ivars Silamikelis[1†], Monta Ustinova[1], Ineta Kalnina[1], Linda Zaharenko[1], Raitis Peculis[1], Ilze Konrade[2], Diana Maria Ciuculete[3], Christina Zhukovsky[3], Dita Gudra[1], Ilze Radovica-Spalvina[1], Davids Fridmanis[1], Valdis Pirags[1], Helgi B. Schiöth[3] and Janis Klovins[1*]

Abstract

Background: Metformin is a widely prescribed antihyperglycemic agent that has been also associated with multiple therapeutic effects in various diseases, including several types of malignancies. There is growing evidence regarding the contribution of the epigenetic mechanisms in reaching metformin's therapeutic goals; however, the effect of metformin on human cells in vivo is not comprehensively studied. The aim of our study was to examine metformin-induced alterations of DNA methylation profiles in white blood cells of healthy volunteers, employing a longitudinal study design.

Results: Twelve healthy metformin-naïve individuals where enrolled in the study. Genome-wide DNA methylation pattern was estimated at baseline, 10 h and 7 days after the start of metformin administration. The whole-genome DNA methylation analysis in total revealed 125 differentially methylated CpGs, of which 11 CpGs and their associated genes with the most consistent changes in the DNA methylation profile were selected: *POFUT2*, *CAMKK1*, *EML3*, *KIAA1614*, *UPF1*, *MUC4*, *LOC727982*, *SIX3*, *ADAM8*, *SNORD12B*, *VPS8*, and several differentially methylated regions as novel potential epigenetic targets of metformin. The main functions of the majority of top-ranked differentially methylated loci and their representative cell signaling pathways were linked to the well-known metformin therapy targets: regulatory processes of energy homeostasis, inflammatory responses, tumorigenesis, and neurodegenerative diseases.

Conclusions: Here we demonstrate for the first time the immediate effect of short-term metformin administration at therapeutic doses on epigenetic regulation in human white blood cells. These findings suggest the DNA methylation process as one of the mechanisms involved in the action of metformin, thereby revealing novel targets and directions of the molecular mechanisms underlying the various beneficial effects of metformin.

Trial registration: EU Clinical Trials Register, 2016-001092-74. Registered 23 March 2017, https://www.clinicaltrialsregister.eu/ctr-search/trial/2016-001092-74/LV.

Keywords: Metformin, Epigenetics, DNA methylation, White blood cells, Longitudinal study

* Correspondence: klovins@biomed.lu.lv
†Ilze Elbere and Ivars Silamikelis contributed equally to this work.
[1]Latvian Biomedical Research and Study Centre, Ratsupites Str. 1 k-1, Riga LV-1067, Latvia
Full list of author information is available at the end of the article

Background

Metformin is the first-line drug for type 2 diabetes (T2D) therapy, used since 1950s [1]. Although there are a great number of various studies on the metformin pharmacogenomics, pharmacokinetics, and lately its interaction with the gut microbiome, the details of the molecular mechanisms of metformin action have not been fully understood.

So far, there are only a few studies within the context of metformin action and changes in one of the most commonly studied epigenetic modifications—DNA methylation. One of the targeted studies has shown that metformin treatment of pregnant rats with gestational diabetes can reduce methylation level of peroxisome proliferator-activated receptor γ coactivator-1A (PPARGC1A), therefore preventing the abnormal glycolipid metabolism in their offspring [2]. In addition, a genome-wide study of metformin effects on lymphoblastoid cell lines has revealed potential biomarkers for metformin's anticancer response [3]. In the context of possible molecular mechanisms of how metformin induce changes in the methylation profile, a recent study has proved that, in cancer cells, metformin can exert its effects via regulation of the H19/SAHH axis [4]. This has been supported by data showing that metformin promotes global methylation by decreasing *S*-adenosylhomocysteine (SAH) intracellular levels in various cell types, including non-cancerous [5]. One of the latest studies have specifically shown metformin's effect on lowering the methylation levels at the metformin transporter genes, resulting in higher expression levels in liver tissue [6]. Studies describing other epigenetic effects of metformin have shown its impact on various histone modifications via multiple mechanisms, mostly AMPK dependent, and effect on expression levels of numerous miRNAs through increase in DICER protein levels as well [7].

Nevertheless, there is a significant lack of information on how metformin affects global epigenetic regulation in non-cancerous cells or in cells obtained from metformin-treated humans. Therefore, our aim was to investigate the short-term effect of metformin on DNA methylation profiles in blood cells from healthy volunteers. Here we compared the changes in DNA methylation in the same subjects before and after the metformin intake.

Results

Characteristics of the study participants

We used Illumina Infinium 450k array to evaluate the effect of metformin on DNA methylation in 12 healthy volunteers. The characteristics of the study group are summarized in Table 1. Samples, for analysis of the methylation levels, from each participant were obtained at three time points, further marked as M0 (before starting a metformin therapy), M10h (10 h after the first metformin intake, before the second tablet), and M7d (time point after 7 days of metformin administration). M10h sample was chosen to evaluate effect of one metformin's dose; to ensure accuracy of this measurement, all study participants were strictly instructed to take the second metformin tablet only after the M10h blood sampling.

Table 1 Characteristics of the study group

Characteristic	Value
Female/male, *n* (%)	7 (58.3%)/5 (41.7%)
Age, years, mean ± SD	31.4 ± 6.7
BMI, mean ± SD	25.3 ± 3.5
ALAT*, U/l, mean ± SD	25 ± 13
Creatinine*, μmol/l, mean ± SD	68 ± 8.9
Fasting plasma glucose*, mmol/l, mean ± SD	5.1 ± 0.3

BMI body mass index, *SD* standard deviation, *ALAT* alanine aminotransferase
*Samples for hematological, biochemical tests were collected before metformin administration

Differentially methylated CpGs

During the data preprocessing stage, 64,512 (13.29%) probes were filtered out, leaving 421,000 probes for downstream analysis. To detect differentially methylated CpG sites/probes (DMPs), we applied limma analysis between contrasts at all three time points, i.e., baseline, after 10 h and 7 days of metformin administration. The model included the methylation values at the contrasted time points, together with the cell-type estimations as covariates. Comparing methylation values at M10h and M0 samples, 72 differentially methylated CpG sites with a false discovery rate (FDR) of < 0.05 were identified after correction for multiple testing using the Benjamini-Hochberg method. In the same way, 52 DMPs were found applying contrast between methylation levels at M7d and M0 and only one (cg07026010—*NUDCD3*) in case of M7d with M10h comparison (full list of significant CpGs is available in Additional file 1). Of these, 43 (59.72%), 24 (46.15%), and 1 (100%) CpG sites were hypermethylated, and 29 (40.28%), 28 (53.85%), and 0 (0%) CpG sites were hypomethylated when contrast analyses were applied for M10h vs M0, M7d vs M0, and M7d vs M10h respectively (Fig. 1). The median absolute difference in beta values, comparing all contrasts, was 0.013 (interquartile range (IQR), 0.006–0.029) for statistically significant differentially methylated probes. The average estimated genomic inflation factor (λ) for all three contrasts before correction was 1.64 ± 0.28, and after including covariates, it was reduced to 1.30 ± 0.15. Additional evaluation of λ with qq-plots depicted the same improvement ensured by including covariates (data not shown).

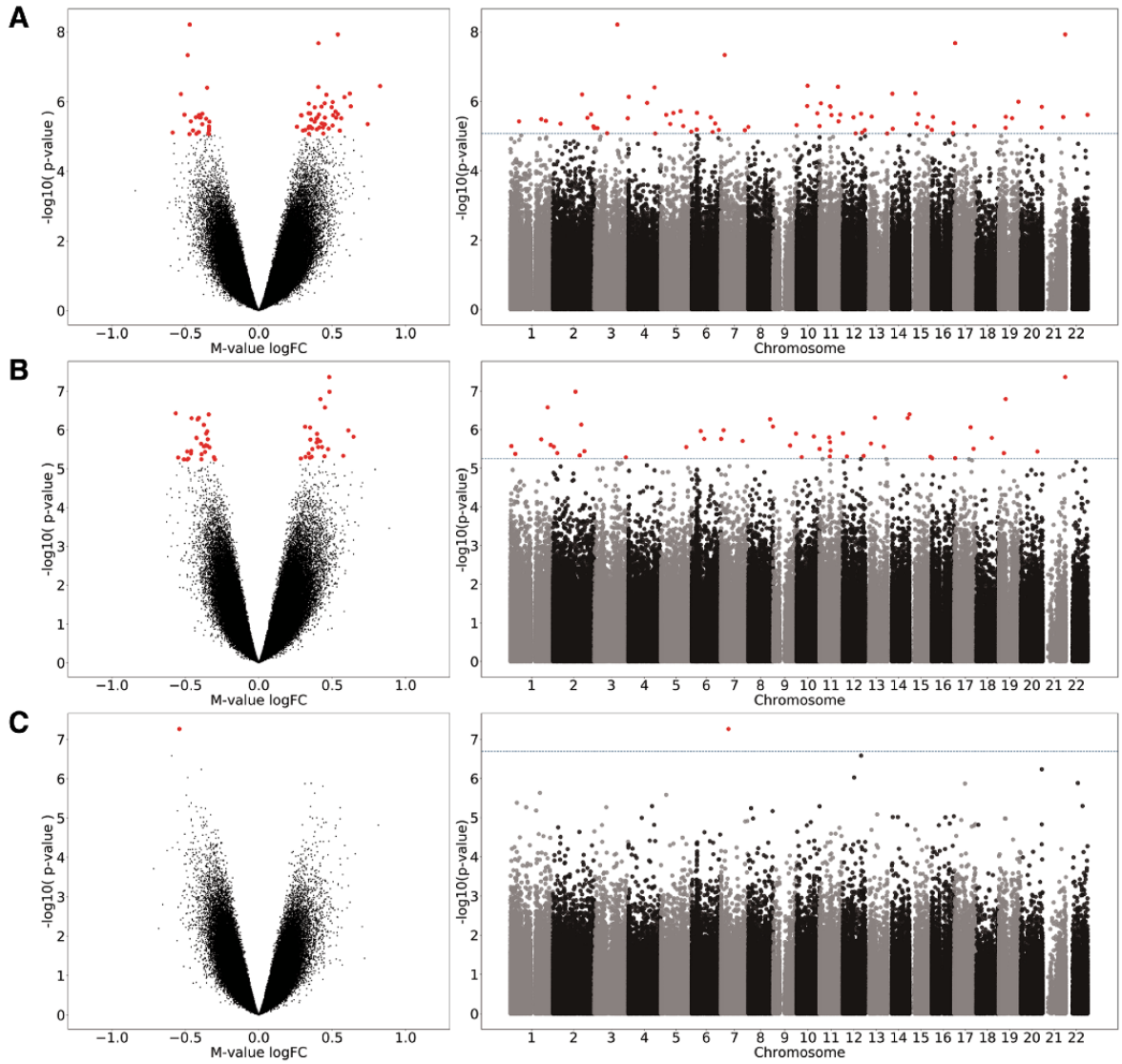


Fig. 1 Differentially methylated positions in all analyzed contrasts. Volcano plot showing raw − log10 (*p* value) versus log-fold change of *M* values and the Manhattan plot showing the position of probes with their corresponding unadjusted *p* values across the genome in **a** M10h vs M0, **b** M7d vs M0, and **c** M7d vs M10h sample comparisons. The significant CpG sites (after FDR correction) are highlighted in red. M0—before starting a metformin therapy; M10h—10 h after the first metformin intake, before the second tablet; M7d—time point after 7 days of metformin administration

Among the identified DMP, a total of 11 CpGs with the most consistent changes in the DNA methylation profile were emphasized (Fig. 2) based on two additional criteria. First, we included all overlapping DMP at both contrasts M10h vs M0 and M7d vs M0 ($n = 5$; cg03515060, cg18394557, cg16013966, cg05638165, cg18824330). Second, we selected probes if their median beta values at time points M10h and M7h overlapped IQRs of M7h and M10h, respectively. Also, IQRs of both time points could not overlap with IQR of time point M0 ($n = 6$; cg12740863, cg16843994, cg12162450, cg19176072, cg01644741, cg02622542). Of these 11 CpGs, 8 (72.73%) CpG sites displayed hypermethylation, while 3 (27.27%) CpG sites showed hypomethylation when comparing methylation levels after the metformin use (at time points M10h and M7d) with methylation levels before the use of metformin (Fig. 2).

All identified 11 CpG sites corresponded to 11 genes according to the 450k annotation file published by Price [8] (Table 2). One of these CpG sites was located in high-density CpG island, 7 CpG sites—in intermediate-density CpG islands with 1 bordering

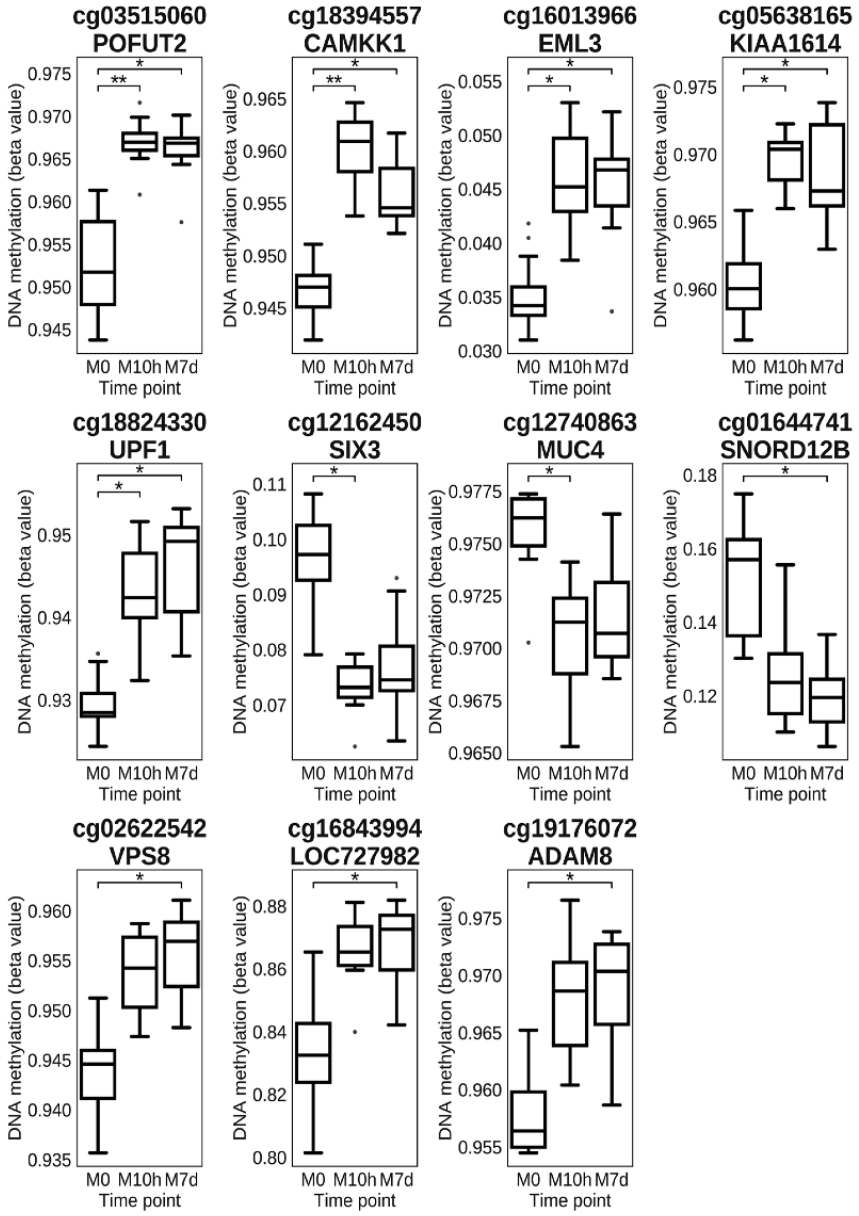


Fig. 2 Methylation levels of the top 11 most significant CpGs across the investigated three time points, i.e., M0 (before starting a metformin therapy), M10h (10 h after the first metformin intake, before the second tablet), and M7d (time point after 7 days of metformin administration), together with their associated genes. Box plots depict median, maximum, minimum, 25th percentile, and 75th percentile. Dots beyond the bounds of the whiskers represent outliers. * and ** denote significance levels 0.05 and 0.01 respectively

high-density CpG island, and 3—in non-islands according to HIL CpG classes.

To analyze the possible influence of circadian changes on the methylation profile, firstly, we searched our DMP list for the most common genes associated with regulation of circadian rhythm, such as *BMAL1*, *PER1*, *PER2*, *PER3*, *ARNTL*, *CRY1*, and *CRY2*. Secondly, we evaluated the main known functional roles of genes associated with the 125 DMPs, and, thirdly, we used the results from pathway enrichment analysis to find any connections with the circadian regulation. In result of these steps, we did not find any significant associations between the DMPs and circadian rhythm.

Table 2 Characterization of the top 11 most significant CpG sites

Filter	CpG site	Chr	logFC M10h vs M0	logFC M7d vs M0	FDR M10h vs M0	FDR M7d vs M0	Gene	Distance to the closest TSS	Gene context[a]	Spearman's correlation between methylation and transcription
Significant in both of the following contrasts: M10h vs M0 and M7d vs M0	cg03515060	21	0.538	0.479	*0.003*	*0.018*	POFUT2	1984	Body	− 0.184
	cg18394557	17	0.406	0.286	*0.003*	*0.047*	CAMKK1	− 8799	Body	− 0.042
	cg16013966	11	0.428	0.395	*0.034*	*0.037*	EML3	− 308	1stExon;5'UTR;TSS1500	− 0.23
	cg05638165	1	0.358	0.351	*0.035*	*0.034*	KIAA1614	14,198	Body	− 0.337
	cg18824330	19	0.363	0.419	*0.043*	*0.022*	UPF1	− 9944	Body	− 0.382
Significant in one of the contrasts and medians for time points M10h or M7d in IQR	cg12740863	3	− 0.359	− 0.260	*0.034*	0.127	MUC4	− 26,158		0.37
	cg16843994	2	0.347	0.404	0.091	*0.038*	LOC727982	− 706		NA
	cg12162450	2	− 0.386	− 0.349	*0.040*	0.059	SIX3	7515		NA
	cg19176072	10	0.462	0.472	0.054	*0.040*	ADAM8	5756	Body	− 0.312
	cg01644741	20	− 0.287	− 0.366	0.137	*0.043*	SNORD12B	39	Body,TSS1500	0.036
	cg02622542	3	0.269	0.348	0.151	*0.047*	VPS8	− 2419		0.166

Statistically significant FDR values are marked in italics
5'UTR 5' untranslated region, *TSS* transcription starting site
[a]TSS1500: Region 200–1500 base pairs upstream of the transcription start site

The correlation between methylation and RNA expression level of the corresponding gene was verified using targeted data form RNA-seq. Out of 11 genes tested, only the expression of *UPF1* (p − 0.024), *MUC4* (p − 0.029), and *KIAA1614* (p − 0.048) showed significant correlation with the methylation of corresponding CpG sites (Table 2).

Differentially methylated regions (DMRs)

During the DMR analysis, we found 13 regions with significant differences in methylation levels (summarized in Table 3). Five of the identified regions overlapped with some of the significant DMPs but not with the 11 sites prioritized by us.

Enrichment analysis

To evaluate the potential biological significance of the impact of differentially methylated CpG sites, we performed a gene set pathway enrichment analysis by using the Ingenuity Pathway Analysis (IPA). All genes associated with significant differentially methylated probes (FDR < 0.05) from different contrasts were selected.

Table 3 Differentially methylated regions

Contrast	Gene	FDR	Number of probes	Chr	Start (bp)[a]	End (bp)[a]	Transcription factors[b]
M10h vs M0	*EPHB1*	1.60E−11	3	3	134,515,421	134,516,302	–
	CDCA7L	3.83E−07	5	7	21,985,276	21,985,628	Nr1h3
	CLVS2	8.21E−07	10	6	123,317,123	123,317,875	Nrsf
	BACE2, MIR3197	1.38E−06	3	21	42,539,960	42,540,409	CTCF
	EXPH5	5.76E−06	6	11	108,464,101	108,464,498	Cmyc; Egr1; FOXA1; MYC; Max; SP1;
	KCNE4	1.50E−05	3	2	223,916,686	223,916,861	USF1
	TTC38	1.50E−05	4	22	46,685,471	46,685,728	NA
	TTC39A	1.51E−05	5	1	51,810,626	51,811,022	–
	NA	2.17E−05	3	4	153,897,215	153,897,453	NA
	NA	2.33E−05	3	10	132,891,318	132,891,371	NA
M7d vs M0	*SFRP2*	1.18E−11	28	4	132,891,371	154,711,183	CTCF; Egr1
	GPR19	4.59E−10	11	12	12,848,515	12,849,588	E2F4; ZBTB33;
	TMEM216	3.46E−07	7	11	61,159,601	61,159,837	CTCF; Egr1; Gabp; Yy1

[a]Physical position (basepair, hg37)
[b]Data from Ensembl 91 regulation resources [98], hg38

Thus, 72 genes were selected from the M10h vs M0 contrast and included in the first pathway analysis, and 52 genes from the M7d vs M0 contrast and included in the second pathway analysis. We did not include the only significant result from the M7d vs M10h contrast. The top enriched canonical pathways are summarized in Table 4.

In addition to the canonical pathways, we identified nine enriched networks in the M10h vs M0 contrast, and four in the M7d vs M0 comparison. The top enriched networks with IPA score > 20 were as follows (score/focus molecules): M10h vs M0—hematological system development and function, cellular movement, cell-to-cell signaling and interaction (28/13); hereditary disorder, neurological disease, organismal injury and abnormalities (23/11). M7d vs M0—cell-to-cell signaling and interaction, cellular assembly and organization, cellular function and maintenance (48/19); cell morphology, cell-to-cell signaling and interaction, cellular assembly and organization (41/17). Two of the most relevant networks are visualized in Fig. 3.

Discussion

The aim of our study was to examine metformin-induced alterations in epigenetic regulation processes by performing genome-wide DNA methylation analysis in human white blood cells followed by estimation of RNA expression levels of identified genes. We conducted our study in order to understand the pathways affected by metformin at real life physiological conditions in humans. This is extremely important taking into account the pleiotropic effects of metformin, and such studies may pinpoint important novel targets not only for treatment of T2D but also for other diseases. Various studies have shown that the evaluated effects in the methylation profile of peripheral blood DNA, that is the only option to access repeated tissue sampling in humans, are highly representative to the changes in other organs [9–11]. It is known that the DNA methylation pattern is highly subject specific and is influenced by many factors making it very difficult to identify the metformin-specific effects in a case-control-based type of study. We therefore selected a longitudinal design for this study, using short response time in order to exclude the influence of other factors. We also involved healthy volunteers to avoid a background of any commonly studied diseases so far related with the beneficial effects of metformin. One of our goals was to detect the fastest practically measurable effect of metformin on DNA methylation. Taking into account the known high variability of metformin pharmacokinetics, the time point when to evaluate the immediate and at the same time most profound effect was chosen to be the impact of one dose, and sampling time was selected at 10 h, before the recommended administration time of the second dose. Furthermore, M10h vs M0 sample comparison revealed the highest number or DMPs, representing the significant effect of one metformin dose.

To our best knowledge, this is the first study showing the metformin-mediated change of DNA methylation in healthy individuals already 10 h after administration. From the pool of 125 significantly modified sites, we prioritized 11 differentially methylated CpG with the largest and most consistent changes in beta values at different contrasts.

We assumed that some methylation changes measured at 10 h (M10h) could be caused by the circadian rhythm, which has been well described before and proven to be a driver of dynamic gene expression [12]. To avoid any false conclusions about the epigenetic targets of metformin, we paid specific attention to the presence of genes involved in the circadian rhythm among regions covering DMPs. We also evaluated this possibility by focusing on two contrasts that represent the different methylation profiles of DNA purified from blood samples that were collected in two distinct time points of the day—M7d vs M0 and M7d vs M10h. We did not observe any overlapping DMPs between the particular contrasts, suggesting no significant influence of the circadian rhythm on the DNA methylation in our data. Surprisingly, we observed only one significant DMP comparing M7d and M10h time points, providing a strong support for the fact that

Table 4 Top enriched canonical pathways by IPA

Contrast	Pathway	*p* value
M10h vs M0	Unfolded protein response	8.82×10^{-3}
	Salvage Pathways of Pyrimidine Deoxyribonucleotides	0.021
	Glycogen Degradation II	0.031
	Glycogen Degradation III	0.036
	Granzyme B Signalling	0.041
	Gα12/13 Signalling	0.046
	Lipid Antigen Presentation by CD1	0.048
M7d vs M0	S-Methyl-5-thio-α-D-ribose 1-phosphate Degradation	6.82×10^{-3}
	Gustation Pathway	0.025

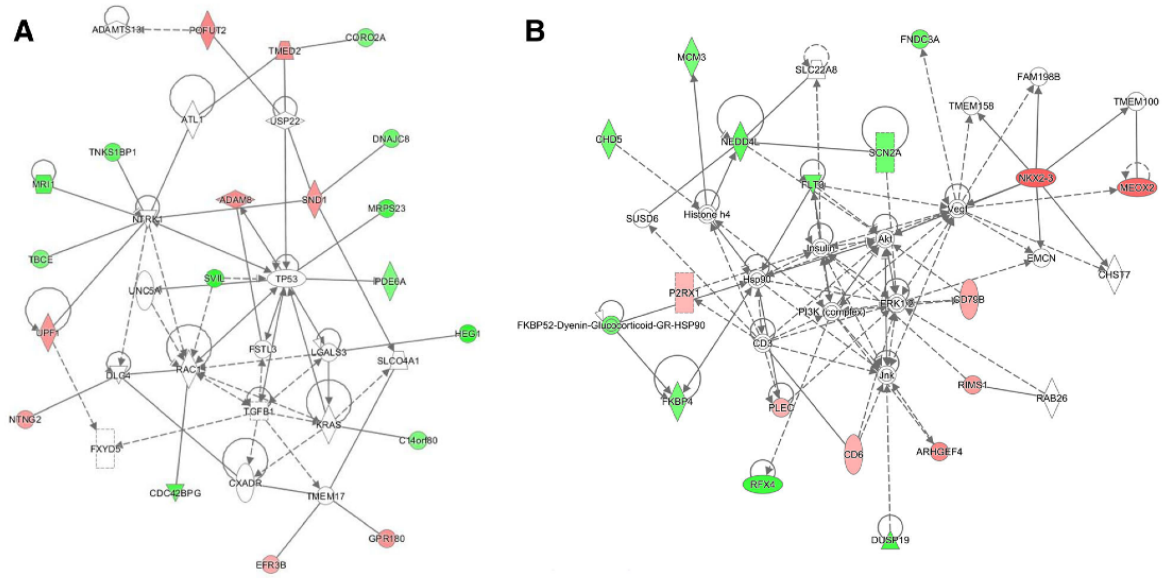


Fig. 3 Top enriched networks from IPA. Green nodes—hypermethylated; red nodes—hypomethylated. **a** Cell-to-cell signaling and interaction, cellular assembly and organization, and cellular function and maintenance (central predicted associated biological functions—tumorigenesis processes) had an IPA score of 48. **b** Cell morphology, cell-to-cell signaling and interaction, and cellular assembly and organization (central predicted associated biological functions—metabolism processes) had an IPA score of 41

observed methylation changes are indeed caused by metformin rather than other factors changing during the trial, such as diet or circadian cycle.

Genes corresponding to the top-ranked DMPs represent the main functional groups associated with previously described targets of metformin therapy: regulatory processes of energy homeostasis, inflammatory responses, tumorigenesis, and neurodegeneration. The criteria based on the comparison of median beta values and IQRs (see the "Results" section) were chosen to avoid bias in prioritization and would allow to include potentially important DMPs in addition to only those being significant at both M10h vs M0 and M7d vs M0 contrasts.

Interestingly, we found DMP within *CAMKK1* gene—one of two highly homologous genes coding for Ca2+/calmodulin-dependent protein kinase kinases (CaMKK) [13]—with CaMKK2 being a known regulator of AMP-activated protein kinase (AMPK). Despite the fact that only CaMKK2 has been proven to form a stable complex with AMPK, both isoforms of the CaMKK are capable of phosphorylating the AMPKα subunit at Thr-172 in vitro [14, 15]. From our data, the differentially methylated CpG close to the *CAMKK1* TSS together with negatively correlated mRNA expression data as the result of metformin administration suggests a potential contribution of CaMKK1 in the AMPK-mediated mechanism of metformin anti-diabetic action.

Furthermore, it is known that metformin exerts its effects also via AMPK-independent mechanisms [16], as shown by CaMKK1 ability to mediate glucose uptake in muscle cells independently from AMPK and Akt [17], in that way suggesting that methylation level changes in CaMKK1 could be a part from an alternative pathway responsible for the therapeutic effects of metformin.

Additionally, we identified a differentially methylated CpG site near the transcription factor coding gene *SIX3* [18]. Downregulation of *SIX3* due to the methylation of the *SIX3* promoter is observed in lung adenocarcinoma tissues and lung cancer cell lines, where mRNA expression of the gene is also associated with higher survival rate [19]. Some research suggest SIX3 linkage to diabetes from genetic studies [20] and show *SIX3* as possible regulator of insulin production in β-cells in an age-dependent manner [21]. Lowered methylation level of CpG near the *SIX3* TSS shown in our data suggests the DNA methylation as another potential epigenetic mechanism involved in *SIX3* expression regulation. *SIX3* is not expressed in human white blood cells [22], explaining the absence of reads corresponding to *SIX3* in our RNA-seq data, but gene expression may manifest in other tissues. So far, normalized insulin production itself has not been considered as a therapeutic effect of metformin, although it might be affected along with metformin-induced improvements of insulin sensitivity [23].

Our data also show ADAM8 as a considerable potential contributor in the anti-inflammatory action of metformin, that is, one of the known beneficial effects of this medication [24]. ADAM8 is a cell surface protease,

mainly expressed in granulocytes and monocytic cells, where it conducts the regulation of monocyte adhesion and migration [25–27]. Its contribution in the inflammatory responses regarding neurodegenerative disorders, allergy, asthma, and acute lung inflammation has been widely described before [28–31]. Our data justify the anti-inflammatory properties of metformin independently of diabetes status [24] and suggest the potential contribution of ADAM8 in the process. Due to its expression in human white blood cells, ADAM8 might be considered a promising biomarker for the detection of metformin-induced anti-inflammatory responses while reflecting inflammatory processes in adipocytes; however, further experimental evidence is required.

Many of the genes linked to the top-ranked DMPs are functionally associated with various malignancies. The most significant DMP in our study appeared to be situated in the body of *POFUT2*. O-Fucosyltransferase 2 encoded by *POFUT2* is proved to restrict epithelial-mesenchymal transition and affect cell motility in mouse embryos [32], and is considered as a useful prognostic biomarker in patients with glioblastoma and adenocarcinoma [33, 34]. To our knowledge, there are no reports yet describing *POFUT2*'s association with the beneficial effects of metformin. Our data also show several more DMPs located within or near the TSS of tumor-related genes, including SNORD12B—previously associated with colorectal and breast cancer pathogenesis [35–37], MUC4—promising prognostic marker and therapeutic target in the case of pancreatic cancer [38–40], KIAA1614 with promoter hypermethylation observed in colon tissues from patients with ulcerative colitis as well as in colon cancer cell lines [41], and UPF1 with indisputably crucial role in the maintenance of genome stability, significantly implicated in various malignancies [42–47].

The functions of two genes from the top DMPs' associated list are poorly defined. We identified increased DNA methylation level close to the TSS of VPS8 gene. VPS8 is an accessory subunit of CORVET complex, necessary for mediating multiple steps in the endocytic pathway and required for fusion of early endosomes [48]. Thus far, there is no conclusive data indicating the possible effects of VPS8 dysregulation on phenotype in humans [49–52]. Likewise, the function of long intergenic non-protein coding RNA 1249 (LINC01249/LOC727982) is still not clear with only few reports on genetic association of SNPs within the gene with infectious disease and blood pressure [53, 54].

Overall, the DNA methylation has a repressive effect on transcription factor binding; therefore, we used ENCODE data on transcription factor binding sites to identify such possible interactions [55, 56]. We detected transcription factors CTCF, CTCFL, and Egr1 binding to the genomic region overlapping the differentially methylated CpG within *EML3* gene; out of these, CTCF is proved to mediate glucagon production [57] and Egr1 is responsible for insulin resistance [58]. Although there are no data available to date, supporting direct metformin impact on EML3 (nuclear microtubule-binding protein) [59] or describing EML3 contribution in metformin therapeutic effects, increased expression of *EML3* in cultured human cells as a result of metformin-1816 small molecule perturbation has been reported before [60]. Likewise, the genomic region within *UPF1* gene, covering the top-ranked CpG site is associated with CTCF, Egr1, and two more transcription factors: MYC—involved in the pathogenesis of diabetes [61], and PU1—initiating insulin resistance as well as regulating lipolysis [62].

The detected DMRs, as well, could essentially be grouped by connection to the processes currently known to be affected by metformin. For example, the most significant DMR was associated with *EPHB1*, which together with other Ephrin receptors forms the largest subgroup of the Eph receptor tyrosine kinase (RTK) family [63]. Underexpression of the EphB1 protein is significantly associated with tumor progression in gastric carcinomas and higher invasiveness of colorectal cancer cells, suggesting a tumor-suppressive role of the protein and possible implication in the beneficial effects of metformin [64, 65].

Another noteworthy DMR was associated with APP-cleaving enzyme 2 coding gene (*BACE2*) encoded protein that cleaves amyloid precursor protein into amyloid beta peptide, and is implicated in the pathogenesis of neurodegenerative diseases including Alzheimer's disease [66–68]. Interestingly, increased β-cell proliferation and glucose-stimulated insulin secretion resulting from reduced Bace2 levels have been previously reported [69]. In a mouse model of T2D, induced by the overexpression of human islet amyloid polypeptide, *BACE2* deficiency improved glucose tolerance, suggesting that *BACE2* inhibition might serve as a potential therapeutic strategy for T2D treatment [70].

Another DMR is associated with *SFRP2*, Secreted Frizzled Related Protein 2. Methylation changes in the promoter region of *SFRP2* have been proposed as a potential noninvasive biomarker for colorectal cancer [71, 72]. Its mRNA is also expressed in mouse and human adipose tissue, and elevated levels have positive correlation with BMI and with abnormal glucose tolerance [73].

The pathway enrichment analysis revealed metformin's association with various pathways some of which already has been described in connection with metformin action but not in the context of epigenetic regulation. The top enriched pathway after one dose of metformin—Unfolded Protein Response (UPR)—has been shown to be one of the main mechanisms of inducing apoptosis by metformin in

acute lymphoblastic leukemia cells [74], and metformin-induced UPR inhibition in kidney cells can explain metformin's beneficial effects [75].

One of the products of the top enriched pathway describing changes after week long metformin administration (*S*-methyl-5-thio-α-D-ribose 1-phosphate Degradation) is L-methionine, an essential amino acid in human organism. Moreover, it is known that L-methionine is used for generation of *S*-adenosylmethionine (SAM) [76], which has been depicted to be an essential part of metformin-induced increase in global methylation levels as it accumulates in cells during metformin therapy [5]. Taking into account the results from enriched pathways and the fact that we mostly observe metformin-induced hypermethylation than hypomethylation, it is possible that activation of this particular canonical pathway may contribute to the previously described increase in SAM levels.

Although enriched networks (Fig. 3) are not directly related to known metformin effects, the downstream molecules of those associated with differential methylation levels in our study group are known to be involved in various pathways related with T2D (e.g., AKT, ERK1/2, JNK, P13K), insulin regulation processes [77], cancer development mechanisms [78], and other.

The correlation between DNA methylation and gene expression is complex and nonlinear [79]. The generally accepted consequence of DNA methylation is transcriptional repression; however, methylation in the transcribed region might also demonstrate positive correlation with mRNA expression [80]. In our study, we did not detect a convincing correlation between DNA methylation of top-ranked loci and transcription level of corresponding genes; however, the influence of methylation as well as gene expression itself are tissue-specific and might be missed by focusing on single type of cells only. Nevertheless, the significant correlation observed between the expression levels of *UPF1*, *MUC4*, *KIAA1614*, and the methylation level of the corresponding CpG sites provide evidence for a crucial contribution of epigenetic regulation in the mechanism of action of metformin, which results in specific alterations of gene expression profiles.

Currently, it is not fully known whether metformin has only an indirect effect on the epigenetic regulation processes in the human organism via the previously described H19/SAHH axis or through linking cellular metabolism to the mechanisms needed for DNA methylation [4, 5]. However, the methylation profile and concentration of metformin used in cell type specific in vitro experiments may significantly differ from the physiological levels and observations in the affected cells in human body. The large variation of SAH and SAM levels in various cell types has been described [5]. In addition, the previous studies evaluating the metformin-induced methylation profile changes mostly have been targeted; thus, it is not surprising that we did not observe the DMPs at the same genes or pathways.

Major limitation of this study is the low sample size even though there are number of reports using the same number of individuals in their studies [81–84]. On the other hand, we believe that this weakness is compensated by the number of strengths in our design. First, we used a longitudinal study design and it has been recognized that, in similar time series studies, individuals can be treated as their own controls before and during treatment and sufficiently increase the power of the study [85] compared to case-control design especially accounting for the inter-individual variability among study participants. Secondly, the longitudinal design combined with observation of methylation changes in the shortest possible time allows us to minimize the effects of other factors that can induce changes in methylation unrelated to the metformin treatment. Thirdly, inclusion of healthy subjects should have minimized false associations and conclusions arising from unaccounted treatment status by metformin or other medications in T2D patients, including the unknown true duration of T2D before diagnosis. Finally, the use of genome-wide methylation analysis allows us to observe unbiased effects and find new metformin targets.

Another limitation in our study is the lack of clinical and biochemical measures at all time points. In the same time, it has been previously shown that metformin has small or no effect of such measures as plasma glucose level in healthy individuals [86, 87], and we decided not to include those in study protocol.

Unfortunately, due to the lack of similar studies, we were not able to support our findings from literature and replication in other cohorts is needed.

Conclusions

This is the first study showing the immediate effect of metformin on white blood cell DNA methylation in humans at therapeutic doses. The gained knowledge about the metformin-induced methylation profile changes in healthy individuals can be used as basis for further in vitro and in vivo studies, which are important due to the growing number of various metformin therapeutic application possibilities in non-diabetic patients.

Methods

Study design

Study group involved 12 healthy metformin-naïve voluntary individuals. The involvement and sample collection was organized in collaboration with the Genome Database of Latvian Population (LGDB) [88]. Exclusion/inclusion criteria (Additional file 2) were defined according to the requirements of concurrently ongoing

clinical trial (registration number: 2016-001092-74 (www.clinicaltrialsregister.eu)), which also involves gut microbiome analysis. Participants were included if they matched the following criteria: have not used antibiotics, immunosuppressive medicaments, corticosteroids, or pharmaceutical-grade probiotics during the time period of the past 2 months; have not been diagnosed with oncological, autoimmune, chronical gastrointestinal tract diseases, or T2D; have not had diarrhea in the past week; and are not taking any other medications incompatible with metformin. The research subjects received an 850-mg metformin tablet (Berlin-Chemie AG) twice a day for a week. Samples for hematological, biochemical tests were collected in certified clinical laboratory at fasting state 1–3 days before starting the metformin administration. Whole blood samples for methylation analysis were collected at three time points: (1) before starting metformin therapy (morning, fasting state)—M0, (2) 10 h after first metformin intake, before the second tablet (evening)—M10h, and (3) after 7 days of metformin administration (morning, fasting state)—M7d. Throughout the article, we have defined the measurement of 10-h sample as the immediate effect of metformin.

Sample analysis

DNA isolation from whole blood samples using the phenol-chloroform extraction method was performed by Genome Database of Latvian Population (briefly described before [89]). DNA samples were quantified with Qubit® 2.0 Fluorometer using Qubit dsDNA HS Assay Kit (TherfmoFisher Scientific, USA). For the bisulfite conversion, the EZ DNA Methylation-Gold TM kit (Zymo research, USA) was used according to the manufacturer's instructions. DNA methylation was determined by the Illumina Infinium HumanMethylation450 BeadChip Array (Illumina, USA), using 500 ng of each bisulfite-treated DNA sample.

Total RNA was isolated from whole blood samples using PerfectPure RNA Blood Kit (5Prime GmbH, Hamburg, Germany). Ribosomal RNS depletion was done with Low Input RiboMinus™ Eukaryote System v2 (Thermo Fisher Scientific, USA). For cDNA library preparation, we used Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific, USA), and sequencing was performed on the Ion Proton™ System and Ion PI™ Chip (Thermo Fisher Scientific, USA).

Data preprocessing and statistical analysis

IDAT files were imported using R package minfi [90]. Cell counts were estimated from methylation data using Houseman algorithm [91] implemented in minfi.

Data preprocessing and normalization was done using Enmix [92]. Briefly, probes with detection p value > 0.05 and probes with a multimodal distribution were filtered out. Background correction was performed with the function preprocessENmix using unused color channels as a background parameter estimate. Probe intensities were normalized using a quantile normalization method, and probe type bias was adjusted using the Regression on Correlated Probes (RCP) method [93]. Probes having a SNP or single base extension annotation in CpG site were excluded. Due to interrupted use of metformin by one of the study subjects, the sample taken after 1 week of metformin administration for that particular subject was discarded.

Batch effect was removed from data using slide and subsequently subjects as covariates as they showed the strongest influence on the probe methylation variability. Batch effect was removed using ComBat [94] wrapped in the Enmix package. Differentially methylated probes between time points were identified using limma [95] on ComBat preprocessed data, adjusting for the following cell types estimated by minfi: CD8T, CD4T, NK, and Gran. Inflation factor of p-value distribution was estimated using R package GenABEL [96]. All analyses were performed using R (3.3.3).

Statistically significant DMRs were identified with DMRcate software [97], FDR < 0.05. Threshold for minimum number of probes within the region was set to three. DMRs were estimated from methylation M values using the individual CpG site significance threshold at FDR < 0.05. The interval between individual significant CpG sites had to be less than 1000 bp in the regions. The bandwidth scaling factor was set as suggested in the manual ($C = 2$). Regulatory information from Ensembl 91 regulation resources was added to identified DMPs and DMRs using Ensembl Regulation API [98].

Pathway enrichment analysis was performed with the IPA tool [99]. Information about enriched canonical pathways and networks was obtained performing the core analysis on all significant DMPs with FDR < 0.05.

RNA-seq data analysis

Reads were mapped against human reference genome GRCh38, and read quantification was performed using STAR (2.5.3a) [100]. Obtained per-gene read counts were normalized using trimmed mean normalization (TMM), and counts per million (CPM) values were calculated with edgeR [101]. ComBat [94] implemented in R package sva [102] was used to adjust CPM values for subject-specific effects, and the Spearman correlation was estimated for the adjusted CPM values and the beta values for 11 selected CpG sites with SciPy [103].

Additional files

Additional file 1: Full results representing all CpGs within the analyzed contrasts with significantly changed methylation levels, identified after

correction for multiple testing using the Benjamini-Hochberg method. (XLSX 21 kb)

Additional file 2: List of inclusion/exclusion criteria. (DOCX 14 kb)

Abbreviations

CPM: Counts per million; DMP: Differentially methylated CpG site/probe; DMR: Differentially methylated region; IPA: Ingenuity Pathway Analysis; IQR: Interquartile range; RCP: Regression on Correlated Probes; T2D: Type 2 diabetes; TMM: Trimmed mean normalization; TSS: Transcription starting site

Acknowledgements

We acknowledge the Genome Database of Latvian Population (LGDB) and Latvian Biomedical Research and Study Centre for organization of participants' recruitment and bio-sample processing and data storage. The authors thank all the volunteers who participated in the clinical trial.

Funding

The work was supported by the European Regional Development Fund under the project "Investigation of interplay between multiple determinants influencing response to metformin: search for reliable predictors for efficacy of type 2 diabetes therapy" (Project Nr.: 1.1.1.1/16/A/091).

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

IKalnina, LZ, DF, HBS, and JK designed the research; IKonrade and VP oversaw patient recruitment; IE, IKalnina, DG, IRS, conducted the research; IE, IS, MU, and RP performed the analyses; IE, IS, MU wrote the manuscript; IK, DMC, CZ, HBS, and JK oversaw the research and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Study has been carried out in accordance with the Declaration of Helsinki and approved by Central Medical Ethics Committee (1/16-05-12) and The State Agency of Medicines of the Republic of Latvia (17-1723), clinical trial registration number: 2016-001092-74 (www.clinicaltrialsregister.eu).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1]Latvian Biomedical Research and Study Centre, Ratsupites Str. 1 k-1, Riga LV-1067, Latvia. [2]Riga East Clinical University Hospital, 2 Hipokrata Street, Riga LV-1038, Latvia. [3]Department of Neuroscience, Functional Pharmacology, Uppsala University, BMC, Box 593, 751 24 Uppsala, Sweden.

Received: 17 April 2018 Accepted: 29 November 2018
Published online: 13 December 2018

References

1. Marshall SM. 60 years of metformin use: a glance at the past and a look to the future. Diabetologia. 2017;60:1561–5.
2. Song AQ, Sun LR, Zhao YX, Gao YH, Chen L. Effect of insulin and metformin on methylation and glycolipid metabolism of peroxisome proliferator-activated receptor gamma coactivator-1A of rat offspring with gestational diabetes mellitus. Asian Pac J Trop Med. 2016;9:91–5.
3. Niu N, Liu T, Cairns J, Ly RC, Tan X, Deng M, Fridley BL, Kalari KR, Abo RP, Jenkins G, et al. Metformin pharmacogenomics: a genome-wide association study to identify genetic and epigenetic biomarkers involved in metformin anticancer response using human lymphoblastoid cell lines. Hum Mol Genet. 2016;25:4819–34.
4. Zhong T, Men Y, Lu L, Geng T, Zhou J, Mitsuhashi A, Shozu M, Maihle NJ, Carmichael GG, Taylor HS, Huang Y. Metformin alters DNA methylation genome-wide via the H19/SAHH axis. Oncogene. 2017;36:2345–54.
5. Cuyas E, Fernandez-Arroyo S, Verdura S, Garcia RA, Stursa J, Werner L, Blanco-Gonzalez E, Montes-Bayon M, Joven J, Viollet B, et al. Metformin regulates global DNA methylation via mitochondrial one-carbon metabolism. Oncogene. 2017;37:963.
6. Garcia-Calzon S, Perfilyev A, Mannisto V, de Mello VD, Nilsson E, Pihlajamaki J, Ling C. Diabetes medication associates with DNA methylation of metformin transporter genes in the human liver. Clin Epigenetics. 2017;9:102.
7. Bridgeman SC, Ellison GC, Melton PE, Newsholme P, Mamotte CDS. Epigenetic effects of metformin: from molecular mechanisms to clinical implications. Diabetes Obes Metab. 2018;20:1553–62.
8. Price ME, Cotton AM, Lam LL, Farre P, Emberly E, Brown CJ, Robinson WP, Kobor MS. Additional annotation enhances potential for biologically-relevant analysis of the Illumina Infinium HumanMethylation450 BeadChip array. Epigenetics Chromatin. 2013;6:4.
9. Crujeiras AB, Diaz-Lagares A, Sandoval J, Milagro FI, Navas-Carretero S, Carreira MC, Gomez A, Hervas D, Monteiro MP, Casanueva FF, et al. DNA methylation map in circulating leukocytes mirrors subcutaneous adipose tissue methylation pattern: a genome-wide analysis from non-obese and obese patients. Sci Rep. 2017;7:41903.
10. Farre P, Jones MJ, Meaney MJ, Emberly E, Turecki G, Kobor MS. Concordant and discordant DNA methylation signatures of aging in human blood and brain. Epigenetics Chromatin. 2015;8:19.
11. Barault L, Ellsworth RE, Harris HR, Valente AL, Shriver CD, Michels KB. Leukocyte DNA as surrogate for the evaluation of imprinted Loci methylation in mammary tissue DNA. PLoS One. 2013;8:e55896.
12. Lim AS, Srivastava GP, Yu L, Chibnik LB, Xu J, Buchman AS, Schneider JA, Myers AJ, Bennett DA, De Jager PL. 24-hour rhythms of DNA methylation and their relation with rhythms of RNA expression in the human dorsolateral prefrontal cortex. PLoS Genet. 2014;10:e1004792.
13. Okuno S, Kitani T, Fujisawa H. Studies on the substrate specificity of Ca2+/calmodulin-dependent protein kinase kinase alpha. J Biochem. 1997;122:337–43.
14. Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M, Carling D. Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. Cell Metab. 2005;2:21–33.
15. Green MF, Anderson KA, Means AR. Characterization of the CaMKKbeta-AMPK signaling complex. Cell Signal. 2011;23:2005–12.
16. Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, Tanti JF, Le Marchand-Brustel Y, Bost F. The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene. 2008;27:3576–86.
17. Witczak CA, Fujii N, Hirshman MF, Goodyear LJ. Ca2+/calmodulin-dependent protein kinase kinase-alpha regulates skeletal muscle glucose uptake independent of AMP-activated protein kinase and Akt activation. Diabetes. 2007;56:1403–9.
18. Granadino B, Gallardo ME, Lopez-Rios J, Sanz R, Ramos C, Ayuso C, Bovolenta P, Rodriguez de Cordoba S. Genomic cloning, structure, expression pattern, and chromosomal location of the human SIX3 gene. Genomics. 1999;55:100–5.
19. Mo ML, Okamoto J, Chen Z, Hirata T, Mikami I, Bosco-Clement G, Li H, Zhou HM, Jablons DM, He B. Down-regulation of SIX3 is associated with clinical outcome in lung adenocarcinoma. PLoS One. 2013;8:e71816.
20. Hwang JY, Sim X, Wu Y, Liang J, Tabara Y, Hu C, Hara K, Tam CH, Cai Q, Zhao Q, et al. Genome-wide association meta-analysis identifies novel variants associated with fasting plasma glucose in East Asians. Diabetes. 2015;64:291–8.
21. Arda HE, Li L, Tsai J, Torre EA, Rosli Y, Peiris H, Spitale RC, Dai C, Gu X, Qu K, et al. Age-dependent pancreatic gene regulation reveals mechanisms governing human beta cell function. Cell Metab. 2016;23:909–20.
22. Harrow J, Frankish A, Gonzalez JM, Tapanari E, Diekhans M, Kokocinski F, Aken BL, Barrell D, Zadissa A, Searle S, et al. GENCODE: the reference human genome annotation for the ENCODE project. Genome Res. 2012;22:1760–74.
23. Giannarelli R, Aragona M, Coppelli A, Del Prato S. Reducing insulin resistance with metformin: the evidence today. Diabetes Metab. 2003;29:6S28–35.

24. Cameron AR, Morrison VL, Levin D, Mohan M, Forteath C, Beall C, McNeilly AD, Balfour DJ, Savinko T, Wong AK, et al. Anti-inflammatory effects of metformin irrespective of diabetes status. Circ Res. 2016;119:652–65.
25. Yoshiyama K, Higuchi Y, Kataoka M, Matsuura K, Yamamoto S. CD156 (human ADAM8): expression, primary amino acid sequence, and gene location. Genomics. 1997;41:56–62.
26. Yoshida S, Setoguchi M, Higuchi Y, Akizuki S, Yamamoto S. Molecular cloning of cDNA encoding MS2 antigen, a novel cell surface antigen strongly expressed in murine monocytic lineage. Int Immunol. 1990;2:585–91.
27. Hodgkinson CP, Ye S. Microarray analysis of peroxisome proliferator-activated receptor-gamma induced changes in gene expression in macrophages. Biochem Biophys Res Commun. 2003;308:505–10.
28. Dreymueller D, Pruessmeyer J, Schumacher J, Fellendorf S, Hess FM, Seifert A, Babendreyer A, Bartsch JW, Ludwig A. The metalloproteinase ADAM8 promotes leukocyte recruitment in vitro and in acute lung inflammation. Am J Physiol Lung Cell Mol Physiol. 2017;313:L602–14.
29. Schlomann U, Rathke-Hartlieb S, Yamamoto S, Jockusch H, Bartsch JW. Tumor necrosis factor alpha induces a metalloprotease-disintegrin, ADAM8 (CD 156): implications for neuron-glia interactions during neurodegeneration. J Neurosci. 2000;20:7964–71.
30. Fourie AM, Coles F, Moreno V, Karlsson L. Catalytic activity of ADAM8, ADAM15, and MDC-L (ADAM28) on synthetic peptide substrates and in ectodomain cleavage of CD23. J Biol Chem. 2003;278:30469–77.
31. Chen J, Jiang X, Duan Y, Long J, Bartsch JW, Deng L. ADAM8 in asthma. Friend or foe to airway inflammation? Am J Respir Cell Mol Biol. 2013;49:875–84.
32. Du J, Takeuchi H, Leonhard-Melief C, Shroyer KR, Dlugosz M, Haltiwanger RS, Holdener BC. O-fucosylation of thrombospondin type 1 repeats restricts epithelial to mesenchymal transition (EMT) and maintains epiblast pluripotency during mouse gastrulation. Dev Biol. 2010;346:25–38.
33. Dong S, Nutt CL, Betensky RA, Stemmer-Rachamimov AO, Denko NC, Ligon KL, Rowitch DH, Louis DN. Histology-based expression profiling yields novel prognostic markers in human glioblastoma. J Neuropathol Exp Neurol. 2005;64:948–55.
34. Aramburu A, Zudaire I, Pajares MJ, Agorreta J, Orta A, Lozano MD, Gurpide A, Gomez-Roman J, Martinez-Climent JA, Jassem J, et al. Combined clinical and genomic signatures for the prognosis of early stage non-small cell lung cancer based on gene copy number alterations. BMC Genomics. 2015;16:752.
35. Gaedcke J, Grade M, Camps J, Sokilde R, Kaczkowski B, Schetter AJ, Difilippantonio MJ, Harris CC, Ghadimi BM, Moller S, et al. The rectal cancer microRNAome--microRNA expression in rectal cancer and matched normal mucosa. Clin Cancer Res. 2012;18:4919–30.
36. Xu L, Ziegelbauer J, Wang R, Wu WW, Shen RF, Juhl H, Zhang Y, Rosenberg A. Distinct profiles for mitochondrial t-RNAs and small nucleolar RNAs in locally invasive and metastatic colorectal cancer. Clin Cancer Res. 2016;22:773–84.
37. Askarian-Amiri ME, Crawford J, French JD, Smart CE, Smith MA, Clark MB, Ru K, Mercer TR, Thompson ER, Lakhani SR, et al. SNORD-host RNA Zfas1 is a regulator of mammary development and a potential marker for breast cancer. RNA. 2011;17:878–91.
38. Kaur S, Kumar S, Momi N, Sasson AR, Batra SK. Mucins in pancreatic cancer and its microenvironment. Nat Rev Gastroenterol Hepatol. 2013;10:607–20.
39. Andrianifahanana M, Moniaux N, Schmied BM, Ringel J, Friess H, Hollingsworth MA, Buchler MW, Aubert JP, Batra SK. Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance. Clin Cancer Res. 2001;7:4033–40.
40. Gautam SK, Kumar S, Cannon A, Hall B, Bhatia R, Nasser MW, Mahapatra S, Batra SK, Jain M. MUC4 mucin- a therapeutic target for pancreatic ductal adenocarcinoma. Expert Opin Ther Targets. 2017;21:657–69.
41. Kang K, Bae JH, Han K, Kim ES, TO K, Yi JM. A genome-wide methylation approach identifies a new hypermethylated gene panel in ulcerative colitis. Int J Mol Sci. 2016;17:1291.
42. Azzalin CM, Lingner J. The human RNA surveillance factor UPF1 is required for S phase progression and genome stability. Curr Biol. 2006;16:433–9.
43. Chawla R, Redon S, Raftopoulou C, Wischnewski H, Gagos S, Azzalin CM. Human UPF1 interacts with TPP1 and telomerase and sustains telomere leading-strand replication. EMBO J. 2011;30:4047–58.
44. Chang L, Li C, Guo T, Wang H, Ma W, Yuan Y, Liu Q, Ye Q, Liu Z. The human RNA surveillance factor UPF1 regulates tumorigenesis by targeting Smad7 in hepatocellular carcinoma. J Exp Clin Cancer Res. 2016;35:8.
45. Li L, Geng Y, Feng R, Zhu Q, Miao B, Cao J, Fei S. The human RNA surveillance factor UPF1 modulates gastric cancer progression by targeting long non-coding RNA MALAT1. Cell Physiol Biochem. 2017;42:2194–206.
46. Liu C, Karam R, Zhou Y, Su F, Ji Y, Li G, Xu G, Lu L, Wang C, Song M, et al. The UPF1 RNA surveillance gene is commonly mutated in pancreatic adenosquamous carcinoma. Nat Med. 2014;20:596–8.
47. Wang D, Zavadil J, Martin L, Parisi F, Friedman E, Levy D, Harding H, Ron D, Gardner LB. Inhibition of nonsense-mediated RNA decay by the tumor microenvironment promotes tumorigenesis. Mol Cell Biol. 2011;31:3670–80.
48. Perini ED, Schaefer R, Stoter M, Kalaidzidis Y, Zerial M. Mammalian CORVET is required for fusion and conversion of distinct early endosome subpopulations. Traffic. 2014;15:1366–89.
49. Lunetta KL, D'Agostino RB Sr, Karasik D, Benjamin EJ, Guo CY, Govindaraju R, Kiel DP, Kelly-Hayes M, Massaro JM, Pencina MJ, et al. Genetic correlates of longevity and selected age-related phenotypes: a genome-wide association study in the Framingham Study. BMC Med Genet. 2007;8(Suppl 1):S13.
50. Pankratz N, Dumitriu A, Hetrick KN, Sun M, Latourelle JC, Wilk JB, Halter C, Doheny KF, Gusella JF, Nichols WC, et al. Copy number variation in familial Parkinson disease. PLoS One. 2011;6:e20988.
51. Antoni G, Oudot-Mellakh T, Dimitromanolakis A, Germain M, Cohen W, Wells P, Lathrop M, Gagnon F, Morange PE, Tregouet DA. Combined analysis of three genome-wide association studies on vWF and FVIII plasma levels. BMC Med Genet. 2011;12:102.
52. Cai DC, Fonteijn H, Guadalupe T, Zwiers M, Wittfeld K, Teumer A, Hoogman M, Arias-Vasquez A, Yang Y, Buitelaar J, et al. A genome-wide search for quantitative trait loci affecting the cortical surface area and thickness of Heschl's gyrus. Genes Brain Behav. 2014;13:675–85.
53. Manjurano A, Sepulveda N, Nadjm B, Mtove G, Wangai H, Maxwell C, Olomi R, Reyburn H, Drakeley CJ, Riley EM, et al. USP38, FREM3, SDC1, DDC, and LOC727982 gene polymorphisms and differential susceptibility to severe malaria in Tanzania. J Infect Dis. 2015;212:1129–39.
54. Simino J, Shi G, Bis JC, Chasman DI, Ehret GB, Gu X, Guo X, Hwang SJ, Sijbrands E, Smith AV, et al. Gene-age interactions in blood pressure regulation: a large-scale investigation with the CHARGE, global BPgen, and ICBP Consortia. Am J Hum Genet. 2014;95:24–38.
55. Maurano MT, Wang H, John S, Shafer A, Canfield T, Lee K, Stamatoyannopoulos JA. Role of DNA methylation in modulating transcription factor occupancy. Cell Rep. 2015;12:1184–95.
56. Rosenbloom KR, Sloan CA, Malladi VS, Dreszer TR, Learned K, Kirkup VM, Wong MC, Maddren M, Fang R, Heitner SG, et al. ENCODE data in the UCSC genome browser: year 5 update. Nucleic Acids Res. 2013;41:D56–63.
57. Saxena R, Saleheen D, Been LF, Garavito ML, Braun T, Bjonnes A, Young R, Ho WK, Rasheed A, Frossard P, et al. Genome-wide association study identifies a novel locus contributing to type 2 diabetes susceptibility in Sikhs of Punjabi origin from India. Diabetes. 2013;62:1746–55.
58. Shen N, Yu X, Pan FY, Gao X, Xue B, Li CJ. An early response transcription factor, Egr-1, enhances insulin resistance in type 2 diabetes with chronic hyperinsulinism. J Biol Chem. 2011;286:14508–15.
59. Tegha-Dunghu J, Neumann B, Reber S, Krause R, Erfle H, Walter T, Held M, Rogers P, Hupfeld K, Ruppert T, et al. EML3 is a nuclear microtubule-binding protein required for the correct alignment of chromosomes in metaphase. J Cell Sci. 2008;121:1718–26.
60. Lamb J, Crawford ED, Peck D, Modell JW, Blat IC, Wrobel MJ, Lerner J, Brunet JP, Subramanian A, Ross KN, et al. The connectivity map: using gene-expression signatures to connect small molecules, genes, and disease. Science. 2006;313:1929–35.
61. Cheung L, Zervou S, Mattsson G, Abouna S, Zhou L, Ifandi V, Pelengaris S, Khan M. c-Myc directly induces both impaired insulin secretion and loss of beta-cell mass, independently of hyperglycemia in vivo. Islets. 2010;2:37–45.
62. Lin L, Pang W, Chen K, Wang F, Gengler J, Sun Y, Tong Q. Adipocyte expression of PU.1 transcription factor causes insulin resistance through upregulation of inflammatory cytokine gene expression and ROS production. Am J Physiol Endocrinol Metab. 2012;302:E1550–9.
63. Himanen JP, Nikolov DB. Eph receptors and ephrins. Int J Biochem Cell Biol. 2003;35:130–4.
64. Wang JD, Dong YC, Sheng Z, Ma HH, Li GL, Wang XL, Lu GM, Sugimura H, Jin J, Zhou XJ. Loss of expression of EphB1 protein in gastric carcinoma associated with invasion and metastasis. Oncology. 2007;73:238–45.
65. Sheng Z, Wang J, Dong Y, Ma H, Zhou H, Sugimura H, Lu G, Zhou X. EphB1 is underexpressed in poorly differentiated colorectal cancers. Pathobiology. 2008;75:274–80.

66. Hussain I, Powell DJ, Howlett DR, Chapman GA, Gilmour L, Murdock PR, Tew DG, Meek TD, Chapman C, Schneider K, et al. ASP1 (BACE2) cleaves the amyloid precursor protein at the beta-secretase site. Mol Cell Neurosci. 2000;16:609–19.
67. Basi G, Frigon N, Barbour R, Doan T, Gordon G, McConlogue L, Sinha S, Zeller M. Antagonistic effects of beta-site amyloid precursor protein-cleaving enzymes 1 and 2 on beta-amyloid peptide production in cells. J Biol Chem. 2003;278:31512–20.
68. Murphy MP, LeVine H 3rd. Alzheimer's disease and the amyloid-beta peptide. J Alzheimers Dis. 2010;19:311–23.
69. Esterhazy D, Stutzer I, Wang H, Rechsteiner MP, Beauchamp J, Dobeli H, Hilpert H, Matile H, Prummer M, Schmidt A, et al. Bace2 is a beta cell-enriched protease that regulates pancreatic beta cell function and mass. Cell Metab. 2011;14:365–77.
70. Alcarraz-Vizan G, Castano C, Visa M, Montane J, Servitja JM, Novials A. BACE2 suppression promotes beta-cell survival and function in a model of type 2 diabetes induced by human islet amyloid polypeptide overexpression. Cell Mol Life Sci. 2017;74:2827–38.
71. Wang DR, Tang D. Hypermethylated SFRP2 gene in fecal DNA is a high potential biomarker for colorectal cancer noninvasive screening. World J Gastroenterol. 2008;14:524–31.
72. Pehlivan S, Artac M, Sever T, Bozcuk H, Kilincarslan C, Pehlivan M. Gene methylation of SFRP2, P16, DAPK1, HIC1, and MGMT and KRAS mutations in sporadic colorectal cancer. Cancer Genet Cytogenet. 2010;201:128–32.
73. Crowley RK, O'Reilly MW, Bujalska IJ, Hassan-Smith ZK, Hazlehurst JM, Foucault DR, Stewart PM, Tomlinson JW. SFRP2 is associated with increased adiposity and VEGF expression. PLoS One. 2016;11:e0163777.
74. Leclerc GM, Leclerc GJ, Kuznetsov JN, DeSalvo J, Barredo JC. Metformin induces apoptosis through AMPK-dependent inhibition of UPR signaling in ALL lymphoblasts. PLoS One. 2013;8:e74420.
75. Theriault JR, Palmer HJ, Pittman DD. Inhibition of the unfolded protein response by metformin in renal proximal tubular epithelial cells. Biochem Biophys Res Commun. 2011;409:500–5.
76. Mato JM, Lu SC. Role of S-adenosyl-L-methionine in liver health and injury. Hepatology. 2007;45:1306–12.
77. Frojdo S, Vidal H, Pirola L. Alterations of insulin signaling in type 2 diabetes: a review of the current evidence from humans. Biochim Biophys Acta. 2009;1792:83–92.
78. Kasznicki J, Sliwinska A, Drzewoski J. Metformin in cancer prevention and therapy. Ann Transl Med. 2014;2:57.
79. Lim YC, Li J, Ni Y, Liang Q, Zhang J, Yeo GSH, Lyu J, Jin S, Ding C. A complex association between DNA methylation and gene expression in human placenta at first and third trimesters. PLoS One. 2017;12:e0181155.
80. Jones PA. The DNA methylation paradox. Trends Genet. 1999;15:34–7.
81. Benton MC, Johnstone A, Eccles D, Harmon B, Hayes MT, Lea RA, Griffiths L, Hoffman EP, Stubbs RS, Macartney-Coxson D. An analysis of DNA methylation in human adipose tissue reveals differential modification of obesity genes before and after gastric bypass and weight loss. Genome Biol. 2015;16:8.
82. Alkhaled Y, Laqqan M, Tierling S, Lo Porto C, Amor H, Hammadeh ME. Impact of cigarette-smoking on sperm DNA methylation and its effect on sperm parameters. Andrologia. 2018;50:e12950.
83. Cheng Q, Zhao B, Huang Z, Su Y, Chen B, Yang S, Peng X, Ma Q, Yu X, Zhao B, Ke X. Epigenome-wide study for the offspring exposed to maternal HBV infection during pregnancy, a pilot study. Gene. 2018;658:76–85.
84. Urdinguio RG, Torro MI, Bayon GF, Alvarez-Pitti J, Fernandez AF, Redon P, Fraga MF, Lurbe E. Longitudinal study of DNA methylation during the first 5 years of life. J Transl Med. 2016;14:160.
85. Goodrich JK, Di Rienzi SC, Poole AC, Koren O, Walters WA, Caporaso JG, Knight R, Ley RE. Conducting a microbiome study. Cell. 2014;158:250–62.
86. Sambol NC, Chiang J, O'Conner M, Liu CY, Lin ET, Goodman AM, Benet LZ, Karam JH. Pharmacokinetics and pharmacodynamics of metformin in healthy subjects and patients with noninsulin-dependent diabetes mellitus. J Clin Pharmacol. 1996;36:1012–21.
87. Tokubuchi I, Tajiri Y, Iwata S, Hara K, Wada N, Hashinaga T, Nakayama H, Mifune H, Yamada K. Beneficial effects of metformin on energy metabolism and visceral fat volume through a possible mechanism of fatty acid oxidation in human subjects and rats. PLoS One. 2017;12:e0171293.
88. Rovite V, Wolff-Sagi Y, Zaharenko L, Nikitina-Zake L, Grens E, Klovins J. Genome Database of the Latvian Population (LGDB): design, goals, and primary results. J Epidemiol. 2018;28:353–60.
89. Ignatovica V, Latkovskis G, Peculis R, Megnis K, Schioth HB, Vaivade I, Fridmanis D, Pirags V, Erglis A, Klovins J. Single nucleotide polymorphisms of the purinergic 1 receptor are not associated with myocardial infarction in a Latvian population. Mol Biol Rep. 2012;39:1917–25.
90. Aryee MJ, Jaffe AE, Corrada-Bravo H, Ladd-Acosta C, Feinberg AP, Hansen KD, Irizarry RA. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics. 2014;30:1363–9.
91. Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, Wiencke JK, Kelsey KT. DNA methylation arrays as surrogate measures of cell mixture distribution. BMC Bioinformatics. 2012;13:86.
92. Xu Z, Niu L, Li L, Taylor JA. ENmix: a novel background correction method for Illumina HumanMethylation450 BeadChip. Nucleic Acids Res. 2016;44:e20.
93. Niu L, Xu Z, Taylor JA. RCP: a novel probe design bias correction method for Illumina Methylation BeadChip. Bioinformatics. 2016;32:2659–63.
94. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics. 2007;8:118–27.
95. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43:e47.
96. Aulchenko YS, Ripke S, Isaacs A, van Duijn CM. GenABEL: an R library for genome-wide association analysis. Bioinformatics. 2007;23:1294–6.
97. Peters TJ, Buckley MJ, Statham AL, Pidsley R, Samaras K, R VL, Clark SJ, Molloy PL. De novo identification of differentially methylated regions in the human genome. Epigenetics Chromatin. 2015;8:6.
98. Zerbino DR, Johnson N, Juettemann T, Sheppard D, Wilder SP, Lavidas I, Nuhn M, Perry E, Raffaillac-Desfosses Q, Sobral D, et al. Ensembl regulation resources. Database (Oxford). 2016;2016:bav119.
99. Kramer A, Green J, Pollard J Jr, Tugendreich S. Causal analysis approaches in ingenuity pathway analysis. Bioinformatics. 2014;30:523–30.
100. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29:15–21.
101. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26:139–40.
102. Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. Bioinformatics. 2012;28:882–3.
103. Olivier BG, Rohwer JM, Hofmeyr JH. Modelling cellular processes with Python and Scipy. Mol Biol Rep. 2002;29:249–54.

III Baseline gut microbiome composition predicts metformin therapy short-term efficacy in newly diagnosed type 2 diabetes patients

Highlights:

1. At the species level, reduction in the abundance of *Clostridium bartlettii* and *Barnesiella intestinihominis*, and an increase in the abundance of *Parabacteroides distasonis* and *Oscillibacter* unclassified overlapped between both T2D patients and healthy individuals.
2. Non-Respoders group had higher abundance of species *Prevotella copri* compared to Responders before starting metformin therapy.
3. The gut microbiome of metformin therapy Responders at baseline was characterized by enrichment of *Enterococcus faecium*, *Lactococcus lactis*, *Odoribacter*, and *Dialister*.

PLOS ONE

RESEARCH ARTICLE

Baseline gut microbiome composition predicts metformin therapy short-term efficacy in newly diagnosed type 2 diabetes patients

Ilze Elbere[1], Ivars Silamikelis[1], Ilze Izabella Dindune[1], Ineta Kalnina[1], Monta Ustinova[1], Linda Zaharenko[1], Laila Silamikele[1], Vita Rovite[1], Dita Gudra[1], Ilze Konrade[1,2], Jelizaveta Sokolovska[3], Valdis Pirags[1,3], Janis Klovins[1]*

1 Latvian Biomedical Research and Study Centre, Riga, Latvia, **2** Riga Stradins University, Riga, Latvia, **3** Faculty of Medicine, University of Latvia, Riga, Latvia

* klovins@biomed.lu.lv

Citation: Elbere I, Silamikelis I, Dindune II, Kalnina I, Ustinova M, Zaharenko L, et al. (2020) Baseline gut microbiome composition predicts metformin therapy short-term efficacy in newly diagnosed type 2 diabetes patients. PLoS ONE 15(10): e0241338. https://doi.org/10.1371/journal.pone.0241338

Editor: Christopher Staley, University of Minnesota Twin Cities, UNITED STATES

Received: April 17, 2020

Accepted: October 13, 2020

Published: October 30, 2020

Data Availability Statement: All shotgun metagenomic sequences are available from the European Nucleotide Archive (ENA) (accession number PRJEB39500).

Funding: The work was supported by the European Regional Development Fund under the project "Investigation of interplay between multiple determinants influencing response to metformin: search for reliable predictors for efficacy of type 2 diabetes therapy" (Project No. 1.1.1.1/16/A/091,

Abstract

Background

The study was conducted to investigate the effects of metformin treatment on the human gut microbiome's taxonomic and functional profile in the Latvian population, and to evaluate the correlation of these changes with therapeutic efficacy and tolerance.

Methods

In this longitudinal observational study, stool samples for shotgun metagenomic sequencing-based analysis were collected in two cohorts. The first cohort included 35 healthy nondiabetic individuals (metformin dose 2x850mg/day) at three time-points during metformin administration. The second cohort was composed of 50 newly-diagnosed type 2 diabetes patients (metformin dose–determined by an endocrinologist) at two concordant times. Patients were defined as Responders if their HbA1c levels during three months of metformin therapy had decreased by ≥12.6 mmol/mol (1%), while in Non-responders HbA1c were decreased by <12.6 mmol/mol (1%).

Results

Metformin reduced the alpha diversity of microbiota in healthy controls ($p = 0.02$) but not in T2D patients. At the species level, reduction in the abundance of *Clostridium bartlettii* and *Barnesiella intestinihominis*, as well as an increase in the abundance of *Parabacteroides distasonis* and *Oscillibacter* unclassified overlapped between both study groups. A large number of group-specific changes in taxonomic and functional profiles was observed. We identified an increased abundance of *Prevotella copri* (FDR = 0.01) in the Non-Responders subgroup, and enrichment of *Enterococcus faecium*, *Lactococcus lactis*, *Odoribacter*, and *Dialister* at baseline in the Responders group. Various taxonomic units were associated with the observed incidence of side effects in both cohorts.

https://ec.europa.eu/regional_policy/en/funding/erdf). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Conclusions

Metformin effects are different in T2D patients and healthy individuals. Therapy induced changes in the composition of gut microbiome revealed possible mediators of observed short-term therapeutic effects. The baseline composition of the gut microbiome may influence metformin therapy efficacy and tolerance in T2D patients and could be used as a powerful prediction tool.

Introduction

Type 2 diabetes (T2D) is a metabolic disease with rapidly increasing prevalence, characterized by variable etiology, clinical presentation, and consequences. Metformin has been used in clinical practice for more than 60 years [1, 2] and is currently considered as a first-choice medication for T2D treatment worldwide. Regardless of its diverse beneficial impact on health, more than 20% of patients fail to reach the glycemic target when on metformin monotherapy [3], and more than 30% experience mostly gastrointestinal (GI) side effects (SE) [4]. These results suggest that the gut microbiome is an intermediary of metformin therapy, which highlight the need for the development of precision medicine-based therapeutic approaches [5]. Because of the complex structure, compositional and functional dynamics, and the host-microbiome interaction, the microbiome has been postulated as a key component of precision medicine approaches [6]. Moreover, latest studies have suggested that metagenomics predictive tools for T2D should be specific for the age and geographical location of the population studied [7]. Several studies on the interaction between the human gut microbiome and metformin have been performed [8–12], but most of these studies used a case-control design involving patients with different diabetes duration and therapy history.

The aim of this study was to investigate the effects of metformin treatment on the taxonomical and functional profile of the human gut microbiome and to evaluate the correlation of these changes with the therapeutic efficacy and tolerance in a prospective cohort of T2D patients. Our research provides novel information on short-term effects induced by metformin with the advantage of longitudinal data, including treatment naïve patients, as well as characterizes the predictive quality of baseline microbiota composition. In addition, the growing evidence on other therapeutic targets of metformin requires more detailed information on metformin effects in non-diabetic populations, therefore, the data from the healthy cohort offer complementary value.

Materials and methods

Study design, sample and data collection

The study involved two longitudinal cohorts of participants: OPTIMED cohort of newly-diagnosed T2D patients (N = 50) recruited within the framework of Genome Database of Latvian population [13], and a cohort of healthy individuals (N = 35). A full list of inclusion/exclusion criteria for both cohorts can be found in the S1 Text. Informed consent was obtained from all participants at the beginning of the study. Healthy individuals received 850mg metformin twice a day for 7 days within the framework of the clinical trial (registration number: 2016-001092-74 (www.clinicaltrialsregister.eu)), while T2D patients were treated with metformin monotherapy according to therapy prescribed by an endocrinologist (individual dosage, titration, etc.). The study was carried out in accordance with the principles of the Declaration of

Helsinki, and approved by the Central Medical Ethics Committee (1/19-10-22) and State Agency of Medicines of the Republic of Latvia (17–1723). The primary and secondary endpoints together with other methodological details (regarding the clinical trial involving healthy volunteers and standard operating procedures in place for stool sample collection) have been described previously [11].

Stool samples were collected in two aliquots at pre-determined time points during the study, depending on the design for each study cohort (Fig 1). Samples were coded as follows: M0 – before metformin treatment, M24h – 24 hours after the first metformin dose (only in the study group of healthy individuals), and M7d – 7 days after starting the therapy. All samples were collected by participants at home, using sterile collection tubes without buffer (collection date and time were marked). Within 24 hours participants delivered samples to the closest clinical or research laboratory where samples were frozen at −80˚C (delivery time was registered).

Blood samples for biochemical/hematological analysis (conducted in a certified clinical laboratory) to evaluate inclusion/exclusion criteria and obtain relevant clinical data were collected from participants within both cohorts. Samples were collected in the fasting state before starting metformin administration. For the patient cohort, a repeated biochemical/hematological analysis was performed three months later (follow-up coded as a time point M3m).

The information on anthropometric measurements, dietary habits, and biochemical/hematological analyses was obtained before starting metformin administration. Healthy volunteers registered their diet during the metformin administration, as well as any observed SE in special questionnaires. Patients of OPTIMED cohort were interviewed via phone by their endocrinologists after the first week of metformin therapy to register possible metformin-induced SE.

For the analysis of gut microbiome mediated metformin's therapy efficacy patients were divided into two subgroups based on the observed reduction of HbA_{1c} during three months long metformin therapy. Patients were defined as Responders if their HbA_{1c} levels had

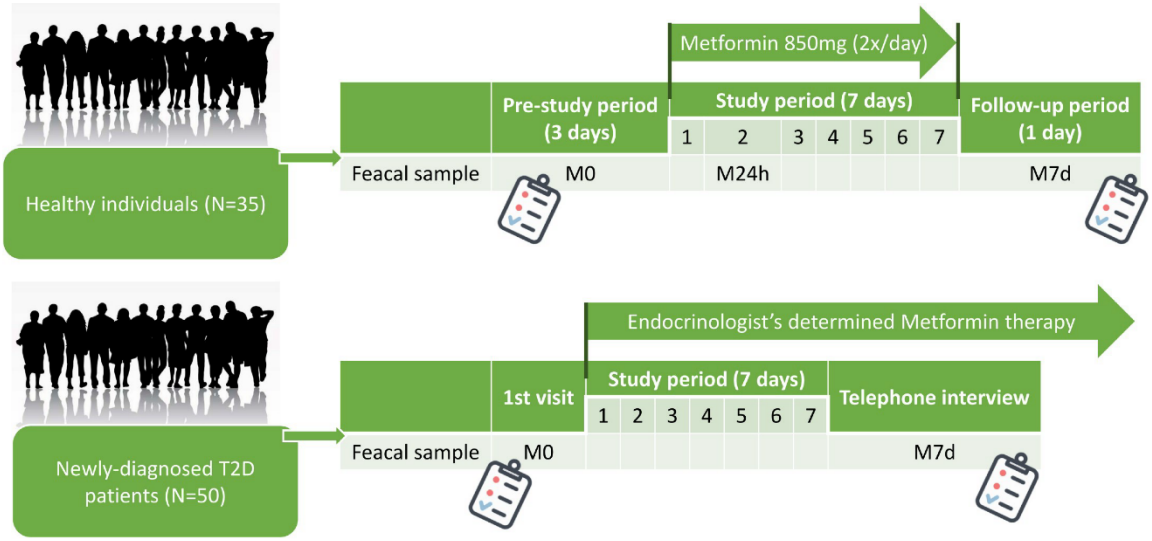


Fig 1. Study design depicting sample collection in both cohorts. Samples were coded as follows: M0 –before starting metformin treatment, M24h – 24 hours after first metformin dose, and M7d – 7 days after the first intake of metformin. T2D –type 2 diabetes.

https://doi.org/10.1371/journal.pone.0241338.g001

decreased by ≥12.6 mmol/mol (1%), or Non-responders if their HbA_{1c} levels had decreased by <12.6 mmol/mol (1%). This threshold has been previously established within a systematic review comparing three months long metformin therapy with placebo and used in other studies as well [14, 15].

Sample processing and sequencing

Microbial DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals) in line with to the manufacturer's instructions [16]. Further shotgun metagenomic library preparation was done by fragmenting the DNA at 300 bp (Covaris) and following the manual of the Ion Plus Fragment Library kit (ThermoFisher Scientific, USA). That included the following sample processing steps: (1) end repair after the physical fragmentation and clean-up with NucleoMag magnetic beads (Macherey-Nagel, Düren, Germany), (2) adaptor ligation, nick-repair, and clean-up, (3) size selection in the range 360–440 bp, performed with BluePippin DNA 2% Dye-Free Agarose gel cassette with V1 Marker, and clean-up, and (4) amplification and clean-up. Samples were sequenced using Ion Proton sequencer with Ion PI Chip Kit v3 (>3000000 reads/sample) [17, 18].

Sequence analysis and statistics

Raw data from the sequencer were processed as follows: adapters were removed with cutadapt 1.16, sequences were trimmed with Trimmomatic v0.38 (5bp window, quality threshold = 20, average quality = 20, minimal length = 75), mapping was performed with bowtie2-2.3.5.1 using Homo sapiens genome Ensembl GRCh38 release-90 reference to remove host DNA sequences. Information on read numbers during sequence preprocessing has been summarized in S3 Table.

Composition and functionality from the remaining sequences of gut microbiome samples were analyzed using the HUMAnN2 pipeline [19], and taxonomic data were obtained with MetaPhlAn2 [20], analyses were performed with default parameters. Species level alpha diversity was calculated as the exponential of the Shannon index resulting in the effective number of species, and beta diversity was analyzed with non-metric multidimensional scaling (NMDS) using Bray-Curtis distances. Results of beta diversity were compared between subgroups with permutational multivariate analysis of variance—PERMANOVA. To explain the effects of environmental variables, adonis function (vegan package) was used to test the significance of individual variables, and complemented with Canonical Correspondence Analysis (CCA) and visualized with biplot using R software (version 3.6.0) [21]. Evaluation of variables of interest was performed in two cases: (1) for all samples–both groups, baseline and follow up–to evaluate the contribution of age, gender and BMI; (2) only for T2D patient samples–to evaluate possible effect of the different prescribed metformin doses. Changes during metformin therapy and differences between study subgroups within the taxonomic and functional profiles were evaluated by R package *limma* using *voom* transformation with sample-specific quality weights (further referred as limma+voom). All tests were adjusted by age, gender, and BMI, false discovery rate (FDR) adjusted values were used. T2D group data were adjusted by baseline HbA_{1c} levels. Only taxa present in ≥10% of samples were included. To compare metformin therapy response groups, the corrected data matrix was used for sparse Partial least squares discriminant analysis (sPLS-DA), a supervised model to reveal microbiota variation between groups. Key taxonomic groups responsible for the differential microbiota structure were detected using the "splsda" function in the R package "mix Omics" [22], tuning of sPLS-DA parameters was performed to determine the main taxonomic groups that enable discrimination of the subgroups with the lowest possible error rate. Taxonomic groups with variable importance in

projection (VIP) > 1.5 were considered to be important contributors to the model. Additional cellular function enrichment analysis and visualization of functional profile data were performed using the Omics Dashboard integrated into MetaCyc. The dashboard computes enrichment p-values using Grossmann's parent-child-union variation of the Fisher-exact test (applying the FDR multiple hypothesis correction) and then transforms each p-value to an enrichment score: -log10 (p-value). Significance threshold <0.05 [23]. Statistical significance for changes/differences of the Shannon index and other analyzed parameters was evaluated by the Wilcoxon signed-rank test. Data normalizations were performed as integrated into the used tools, paired comparisons were used when appropriate.

Validation cohort

To validate the results of the performed sPLS-DA analysis, we included another independent cohort of 58 newly diagnosed T2D patients. Inclusion/exclusion criteria, sample collection guidelines and design were the same as for the OPTIMED cohort, however, data were obtained from a different sequencing platform. Sequencing data preprocessing and statistical analysis were performed as described for OPTIMED cohort. Detailed information on methods for sample and data processing of Validation cohort is provided in S2 Text.

Results

Characterization of study cohorts

In total 100 samples were collected and analyzed from the OPTIMED cohort, and 103 samples from the healthy individuals. The characterization of the analyzed groups is summarized in Table 1. The average ±SD sequencing depth was 4.6 M ± 2.4 M raw reads per sample. During the clinical trial, two healthy participants withdrew from the study prematurely due to GI-SE, therefore no data on M7d time point were available for these two participants.

Retrospective analyses of the questionnaire data from the OPTIMED cohort revealed that few patients had some deviations from the expected study design: one patient was assigned to diet change in the first week, without any drug treatment; another used sulfonylurea group medication. These participants were excluded from the study group, leaving 48 T2D patients for further analysis.

Table 1. Characteristics of the analyzed cohorts.

Characteristic	*Healthy individuals, N = 35*	*T2D patients, N = 50*	*Validation cohort (T2D patients), N = 58*
Males/females, n (%)	10 (28.6%) / 25 (71.4%)	22 (44%) / 28 (56%)	30 (51.7%) / 28 (28.3%)
Age (years), mean ± SD	31.5 ± 10.2	58.6 ±12.5	58.2 ± 10.3
BMI, mean ± SD	24.5 ± 3.2	34.8 ± 6.7	34.0 ± 5.9
HbA_{1c} (mmol/mol) mean ± SD	32.2 ± 1.8	66.1 ± 0.5	59.6 ± 0.5
HbA_{1c} (%), mean ± SD	5.1 ± 0.5	8.2 ± 2.1	7.6 ± 2.0
Creatinine (μmol/l), mean ± SD	67.6 ± 11.6	68.6 ± 13.7	67.3 ± 17.0
ALAT (U.l), mean ± SD	23.5 ± 10.7	40.3 ± 21.8	46.0 ± 30.7
TG (mmol/l), mean ± SD	1.3 ± 1.0	2.4 ±1.8	2.4 ± 1.6
HDL-C (mmol/l), mean ± SD	1.6 ± 0.4	1.2 ± 0.3	1.3 ± 0.4
LDL-C (mmol/l), mean ± SD	2.8 ± 0.8	3.6 ± 1.0	3.4 ± 1.3
Metformin dose (mg/day), mean ± SD	1700 ± 0	1146 ± 702	1006 ± 455

T2D - type 2 diabetes; SD–standard deviation; BMI–body mass index; ALAT—alanine transaminase, TG – triglycerides, HDL-C–High-density lipoprotein cholesterol, LDL-C – Low-density lipoprotein cholesterol.

https://doi.org/10.1371/journal.pone.0241338.t001

When evaluating sample composition between both analyzed cohorts at baseline (M0 time point), it was possible to distinguish these groups based on beta diversity (Fig 2A). Differences were statistically significant (PERMANOVA: $R^2 = 0.035$, p = 0.0015). Moreover, alpha diversity (Fig 2B) was significantly higher in the healthy cohort. To characterize the possible effect of available covariates and their contribution to the variation in the taxonomical composition, CCA was performed on all collected samples. We found the corresponding contribution of the analyzed cofactors: age (1.5%, p = 0.001), BMI (4.1%, p = 0.001), gender (1.4%, p = 0.001).

Metformin-induced changes in the taxonomic profile

Metformin induced a significant decrease in effective species number in healthy individuals (M0 vs M7d –median M0 = 12.9, median M7d = 11.8; p = 0.024), supporting the results from our pilot study in a smaller group [11]. In T2D patients we observed a slight increase in the effective species number at the same time points (M0 vs M7d –median M0 = 15.6, median M7d = 12.1; p = 0.35) but this change was not significant (Fig 2B).

After a weeklong metformin treatment, the healthy group showed 115 significantly changed features at various taxonomic levels, and the OPTIMED cohort showed 26 changed features (Fig 3, S1 and S2 Figs, S2 Table). At species level, only four alterations overlapped between both study groups–a decrease in the abundance of *Clostridium bartlettii* and *Barnesiella intestinihominis*, and an increase in the abundance of *Parabacteroides distasonis* and *Oscillibacter* unclassified–while other changes in the taxonomic profile were specific to the analyzed cohorts.

To ensure the accuracy of the results, we additionally tested the possible effects of different metformin doses. When analyzed by CCA, the dose of metformin was not a significant co-factor in influencing the microbiome composition (0.9%, p = 0.56) in the patient cohort.

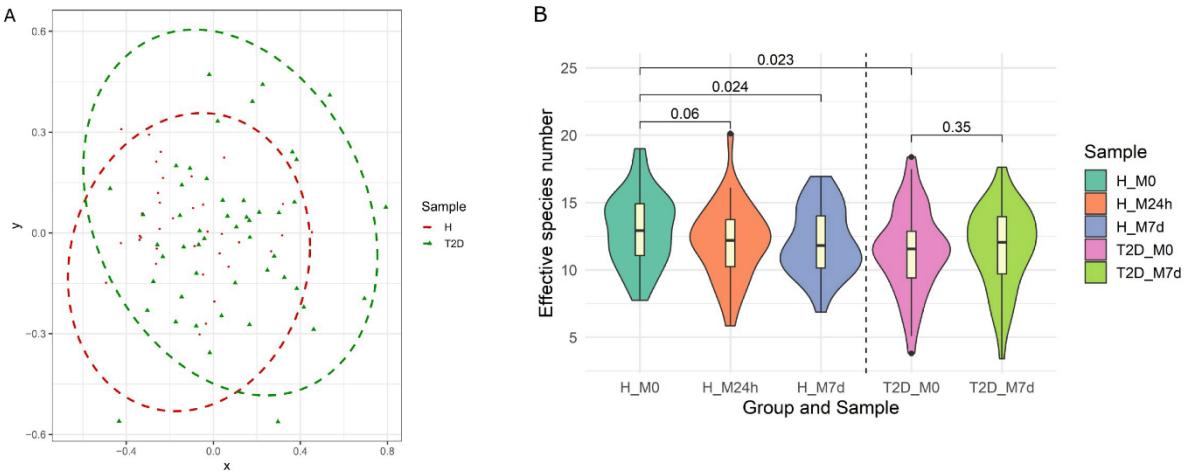


Fig 2. Diversity characteristics of analyzed samples. (A) Beta diversity characterizing and comparing samples before metformin therapy between healthy individuals (H) and OPTIMED cohort patients (T2D). Ellipses represent the 95% confidence interval surrounding each group of samples. Different symbols represent the participants of the study. Red circles correspond to healthy individuals while green triangles represent type 2 diabetes patients. (B) Alpha diversity calculated in all analyzed time points. Groups marked as follows: H–healthy individuals; T2D – type 2 diabetes patients. Samples: M0—before starting metformin treatment; M24h – 24 hours after the first intake of metformin; M7d –after 7 days treatment with metformin. Violin plot representing the effective number of species combines boxplots, depicting the median value and interquartile ranges, with Kernel density plots.

https://doi.org/10.1371/journal.pone.0241338.g002

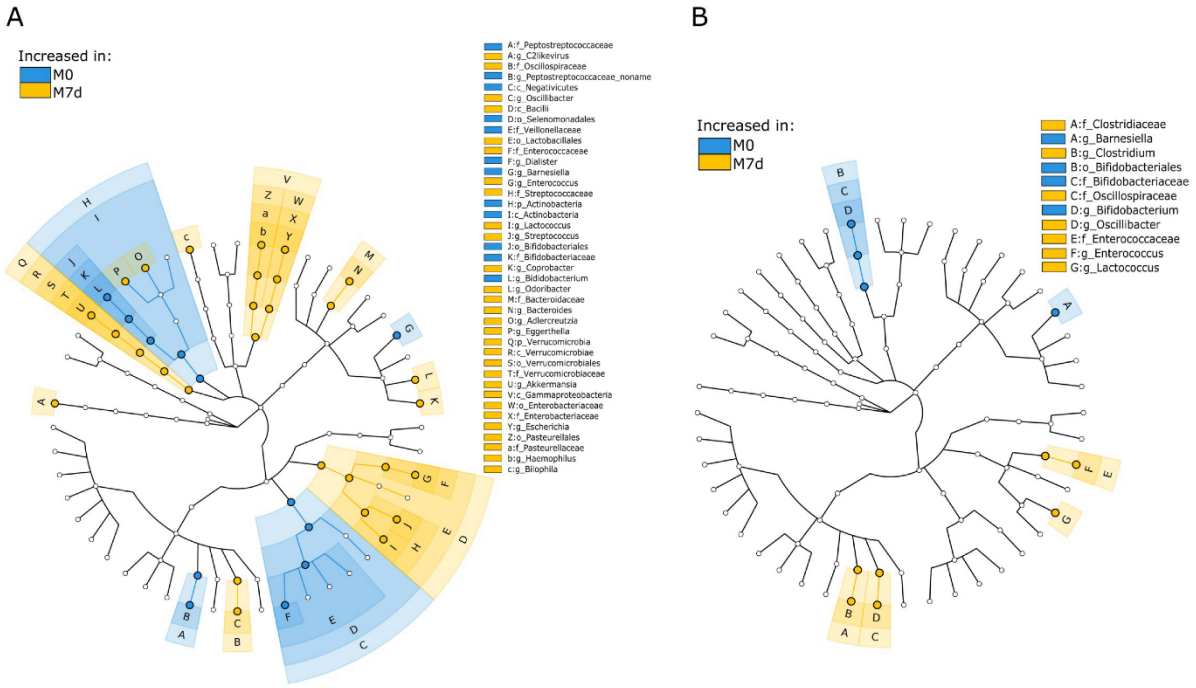


Fig 3. GraPhlAn cladogram for taxonomic composition changes during a week-long metformin administration. (A) healthy individuals, (B) type 2 diabetes patients. Samples are marked as follows: M0—before starting metformin treatment (blue); M7d –after 7 days treatment with metformin (yellow). Colors of nodes and shading indicate the microbial lineages that are enriched within corresponding samples. Only differentially abundant taxa at the genus or higher taxonomic ranks are indicated. For detailed results in lower taxonomical levels, see S1 and S2 Figs.

https://doi.org/10.1371/journal.pone.0241338.g003

Taxonomic differences associated with the treatment side effects and efficacy

During the study, all observed SE were registered in the study group-specific questionnaires. Study subjects from each cohort were divided into two groups according to the type of registered SE during the usage of metformin. The first group included participants with no or mild SE defined by headache, meteorism (tympanites), stomach ache, nausea, and loss of appetite; and the second group included individuals with severe SE defined by loose stools 1–3 times a day, diarrhea, vomiting. In the OPTIMED cohort, nine individuals had severe SE and 39 did not report any SE while in the group of healthy individuals 21 participants had mild or no SE and 14 had severe intolerance. For a detailed analysis of possible microbiome mediated mechanisms and predictors of metformin-induced GI-SE, we performed a comparison of taxonomic profiles between these defined groups at the analyzed study time points (Fig 4).

As a next step, we evaluated the association between the presence of specific taxonomic groups before therapy start (M0) and the efficacy of metformin therapy (changes in HbA_{1c} levels during the first three months of therapy) in the cohort of T2D. Two of the patients had withdrawn from the OPTIMED study before the three-month time point (M3m), therefore, they were excluded from this analysis. We divided the remaining OPTIMED cohort (N = 46) into two groups characterized in Table 2. Metformin's therapeutic effects induced a statistically significant reduction in HbA_{1c} levels during the first three months of therapy in both groups

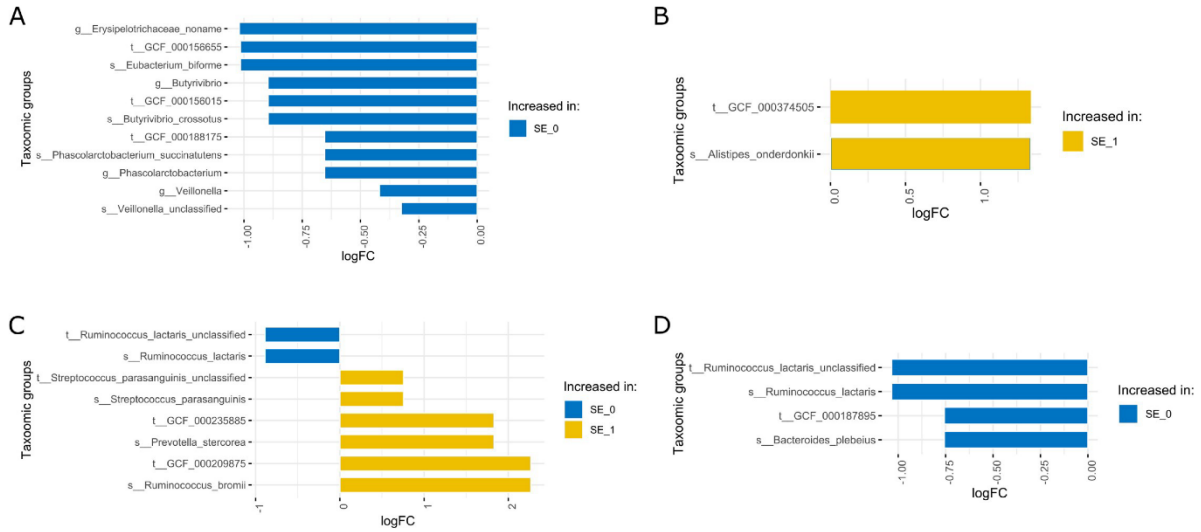


Fig 4. Differences in gut microbiome taxonomic composition between patients with severe side effects (SE_1) and no or mild reported side effects (SE_0) in both cohorts at all analyzed time points. (A) Healthy individuals at M0, (B) healthy individuals at M7d, (C) OPTIMED cohort at M0, (D) OPTIMED cohort at M7d. Taxa enriched in patients with severe side effects are indicated with yellow color, and taxa enriched in patients with no or mild side effects are in blue. Samples: M0 – before starting metformin treatment; M7d –after 7 days treatment with metformin.

https://doi.org/10.1371/journal.pone.0241338.g004

(Responders (p = 0.0002), Non-responders (p = 0.001)), but not on BMI. For any further analyses, we performed a correction by baseline HbA_{1c} value.

Firstly, we tested for differentially abundant taxonomic groups between both OPTIMED subgroups. When comparing taxonomic profiles we observed an increased abundance of species *Prevotella copri* in Non-Responders group (logFC = -2.8, FDR = 0.01) at M0 time point. No significant differences in the effective species number were detected when comparing these subgroups.

Secondly, we performed an additional sPLS-DA model to explore which taxonomic groups could discriminate patients belonging to one of the defined subgroups, and the VIP score was used to assess the contribution of each analyzed taxonomic unit (Fig 5). In total 43 taxonomic groups were detected with VIP score >1.5 (the full list is summarized in S1 Table).

Table 2. Characteristics of OPTIMED cohort's subgroups divided by response to metformin therapy during the first three months of therapy.

Characteristic	*Responders, N = 18*	*Non-responders, N = 28*	*p-value*
Males/females, n (%)	12 (66.7%) / 6 (33.3%)	8 (28.6%) / 20 (71.4%)	-
Age (years), mean ± SD	53.6 ± 10.5	61.3 ± 12.5	0.02
M0 BMI, mean ± SD	35.8 ± 7.8	34.6 ± 5.4	0.50
M3m BMI, mean ± SD	35.1 ± 7.2	34.0 ± 5.8	0.77
M0 HbA_{1c} (mmol/mol), mean ± SD	83.6 ± 4.9	50.8 ± 12.6	2.36 E-7
M0 HbA_{1c} (%), mean ± SD	9.8 ± 1.7	6.8 ± 1.0	2.36 E-7
M3m HbA_{1c} (mmol/mol), mean ± SD	53.0 ± 13.7	48.6 ± 12.6	0.04
M3m HbA_{1c} (%), mean ± SD	7.0 ± 0.9	6.6 ± 1.0	0.04

SD–standard deviation; BMI–body mass index; M0 –before starting metformin treatment; M3m –after three months of metformin treatment.

https://doi.org/10.1371/journal.pone.0241338.t002

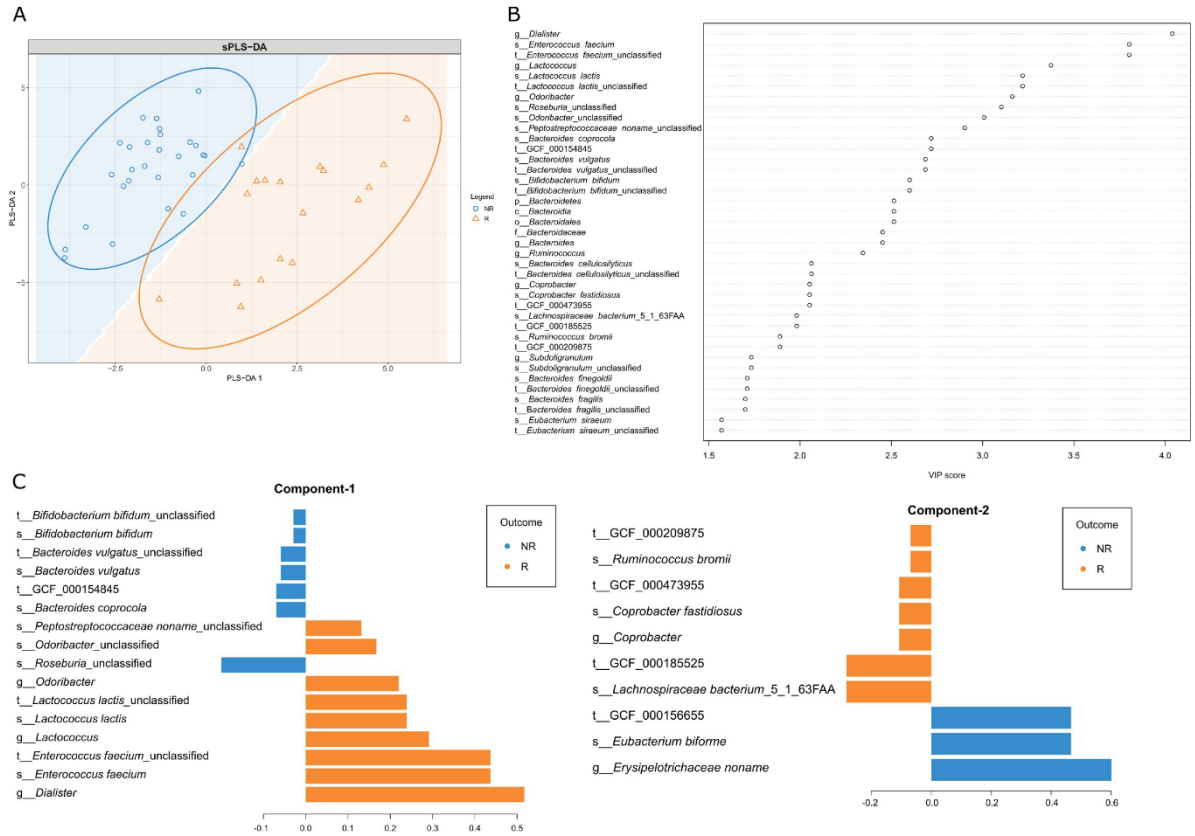


Fig 5. Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) of OPTIMED cohort subgroups at M0 time point. (A) Sample plot depicting the first two sPLS-DA components with 95% confidence level ellipse plots. The background coloring describes the predicted area for each class, defined as the 2D surface where all points are predicted to be of the same class. Samples and subgroups coded as follows: R–Responders, orange triangles; NR–Non-Responders, blue circles. (B) VIP (variable importance projection) score dot-chart classified by sPLS-DA. Depicted taxonomic groups with VIP≥1.5 in the first component. (C) The contribution of each taxonomic group on the first and second components, the length of the bar represents the importance of each feature to the component (importance from bottom to top). Colors indicate the patient subgroup (NR (blue) vs R (orange)) in which the taxonomic group is most abundant.

https://doi.org/10.1371/journal.pone.0241338.g005

Finally, we performed the same analysis on the independent validation cohort (characteristics summarized in Table 1). In result, for sPLS-DA model six taxonomic units overlapped: species *Bacteroides vulgatus* and its strain *Bacteroides vulgatus* unclassified, genus *Erysipelotrichaceae* noname and its species *Eubacterium biforme*, and its strain GCF 000156655, and species *Ruminococcus obeum*.

Functional analysis

Using the advantage of shotgun metagenomics data, we further evaluated the changes in possible functions of the analyzed gut microbiomes. This task was performed by analyzing the differential abundance of signaling pathways calculated by HUMAnN2 (proportional to the number of complete "copies" of the pathway in the community) within both studied cohorts. As a result, we identified 24 significant features in the OPTIMED cohort and 118 features in the healthy group (the specific pathways depicted in S3 and S4 Figs) with changed abundance during the metformin

therapy. To gain a detailed insight of general biological meaning represented by these functional changes we performed a cellular function enrichment analysis (Fig 6). Enrichment scores were calculated from logFC and adjusted p-values obtained from differential pathway abundance analysis comparing M7d versus M0 samples both in healthy and in T2D cohorts.

To perform targeted evaluation of some specific metabolic functions previously described to be associated with metformin, we took a detailed look under some of the enrichment categories. Firstly, under Cell Structure Biosynthesis category, increased peptidoglycan biosynthesis was observed in both analyzed cohorts, and increment in pathways dedicated to biosynthesis of LPS precursor Lipid IVa was detected only in the healthy cohort.

Secondly, after detailed analysis of enriched pathways under Cofactor, Carrier, and Vitamin Biosynthesis (Fig 6), in vitamin biosynthesis subcategory we observed changes only in the healthy cohort–reduced folate biosynthesis, increased thiamine and vitamin B6 biosynthesis.

Discussion

Our study has added new data on several most likely universal metformin effects on the human gut microbiome profile and presents novel data for therapeutic efficacy and tolerance prediction in newly diagnosed T2D patients. Also, we have characterized the differences representing metformin effects in T2D patients and healthy individuals, accenting the need for additional microbiome studies in groups with different responses to metformin therapy, both, in context of geographical localization and metformin targets outside the T2D.

The main strengths of our study are the longitudinal design examining the short-term metformin therapy effects on well-characterized treatment naïve patients and the additional study of healthy individuals receiving metformin. The used methodology of shotgun metagenome sequencing also improves the quality of study allowing discussing species-level data and changes in the functional profile. The main limitation is the relatively small sizes of study groups, however, we are the first to present short-term metformin effects observed after a weeklong therapy in newly diagnosed patients and it should be noted that the previously published longitudinal studies are similarly sized or even smaller [9, 10]. We also did not include a placebo arm and blinded design that could uncover possible metformin independent effects. In addition, the higher metformin dose given to the participants of the healthy cohort is a confounding limitation for comparison of both analyzed groups. Long-term follow-up for our study groups would also provide additional opportunity to evaluate the stability of observed effects, however, specifically, the short-term therapy results are with high clinical significance as it is known that the highest incidence of SE is observed during the first weeks [24].

In both healthy individuals and newly diagnosed T2D patients, we observed a metformin-induced reduction in the abundance of *Clostridium bartlettii* (also called *Intestinibacter bartlettii*–latest classification [25]). The role of this species is still unclear as its abundance has shown a negative correlation with markers for insulin resistance [26], but in other studies, it has been described as a robust biomarker for Crohn's disease and ulcerative colitis [27]. Importantly, a reduced abundance of *Intestinibacter* genus has been observed in previous metformin studies [8, 9, 11], thus, suggesting it to be one of the universal markers characterizing metformin effects on the gut microbiome.

One of the most intriguing findings was the increased abundance of *Parabacteroides distasonis* species. This taxonomic group has been recently associated with improved insulin sensitivity in obese human subjects [28], alleviated obesity and metabolic dysfunctions in mice [29], and has been proven to negatively correlate with fasting blood glucose levels [30]. In addition, some recent studies of metformin effects have observed an increase in the abundance of this species or obtained associations with therapy outcomes [31, 32].

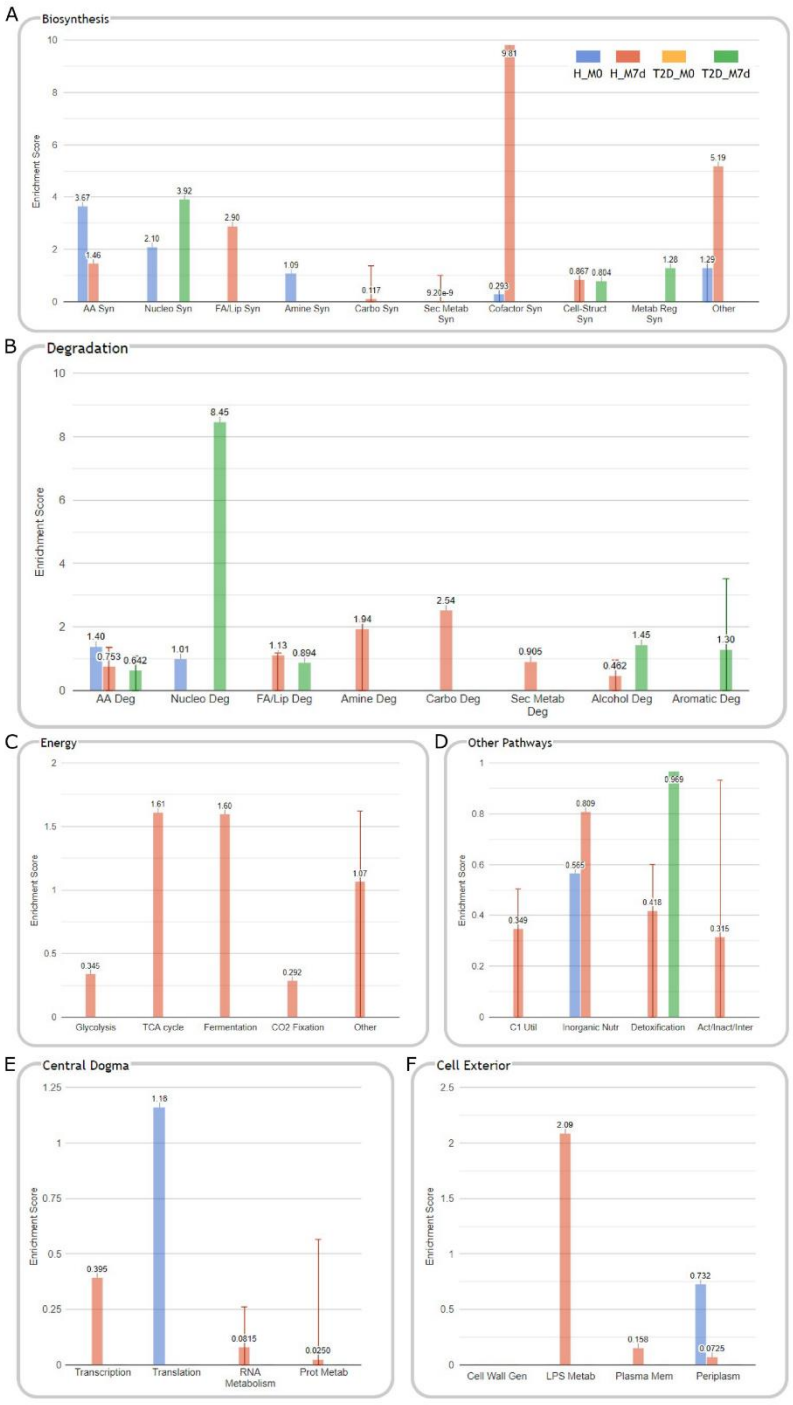

A
Biosynthesis
H_M0 H_M7d T2D_M0 T2D_M7d
Enrichment Score
AA Syn
Nucleo Syn
FA/Lip Syn
Amine Syn
Carbo Syn
Sec Metab Syn
Cofactor Syn
Cell-Struct Syn
Metab Reg Syn
Other
B
Degradation
AA Deg
Nucleo Deg
FA/Lip Deg
Amine Deg
Carbo Deg
Sec Metab Deg
Alcohol Deg
Aromatic Deg
C
Energy
Glycolysis
TCA cycle
Fermentation
CO2 Fixation
Other
D
Other Pathways
C1 Util
Inorganic Nutr
Detoxification
Act/Inact/Inter
E
Central Dogma
Transcription
Translation
RNA Metabolism
Prot Metab
F
Cell Exterior
Cell Wall Gen
LPS Metab
Plasma Mem
Periplasm

Fig 6. Cellular function enrichment analysis comparing functional profiles before and after 7 days long metformin therapy both in the OPTIMED cohort and in healthy individuals. Respectively, H_M0 (blue) and T2D_M0 (yellow) results represent data on pathways and functions reduced during metformin therapy and H_M7d (red) and T2D_M7d (green) – pathways and functions increased, only significant ($p<0.05$) enrichment categories are shown. Enrichment results are depicted in the following set of functional panels: (A) Biosynthesis, (B) Degradation, (C) Energy, (D) Other pathways, (E) Central dogma, (F) Cell exterior. Groups marked as follows: H–healthy individuals; T2D – type 2 diabetes patients. Samples: M0—before starting metformin treatment; M7d –after 7 days treatment with metformin. Pathway abbreviations: (A) AA Syn – amino acid biosynthesis; Nucleo Syn – nucleoside and nucleotide biosynthesis; FA/Lip Syn–fatty acid and lipid biosynthesis; Amine Syn–amine and polyamine biosynthesis; Carbo Syn – carbohydrate biosynthesis; Sec Metab Syn–secondary metabolite biosynthesis; Cofactor Syn–cofactor, carrier, and vitamin biosynthesis; Cell-Struct Syn–cell structure biosynthesis; Metab Reg Syn–metabolic regulator biosynthesis. (B) AA Deg–amino acid degradation; Nucleo Deg–nucleoside and nucleotide degradation; FA/Lip Deg–fatty acid and lipid degradation; Amine Deg–amine and polyamine degradation; Carbo Deg –carbohydrates and carboxylates degradation; Sec Metab Deg–secondary metabolite degradation; Alcochol Deg–alcohol degradation; Aromatic Deg–aromatic compound degradation. (D) C1 Util–C1 compound utilization and assimilation; Inorganic Nutr – inorganic nutrient metabolism; Act/Inact/Inter—Activation/Inactivation/Interconversion. (E) Prot Metab–protein metabolism. (F) Cell Wall Gen–cell wall biogenesis/organization proteins; LPS Metab – Lipopolysaccharide Metabolism Proteins; Plasma Mem–plasma membrane proteins; Periplasm–periplasmic proteins.

https://doi.org/10.1371/journal.pone.0241338.g006

At genus level, as possibly negative metformin effect, decrement in *Bifidobacterium* was observed in both analyzed cohorts. This taxon plays an important role in human health maintenance and is widely used as probiotics, as well as its reduction can be used as a biomarker for certain diseases [33]. Co-administration of *Bifidobacterium bifidum* G9-1 with metformin even has shown beneficial effects on reducing GI-SE [34]. However, the observed reduction in this genus needs more research, as effects on health are most likely species and strain specific, and our result is in contradiction with results of previous studies, for example, previously it has been reported that metformin enhances growth of species *Bifidobacterium adolescentis* in pure cultures [9].

We confirmed the previously observed increase in the abundance of *Escherichia coli* [9, 11] only in the group of healthy individuals. In this study cohort, we observed the highest incidence of SE, therefore, contributing the hypothesis of these changes as a possible basis for metformin intolerance. Interestingly, in previous studies, the highest SE occurrences (up to 50% and higher) have been observed during metformin administration in non-diabetic cohorts, such as healthy volunteers [10, 11] or polycystic ovary syndrome patients [35] compared to T2D. Regarding the possible beneficial effects of metformin specific to the healthy cohort, we found a metformin-induced reduction in *Dialister invisus* and *Bifidobacterium longum*, both associated with intestinal permeability and compromised gut health [36].

In the search for possible microbiome signatures describing or predicting the therapy tolerance, we observed different profiles in both analyzed cohorts. One of the taxa specific to the subgroup with severe SE in OPTIMED cohort (M0 time point) was *Streptococcus parasanguinis*, previously shown to be increased in individuals who use platelet aggregation inhibitors and proton pump inhibitors [37], which both are groups of medications frequently prescribed for the treatment of diabetes comorbidities. This result could indicate the possible effects of polypharmacy on the metformin's interaction with gut microbiome and the subsequent therapy tolerance. In contrast, species *Ruminococcus lactaris* which was enriched in OPTIMED subgroup with no or mild SE both at M0 and M7d samples, has been enriched in healthy individuals compared to T2D patients or obese subjects in various populations [38, 39], as well as negatively associated with statin use [40]. Within the healthy cohort (subgroup with no or mild SE) both before and after metformin use was characterized by an increased abundance of *Phascolarctobacterium succinatutens*, a succinate-consumer and substantial producer of short-chain fatty acids acetate and propionate. Therefore, this taxon has been associated with the metabolic state and even the mood of the host [41]. Some of our observed taxa have been

previously characterized as discriminants for SE development in a healthy cohort, such as *Alistipes* [10]. Altogether, these results give new insights into possible microbiome signatures for future prediction of therapy tolerance, but additional studies in larger patient cohorts with a well-characterized incidence of SE are needed to confirm our data.

Taking into account the widely known variation of metformin's therapeutic efficacy [42] we used the advantage of our longitudinal and well described OPTIMED cohort to search for potential microbiome-based markers as predictors. Firstly, we compared the taxonomic profiles in the subgroups crossectionally–at M0 time point before starting metformin therapy–with a fitted linear model for a series of arrays. The main finding in this step was the increment in abundance of *Prevotella copri* in the samples from Non-Responders to the therapy. Previous studies describing the possible functionality of *P.copri* species present contradictory data. Research results suggest both *P.copri* mediated beneficial effects on the host's metabolic profile as a succinate producer [43] and its induced increase in insulin resistance, glucose intolerance, and lipopolysaccharides plasma levels [44, 45]. However, the latest data indicate that strain-specific effects most likely explain this controversy, and the strain-level composition might be diet dependent [46]. Thus for the future development of biomarker-based approaches, strain-level data should be analyzed to account for the population and lifestyle specific microbiome composition with an aim to precisely predict its dependent functionality.

Secondly, the performed sPLS-DA analysis revealed a broad list of key taxa discriminating both therapy response subgroups at M0 time point. More precisely, the microbiome of the Responders group at baseline was enriched with various taxonomic groups characterized as potentially probiotic. For example (1) *Enterococcus faecium* significantly decreased body weight, serum lipid levels, blood glucose level, and insulin resistance in rats fed with a high-fat diet [47]; (2) several *Lactococcus lactis* strains have shown the ability to reduce hyperglycemia, improve glucose tolerance and insulin secretion [48, 49]; (3) bacteria from *Odoribacter* genus have been associated with a healthy fasting serum lipid profile [26], and displayed a negative correlation with insulin resistance [50]. The top result from this analysis–genus *Dialister* – has been characterized as a taxon possibly mediating the beneficial effects on the metabolic profile of whole-grains [51], however more data on underlying species are needed. In contrast, the various species from the predominant genus *Bacteroides*, found to be specific to the group of Non-responders, has been previously described in higher abundance in type 1 and T2D patients [52, 53], as well as associated with a negative impact on metabolic health [44]. Nevertheless, it is important to accent that the possible biological role of this genus is highly variable due to numerous species and strains within it, therefore, the interaction with the host can be both beneficial and harmful [54] and need to be further studied in the context of metformin response.

Data validation of sPLS-DA model in the independent cohort highlighted six taxonomic groups from three phylogenetic branches that have been previously associated with T2D, glucose tolerance, insulin resistance, and blood glucose levels, however, the results are highly conflicting and mostly population specific [45, 55–57]. These results highlight the urgent need for further population specific clinical studies to develop highly precise microbiome-based prediction tools for therapy efficacy.

To our best knowledge, we are the first to report the results of such analysis combining metformin therapy efficacy and microbiome profile data from newly diagnosed and treatment naïve T2D patients. Most importantly, comparing to studies with a similar design that compare Responders and Non-Responders to the antidiabetic therapy but analyze other targets, we have performed data correction by baseline HbA_{1c} measurement, to reduce biases created by frequently observed higher baseline values in the Responders group, as suggested previously [58]. Nevertheless, it is important to point out that other therapy efficacy influencing

indicators should be evaluated to more precisely distinguish microbiome-related effects from e.g. presence of genetic factors previously associated with efficacy.

As for the functional profile, only a portion of the observed results has been previously characterized. A large number of the significantly changed pathways and subsequently the results of enrichment analysis during metformin use were representing the increment in pathways characterizing synthesis of lipopolysaccharides and peptidoglycans (under *Cell-Struct Syn* in Fig 6), which is another signature of metformin effects [9, 59]. These changes were mainly found in the healthy cohort, most likely accounting for the high number of observed fluctuations in the taxonomical profiles. For example, a recent study employing genome-scale metabolic modelling has shown that lipopolysaccharide synthesis, nucleotide sugar metabolism, and amino acid metabolism (under *Cell-Struct Syn*, *Nucleo Syn*, *Nucleo Deg*, *AA Syn*, *AA deg* in Fig 6) are pathways most likely effected by abundance changes in such taxa as *Escherichia* spp. and *A*. *muciniphila* [60]. Compared to other analyses of metformin-induced changes in the functional profile performed in T2D cohorts, we observed similar changes, such as an increase in lysine and threonine degradation (in healthy cohort), and sugar nucleotide biosynthesis (in OPTIMED cohort) [9]. Interestingly, the enriched cellular functions appeared to be cohort-specific and in cases when similar functional changes are observed, the observed underlying mechanisms differed.

In addition, as metformin is known to be associated with vitamins B level alterations and even deficiencies [61, 62], we as well observed metformin induced changes in various vitamin B pathways, however, only in the healthy cohort. The inhibition of folate metabolism have been characterized as one of the mechanisms behind metformin effects on increased lifespan in *C.elegans* [63]. However, the suppression of folate producing bacteria has been proposed as one of the causes for GI-SE [64], therefore, indicating a possible explanation for the high prevalence of SE observed specifically in the healthy cohort.

The large disparity in the observed microbiome profile changes in both cohorts could be explained by initial differences (as depicted in Fig 2). Moreover, as the T2D cohort is expected to be more heterogeneous [65], it explains the smaller number of significantly changed features in OPTIMED patients. In addition, our results have approved some seemingly universal microbiome signatures for metformin therapy and displayed new data on microbiome changes, most likely responsible for population-specific effects dependent on health status as well as geographical localization. Our results on the prediction of therapy tolerance and efficacy may reveal novel biomarkers, which need to be further studied to fully characterize the strain-level dynamics and validated in larger cohorts. These results highlight the need to develop personalized medicine based approaches based on gut microbiome testing before starting the therapy and will serve as the basis for further studies on microbiome modulation techniques to improve both metformin therapeutic efficacy and tolerance and, therefore, the quality of life of patients.

Supporting information

S1 Text. List of inclusion/exclusion criteria.
(DOCX)

S2 Text. Methodological description of validation cohort sample and data processing.
(DOCX)

S1 Fig. Metformin induced differences in gut microbiome taxonomic composition in T2D cohort. Differentiating feature analysis was carried out with limma+voom, adjusted p-value cut-off = 0.05. Samples coded as follows: M0 –before starting metformin treatment (blue,

negative logFC), and M7d – 7 days after the first intake of metformin (yellow, positive logFC). logFC–log fold change.
(TIF)

S2 Fig. Metformin induced differences in gut microbiome taxonomic composition in healthy cohort. Differentiating feature analysis was carried out with limma+voom, adjusted p-value cut-off = 0.05. Samples coded as follows: M0 –before starting metformin treatment (blue, negative logFC), and M7d – 7 days after the first intake of metformin (yellow, positive logFC). logFC–log fold change.
(TIF)

S3 Fig. Differences in abundance of signaling pathways induced by metformin therapy in T2D patient cohort. Differentiating feature analysis was carried out with limma+voom, adjusted p-value cut off = 0.05. Samples coded as follows: M7d – 7 days after the first intake of metformin (blue, positive logFC). logFC–log fold change.
(TIF)

S4 Fig. Differences in abundance of signaling pathways induced by metformin therapy in healthy individuals. Differentiating feature analysis was carried out with limma+voom, adjusted p-value cut off = 0.05. Samples coded as follows: M0 –before starting metformin treatment (blue, negative logFC), and M7d – 7 days after the first intake of metformin (yellow, positive logFC). logFC–log fold change.
(TIF)

S1 Table. Taxonomic groups with VIP score >1.5 in at least one of the first two components from sPLS-DA analysis.
(DOCX)

S2 Table. Effect sizes and p-values of performed comparisons with limma+voom analysis.
(XLSX)

S3 Table. Sequence preprocessing information.
(XLSX)

Acknowledgments

We acknowledge the Genome Database of Latvian Population and Latvian Biomedical Research and Study Centre for the organization of participants' recruitment and bio-sample processing and data storage. The authors thank all the volunteers who participated in the clinical trial.

Author Contributions

Conceptualization: Ineta Kalnina, Monta Ustinova, Linda Zaharenko, Janis Klovins.

Data curation: Ilze Elbere, Ilze Izabella Dindune, Ineta Kalnina, Monta Ustinova, Linda Zaharenko, Laila Silamikele, Dita Gudra, Jelizaveta Sokolovska.

Formal analysis: Ilze Elbere, Ivars Silamikelis, Ilze Izabella Dindune.

Funding acquisition: Valdis Pirags, Janis Klovins.

Investigation: Monta Ustinova, Laila Silamikele.

Methodology: Ilze Elbere, Ivars Silamikelis, Ineta Kalnina, Dita Gudra, Valdis Pirags.

Project administration: Vita Rovite, Valdis Pirags, Janis Klovins.

Resources: Vita Rovite, Dita Gudra, Ilze Konrade, Jelizaveta Sokolovska, Valdis Pirags.

Software: Ivars Silamikelis.

Supervision: Ilze Konrade, Jelizaveta Sokolovska, Valdis Pirags, Janis Klovins.

Validation: Vita Rovite.

Visualization: Ilze Elbere.

Writing – original draft: Ilze Elbere.

Writing – review & editing: Janis Klovins.

References

1. Skyler JS, Bakris GL, Bonifacio E, Darsow T, Eckel RH, Groop L, et al. Differentiation of Diabetes by Pathophysiology, Natural History, and Prognosis. Diabetes. 2017; 66(2):241–55. https://doi.org/10.2337/db16-0806 PMID: 27980006
2. Zhou J, Massey S, Story D, Li L. Metformin: An Old Drug with New Applications. Int J Mol Sci. 2018; 19 (10). https://doi.org/10.3390/ijms19102863 PMID: 30241400
3. Kahn SE, Haffner SM, Heise MA, Herman WH, Holman RR, Jones NP, et al. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. N Engl J Med. 2006; 355(23):2427–43. https://doi.org/10.1056/NEJMoa066224 PMID: 17145742
4. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med. 2002; 346(6):393–403. https://doi.org/10.1056/NEJMoa012512 PMID: 11832527
5. Whang A, Nagpal R, Yadav H. Bi-directional drug-microbiome interactions of anti-diabetics. EBioMedicine. 2019; 39:591–602. https://doi.org/10.1016/j.ebiom.2018.11.046 PMID: 30553752
6. Petrosino JF. The microbiome in precision medicine: the way forward. Genome Med. 2018; 10(1):12. https://doi.org/10.1186/s13073-018-0525-6 PMID: 29471863
7. Karlsson FH, Tremaroli V, Nookaew I, Bergstrom G, Behre CJ, Fagerberg B, et al. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature. 2013; 498(7452):99–103. https://doi.org/10.1038/nature12198 PMID: 23719380
8. Forslund K, Hildebrand F, Nielsen T, Falony G, Le Chatelier E, Sunagawa S, et al. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. Nature. 2015; 528 (7581):262–6. https://doi.org/10.1038/nature15766 PMID: 26633628
9. Wu H, Esteve E, Tremaroli V, Khan MT, Caesar R, Manneras-Holm L, et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. Nat Med. 2017. https://doi.org/10.1038/nm.4345 PMID: 28530702
10. Bryrup T, Thomsen CW, Kern T, Allin KH, Brandslund I, Jorgensen NR, et al. Metformin-induced changes of the gut microbiota in healthy young men: results of a non-blinded, one-armed intervention study. Diabetologia. 2019; 62(6):1024–35. https://doi.org/10.1007/s00125-019-4848-7 PMID: 30904939
11. Elbere I, Kalnina I, Silamikelis I, Konrade I, Zaharenko L, Sekace K, et al. Association of metformin administration with gut microbiome dysbiosis in healthy volunteers. PLoS One. 2018. https://doi.org/10.1371/journal.pone.0204317 PMID: 30261008
12. de la Cuesta-Zuluaga J, Mueller NT, Corrales-Agudelo V, Velasquez-Mejia EP, Carmona JA, Abad JM, et al. Metformin Is Associated With Higher Relative Abundance of Mucin-Degrading Akkermansia muciniphila and Several Short-Chain Fatty Acid-Producing Microbiota in the Gut. Diabetes Care. 2017; 40 (1):54–62. https://doi.org/10.2337/dc16-1324 PMID: 27999002
13. Rovite V, Wolff-Sagi Y, Zaharenko L, Nikitina-Zake L, Grens E, Klovins J. Genome Database of the Latvian Population (LGDB): Design, Goals, and Primary Results. J Epidemiol. 2018.
14. Sherifali D, Nerenberg K, Pullenayegum E, Cheng JE, Gerstein HC. The effect of oral antidiabetic agents on A1C levels: a systematic review and meta-analysis. Diabetes Care. 2010; 33(8):1859–64. https://doi.org/10.2337/dc09-1727 PMID: 20484130
15. Kashi Z, Mahrooz A, Kianmehr A, Alizadeh A. The Role of Metformin Response in Lipid Metabolism in Patients with Recent-Onset Type 2 Diabetes: HbA1c Level as a Criterion for Designating Patients as

Responders or Nonresponders to Metformin. PLoS One. 2016; 11(3):e0151543. https://doi.org/10.1371/journal.pone.0151543 PMID: 26978661

16. Burbach K, Seifert J, Pieper DH, Camarinha-Silva A. Evaluation of DNA extraction kits and phylogenetic diversity of the porcine gastrointestinal tract based on Illumina sequencing of two hypervariable regions. Microbiologyopen. 2016; 5(1):70–82. https://doi.org/10.1002/mbo3.312 PMID: 26541370

17. Janes VA, van der Laan JS, Matamoros S, Mende DR, de Jong MD, Schultsz C. Thermus thermophilus DNA can be used as internal control for process monitoring of clinical metagenomic next-generation sequencing of urine samples. J Microbiol Methods. 2020; 176:106005. https://doi.org/10.1016/j.mimet.2020.106005 PMID: 32687865

18. Walsh AM, Crispie F, O'Sullivan O, Finnegan L, Claesson MJ, Cotter PD. Species classifier choice is a key consideration when analysing low-complexity food microbiome data. Microbiome. 2018; 6(1):50. https://doi.org/10.1186/s40168-018-0437-0 PMID: 29554948

19. Franzosa EA, McIver LJ, Rahnavard G, Thompson LR, Schirmer M, Weingart G, et al. Species-level functional profiling of metagenomes and metatranscriptomes. Nat Methods. 2018; 15(11):962–8. https://doi.org/10.1038/s41592-018-0176-y PMID: 30377376

20. Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, et al. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. Nat Methods. 2015; 12(10):902–3. https://doi.org/10.1038/nmeth.3589 PMID: 26418763

21. Torondel B, Ensink JH, Gundogdu O, Ijaz UZ, Parkhill J, Abdelahi F, et al. Assessment of the influence of intrinsic environmental and geographical factors on the bacterial ecology of pit latrines. Microb Biotechnol. 2016; 9(2):209–23. https://doi.org/10.1111/1751-7915.12334 PMID: 26875588

22. Le Cao KA, Boitard S, Besse P. Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems. BMC Bioinformatics. 2011; 12:253. https://doi.org/10.1186/1471-2105-12-253 PMID: 21693065

23. Paley S, Parker K, Spaulding A, Tomb JF, O'Maille P, Karp PD. The Omics Dashboard for interactive exploration of gene-expression data. Nucleic Acids Res. 2017; 45(21):12113–24. https://doi.org/10.1093/nar/gkx910 PMID: 29040755

24. Haupt E, Knick B, Koschinsky T, Liebermeister H, Schneider J, Hirche H. Oral antidiabetic combination therapy with sulphonylureas and metformin. Diabete Metab. 1991; 17(1 Pt 2):224–31. PMID: 1936481

25. Gerritsen J, Fuentes S, Grievink W, van Niftrik L, Tindall BJ, Timmerman HM, et al. Characterization of Romboutsia ilealis gen. nov., sp. nov., isolated from the gastro-intestinal tract of a rat, and proposal for the reclassification of five closely related members of the genus Clostridium into the genera Romboutsia gen. nov., Intestinibacter gen. nov., Terrisporobacter gen. nov. and Asaccharospora gen. nov. Int J Syst Evol Microbiol. 2014; 64(Pt 5):1600–16. https://doi.org/10.1099/ijs.0.059543-0 PMID: 24480908

26. Brahe LK, Le Chatelier E, Prifti E, Pons N, Kennedy S, Hansen T, et al. Specific gut microbiota features and metabolic markers in postmenopausal women with obesity. Nutr Diabetes. 2015; 5:e159. https://doi.org/10.1038/nutd.2015.9 PMID: 26075636

27. Wingfield B, Coleman S, McGinnity TM, Bjourson A. Robust Microbial Markers for Non-Invasive Inflammatory Bowel Disease Identification. IEEE/ACM Trans Comput Biol Bioinform. 2018. https://doi.org/10.1109/TCBB.2018.2831212 PMID: 29994028

28. Haro C, Montes-Borrego M, Rangel-Zuniga OA, Alcala-Diaz JF, Gomez-Delgado F, Perez-Martinez P, et al. Two Healthy Diets Modulate Gut Microbial Community Improving Insulin Sensitivity in a Human Obese Population. J Clin Endocrinol Metab. 2016; 101(1):233–42. https://doi.org/10.1210/jc.2015-3351 PMID: 26505825

29. Wang K, Liao M, Zhou N, Bao L, Ma K, Zheng Z, et al. Parabacteroides distasonis Alleviates Obesity and Metabolic Dysfunctions via Production of Succinate and Secondary Bile Acids. Cell Rep. 2019; 26(1):222–35 e5. https://doi.org/10.1016/j.celrep.2018.12.028 PMID: 30605678

30. Wei S, Han R, Zhao J, Wang S, Huang M, Wang Y, et al. Intermittent administration of a fasting-mimicking diet intervenes in diabetes progression, restores beta cells and reconstructs gut microbiota in mice. Nutr Metab (Lond). 2018; 15:80. https://doi.org/10.1186/s12986-018-0318-3 PMID: 30479647

31. Zhang Y, Cheng Y, Zuo J, Yan L, Li Q, Thring RW, et al. Metformin alleviates insulin resistance in obese-induced mice via remodeling gut microbiota and intestinal metabolites. bioRxiv [Preprint] [Internet]. 2020:[2020.05.26.116715 p.]. Available from: https://www.biorxiv.org/content/biorxiv/early/2020/06/19/2020.05.26.116715.full.pdf.

32. Palacios T, Vitetta L, Coulson S, Madigan CD, Lam YY, Manuel R, et al. Targeting the Intestinal Microbiota to Prevent Type 2 Diabetes and Enhance the Effect of Metformin on Glycaemia: A Randomised Controlled Pilot Study. Nutrients. 2020; 12(7). https://doi.org/10.3390/nu12072041 PMID: 32660025

33. Arboleya S, Watkins C, Stanton C, Ross RP. Gut Bifidobacteria Populations in Human Health and Aging. Front Microbiol. 2016; 7:1204. https://doi.org/10.3389/fmicb.2016.01204 PMID: 27594848

34. Makizaki Y, Maeda A, Yamamoto M, Tamura S, Tanaka Y, Nakajima S, et al. Bifidobacterium bifidum G9-1 ameliorates soft feces induced by metformin without affecting its antihyperglycemic action. Biosci Microbiota Food Health. 2020; 39(3):145–51. https://doi.org/10.12938/bmfh.2019-022 PMID: 32775133

35. Lord JM, Flight IH, Norman RJ. Metformin in polycystic ovary syndrome: systematic review and meta-analysis. BMJ. 2003; 327(7421):951–3. https://doi.org/10.1136/bmj.327.7421.951 PMID: 14576245

36. Zheng P, Li Z, Zhou Z. Gut microbiome in type 1 diabetes: A comprehensive review. Diabetes Metab Res Rev. 2018; 34(7):e3043. https://doi.org/10.1002/dmrr.3043 PMID: 29929213

37. Weersma RK, Zhernakova A, Fu J. Interaction between drugs and the gut microbiome. Gut. 2020; 69 (8):1510–9. https://doi.org/10.1136/gutjnl-2019-320204 PMID: 32409589

38. Doumatey AP, Adeyemo A, Zhou J, Lei L, Adebamowo SN, Adebamowo C, et al. Gut Microbiome Profiles Are Associated With Type 2 Diabetes in Urban Africans. Front Cell Infect Microbiol. 2020; 10:63. https://doi.org/10.3389/fcimb.2020.00063 PMID: 32158702

39. Yasir M, Angelakis E, Bibi F, Azhar EI, Bachar D, Lagier JC, et al. Comparison of the gut microbiota of people in France and Saudi Arabia. Nutr Diabetes. 2015; 5:e153. https://doi.org/10.1038/nutd.2015.3 PMID: 25915742

40. Zhernakova A, Kurilshikov A, Bonder MJ, Tigchelaar EF, Schirmer M, Vatanen T, et al. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. Science. 2016; 352(6285):565–9. https://doi.org/10.1126/science.aad3369 PMID: 27126040

41. Connors J, Dawe N, Van Limbergen J. The Role of Succinate in the Regulation of Intestinal Inflammation. Nutrients. 2018; 11(1). https://doi.org/10.3390/nu11010025 PMID: 30583500

42. Rashid M, Shahzad M, Mahmood S, Khan K. Variability in the therapeutic response of Metformin treatment in patients with type 2 diabetes mellitus. Pak J Med Sci. 2019; 35(1):71–6. https://doi.org/10.12669/pjms.35.1.100 PMID: 30881399

43. De Vadder F, Kovatcheva-Datchary P, Zitoun C, Duchampt A, Backhed F, Mithieux G. Microbiota-Produced Succinate Improves Glucose Homeostasis via Intestinal Gluconeogenesis. Cell Metab. 2016; 24 (1):151–7. https://doi.org/10.1016/j.cmet.2016.06.013 PMID: 27411015

44. Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BA, et al. Human gut microbes impact host serum metabolome and insulin sensitivity. Nature. 2016; 535(7612):376–81. https://doi.org/10.1038/nature18646 PMID: 27409811

45. Leite AZ, Rodrigues NC, Gonzaga MI, Paiolo JCC, de Souza CA, Stefanutto NAV, et al. Detection of Increased Plasma Interleukin-6 Levels and Prevalence of Prevotella copri and Bacteroides vulgatus in the Feces of Type 2 Diabetes Patients. Front Immunol. 2017; 8:1107. https://doi.org/10.3389/fimmu.2017.01107 PMID: 28966614

46. De Filippis F, Pasolli E, Tett A, Tarallo S, Naccarati A, De Angelis M, et al. Distinct Genetic and Functional Traits of Human Intestinal Prevotella copri Strains Are Associated with Different Habitual Diets. Cell Host Microbe. 2019; 25(3):444–53 e3. https://doi.org/10.1016/j.chom.2019.01.004 PMID: 30799264

47. Zhang F, Qiu L, Xu X, Liu Z, Zhan H, Tao X, et al. Bene fi cial effects of probiotic cholesterol-lowering strain of Enterococcus faecium WEFA23 from infants on diet-induced metabolic syndrome in rats. J Dairy Sci. 2017; 100(3):1618–28. https://doi.org/10.3168/jds.2016-11870 PMID: 28041735

48. Ma Y, Liu J, Hou J, Dong Y, Lu Y, Jin L, et al. Oral administration of recombinant Lactococcus lactis expressing HSP65 and tandemly repeated P277 reduces the incidence of type I diabetes in non-obese diabetic mice. PLoS One. 2014; 9(8):e105701. https://doi.org/10.1371/journal.pone.0105701 PMID: 25157497

49. Zeng Z, Yu R, Zuo F, Zhang B, Ma H, Chen S. Recombinant Lactococcus lactis expressing bioactive exendin-4 to promote insulin secretion and beta-cell proliferation in vitro. Appl Microbiol Biotechnol. 2017; 101(19):7177–86. https://doi.org/10.1007/s00253-017-8410-6 PMID: 28828521

50. Yamashita M, Okubo H, Kobuke K, Ohno H, Oki K, Yoneda M, et al. Alteration of gut microbiota by a Westernized lifestyle and its correlation with insulin resistance in non-diabetic Japanese men. J Diabetes Investig. 2019; 10(6):1463–70. https://doi.org/10.1111/jdi.13048 PMID: 30901505

51. Walter J, Martinez I, Rose DJ. Holobiont nutrition: considering the role of the gastrointestinal microbiota in the health benefits of whole grains. Gut Microbes. 2013; 4(4):340–6. https://doi.org/10.4161/gmic.24707 PMID: 23645316

52. Ganesan K, Chung SK, Vanamala J, Xu B. Causal Relationship between Diet-Induced Gut Microbiota Changes and Diabetes: A Novel Strategy to Transplant Faecalibacterium prausnitzii in Preventing Diabetes. Int J Mol Sci. 2018; 19(12). https://doi.org/10.3390/ijms19123720 PMID: 30467295

53. Siljander H, Honkanen J, Knip M. Microbiome and type 1 diabetes. EBioMedicine. 2019; 46:512–21. https://doi.org/10.1016/j.ebiom.2019.06.031 PMID: 31257149

54. Wexler AG, Goodman AL. An insider's perspective: Bacteroides as a window into the microbiome. Nat Microbiol. 2017; 2:17026. https://doi.org/10.1038/nmicrobiol.2017.26 PMID: 28440278

55. Wang Y, Luo X, Mao X, Tao Y, Ran X, Zhao H, et al. Gut microbiome analysis of type 2 diabetic patients from the Chinese minority ethnic groups the Uygurs and Kazaks. PLoS One. 2017; 12(3):e0172774. https://doi.org/10.1371/journal.pone.0172774 PMID: 28328990

56. Gurung M, Li Z, You H, Rodrigues R, Jump DB, Morgun A, et al. Role of gut microbiota in type 2 diabetes pathophysiology. EBioMedicine. 2020; 51:102590. https://doi.org/10.1016/j.ebiom.2019.11.051 PMID: 31901868

57. Zhang X, Shen D, Fang Z, Jie Z, Qiu X, Zhang C, et al. Human gut microbiota changes reveal the progression of glucose intolerance. PLoS One. 2013; 8(8):e71108. https://doi.org/10.1371/journal.pone.0071108 PMID: 24013136

58. Jones AG, Lonergan M, Henley WE, Pearson ER, Hattersley AT, Shields BM. Should Studies of Diabetes Treatment Stratification Correct for Baseline HbA1c? PLoS One. 2016; 11(4):e0152428. https://doi.org/10.1371/journal.pone.0152428 PMID: 27050911

59. Ma W, Chen J, Meng Y, Yang J, Cui Q, Zhou Y. Metformin Alters Gut Microbiota of Healthy Mice: Implication for Its Potential Role in Gut Microbiota Homeostasis. Front Microbiol. 2018; 9:1336. https://doi.org/10.3389/fmicb.2018.01336 PMID: 29988362

60. Rosario D, Benfeitas R, Bidkhori G, Zhang C, Uhlen M, Shoaie S, et al. Understanding the Representative Gut Microbiota Dysbiosis in Metformin-Treated Type 2 Diabetes Patients Using Genome-Scale Metabolic Modeling. Front Physiol. 2018; 9:775. https://doi.org/10.3389/fphys.2018.00775 PMID: 29988585

61. Porter KM, Ward M, Hughes CF, O'Kane M, Hoey L, McCann A, et al. Hyperglycemia and Metformin Use Are Associated With B Vitamin Deficiency and Cognitive Dysfunction in Older Adults. J Clin Endocrinol Metab. 2019; 104(10):4837–47. https://doi.org/10.1210/jc.2018-01791 PMID: 30920623

62. Wakeman M, Archer DT. Metformin and Micronutrient Status in Type 2 Diabetes: Does Polypharmacy Involving Acid-Suppressing Medications Affect Vitamin B12 Levels? Diabetes Metab Syndr Obes. 2020; 13:2093–108. https://doi.org/10.2147/DMSO.S237454 PMID: 32606868

63. Cabreiro F, Au C, Leung KY, Vergara-Irigaray N, Cocheme HM, Noori T, et al. Metformin retards aging in C. elegans by altering microbial folate and methionine metabolism. Cell. 2013; 153(1):228–39. https://doi.org/10.1016/j.cell.2013.02.035 PMID: 23540700

64. Olgun A. "Metformin-resistant" folic acid producing probiotics or folic acid against metformin's adverse effects like diarrhea. Med Hypotheses. 2017; 106:33–4. https://doi.org/10.1016/j.mehy.2017.07.009 PMID: 28818268

65. Asquith M, Rosenbaum JT. The interaction between host genetics and the microbiome in the pathogenesis of spondyloarthropathies. Curr Opin Rheumatol. 2016; 28(4):405–12. https://doi.org/10.1097/BOR.0000000000000299 PMID: 27152700

4. DISCUSSION

Type 2 diabetes is a complex and heterogeneous group of metabolic disorders with increasing incidence and prevalence worldwide and it has been recognized as a global burden for healthcare and economics. Therefore, timely identification of individuals at risk, improved detection and monitoring of T2D patients, effective treatment with reduced incidence of comorbidities, and improved awareness are the key elements for decreasing the future burden of this disease (Alzaid *et al.* 2020). Metformin is a first-line antidiabetic drug used for more than 60 years, moreover, its pleiotropic effects have shown significant results in the treatment of many other diseases outside T2D (Lv and Guo 2020). In addition, metformin has been characterized by a high safety profile, low costs, and even a protective effect on various diseases. Despite the high number of studies on metformin effects, the pharmacodynamic actions of this medication are not fully understood and variance of the therapy efficacy and tolerance exists (Rashid *et al.* 2019, Gedawy *et al.* 2020).

The gut microbiome has been proven to be a significant mediator and as well as a place of action for metformin pharmacodynamics. Many high-quality studies have been performed worldwide, however, results are often controversial and discovery of new confounding factors interferes with the certainty of some findings. Such factors as a dose of medication, effects of other drugs, comorbidities, different experimental designs, variation across individuals and study populations have been highlighted as some of the most significant reasons for the diverse results (Zhang and Hu 2020).

The strongest and most consistent observation validated by the results in gut microbiome of both analysed study populations from this thesis, healthy individuals and newly diagnosed T2D patients, is the reduction in the abundance of *Peptostreptococcaceae* family. As expected, the study employing shotgun metagenomics analysis allowed to identify these changes even at the species level as a reduction of *Clostridium bartlettii* (latest classification: *Intestinibacter bartlettii* (Gerritsen *et al.* 2014)). Most of the other human microbiome studies performed have confirmed these metformin-induced changes as well (Forslund *et al.* 2015, Wu *et al.* 2017, Bryrup *et al.* 2019), therefore, suggesting it to be a pronounced effect, most likely independent from the population or disease specific background.

Similarly, our results from the healthy cohort were among the first in series of findings observing metformin-induced increase in abundance of *Escherichia* spp. (or *Escherichia/Shigella* spp. in studies based on *16S rRNA*) (Forslund *et al.* 2015, Wu *et al.* 2017, Bryrup *et al.* 2019, Ejtahed *et al.* 2019) while it was not significant in our T2D cohort. This

increase has been thought to be one of the mechanisms responsible for the metformin-induced gastrointestinal side effects (Forslund *et al.* 2015). However, a recent study proposed that this increase could also be related to metformin's beneficial effects on weight reduction as in some cohorts a negative correlation of *Escherichia coli* abundance with BMI has been observed. The same study hypothesized that the underlying mechanism could be related to possible satietogenic protein release from commensal *Escherichia coli* (Ejtahed *et al.* 2019). Additional longitudinal studies in T2D patients or obese individuals are needed to validate this hypothesis. Moreover, the observed differences between metformin effects in the healthy individuals and the newly diagnosed T2D patients support the previously reported variation of metformin effects (Zhang and Hu 2020), thus, emphasising the need for new high quality studies in diseases other than T2D for which metformin beneficial effects have been proposed.

Interestingly, we observed a significant increase in the abundance of *Akkermansia muciniphila*, considered one of the most beneficial gut microbiome species, only with the more sensitive shotgun metagenomics approach and only in the cohort of healthy individuals. This species has been widely characterized in the context of metformin therapy and its increase has been proposed as one of the most significant metformin effects in the gut (Rodriguez *et al.* 2018), as *A. muciniphila* mediates many beneficial effects on the host (Macchione *et al.* 2019). Our results indicate this species' relatively low presence in our cohorts, which might be one of the Latvian population-specific microbiome signatures.

More than 20% of patients fail to reach the glycaemic target when on metformin monotherapy (Kahn *et al.* 2006) and more than 30% experience gastrointestinal side effects (Knowler *et al.* 2002). Therefore, the use of specific microbiome signatures in baseline samples (collected before starting metformin) to predict therapy efficacy and tolerance is a promising approach and can significantly improve the treatment algorithms. Such knowledge, combined with the wide range of possibilities to modify the gut microbiome (Quigley and Gajula 2020, Deehan *et al.* 2021), can help to develop microbiome modulation approaches improving the metformin's therapeutic efficacy and reducing the incidence of gastrointestinal side effects. This would significantly improve the quality of life for patients and increase adherence to the prescribed therapy. For example, a combination of metformin therapy with prebiotic mannan-oligosaccharides suggested augmentation of metformin's hypoglycaemic effects in mice (Zheng *et al.* 2018). Also, the addition of a prebiotic gastrointestinal microbiome modulator compared to placebo significantly reduced metformin intolerance in T2D patients (Burton *et al.* 2015). However, in obese patients metformin treatment has been shown to compromise microbiome changes and even the metabolic improvements observed from the

probiotic intervention (Hiel *et al.* 2020), highlighting some challenges of possible metformin therapy combinations with the microbiome modulation approaches.

Our results (Elbere *et al.* 2020) offer novel knowledge for the creation of such microbiome-based prediction algorithms as we were the first to report results of analysis combining metformin therapy efficacy and microbiome profile data from newly diagnosed and treatment naïve T2D patients. Classification of T2D patients as metformin therapy Responders and Non-responders according to changes HbA1c has been done before only in the context of other parameters, like genetic polymorphisms, urine metabolites, or DNA methylation (Mahrooz *et al.* 2015, Park *et al.* 2018, Ebid *et al.* 2019, Garcia-Calzon *et al.* 2020) largely ignoring the potential of microbiota composition in this regard. Future studies including a combination of various markers would be significant for defining the complex mechanisms behind metformin therapy response.

It is important that the different microbiome analysis methods (*16S rRNA* amplicon vs. shotgun metagenome sequencing) applied for the two publications in this thesis present highly similar results despite the different sample sizes, providing additional integrity of the obtained results. The level of consistent results across these methods has been tested and demonstrated before (Rausch *et al.* 2019). Our most recent study using the shotgun metagenomics approach highlights the possible species and even strain-specific effects, which is in line with the latest understanding regarding the significance of strain-level epidemiology in the human microbiome (Yan *et al.* 2020). These results emphasize the advantages of this method for explaining metformin pharmacodynamic effects in the gut microbiome and predictive biomarker detection that could be further used for the development of precision medicine based therapy algorithms and approaches.

At the time of publication, our study about metformin effects on DNA methylation in the healthy individual group was the first to report the immediate effect of metformin on white blood cell DNA methylation in humans at therapeutic doses. Therefore, the discussion about similar or controversial findings was limited. New data has been published in this field during the previous two years, including several studies in human subjects. In a targeted study metformin therapy during pregnancy prevented DNA methylation changes of offspring associated with the intrauterine PCOS environment (Echiburu *et al.* 2020). Two studies employing genome-wide methylation profile analysis showed interesting results in T2D patients. Firstly, metformin treated versus newly diagnosed T2D patients showed significantly different DNA methylation profiles with mostly observed metformin-associated hypermethylation (Solomon *et al.* 2020). Secondly, in a pre-print paper metformin anti-aging

effects have been studied in the context of the epigenetic clock and show enrichment in cellular pathways related to the aging process in T2D patients (Li *et al.* 2021). Similar to the first above-mentioned genome-wide methylation study, our results depicted mostly DNA hypermethylation associated with metformin therapy, in contrast to results observed by Li and colleagues (Li *et al.* 2021). In a previous analysis of genome-scale DNA methylation changes *in vitro*, metformin effects on the epigenetic regulation showed a combination of hyper, hypo, and non-differentially methylated CpG sites, therefore, highlighting the differential effects of metformin on gene methylation (Zhong *et al.* 2017). However, the results about specific CpGs or DMRs observed in our study have not yet been replicated by any of the human studies performed later. One of the explanations might be the population-specific difference in patient cohorts. Also, it is important to note that all of the recent human studies have been performed in case-control design comparing metformin users versus non-users, and any longitudinal data to which compare our results are missing, hindering many patient-specific changes in methylation profile.

In addition to metformin's effects on DNA methylation and similarly to the gut microbiome, some studies have focused on the possible use of DNA methylation profile as a prediction tool for metformin's therapy efficacy or tolerance (Garcia-Calzon *et al.* 2020). As the paper mentioned above includes data from our OPTIMED cohort, future research could combine gut microbiome and DNA methylation biomarkers for prediction purposes. Even other -omics-based data collected from healthy and T2D cohorts could take a step closer to more precise and personalized prediction tools.

Importantly, when comparing *in vitro* and animal studies with those involving human subjects the discussion regarding metformin concentration used in such studies remains inconclusive. The doses are often not comparable (even 10 - 100 times higher than maximally achievable therapeutic concentrations), therefore, questioning the possible transfer of results from these studies to effects in clinical trials and everyday treatment strategies (He and Wondisford 2015). Thus, the growing number of metformin pharmacodynamic studies involving humans in combination with the preclinical experiments creates a reliable knowledge base for improving treatment approaches in clinical practice.

Multi-omics approaches have been widely used for metformin pharmacodynamic studies both individually and in combination. Nevertheless, the complex interaction among the metformin pharmacodynamic effects, microbiome, and DNA methylation altogether remains elusive. Many effector molecules derived from the gut microbiome have a significant impact on host epigenetics. The most known of them are various methyl donors (folate, choline, vitamin B12,

etc.), as well as SCFAs (Ye *et al.* 2017). Interestingly, metformin is known to be associated with level alterations and even deficiencies of B-group vitamins, especially vitamin B12 (Yang *et al.* 2019). Together with other previous studies, our results have shown metformin associated alterations in microbiome functional pathways related to B-group vitamin synthesis and metabolism (Olgun 2017, Rosario *et al.* 2018). Previous research has characterized such gut microbial communities as *Lactobacillus* and *Bifidobacteria* to be important regulators of these methyl donor nutrients (Rossi *et al.* 2011), and for some of the species, their effects on DNA methylation have been proved *in vitro* (Cortese *et al.* 2016). Moreover, most of the studies in animal models or humans analysing metformin effects on the gut microbiome report increase in at least one of these taxa, and direct metformin effects on bacterial growth have been shown in the case of *Bifidobacterium adolescentis* (Wu *et al.* 2017, Zhang and Hu 2020). Therefore, it could be hypothesized that both direct and indirect effects of metformin on the gut microbiome composition and functionality could be another pathway for alterations related to B-group vitamin metabolism and even a significant mediator for available methyl donors and the observed changes in the epigenetic profile of the host.

One of the main and beneficial effects observed in the human gut microbiome during metformin use is an increase in the abundance of *Akkermansia muciniphila* and other SCFA-producing bacteria. Despite the described mechanisms of SCFA induced phosphorylation of ERK, which results in downregulation of DNMT1 and further demethylation of specific genes (Sarkar *et al.* 2011), metformin therapy has been associated with both global DNA hypermethylation and hypomethylation (Elbere *et al.* 2018, Solomon *et al.* 2020). Moreover, the reduced activity of DNMT1 that leads to demethylation of specific tumour suppressor genes has been proposed as one of the mechanisms of action for anticancer and antidiabetic effects of metformin (Bridgeman *et al.* 2018). In the case of metformin effects on *A. muciniphila* abundance, some population-specific microbiome diversity needs to be taken into account, as in our study groups we observed that almost a half of participants did not have detectable levels of these species. Nevertheless, other SCFA producing taxa could fill the niche and employ the microbiome mediated beneficial effects of metformin treatment.

In addition, although the mechanisms are not clear, a study showed that commensal microbiota increases DNA methylation level in the Toll-Like Receptor 4 (*TLR4)* gene, which usually recognizes lipopolysaccharides (LPS) and further activates the innate immune system. This increased methylation then leads to decreased responsiveness to LPS to ensure maintenance of bacterial insensitivity in the colon (Takahashi *et al.* 2011). In our study employing shotgun metagenomics, we observed metformin induced increase in abundance of various pathways

involved in biosynthesis and metabolism of LPS, similarly to results reported previously (Wu *et al.* 2017), however, only in healthy individuals. Interestingly, in our methylation study (that includes a subgroup of the same healthy individuals analysed in the microbiome study) we found increased methylation level for the most significantly changed probe representing the *TLR4* gene (both contrasts: M10h vs M0 and M7d vs M0). This change was unfortunately not significant after correction (unpublished results). In intestinal epithelial cells of healthy individuals, such increase of methylation in *TLR4* gene represents a significant example of a mechanism contributing to the maintenance of intestinal symbiosis (Takahashi *et al.* 2011). Nevertheless, the absence of significant results in our study could be explained not only by the small sample size but also by the different cell types analysed compared to the literature. These data offer insight into correlations that need to be tested in future studies employing a bigger cohort as well as additional statistical analysis. Altogether, the possible microbiome mediated effects on the epigenetic profile of the host highlight the need for more extensive multi-omics studies as well as gives insight into the complex nature of multi-level interactions between various sites depicting metformin's pharmacodynamic mechanisms of action.

The main limitation of our results is the relatively small study groups. Nevertheless, both the microbiome profile and DNA methylation signatures are dynamic and highly subject-specific, therefore, the applied longitudinal design represents one of the main advantages of the studies included in this thesis. The repeated sampling increases the statistical power allowing the baseline samples from the same individuals to be treated as controls. This as well controls for a number of possible confounding factors, such as age, gender, diet, etc. (Goodrich *et al.* 2014). Moreover, the unique cohort of newly diagnosed and antidiabetic treatment naïve patients ensures a higher homogeneity of the group.

A significant strength of our study is the short period between the collected samples which allows evaluating immediate effects of the metformin. While additional data from samples collected during a longer period would definitely benefit the results, it is important to note that when analysing samples from patients with stable metformin therapy for more than three months, it would be challenging to distinguish metformin-mediated effects from those secondary to the metabolic improvements due to therapy.

Moving forward, a new analysis could be performed employing the existing data and the continuously growing sample size of the OPTIMED cohort to:

(1) Validate findings presented in this theses using bigger sample size;

(2) Obtain more precise results by performing microbiome analysis with higher sequencing depth;
(3) Confirm the proposed hypothesis regarding the interaction between the gut microbiome and the DNA methylation profile.

In addition, functional studies implemented with mice models, *in vitro* experiments, or the new state-of-the-art microfluidic systems like gut-on-a-chip should be needed to move from the observed associations to evidence about causality.

5. CONCLUSIONS

1. Metformin-induced reduction in the abundance of *Peptostreptococcaceae* family (and *Intestinibacter bartlettii* species) is the most pronounced effect of metformin on the gut microbiota.
2. Changes in gut microbiome diversity, composition, and functional profile during metformin use are group-specific.
3. The increase in the abundance of opportunistic pathogens represents a possible trigger for the occurrence of side effects.
4. Metformin induces significant effects on peripheral blood cell DNA methylation profile already after one dose.
5. Changes in peripheral blood cell DNA methylation profile of healthy individuals represent the main functional groups associated with previously described targets of metformin therapy: regulatory processes of energy homeostasis, inflammatory responses, tumorigenesis, and neurodegeneration.
6. Gut microbiome composition enriched with various probiotic species is a significant biomarker for increased therapeutic efficacy of metformin.
7. The baseline composition of the gut microbiome may influence metformin therapy efficacy and tolerance in T2D patients and could be used as a powerful prediction tool.

6. THESIS

1. Metformin pharmacodynamic effects on the human gut microbiome and DNA methylation profile are immediate and can be observed already within the first 24 hours after its administration.
2. Metformin-affected microbiota contributes significantly to the development of gastrointestinal side effects.
3. Gut microbiome composition before the antidiabetic treatment has a high potential to be used as an effective prediction tool for efficacy and tolerance of metformin therapy.
4. Changes in DNA methylation profile depict new mechanisms of metformin's action.

7. PUBLICATIONS

I Elbere I, Kalnina I, Silamikelis I, Konrade I, Zaharenko L, Sekace K, Radovica-Spalvina I, Fridmanis D, Gudra D, Pirags V, Klovins J. Association of metformin administration with gut microbiome dysbiosis in healthy volunteers. PLoS One. 2018 Sep 27;13(9):e0204317. doi: 10.1371/journal.pone.0204317.

II Elbere I, Silamikelis I, Ustinova M, Kalnina I, Zaharenko L, Peculis R, Konrade I, Ciuculete DM, Zhukovsky C, Gudra D, Radovica-Spalvina I, Fridmanis D, Pirags V, Schiöth HB, Klovins J. Significantly altered peripheral blood cell DNA methylation profile as a result of immediate effect of metformin use in healthy individuals. Clin Epigenetics. 2018 Dec 13;10(1):156. doi: 10.1186/s13148-018-0593-x.

III Elbere I, Silamikelis I, Dindune II, Kalnina I, Ustinova M, Zaharenko L, Silamikele L, Rovite V, Gudra D, Konrade I, Sokolovska J, Pirags V, Klovins J. Baseline gut microbiome composition predicts metformin therapy short-term efficacy in newly diagnosed type 2 diabetes patients. PLoS One. 2020 Oct 30;15(10):e0241338. doi: 10.1371/journal.pone.0241338.

8. APPROBATION OF RESEARCH

1) **Elbere I**, Silamiķelis I, Dindune I. I, Kalniņa I, Ustinova M, Silamiķele L, Rovīte V, Gudrā D, Konrāde I, Sokolovska J, Pīrags V, Kloviņš J. *Metformīna terapijas efektivitātes un tolerances biomarķieri cilvēka zarnu mikrobiomā.* 31.01.20220. LU 78. Zinātniskā konference. Mutiska prezentācija.
2) Klovins J, **Elbere I**, Ustinova M, Silamikelis I, Konrade I, Pirags V, *Metformin mediated gut microbiome modulation in T2D patients and controls induces specific changes in bacterial content*. 02.-06.12.2019. International Diabetes Federation Congress 2019, Busan, Korea. Abstract book.
3) **Elbere I**, Dindune I. I, Kalniņa I, Silamiķelis I, Gulbinska L, Konrāde I, Zaharenko L, Radoviča-Spalviņa I, Ustinova M, Fridmanis D, Gudrā D, Pīrāgs V, Kloviņš J. *Functional profile of the gut microbiome and its changes during metformin therapy in healthy individuals and newly-diagnosed type 2 diabetes patients*. 17.-19.06.2019. FEBS3+ conference, Rīga, Latvia. Oral presentation.
4) **Elbere I**, Dindune I.I, Kalnina I, Silamikelis I, Konrade I, Gulbinska L, Ustinova M, Zaharenko L, Radovica-Spalvina I, Fridmanis D, Gudra D, Pirags V, Klovins J. *Association of type 2 diabetes mellitus and metformin pharmacodynamics with the human gut microbiome composition*. 12.-16.08.2019. Metabolic Pathway Analysis, Rīga, Latvia. Abstract book.
5) **Elbere I**, Kalnina I, Silamikelis I, Dindune I. I, Gulbinska L, Zaharenko L, Radovica-Spalvina I, Gudra D, Fridmanis D, Konrade I, Pirags V, Klovins J. *Metformin induced changes in gut microbiome composition in healthy individuals and newly-diagnosed type 2 diabetes patients*. European Human Genetics Conference 2018, Milan, Italy. Abstract book.
6) Klovins J, **Elbere I**, Peculis R, Kalnina I, Zaharenko L, Konrade I & Pirags V, *Alterations in DNA methylation from peripheral blood cells in humans threated with metformin*. 19th European Congress of Endocrinology, Lisbon, Portugal. Oral Presentation. Endocrine Abstracts (2017) 49 OC6.3.
7) **Elbere I**, Kalnina I, Silamikelis I, Konrade I, Zaharenko L, Radovica I, Fridmanis F, Gudra D, Pirags V, Klovins J. *Changes induced by a weeklong metformin treatment in the human gut microbiome.* AZ Nordic Baltic Diabetes Science Forum. Gothenburg, Sweden, 19.-20.01.2017. Abstract book.
8) **Elbere I**, Kalnina I, Silamikelis I, Konrade I, Zaharenko L, Radovica I, Fridmanis D, Gudra D, Pirags V, Klovins J. *Association of metformin treatment with gut microbiome*

dysbiosis in healthy volunteers. 2nd annual Europan Microbiome Congress. London, UK, 30.11-01.12.2016. Abstract book.

AWARDS AND SCHOLARSHIPS

1) Study year 2017/2018 – Study year 2019/2020: "Mikrotikls Ltd" scholarship for PhD students in the fields of the natural, life, and medical sciences (administrated by University of Latvia Foundation)

2) 2019 - Letter of appreciation by the President of Latvian Academy of Sciences about the study "Discovered new mechanisms and efficacy markers for the treatment of type 2 diabetes in the gut microbiome of patients" (**Mg.biol. Ilze Elbere**, Mg.biol. Monta Ustinova, Mg.biol. Ivars Silamiķelis, Mg.biol. Laila Silamiķele, LZA correspondent member Ilze Konrāde, LZA academic Valdis Pīrāgs, LZA academic Jānis Kloviņš)

3) Study year 2016/2017 – scholarship of Alfreds Raisters for new researchers by University of Latvia Foundation

4) Study year 2014/2015 – scholarship of Kristaps Morbergs by University of Latvia Foundation

5) Study year 2013/2014 – scholarship of Kristaps Morbergs by University of Latvia Foundation

6) Study year 2012/2013 – scholarship "Ceļamaize" by University of Latvia Foundation

9. ACKNOWLEDGEMENTS

This research was supported by the European Regional Development Fund under the project "Investigation of interplay between multiple determinants influencing response to metformin: search for reliable predictors for efficacy of type 2 diabetes therapy" (Project No. 1.1.1.1/16/A/091).

I kindly thank to the Genome Database of Latvian Population for the involvement in participants' recruitment and bio-sample processing and data storage. I thank all the volunteers who participated in the clinical trial.

I am very thankful to the Ltd "MikroTik" and Foundation of University of Latvia for the honour and financial support with scholarship for PhD students in the fields of the natural, life, and medical sciences.

I would like to express a special gratitude to my supervisor Prof. Jānis Kloviņš for advice, support, and motivation during my research and the professional growth. I am thankful to my first supervisors during bachelor studies Raitis Pečulis and Ineta Kalniņa for the patience, encouragement, and the shared knowledge. I am also thankful to all my colleagues at Human Genetics and Molecular Medicine group, especially Monta Ustinova for the companionship during the implementation of metformin studies.

Finally, I give special thanks to my husband, family and friends for the endless support, encouragement, and understanding.

REFERENCES

1. Aguirre de Carcer, D. 2018. The human gut pan-microbiome presents a compositional core formed by discrete phylogenetic units. - Sci Rep, 8 (1): 14069.
2. Alegria-Torres, J. A., Baccarelli, A. and Bollati, V. 2011. Epigenetics and lifestyle. - Epigenomics, 3 (3): 267-77.
3. Allan, J., Hartman, P. G., Crane-Robinson, C. and Aviles, F. X. 1980. The structure of histone H1 and its location in chromatin. - Nature, 288 (5792): 675-9.
4. Allen, J. and Sears, C. L. 2019. Impact of the gut microbiome on the genome and epigenome of colon epithelial cells: contributions to colorectal cancer development. - Genome Med, 11 (1): 11.
5. Allis, C. D. and Jenuwein, T. 2016. The molecular hallmarks of epigenetic control. - Nat Rev Genet, 17 (8): 487-500.
6. Alzaid, A., Ladron de Guevara, P., Beillat, M., Lehner Martin, V. and Atanasov, P. 2020. Burden of disease and costs associated with type 2 diabetes in emerging and established markets: systematic review analyses. - Expert Rev Pharmacoecon Outcomes Res, 1-14.
7. Aryee, M. J., Jaffe, A. E., Corrada-Bravo, H., Ladd-Acosta, C., Feinberg, A. P., Hansen, K. D. and Irizarry, R. A. 2014. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. - Bioinformatics, 30 (10): 1363-9.
8. Aschner, P. 2017. New IDF clinical practice recommendations for managing type 2 diabetes in primary care. - Diabetes Res Clin Pract, 132 169-170.
9. Aulchenko, Y. S., Ripke, S., Isaacs, A. and van Duijn, C. M. 2007. GenABEL: an R library for genome-wide association analysis. - Bioinformatics, 23 (10): 1294-6.
10. Bahne, E., Sun, E. W. L., Young, R. L., Hansen, M., Sonne, D. P., Hansen, J. S., Rohde, U., Liou, A. P., Jackson, M. L., de Fontgalland, D., Rabbitt, P., Hollington, P., Sposato, L., Due, S., Wattchow, D. A., Rehfeld, J. F., Holst, J. J., Keating, D. J., Vilsboll, T. and Knop, F. K. 2018. Metformin-induced glucagon-like peptide-1 secretion contributes to the actions of metformin in type 2 diabetes. - JCI Insight, 3 (23):
11. Bailey, C. J., Wilcock, C. and Scarpello, J. H. 2008. Metformin and the intestine. - Diabetologia, 51 (8): 1552-3.
12. Bao, W., Tobias, D. K., Bowers, K., Chavarro, J., Vaag, A., Grunnet, L. G., Strom, M., Mills, J., Liu, A., Kiely, M. and Zhang, C. 2014. Physical activity and sedentary behaviors associated with risk of progression from gestational diabetes mellitus to type 2 diabetes mellitus: a prospective cohort study. - JAMA Intern Med, 174 (7): 1047-55.
13. Bashiardes, S., Zilberman-Schapira, G. and Elinav, E. 2016. Use of Metatranscriptomics in Microbiome Research. - Bioinform Biol Insights, 10 19-25.
14. Belizario, J. E. and Napolitano, M. 2015. Human microbiomes and their roles in dysbiosis, common diseases, and novel therapeutic approaches. - Front Microbiol, 6 1050.
15. Bellou, V., Belbasis, L., Tzoulaki, I. and Evangelou, E. 2018. Risk factors for type 2 diabetes mellitus: An exposure-wide umbrella review of meta-analyses. - PLoS One, 13 (3): e0194127.
16. Berg, G., Rybakova, D., Fischer, D., Cernava, T., Verges, M. C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G. H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J. A., Maguin, E., Mauchline, T., McClure, R., Mitter, B., Ryan, M., Sarand, I., Smidt, H., Schelkle, B., Roume, H., Kiran, G. S., Selvin, J., Souza, R. S. C., van Overbeek, L., Singh, B. K., Wagner, M., Walsh, A., Sessitsch, A. and Schloter, M. 2020. Microbiome definition re-visited: old concepts and new challenges. - Microbiome, 8 (1): 103.
17. Borchiellini, M., Ummarino, S. and Di Ruscio, A. 2019. The Bright and Dark Side of DNA Methylation: A Matter of Balance. - Cells, 8 (10):
18. Brestoff, J. R. and Artis, D. 2013. Commensal bacteria at the interface of host metabolism and the immune system. - Nat Immunol, 14 (7): 676-84.
19. Bridgeman, S. C., Ellison, G. C., Melton, P. E., Newsholme, P. and Mamotte, C. D. S. 2018. Epigenetic effects of metformin: From molecular mechanisms to clinical implications. - Diabetes Obes Metab, 20 (7): 1553-1562.
20. Bryrup, T., Thomsen, C. W., Kern, T., Allin, K. H., Brandslund, I., Jorgensen, N. R., Vestergaard, H., Hansen, T., Hansen, T. H., Pedersen, O. and Nielsen, T. 2019. Metformin-induced changes of the gut microbiota in healthy young men: results of a non-blinded, one-armed intervention study. - Diabetologia, 62 (6): 1024-1035.
21. Brunkwall, L. and Orho-Melander, M. 2017. The gut microbiome as a target for prevention and treatment of hyperglycaemia in type 2 diabetes: from current human evidence to future possibilities. - Diabetologia, 60 (6): 943-951.
22. Burton, J. H., Johnson, M., Johnson, J., Hsia, D. S., Greenway, F. L. and Heiman, M. L. 2015. Addition of a Gastrointestinal Microbiome Modulator to Metformin Improves Metformin Tolerance and Fasting Glucose Levels. - J Diabetes Sci Technol, 9 (4): 808-14.

23. Cani, P. D. and de Vos, W. M. 2017. Next-Generation Beneficial Microbes: The Case of Akkermansia muciniphila. - Front Microbiol, 8 1765.
24. Carbonero, F. 2017. Human epigenetics and microbiome: the potential for a revolution in both research areas by integrative studies. - Future Sci OA, 3 (3): FSO207.
25. Cavalli, G. and Heard, E. 2019. Advances in epigenetics link genetics to the environment and disease. - Nature, 571 (7766): 489-499.
26. Chandra, S., Alam, M. T., Dey, J., Chakrapani, P. S. B., Ray, U., Srivastava, A. K., Gandhi, S. and Tripathi, P. P. 2020. Healthy Gut, Healthy Brain: The Gut Microbiome in Neurodegenerative Disorders. - Curr Top Med Chem,
27. Cortese, R., Lu, L., Yu, Y., Ruden, D. and Claud, E. C. 2016. Epigenome-Microbiome crosstalk: A potential new paradigm influencing neonatal susceptibility to disease. - Epigenetics, 11 (3): 205-15.
28. de la Cuesta-Zuluaga, J., Mueller, N. T., Corrales-Agudelo, V., Velasquez-Mejia, E. P., Carmona, J. A., Abad, J. M. and Escobar, J. S. 2017. Metformin Is Associated With Higher Relative Abundance of Mucin-Degrading Akkermansia muciniphila and Several Short-Chain Fatty Acid-Producing Microbiota in the Gut. - Diabetes Care, 40 (1): 54-62.
29. Deehan, E. C., Colin-Ramirez, E., Triador, L., Madsen, K. L., Prado, C. M., Field, C. J., Ball, G. D. C., Tan, Q., Orsso, C., Dinu, I., Pakseresht, M., Rubin, D., Sharma, A. M., Tun, H., Walter, J., Newgard, C. B., Freemark, M., Wine, E. and Haqq, A. M. 2021. Efficacy of metformin and fermentable fiber combination therapy in adolescents with severe obesity and insulin resistance: study protocol for a double-blind randomized controlled trial. - Trials, 22 (1): 148.
30. Dempsey, J., Zhang, A. and Cui, J. Y. 2018. Coordinate regulation of long non-coding RNAs and protein-coding genes in germ-free mice. - BMC Genomics, 19 (1): 834.
31. Devaux, C. A. and Raoult, D. 2018. The Microbiological Memory, an Epigenetic Regulator Governing the Balance Between Good Health and Metabolic Disorders. - Front Microbiol, 9 1379.
32. Dimas, A. S., Lagou, V., Barker, A., Knowles, J. W., Magi, R., Hivert, M. F., Benazzo, A., Rybin, D., Jackson, A. U., Stringham, H. M., Song, C., Fischer-Rosinsky, A., Boesgaard, T. W., Grarup, N., Abbasi, F. A., Assimes, T. L., Hao, K., Yang, X., Lecoeur, C., Barroso, I., Bonnycastle, L. L., Bottcher, Y., Bumpstead, S., Chines, P. S., Erdos, M. R., Graessler, J., Kovacs, P., Morken, M. A., Narisu, N., Payne, F., Stancakova, A., Swift, A. J., Tonjes, A., Bornstein, S. R., Cauchi, S., Froguel, P., Meyre, D., Schwarz, P. E., Haring, H. U., Smith, U., Boehnke, M., Bergman, R. N., Collins, F. S., Mohlke, K. L., Tuomilehto, J., Quertemous, T., Lind, L., Hansen, T., Pedersen, O., Walker, M., Pfeiffer, A. F., Spranger, J., Stumvoll, M., Meigs, J. B., Wareham, N. J., Kuusisto, J., Laakso, M., Langenberg, C., Dupuis, J., Watanabe, R. M., Florez, J. C., Ingelsson, E., McCarthy, M. I., Prokopenko, I. and Investigators, M. 2014. Impact of type 2 diabetes susceptibility variants on quantitative glycemic traits reveals mechanistic heterogeneity. - Diabetes, 63 (6): 2158-71.
33. Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M. and Gingeras, T. R. 2013. STAR: ultrafast universal RNA-seq aligner. - Bioinformatics, 29 (1): 15-21.
34. Doestzada, M., Vila, A. V., Zhernakova, A., Koonen, D. P. Y., Weersma, R. K., Touw, D. J., Kuipers, F., Wijmenga, C. and Fu, J. 2018. Pharmacomicrobiomics: a novel route towards personalized medicine? - Protein Cell, 9 (5): 432-445.
35. Doyle-Delgado, K., Chamberlain, J. J., Shubrook, J. H., Skolnik, N. and Trujillo, J. 2020. Pharmacologic Approaches to Glycemic Treatment of Type 2 Diabetes: Synopsis of the 2020 American Diabetes Association's Standards of Medical Care in Diabetes Clinical Guideline. - Ann Intern Med, 173 (10): 813-821.
36. Ducarmon, Q. R., Zwittink, R. D., Hornung, B. V. H., van Schaik, W., Young, V. B. and Kuijper, E. J. 2019. Gut Microbiota and Colonization Resistance against Bacterial Enteric Infection. - Microbiol Mol Biol Rev, 83 (3):
37. Ebid, A. I. M., Ehab, M., Ismail, A., Soror, S. and Mahmoud, M. A. 2019. The influence of SLC22A1 rs622342 and ABCC8 rs757110 genetic variants on the efficacy of metformin and glimepiride combination therapy in Egyptian patients with type 2 diabetes. - J Drug Assess, 8 (1): 115-121.
38. Echiburu, B., Milagro, F., Crisosto, N., Perez-Bravo, F., Flores, C., Arpon, A., Salas-Perez, F., Recabarren, S. E., Sir-Petermann, T. and Maliqueo, M. 2020. DNA methylation in promoter regions of genes involved in the reproductive and metabolic function of children born to women with PCOS. - Epigenetics, 15 (11): 1178-1194.
39. Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. and Knight, R. 2011. UCHIME improves sensitivity and speed of chimera detection. - Bioinformatics, 27 (16): 2194-200.
40. Ejtahed, H. S., Tito, R. Y., Siadat, S. D., Hasani-Ranjbar, S., Hoseini-Tavassol, Z., Rymenans, L., Verbeke, K., Soroush, A. R., Raes, J. and Larijani, B. 2019. Metformin induces weight loss associated with gut microbiota alteration in non-diabetic obese women: a randomized double-blind clinical trial. - Eur J Endocrinol, 180 (3): 165-176.
41. Elbere, I., Silamikelis, I., Dindune, II, Kalnina, I., Ustinova, M., Zaharenko, L., Silamikele, L., Rovite, V., Gudra, D., Konrade, I., Sokolovska, J., Pirags, V. and Klovins, J. 2020. Baseline gut microbiome composition

predicts metformin therapy short-term efficacy in newly diagnosed type 2 diabetes patients. - PLoS One, 15 (10): e0241338.
42. Elbere, I., Silamikelis, I., Ustinova, M., Kalnina, I., Zaharenko, L., Peculis, R., Konrade, I., Ciuculete, D. M., Zhukovsky, C., Gudra, D., Radovica-Spalvina, I., Fridmanis, D., Pirags, V., Schioth, H. B. and Klovins, J. 2018. Significantly altered peripheral blood cell DNA methylation profile as a result of immediate effect of metformin use in healthy individuals. - Clin Epigenetics, 10 (1): 156.
43. Engevik, M. and Versalovic, J. 2019. Taking a Closer Look at the Biogeography of the Human Gastrointestinal Microbiome. - Gastroenterology, 157 (4): 927-929.
44. Forslund, K., Hildebrand, F., Nielsen, T., Falony, G., Le Chatelier, E., Sunagawa, S., Prifti, E., Vieira-Silva, S., Gudmundsdottir, V., Pedersen, H. K., Arumugam, M., Kristiansen, K., Voigt, A. Y., Vestergaard, H., Hercog, R., Costea, P. I., Kultima, J. R., Li, J., Jorgensen, T., Levenez, F., Dore, J., Meta, H. I. T. c., Nielsen, H. B., Brunak, S., Raes, J., Hansen, T., Wang, J., Ehrlich, S. D., Bork, P. and Pedersen, O. 2015. Disentangling type 2 diabetes and metformin treatment signatures in the human gut microbiota. - Nature, 528 (7581): 262-266.
45. Franzosa, E. A., McIver, L. J., Rahnavard, G., Thompson, L. R., Schirmer, M., Weingart, G., Lipson, K. S., Knight, R., Caporaso, J. G., Segata, N. and Huttenhower, C. 2018. Species-level functional profiling of metagenomes and metatranscriptomes. - Nat Methods, 15 (11): 962-968.
46. Garcia-Calzon, S., Perfilyev, A., Mannisto, V., de Mello, V. D., Nilsson, E., Pihlajamaki, J. and Ling, C. 2017. Diabetes medication associates with DNA methylation of metformin transporter genes in the human liver. - Clin Epigenetics, 9 102.
47. Garcia-Calzon, S., Perfilyev, A., Martinell, M., Ustinova, M., Kalamajski, S., Franks, P. W., Bacos, K., Elbere, I., Pihlajamaki, J., Volkov, P., Vaag, A., Groop, L., Maziarz, M., Klovins, J., Ahlqvist, E. and Ling, C. 2020. Epigenetic markers associated with metformin response and intolerance in drug-naive patients with type 2 diabetes. - Sci Transl Med, 12 (561):
48. Gedawy, A., Al-Salami, H. and Dass, C. R. 2020. Role of metformin in various pathologies: state-of-the-art microcapsules for improving its pharmacokinetics. - Ther Deliv, 11 (11): 733-753.
49. Gerritsen, J., Fuentes, S., Grievink, W., van Niftrik, L., Tindall, B. J., Timmerman, H. M., Rijkers, G. T. and Smidt, H. 2014. Characterization of Romboutsia ilealis gen. nov., sp. nov., isolated from the gastro-intestinal tract of a rat, and proposal for the reclassification of five closely related members of the genus Clostridium into the genera Romboutsia gen. nov., Intestinibacter gen. nov., Terrisporobacter gen. nov. and Asaccharospora gen. nov. - Int J Syst Evol Microbiol, 64 (Pt 5): 1600-1616.
50. Gong, L., Goswami, S., Giacomini, K. M., Altman, R. B. and Klein, T. E. 2012. Metformin pathways: pharmacokinetics and pharmacodynamics. - Pharmacogenet Genomics, 22 (11): 820-7.
51. Goodrich, J. K., Di Rienzi, S. C., Poole, A. C., Koren, O., Walters, W. A., Caporaso, J. G., Knight, R. and Ley, R. E. 2014. Conducting a microbiome study. - Cell, 158 (2): 250-262.
52. Graham, G. G., Punt, J., Arora, M., Day, R. O., Doogue, M. P., Duong, J. K., Furlong, T. J., Greenfield, J. R., Greenup, L. C., Kirkpatrick, C. M., Ray, J. E., Timmins, P. and Williams, K. M. 2011. Clinical pharmacokinetics of metformin. - Clin Pharmacokinet, 50 (2): 81-98.
53. Gurung, M., Li, Z., You, H., Rodrigues, R., Jump, D. B., Morgun, A. and Shulzhenko, N. 2020. Role of gut microbiota in type 2 diabetes pathophysiology. - EBioMedicine, 51 102590.
54. He, Y., Wu, W., Zheng, H. M., Li, P., McDonald, D., Sheng, H. F., Chen, M. X., Chen, Z. H., Ji, G. Y., Zheng, Z. D., Mujagond, P., Chen, X. J., Rong, Z. H., Chen, P., Lyu, L. Y., Wang, X., Wu, C. B., Yu, N., Xu, Y. J., Yin, J., Raes, J., Knight, R., Ma, W. J. and Zhou, H. W. 2018. Regional variation limits applications of healthy gut microbiome reference ranges and disease models. - Nat Med, 24 (10): 1532-1535.
55. He, L., Sabet, A., Djedjos, S., Miller, R., Sun, X., Hussain, M. A., Radovick, S. and Wondisford, F. E. 2009. Metformin and insulin suppress hepatic gluconeogenesis through phosphorylation of CREB binding protein. - Cell, 137 (4): 635-46.
56. He, L. and Wondisford, F. E. 2015. Metformin action: concentrations matter. - Cell Metab, 21 (2): 159-162.
57. Hiel, S., Gianfrancesco, M. A., Rodriguez, J., Portheault, D., Leyrolle, Q., Bindels, L. B., Gomes da Silveira Cauduro, C., Mulders, M., Zamariola, G., Azzi, A. S., Kalala, G., Pachikian, B. D., Amadieu, C., Neyrinck, A. M., Loumaye, A., Cani, P. D., Lanthier, N., Trefois, P., Klein, O., Luminet, O., Bindelle, J., Paquot, N., Cnop, M., Thissen, J. P. and Delzenne, N. M. 2020. Link between gut microbiota and health outcomes in inulin -treated obese patients: Lessons from the Food4Gut multicenter randomized placebo-controlled trial. - Clin Nutr, 39 (12): 3618-3628.
58. Holst, J. J. 2007. The physiology of glucagon-like peptide 1. - Physiol Rev, 87 (4): 1409-39.
59. Houghton, D., Hardy, T., Stewart, C., Errington, L., Day, C. P., Trenell, M. I. and Avery, L. 2018. Systematic review assessing the effectiveness of dietary intervention on gut microbiota in adults with type 2 diabetes. - Diabetologia, 61 (8): 1700-1711.
60. Houseman, E. A., Accomando, W. P., Koestler, D. C., Christensen, B. C., Marsit, C. J., Nelson, H. H., Wiencke, J. K. and Kelsey, K. T. 2012. DNA methylation arrays as surrogate measures of cell mixture distribution. - BMC Bioinformatics, 13 86.

61. Howell, J. J., Hellberg, K., Turner, M., Talbott, G., Kolar, M. J., Ross, D. S., Hoxhaj, G., Saghatelian, A., Shaw, R. J. and Manning, B. D. 2017. Metformin Inhibits Hepatic mTORC1 Signaling via Dose-Dependent Mechanisms Involving AMPK and the TSC Complex. - Cell Metab, 25 (2): 463-471.
62. Hundal, R. S., Krssak, M., Dufour, S., Laurent, D., Lebon, V., Chandramouli, V., Inzucchi, S. E., Schumann, W. C., Petersen, K. F., Landau, B. R. and Shulman, G. I. 2000. Mechanism by which metformin reduces glucose production in type 2 diabetes. - Diabetes, 49 (12): 2063-9.
63. Hunter, R. W., Hughey, C. C., Lantier, L., Sundelin, E. I., Peggie, M., Zeqiraj, E., Sicheri, F., Jessen, N., Wasserman, D. H. and Sakamoto, K. 2018. Metformin reduces liver glucose production by inhibition of fructose-1-6-bisphosphatase. - Nat Med, 24 (9): 1395-1406.
64. International Diabetes Federation 2019. IDF Diabetes Atlas, 9th edn. Brussels,
65. Yan, Y., Nguyen, L. H., Franzosa, E. A. and Huttenhower, C. 2020. Strain-level epidemiology of microbial communities and the human microbiome. - Genome Med, 12 (1): 71.
66. Yang, W., Cai, X., Wu, H. and Ji, L. 2019. Associations between metformin use and vitamin B12 levels, anemia, and neuropathy in patients with diabetes: a meta-analysis. - J Diabetes, 11 (9): 729-743.
67. Ye, J., Wu, W., Li, Y. and Li, L. 2017. Influences of the Gut Microbiota on DNA Methylation and Histone Modification. - Dig Dis Sci, 62 (5): 1155-1164.
68. Yuille, S., Reichardt, N., Panda, S., Dunbar, H. and Mulder, I. E. 2018. Human gut bacteria as potent class I histone deacetylase inhibitors in vitro through production of butyric acid and valeric acid. - PLoS One, 13 (7): e0201073.
69. Johnson, W. E., Li, C. and Rabinovic, A. 2007. Adjusting batch effects in microarray expression data using empirical Bayes methods. - Biostatistics, 8 (1): 118-27.
70. Kahn, S. E., Haffner, S. M., Heise, M. A., Herman, W. H., Holman, R. R., Jones, N. P., Kravitz, B. G., Lachin, J. M., O'Neill, M. C., Zinman, B., Viberti, G. and Group, A. S. 2006. Glycemic durability of rosiglitazone, metformin, or glyburide monotherapy. - N Engl J Med, 355 (23): 2427-43.
71. Kashi, Z., Mahrooz, A., Kianmehr, A. and Alizadeh, A. 2016. The Role of Metformin Response in Lipid Metabolism in Patients with Recent-Onset Type 2 Diabetes: HbA1c Level as a Criterion for Designating Patients as Responders or Nonresponders to Metformin. - PLoS One, 11 (3): e0151543.
72. Kebede, A. F., Schneider, R. and Daujat, S. 2015. Novel types and sites of histone modifications emerge as players in the transcriptional regulation contest. - FEBS J, 282 (9): 1658-74.
73. Kharroubi, A. T. and Darwish, H. M. 2015. Diabetes mellitus: The epidemic of the century. - World J Diabetes, 6 (6): 850-67.
74. Kho, Z. Y. and Lal, S. K. 2018. The Human Gut Microbiome - A Potential Controller of Wellness and Disease. - Front Microbiol, 9 1835.
75. Kim, H., Sitarik, A. R., Woodcroft, K., Johnson, C. C. and Zoratti, E. 2019. Birth Mode, Breastfeeding, Pet Exposure, and Antibiotic Use: Associations With the Gut Microbiome and Sensitization in Children. - Curr Allergy Asthma Rep, 19 (4): 22.
76. Kim, Y. D., Park, K. G., Lee, Y. S., Park, Y. Y., Kim, D. K., Nedumaran, B., Jang, W. G., Cho, W. J., Ha, J., Lee, I. K., Lee, C. H. and Choi, H. S. 2008. Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase-dependent regulation of the orphan nuclear receptor SHP. - Diabetes, 57 (2): 306-14.
77. Kim, S., Covington, A. and Pamer, E. G. 2017. The intestinal microbiota: Antibiotics, colonization resistance, and enteric pathogens. - Immunol Rev, 279 (1): 90-105.
78. Kyriachenko, Y., Falalyeyeva, T., Korotkyi, O., Molochek, N. and Kobyliak, N. 2019. Crosstalk between gut microbiota and antidiabetic drug action. - World J Diabetes, 10 (3): 154-168.
79. Knowler, W. C., Barrett-Connor, E., Fowler, S. E., Hamman, R. F., Lachin, J. M., Walker, E. A., Nathan, D. M. and Diabetes Prevention Program Research, G. 2002. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. - N Engl J Med, 346 (6): 393-403.
80. Kootte, R. S., Levin, E., Salojarvi, J., Smits, L. P., Hartstra, A. V., Udayappan, S. D., Hermes, G., Bouter, K. E., Koopen, A. M., Holst, J. J., Knop, F. K., Blaak, E. E., Zhao, J., Smidt, H., Harms, A. C., Hankemeijer, T., Bergman, J., Romijn, H. A., Schaap, F. G., Olde Damink, S. W. M., Ackermans, M. T., Dallinga-Thie, G. M., Zoetendal, E., de Vos, W. M., Serlie, M. J., Stroes, E. S. G., Groen, A. K. and Nieuwdorp, M. 2017. Improvement of Insulin Sensitivity after Lean Donor Feces in Metabolic Syndrome Is Driven by Baseline Intestinal Microbiota Composition. - Cell Metab, 26 (4): 611-619 e6.
81. Kouzarides, T. 2007. Chromatin modifications and their function. - Cell, 128 (4): 693-705.
82. Kramer, A., Green, J., Pollard, J., Jr. and Tugendreich, S. 2014. Causal analysis approaches in Ingenuity Pathway Analysis. - Bioinformatics, 30 (4): 523-30.
83. Krautkramer, K. A., Kreznar, J. H., Romano, K. A., Vivas, E. I., Barrett-Wilt, G. A., Rabaglia, M. E., Keller, M. P., Attie, A. D., Rey, F. E. and Denu, J. M. 2016. Diet-Microbiota Interactions Mediate Global Epigenetic Programming in Multiple Host Tissues. - Mol Cell, 64 (5): 982-992.
84. Kronfol, M. M., Dozmorov, M. G., Huang, R., Slattum, P. W. and McClay, J. L. 2017. The role of epigenomics in personalized medicine. - Expert Rev Precis Med Drug Dev, 2 (1): 33-45.

85. Lagier, J. C., Dubourg, G., Million, M., Cadoret, F., Bilen, M., Fenollar, F., Levasseur, A., Rolain, J. M., Fournier, P. E. and Raoult, D. 2018. Culturing the human microbiota and culturomics. - Nat Rev Microbiol, 16 540-550.
86. Lagkouvardos, I., Overmann, J. and Clavel, T. 2017. Cultured microbes represent a substantial fraction of the human and mouse gut microbiota. - Gut Microbes, 8 (5): 493-503.
87. Le Cao, K. A., Boitard, S. and Besse, P. 2011. Sparse PLS discriminant analysis: biologically relevant feature selection and graphical displays for multiclass problems. - BMC Bioinformatics, 12 253.
88. Lee, J., Papa, F., Jaini, P. A., Alpini, S. and Kenny, T. 2020. An Epigenetics-Based, Lifestyle Medicine-Driven Approach to Stress Management for Primary Patient Care: Implications for Medical Education. - Am J Lifestyle Med, 14 (3): 294-303.
89. Leek, J. T., Johnson, W. E., Parker, H. S., Jaffe, A. E. and Storey, J. D. 2012. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. - Bioinformatics, 28 (6): 882-3.
90. Li, M., Duan, L., Cai, Y., Hao, B., Chen, J. and Liu, H. 2021. Effect of Metformin on the Epigenetic Age of Peripheral Blood in Patients With Diabetes Mellitus (DM). - Research Square,
91. Liebl, K. and Zacharias, M. 2019. How methyl-sugar interactions determine DNA structure and flexibility. - Nucleic Acids Res, 47 (3): 1132-1140.
92. Lister, R., Pelizzola, M., Dowen, R. H., Hawkins, R. D., Hon, G., Tonti-Filippini, J., Nery, J. R., Lee, L., Ye, Z., Ngo, Q. M., Edsall, L., Antosiewicz-Bourget, J., Stewart, R., Ruotti, V., Millar, A. H., Thomson, J. A., Ren, B. and Ecker, J. R. 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. - Nature, 462 (7271): 315-22.
93. Liu, Y. X., Qin, Y., Chen, T., Lu, M., Qian, X., Guo, X. and Bai, Y. 2020. A practical guide to amplicon and metagenomic analysis of microbiome data. - Protein Cell,
94. Liu, N. and Pan, T. 2015. RNA epigenetics. - Transl Res, 165 (1): 28-35.
95. Lloyd-Price, J., Abu-Ali, G. and Huttenhower, C. 2016. The healthy human microbiome. - Genome Med, 8 (1): 51.
96. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F. and Richmond, T. J. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. - Nature, 389 (6648): 251-60.
97. Luizon, M. R., Eckalbar, W. L., Wang, Y., Jones, S. L., Smith, R. P., Laurance, M., Lin, L., Gallins, P. J., Etheridge, A. S., Wright, F., Zhou, Y., Molony, C., Innocenti, F., Yee, S. W., Giacomini, K. M. and Ahituv, N. 2016. Genomic Characterization of Metformin Hepatic Response. - PLoS Genet, 12 (11): e1006449.
98. Lv, Z. and Guo, Y. 2020. Metformin and Its Benefits for Various Diseases. - Front Endocrinol (Lausanne), 11 191.
99. Macchione, I. G., Lopetuso, L. R., Ianiro, G., Napoli, M., Gibiino, G., Rizzatti, G., Petito, V., Gasbarrini, A. and Scaldaferri, F. 2019. Akkermansia muciniphila: key player in metabolic and gastrointestinal disorders. - Eur Rev Med Pharmacol Sci, 23 (18): 8075-8083.
100. Madiraju, A. K., Erion, D. M., Rahimi, Y., Zhang, X. M., Braddock, D. T., Albright, R. A., Prigaro, B. J., Wood, J. L., Bhanot, S., MacDonald, M. J., Jurczak, M. J., Camporez, J. P., Lee, H. Y., Cline, G. W., Samuel, V. T., Kibbey, R. G. and Shulman, G. I. 2014. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. - Nature, 510 (7506): 542-6.
101. Mahrooz, A., Parsanasab, H., Hashemi-Soteh, M. B., Kashi, Z., Bahar, A., Alizadeh, A. and Mozayeni, M. 2015. The role of clinical response to metformin in patients newly diagnosed with type 2 diabetes: a monotherapy study. - Clin Exp Med, 15 (2): 159-65.
102. Maynard, C. L., Elson, C. O., Hatton, R. D. and Weaver, C. T. 2012. Reciprocal interactions of the intestinal microbiota and immune system. - Nature, 489 (7415): 231-41.
103. Mannucci, E., Tesi, F., Bardini, G., Ognibene, A., Petracca, M. G., Ciani, S., Pezzatini, A., Brogi, M., Dicembrini, I., Cremasco, F., Messeri, G. and Rotella, C. M. 2004. Effects of metformin on glucagon-like peptide-1 levels in obese patients with and without Type 2 diabetes. - Diabetes Nutr Metab, 17 (6): 336-42.
104. Marchesi, J. R. and Ravel, J. 2015. The vocabulary of microbiome research: a proposal. - Microbiome, 3 31.
105. Marin-Penalver, J. J., Martin-Timon, I., Sevillano-Collantes, C. and Del Canizo-Gomez, F. J. 2016. Update on the treatment of type 2 diabetes mellitus. - World J Diabetes, 7 (17): 354-95.
106. Marin, T. L., Gongol, B., Zhang, F., Martin, M., Johnson, D. A., Xiao, H., Wang, Y., Subramaniam, S., Chien, S. and Shyy, J. Y. 2017. AMPK promotes mitochondrial biogenesis and function by phosphorylating the epigenetic factors DNMT1, RBBP7, and HAT1. - Sci Signal, 10 (464):
107. Mark Welch, J. L., Hasegawa, Y., McNulty, N. P., Gordon, J. I. and Borisy, G. G. 2017. Spatial organization of a model 15-member human gut microbiota established in gnotobiotic mice. - Proc Natl Acad Sci U S A, 114 (43): E9105-E9114.
108. McCreight, L. J., Bailey, C. J. and Pearson, E. R. 2016. Metformin and the gastrointestinal tract. - Diabetologia, 59 (3): 426-35.
109. Meng, Y., Xiang, R., Yan, H., Zhou, Y., Hu, Y., Yang, J., Zhou, Y. and Cui, Q. 2020. Transcriptomic landscape profiling of metformin-treated healthy mice: Implication for potential hypertension risk when prophylactically used. - J Cell Mol Med, 24 (14): 8138-8150.

110. Milani, C., Hevia, A., Foroni, E., Duranti, S., Turroni, F., Lugli, G. A., Sanchez, B., Martin, R., Gueimonde, M., van Sinderen, D., Margolles, A. and Ventura, M. 2013. Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. - PLoS One, 8 (7): e68739.
111. Mischke, M. and Plosch, T. 2013. More than just a gut instinct-the potential interplay between a baby's nutrition, its gut microbiome, and the epigenome. - Am J Physiol Regul Integr Comp Physiol, 304 (12): R1065-9.
112. Moore, R. E. and Townsend, S. D. 2019. Temporal development of the infant gut microbiome. - Open Biol, 9 (9): 190128.
113. Natividad, J. M. and Verdu, E. F. 2013. Modulation of intestinal barrier by intestinal microbiota: pathological and therapeutic implications. - Pharmacol Res, 69 (1): 42-51.
114. Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W. and Pettersson, S. 2012. Host-gut microbiota metabolic interactions. - Science, 336 (6086): 1262-7.
115. Niu, L., Xu, Z. and Taylor, J. A. 2016. RCP: a novel probe design bias correction method for Illumina Methylation BeadChip. - Bioinformatics, 32 (17): 2659-63.
116. O'Neill, C. A., Monteleone, G., McLaughlin, J. T. and Paus, R. 2016. The gut-skin axis in health and disease: A paradigm with therapeutic implications. - Bioessays, 38 (11): 1167-1176.
117. Olgun, A. 2017. "Metformin-resistant" folic acid producing probiotics or folic acid against metformin's adverse effects like diarrhea. - Med Hypotheses, 106 33-34.
118. Olivier, B. G., Rohwer, J. M. and Hofmeyr, J. H. 2002. Modelling cellular processes with Python and Scipy. - Mol Biol Rep, 29 (1-2): 249-54.
119. Osadchiy, V., Martin, C. R. and Mayer, E. A. 2019. The Gut-Brain Axis and the Microbiome: Mechanisms and Clinical Implications. - Clin Gastroenterol Hepatol, 17 (2): 322-332.
120. Paley, S., Parker, K., Spaulding, A., Tomb, J. F., O'Maille, P. and Karp, P. D. 2017. The Omics Dashboard for interactive exploration of gene-expression data. - Nucleic Acids Res, 45 (21): 12113-12124.
121. Papatheodorou, K., Banach, M., Bekiari, E., Rizzo, M. and Edmonds, M. 2018. Complications of Diabetes 2017. - J Diabetes Res, 2018 3086167.
122. Park, J. E., Jeong, G. H., Lee, I. K., Yoon, Y. R., Liu, K. H., Gu, N. and Shin, K. H. 2018. A Pharmacometabolomic Approach to Predict Response to Metformin in Early-Phase Type 2 Diabetes Mellitus Patients. - Molecules, 23 (7):
123. Paun, A. and Danska, J. S. 2016. Modulation of type 1 and type 2 diabetes risk by the intestinal microbiome. - Pediatr Diabetes, 17 (7): 469-477.
124. Perez-Munoz, M. E., Arrieta, M. C., Ramer-Tait, A. E. and Walter, J. 2017. A critical assessment of the "sterile womb" and "in utero colonization" hypotheses: implications for research on the pioneer infant microbiome. - Microbiome, 5 (1): 48.
125. Peschansky, V. J. and Wahlestedt, C. 2014. Non-coding RNAs as direct and indirect modulators of epigenetic regulation. - Epigenetics, 9 (1): 3-12.
126. Peters, T. J., Buckley, M. J., Statham, A. L., Pidsley, R., Samaras, K., R, V. L., Clark, S. J. and Molloy, P. L. 2015. De novo identification of differentially methylated regions in the human genome. - Epigenetics Chromatin, 8 6.
127. Plows, J. F., Stanley, J. L., Baker, P. N., Reynolds, C. M. and Vickers, M. H. 2018. The Pathophysiology of Gestational Diabetes Mellitus. - Int J Mol Sci, 19 (11):
128. Przewlocka, K., Folwarski, M., Kazmierczak-Siedlecka, K., Skonieczna-Zydecka, K. and Kaczor, J. J. 2020. Gut-Muscle AxisExists and May Affect Skeletal Muscle Adaptation to Training. - Nutrients, 12 (5):
129. Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J. M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H. B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Li, S., Jian, M., Zhou, Y., Li, Y., Zhang, X., Li, S., Qin, N., Yang, H., Wang, J., Brunak, S., Dore, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Meta, H. I. T. C., Bork, P., Ehrlich, S. D. and Wang, J. 2010. A human gut microbial gene catalogue established by metagenomic sequencing. - Nature, 464 (7285): 59-65.
130. Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J. and Glockner, F. O. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. - Nucleic Acids Res, 41 (Database issue): D590-6.
131. Quattrocchi, E., Goldberg, T. and Marzella, N. 2020. Management of type 2 diabetes: consensus of diabetes organizations. - Drugs Context, 9 212607.
132. Quigley, E. M. M. and Gajula, P. 2020. Recent advances in modulating the microbiome. - F1000Res, 9
133. Rashid, M., Shahzad, M., Mahmood, S. and Khan, K. 2019. Variability in the therapeutic response of Metformin treatment in patients with type 2 diabetes mellitus. - Pak J Med Sci, 35 (1): 71-76.
134. Rausch, P., Ruhlemann, M., Hermes, B. M., Doms, S., Dagan, T., Dierking, K., Domin, H., Fraune, S., von Frieling, J., Hentschel, U., Heinsen, F. A., Hoppner, M., Jahn, M. T., Jaspers, C., Kissoyan, K. A. B., Langfeldt, D., Rehman, A., Reusch, T. B. H., Roeder, T., Schmitz, R. A., Schulenburg, H., Soluch, R., Sommer, F., Stukenbrock, E., Weiland-Brauer, N., Rosenstiel, P., Franke, A., Bosch, T. and Baines, J. F.

2019. Comparative analysis of amplicon and metagenomic sequencing methods reveals key features in the evolution of animal metaorganisms. - Microbiome, 7 (1): 133.
135. Rena, G., Hardie, D. G. and Pearson, E. R. 2017. The mechanisms of action of metformin. - Diabetologia, 60 (9): 1577-1585.
136. Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G. A. D., Gasbarrini, A. and Mele, M. C. 2019. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. - Microorganisms, 7 (1):
137. Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W. and Smyth, G. K. 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies. - Nucleic Acids Res, 43 (7): e47.
138. Rizkallah, M. R., Saad, R. and Aziz, R. K. 2010. The Human Microbiome Project, Personalized Medicine and the Birth of Pharmacomicrobiomics. - Current Pharmacogenomics and Personalized Medicine, 8 (3): 182-193.
139. Robinson, M. D., McCarthy, D. J. and Smyth, G. K. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. - Bioinformatics, 26 (1): 139-40.
140. Rodriguez, J., Hiel, S. and Delzenne, N. M. 2018. Metformin: old friend, new ways of action-implication of the gut microbiome? - Curr Opin Clin Nutr Metab Care, 21 (4): 294-301.
141. Rojo, D., Mendez-Garcia, C., Raczkowska, B. A., Bargiela, R., Moya, A., Ferrer, M. and Barbas, C. 2017. Exploring the human microbiome from multiple perspectives: factors altering its composition and function. - FEMS Microbiol Rev, 41 (4): 453-478.
142. Romano, K. A., Martinez-Del Campo, A., Kasahara, K., Chittim, C. L., Vivas, E. I., Amador-Noguez, D., Balskus, E. P. and Rey, F. E. 2017. Metabolic, Epigenetic, and Transgenerational Effects of Gut Bacterial Choline Consumption. - Cell Host Microbe, 22 (3): 279-290 e7.
143. Romano, K. A., Vivas, E. I., Amador-Noguez, D. and Rey, F. E. 2015. Intestinal microbiota composition modulates choline bioavailability from diet and accumulation of the proatherogenic metabolite trimethylamine-N-oxide. - mBio, 6 (2): e02481.
144. Rosario, D., Benfeitas, R., Bidkhori, G., Zhang, C., Uhlen, M., Shoaie, S. and Mardinoglu, A. 2018. Understanding the Representative Gut Microbiota Dysbiosis in Metformin-Treated Type 2 Diabetes Patients Using Genome-Scale Metabolic Modeling. - Front Physiol, 9 775.
145. Rossi, M., Amaretti, A. and Raimondi, S. 2011. Folate production by probiotic bacteria. - Nutrients, 3 (1): 118-34.
146. Rothbart, S. B. and Strahl, B. D. 2014. Interpreting the language of histone and DNA modifications. - Biochim Biophys Acta, 1839 (8): 627-43.
147. Rovite, V., Wolff-Sagi, Y., Zaharenko, L., Nikitina-Zake, L., Grens, E. and Klovins, J. 2018. Genome Database of the Latvian Population (LGDB): Design, Goals, and Primary Results. - Journal of epidemiology, 28 (8): 353-360.
148. Saberzadeh-Ardestani, B., Karamzadeh, R., Basiri, M., Hajizadeh-Saffar, E., Farhadi, A., Shapiro, A. M. J., Tahamtani, Y. and Baharvand, H. 2018. Type 1 Diabetes Mellitus: Cellular and Molecular Pathophysiology at A Glance. - Cell J, 20 (3): 294-301.
149. Safai, N., Suvitaival, T., Ali, A., Spegel, P., Al-Majdoub, M., Carstensen, B., Vestergaard, H., Ridderstrale, M. and Group, C. T. 2018. Effect of metformin on plasma metabolite profile in the Copenhagen Insulin and Metformin Therapy (CIMT) trial. - Diabet Med, 35 (7): 944-953.
150. Sarkar, S., Abujamra, A. L., Loew, J. E., Forman, L. W., Perrine, S. P. and Faller, D. V. 2011. Histone deacetylase inhibitors reverse CpG methylation by regulating DNMT1 through ERK signaling. - Anticancer Res, 31 (9): 2723-32.
151. Saxonov, S., Berg, P. and Brutlag, D. L. 2006. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. - Proc Natl Acad Sci U S A, 103 (5): 1412-7.
152. Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J. and Weber, C. F. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. - Appl Environ Microbiol, 75 (23): 7537-41.
153. Schoch, C. L., Seifert, K. A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A., Chen, W., Fungal Barcoding, C. and Fungal Barcoding Consortium Author, L. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. - Proc Natl Acad Sci U S A, 109 (16): 6241-6.
154. Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S. and Huttenhower, C. 2011. Metagenomic biomarker discovery and explanation. - Genome Biol, 12 (6): R60.
155. Shannon, C. E. 1948. A Mathematical Theory of Communication. - Bell System Technical Journal, 27 (3): 379-423.
156. Sharpton, T. J. 2014. An introduction to the analysis of shotgun metagenomic data. - Front Plant Sci, 5 209.
157. Sherifali, D., Nerenberg, K., Pullenayegum, E., Cheng, J. E. and Gerstein, H. C. 2010. The effect of oral antidiabetic agents on A1C levels: a systematic review and meta-analysis. - Diabetes Care, 33 (8): 1859-64.

158. Solomon, W. L., Hector, S. B. E., Raghubeer, S., Erasmus, R. T., Kengne, A. P. and Matsha, T. E. 2020. Genome-Wide DNA Methylation and LncRNA-Associated DNA Methylation in Metformin-Treated and -Untreated Diabetes. - Epigenomes, 4 (3): 19.
159. Sun, J. and Buys, N. J. 2016. Glucose- and glycaemic factor-lowering effects of probiotics on diabetes: a meta-analysis of randomised placebo-controlled trials. - Br J Nutr, 115 (7): 1167-77.
160. Sun, L., Xie, C., Wang, G., Wu, Y., Wu, Q., Wang, X., Liu, J., Deng, Y., Xia, J., Chen, B., Zhang, S., Yun, C., Lian, G., Zhang, X., Zhang, H., Bisson, W. H., Shi, J., Gao, X., Ge, P., Liu, C., Krausz, K. W., Nichols, R. G., Cai, J., Rimal, B., Patterson, A. D., Wang, X., Gonzalez, F. J. and Jiang, C. 2018. Gut microbiota and intestinal FXR mediate the clinical benefits of metformin. - Nat Med, 24 (12): 1919-1929.
161. Sun, X., Jiao, X., Ma, Y., Liu, Y., Zhang, L., He, Y. and Chen, Y. 2016. Trimethylamine N-oxide induces inflammation and endothelial dysfunction in human umbilical vein endothelial cells via activating ROS-TXNIP-NLRP3 inflammasome. - Biochem Biophys Res Commun, 481 (1-2): 63-70.
162. Takahashi, K., Sugi, Y., Nakano, K., Tsuda, M., Kurihara, K., Hosono, A. and Kaminogawa, S. 2011. Epigenetic control of the host gene by commensal bacteria in large intestinal epithelial cells. - J Biol Chem, 286 (41): 35755-35762.
163. Tanaka, M. and Nakayama, J. 2017. Development of the gut microbiota in infancy and its impact on health in later life. - Allergol Int, 66 (4): 515-522.
164. Tang, M. S., Poles, J., Leung, J. M., Wolff, M. J., Davenport, M., Lee, S. C., Lim, Y. A., Chua, K. H., Loke, P. and Cho, I. 2015. Inferred metagenomic comparison of mucosal and fecal microbiota from individuals undergoing routine screening colonoscopy reveals similar differences observed during active inflammation. - Gut Microbes, 6 (1): 48-56.
165. Teissandier, A. and Bourc'his, D. 2017. Gene body DNA methylation conspires with H3K36me3 to preclude aberrant transcription. - EMBO J, 36 (11): 1471-1473.
166. Tiderencel, K. A., Hutcheon, D. A. and Ziegler, J. 2020. Probiotics for the treatment of type 2 diabetes: A review of randomized controlled trials. - Diabetes Metab Res Rev, 36 (1): e3213.
167. Torondel, B., Ensink, J. H., Gundogdu, O., Ijaz, U. Z., Parkhill, J., Abdelahi, F., Nguyen, V. A., Sudgen, S., Gibson, W., Walker, A. W. and Quince, C. 2016. Assessment of the influence of intrinsic environmental and geographical factors on the bacterial ecology of pit latrines. - Microb Biotechnol, 9 (2): 209-23.
168. Tripathi, A., Debelius, J., Brenner, D. A., Karin, M., Loomba, R., Schnabl, B. and Knight, R. 2018. The gut-liver axis and the intersection with the microbiome. - Nat Rev Gastroenterol Hepatol, 15 (7): 397-411.
169. Truong, D. T., Franzosa, E. A., Tickle, T. L., Scholz, M., Weingart, G., Pasolli, E., Tett, A., Huttenhower, C. and Segata, N. 2015. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. - Nat Methods, 12 (10): 902-3.
170. Udhane, S. S., Legeza, B., Marti, N., Hertig, D., Diserens, G., Nuoffer, J. M., Vermathen, P. and Fluck, C. E. 2017. Combined transcriptome and metabolome analyses of metformin effects reveal novel links between metabolic networks in steroidogenic systems. - Sci Rep, 7 (1): 8652.
171. Ustinova, M., Ansone, L., Silamikelis, I., Rovite, V., Elbere, I., Silamikele, L., Kalnina, I., Fridmanis, D., Sokolovska, J., Konrade, I., Pirags, V. and Klovins, J. 2020. Whole-blood transcriptome profiling reveals signatures of metformin and its therapeutic response. - PLoS One, 15 (8): e0237400.
172. Ustinova, M., Silamikelis, I., Kalnina, I., Ansone, L., Rovite, V., Elbere, I., Radovica-Spalvina, I., Fridmanis, D., Aladyeva, J., Konrade, I., Pirags, V. and Klovins, J. 2019. Metformin strongly affects transcriptome of peripheral blood cells in healthy individuals. - PLoS One, 14 (11): e0224835.
173. Vallianou, N. G., Stratigou, T. and Tsagarakis, S. 2019. Metformin and gut microbiota: their interactions and their impact on diabetes. - Hormones (Athens), 18 (2): 141-144.
174. Vial, G., Detaille, D. and Guigas, B. 2019. Role of Mitochondria in the Mechanism(s) of Action of Metformin. - Front Endocrinol (Lausanne), 10 294.
175. Virtue, A. T., McCright, S. J., Wright, J. M., Jimenez, M. T., Mowel, W. K., Kotzin, J. J., Joannas, L., Basavappa, M. G., Spencer, S. P., Clark, M. L., Eisennagel, S. H., Williams, A., Levy, M., Manne, S., Henrickson, S. E., Wherry, E. J., Thaiss, C. A., Elinav, E. and Henao-Mejia, J. 2019. The gut microbiota regulates white adipose tissue inflammation and obesity via a family of microRNAs. - Sci Transl Med, 11 (496):
176. Visone, R., Bacalini, M. G., Di Franco, S., Ferracin, M., Colorito, M. L., Pagotto, S., Laprovitera, N., Licastro, D., Di Marco, M., Scavo, E., Bassi, C., Saccenti, E., Nicotra, A., Grzes, M., Garagnani, P., De Laurenzi, V., Valeri, N., Mariani-Costantini, R., Negrini, M., Stassi, G. and Veronese, A. 2019. DNA methylation of shelf, shore and open sea CpG positions distinguish high microsatellite instability from low or stable microsatellite status colon cancer stem cells. - Epigenomics, 11 (6): 587-604.
177. Vivarelli, S., Salemi, R., Candido, S., Falzone, L., Santagati, M., Stefani, S., Torino, F., Banna, G. L., Tonini, G. and Libra, M. 2019. Gut Microbiota and Cancer: From Pathogenesis to Therapy. - Cancers (Basel), 11 (1):
178. Waddington, C. H. 1942. The epigenotype. - Endeavour, 1 18 - 20.
179. Wang, Q., Garrity, G. M., Tiedje, J. M. and Cole, J. R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. - Appl Environ Microbiol, 73 (16): 5261-7.

180. Wang, Q., Wang, K., Wu, W., Giannoulatou, E., Ho, J. W. K. and Li, L. 2019. Host and microbiome multi-omics integration: applications and methodologies. - Biophys Rev, 11 (1): 55-65.
181. Weinshilboum, R. M. and Wang, L. 2017. Pharmacogenomics: Precision Medicine and Drug Response. - Mayo Clin Proc, 92 (11): 1711-1722.
182. Woese, C. R. and Fox, G. E. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. - Proc Natl Acad Sci U S A, 74 (11): 5088-90.
183. Woo, V. and Alenghat, T. 2017. Host-microbiota interactions: epigenomic regulation. - Curr Opin Immunol, 44 52-60.
184. Wu, D., Hu, D., Chen, H., Shi, G., Fetahu, I. S., Wu, F., Rabidou, K., Fang, R., Tan, L., Xu, S., Liu, H., Argueta, C., Zhang, L., Mao, F., Yan, G., Chen, J., Dong, Z., Lv, R., Xu, Y., Wang, M., Ye, Y., Zhang, S., Duquette, D., Geng, S., Yin, C., Lian, C. G., Murphy, G. F., Adler, G. K., Garg, R., Lynch, L., Yang, P., Li, Y., Lan, F., Fan, J., Shi, Y. and Shi, Y. G. 2018. Glucose-regulated phosphorylation of TET2 by AMPK reveals a pathway linking diabetes to cancer. - Nature, 559 (7715): 637-641.
185. Wu, H., Esteve, E., Tremaroli, V., Khan, M. T., Caesar, R., Manneras-Holm, L., Stahlman, M., Olsson, L. M., Serino, M., Planas-Felix, M., Xifra, G., Mercader, J. M., Torrents, D., Burcelin, R., Ricart, W., Perkins, R., Fernandez-Real, J. M. and Backhed, F. 2017. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. - Nat Med, 23 (7): 850-858.
186. Xu, Z., Niu, L., Li, L. and Taylor, J. A. 2016. ENmix: a novel background correction method for Illumina HumanMethylation450 BeadChip. - Nucleic Acids Res, 44 (3): e20.
187. Zerbino, D. R., Johnson, N., Juetteman, T., Sheppard, D., Wilder, S. P., Lavidas, I., Nuhn, M., Perry, E., Raffaillac-Desfosses, Q., Sobral, D., Keefe, D., Graf, S., Ahmed, I., Kinsella, R., Pritchard, B., Brent, S., Amode, R., Parker, A., Trevanion, S., Birney, E., Dunham, I. and Flicek, P. 2016. Ensembl regulation resources. - Database (Oxford), 2016
188. Zhang, C. S., Li, M., Ma, T., Zong, Y., Cui, J., Feng, J. W., Wu, Y. Q., Lin, S. Y. and Lin, S. C. 2016. Metformin Activates AMPK through the Lysosomal Pathway. - Cell Metab, 24 (4): 521-522.
189. Zhang, Q. and Hu, N. 2020. Effects of Metformin on the Gut Microbiota in Obesity and Type 2 Diabetes Mellitus. - Diabetes Metab Syndr Obes, 13 5003-5014.
190. Zhang, T., Li, Q., Cheng, L., Buch, H. and Zhang, F. 2019. Akkermansia muciniphila is a promising probiotic. - Microb Biotechnol, 12 (6): 1109-1125.
191. Zheng, Y., Ley, S. H. and Hu, F. B. 2018. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. - Nat Rev Endocrinol, 14 (2): 88-98.
192. Zheng, J., Li, H., Zhang, X., Jiang, M., Luo, C., Lu, Z., Xu, Z. and Shi, J. 2018. Prebiotic Mannan-Oligosaccharides Augment the Hypoglycemic Effects of Metformin in Correlation with Modulating Gut Microbiota. - J Agric Food Chem, 66 (23): 5821-5831.
193. Zhong, T., Men, Y., Lu, L., Geng, T., Zhou, J., Mitsuhashi, A., Shozu, M., Maihle, N. J., Carmichael, G. G., Taylor, H. S. and Huang, Y. 2017. Metformin alters DNA methylation genome-wide via the H19/SAHH axis. - Oncogene, 36 (17): 2345-2354.
194. Zhou, K., Yee, S. W., Seiser, E. L., van Leeuwen, N., Tavendale, R., Bennett, A. J., Groves, C. J., Coleman, R. L., van der Heijden, A. A., Beulens, J. W., de Keyser, C. E., Zaharenko, L., Rotroff, D. M., Out, M., Jablonski, K. A., Chen, L., Javorsky, M., Zidzik, J., Levin, A. M., Williams, L. K., Dujic, T., Semiz, S., Kubo, M., Chien, H. C., Maeda, S., Witte, J. S., Wu, L., Tkac, I., Kooy, A., van Schaik, R. H. N., Stehouwer, C. D. A., Logie, L., MetGen, I., Investigators, D. P. P., Investigators, A., Sutherland, C., Klovins, J., Pirags, V., Hofman, A., Stricker, B. H., Motsinger-Reif, A. A., Wagner, M. J., Innocenti, F., t Hart, L. M., Holman, R. R., McCarthy, M. I., Hedderson, M. M., Palmer, C. N. A., Florez, J. C., Giacomini, K. M. and Pearson, E. R. 2016. Variation in the glucose transporter gene SLC2A2 is associated with glycemic response to metformin. - Nat Genet, 48 (9): 1055-1059.
195. Zou, Y., Xue, W., Luo, G., Deng, Z., Qin, P., Guo, R., Sun, H., Xia, Y., Liang, S., Dai, Y., Wan, D., Jiang, R., Su, L., Feng, Q., Jie, Z., Guo, T., Xia, Z., Liu, C., Yu, J., Lin, Y., Tang, S., Huo, G., Xu, X., Hou, Y., Liu, X., Wang, J., Yang, H., Kristiansen, K., Li, J., Jia, H. and Xiao, L. 2019. 1,520 reference genomes from cultivated human gut bacteria enable functional microbiome analyses. - Nat Biotechnol, 37 (2): 179-185.

APPENDICES

Appendix 1. List of inclusion/exclusion criteria of the studied cohorts

1. List of inclusion/exclusion criteria for the cohort oh healthy individuals

1.1. Inclusion criteria:

a) A healthy individual:

(1) With no known illnesses at the time of application to this study that could affect the result of the study;

(2) Whose body characteristics are within the healthy reference interval (e.g., BMI range is 18.5 – 29.9);

(3) Whose mental state allows him to understand the research process, and give a legal consent for the participation in it;

(4) Whose physical state allows complying with the needs of the study protocol.

b) Age: 18 – 64 years;

c) European descent;

d) Both males and females with reproductive potential match the contraception requirements of the study protocol.

e) Prior to the study-related procedures, the consent of a person's participation in the clinical trial is received by submitting a signed and dated informed consent document.

1.2. Exclusion criteria:

a) Hypersensitivity to any of the components in *Metforal* 850mg;

b) Use of any medication that is not compatible with *Metforal* 850mg therapy (according to *Metforal* description);

c) Pregnancy or lactation;

d) Diagnosis of type 1 or type 2 *diabetes mellitus*, pancreatogenic diabetes, impaired glucose tolerance (evaluated by HbA_{1c} and fasting glucose levels);

e) Polycystic ovary syndrome;

f) Chronical gastrointestinal, oncological, or autoimmune diseases;

g) Renal failure or dysfunction (evaluated by glomerular filtration rate - Cockcroft-Gault formula);

h) Liver dysfunction (ALAT results are not in the reference interval) or alcoholism;

i) Acute conditions with possible effects on kidney functions (dehydration, severe infection, shock);

j) Acute or chronical diseases that could cause tissue hypoxia, (e.g., heart or breathing failure, recent myocardial infarct, shock);

k) Diarrhea during the week before the study;
l) Previous long term use of metformin;
m) Use of any of the following medications in the past two months:
    i. Antibiotics;
    ii. Pharmaceutical-grade probiotics;
    iii. Proton pump inhibitors (e.g. omeprazole, lansoprazole, pantoprazole, etc.);
    iv. Immunosuppressive drugs (methotrexate, etc.);
    v. Corticosteroids (e.g. cortisone, hydrocortisone, prednisolone, etc.);
n) Concurrently to the study, any radiologic procedures involving intravascular administration of iodinated contrast materials are intended.

2. List of inclusion/exclusion criteria for the OPTIMED cohort

2.1. Inclusion criteria

a) Newly diagnosed type 2 diabetes mellitus and initiation of oral antidiabetic therapy;
b) Previous diagnosis of type 2 diabetes mellitus and no oral antidiabetic or insulin therapy used in the previous three months;
c) Newly diagnosed patients for glycemic control for an acute on-site intensive insulin therapy up to five days, continued afterwards;
d) Patients unavailable and not optimized in drug trials;
e) Age of 18;
f) Patients meeting the diagnostic criteria for type 2 diabetes mellitus:
    i. Fasting blood glucose ≥7 mmol / l;
    ii. Blood glucose two hours after OGTT with 75 g glucose ≥11.1 mmol / l.
g) Prior to the study-related procedures, the consent of a person's participation in the clinical trial is received by submitting a signed and dated informed consent document.

2.2. Exclusion criteria:

a) Use of peroral antidiabetic therapy;
b) Use of Type 2 *diabetes mellitus* insulin therapy;
c) Pregnancy.

ALAT - alanine aminotransferase; HbA_{1c} - hemoglobin A1c; OGTT – oral glucose tolerance test.