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Changes in the gut microbiome influence the hypoglycemic effect of metformin through the altered metabolism of branched-chain and nonessential amino acids

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ABSTRACT

Aims: Although metformin has been reported to affect the gut microbiome, the mechanism has not been fully determined. We explained the potential underlying mechanisms of metformin through a multiomics approach.

Methods: An open-label and single-arm clinical trial involving 20 healthy Korean was conducted. Serum glucose and insulin concentrations were measured, and stool samples were collected to analyze the microbiome. Untargeted metabolomic profiling of plasma, urine, and stool samples was performed by GC-TOF-MS. Network analysis was applied to infer the mechanism of the hypoglycemic effect of metformin.

Results: The relative abundances of *Escherichia*, *Romboutsia*, *Intestinibacter*, and *Clostridium* were changed by metformin treatment. Additionally, the relative abundances of metabolites, including carbohydrates, amino acids, and fatty acids, were changed. These changes were correlated with energy metabolism, gluconeogenesis, and branched-chain amino acid metabolism, which are major metabolic pathways related to the hypoglycemic effect.

Conclusions: We observed that specific changes in metabolites may affect hypoglycemic effects through both pathways related to AMPK activation and microbial changes. Energy metabolism was mainly related to hypoglycemic effects. In particular, branched-chain amino acid metabolism and gluconeogenesis were related to microbial metabolites. Our results will help uncover the potential underlying mechanisms of metformin through AMPK and the microbiome.

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1. Introduction

Metformin is the most widely used antidiabetic drug for treating individuals with type 2 diabetes (T2D) and is recommended as a first-line therapy because of its distinct hypoglycemic effect, relative safety, and low cost [1,2]. Metformin is known to increase glucose utilization and decrease gluconeogenesis through the activation of AMP-mediated protein kinase (AMPK) in the liver by entering hepatocytes through organic cation transporter 1 (OCT1) [3,4]. As a result of AMPK activation by metformin, the activity of enzymes involved in the biosynthesis of fatty acids is reduced, and glucose production is inhibited [4].

Metformin is an orally administered drug that is absorbed in the small intestine. The absolute oral bioavailability of metformin is approximately 40–60% [5]. In addition, the concentration of metformin in the human intestine is typically 30–300 times higher than that in plasma [6], and a study using [¹⁴C]metformin positron emission tomography (PET) showed that the concentration of orally administered metformin was high in the intestines [3]. A previous study showed that metformin administered intravenously to rats and humans had fewer hypoglycemic effects than that administered orally [7,8]. Thus, the possibility cannot be excluded that the human intestine is a crucial organ involved in the effect of metformin to improve hyperglycemia.

The intestines play a number of roles in regulating blood glucose levels, such as secreting glucagon-like peptide 1 and peptide YY, regulating bile acid metabolism, and affecting the growth and composition of the gut microbiome [9,10]. Some studies have shown that metformin can change the gut microbial composition [1,2,11] and have suggested that the microbiota contributes to the hypoglycemic effect of metformin. Nevertheless, the mechanism of metformin's effect on the gut microbiome has not been fully determined.

To explain the underlying potential mechanism of the hypoglycemic effect, we applied a global metabolomic approach as a tool for biomarker discovery through the use of biofluids, cells, and tissues. The global metabolomic approach has made it possible to understand the systemic effects of metabolites, thereby affording new insight into their possible underlying mechanisms under various physiological conditions and in various diseases [12]. In this study, we conducted a clinical study in healthy individuals to determine the hypoglycemic effect of metformin and attempted to demonstrate the underlying potential mechanism of the effect through metagenomic and global metabolomic approaches.

2. Methods

2.1. Subjects

This study was performed according to Korean Good Clinical Practices (KGCPs) and the ethical guidelines of the Declaration of Helsinki. The study was conducted after receiving approval from the institutional review board of Seoul National University Bundang Hospital (B-1809-492-003) and the Korean

Ministry of Food and Drug Safety (ClinicalTrials.gov Identifier: NCT03809260).

We recruited and included 20 healthy adult male subjects who were 19–33 years old and had a body mass index (BMI) of 23.66 ± 2.72 kg/m² (data are mean \pm s.d.) during the screening visit. Subjects with active or a history of clinically significant diseases of the kidney or the digestive, nervous, endocrine, or immune systems were excluded from the study. In addition, subjects with a history of gastrointestinal disorders or surgery that could affect the absorption of metformin were also excluded. Subjects with defecation less than five times a week or more than three times a day or who had excessively hard or soft stools were excluded from the study. Subjects whose blood aspartate aminotransferase (AST) and alanine aminotransferase (ALT) values exceeded 1.5 times the upper limit of the normal range during the screening visit or whose estimated glomerular filtration rate (eGFR) calculated by the Modification of Diet in Renal Disease (MDRD) was less than 80 mL/min/1.73 m² were also excluded.

2.2. Study design

This was an open-label, single-arm study (Supplementary Fig. 1). The subjects received the first dose of 500 mg of oral metformin on day 1 at 9 a.m. for the safety of the subjects, and then they received 1000 mg twice daily from day 1 (1 d, 1:30 p.m.) to day 4 (4 d) in the morning. Plasma samples for the pharmacodynamic evaluation of metformin were collected before the first metformin dose (baseline) and on day 4 after the last metformin dose (postmetformin). Stool samples for metagenomics were collected on the morning of day 1 before the first metformin dose and on day 4 after the last metformin dose. The sample used for analysis was from the first stool in the morning. Urine samples were collected on day –1 and day 4. Additionally, plasma, stool, and urine samples were used for untargeted metabolomic analysis.

We provided a normal diet, not a high-fat, high-fiber diet, that met the recommended daily caloric intake of approximately 2700 kcal for adult men, and we limited the intake of foods containing lactic acid bacteria, grapefruit, and caffeine. In addition, we asked the participants to eat the full meal during hospitalization, and any meals other than the provided meals were prohibited.

2.3. Pharmacodynamic (PD) assessments of metformin

For pharmacodynamic (PD) evaluation of metformin, an oral glucose tolerance test (OGTT) was performed, and the serum insulin concentration was measured at baseline (before the first dose of metformin) and postmetformin (2 h after the last dose of metformin) (Supplementary Fig. 1). In brief, a solution containing 75 g glucose was administered to the subjects on an empty stomach, and samples for determining the serum glucose concentration were collected at 0 (before administration of the solution containing 75 g glucose), 0.25, 0.5, 0.75, 1, 1.5, and 2 h. The serum insulin concentration was measured only at 0 h (before administration of the solution containing 75 g glucose).

To evaluate glucose parameters, the maximum serum glucose concentration (G_{\max}) was presented as the actual observed value, and the area under the glucose curve (AUGC) was calculated by the linear-linear trapezoidal method. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as (glucose \cdot insulin)/405.

The baseline corrected PD parameters, including ΔG_{\max} , Δ AUGC, and Δ HOMA-IR, after the last metformin administration were defined by subtracting the baseline values from the postmetformin values (i.e., G_{\max} at postmetformin – G_{\max} at baseline). Smaller Δ AUGC, ΔG_{\max} , and Δ HOMA-IR values, i.e., larger absolute values of the parameters, were interpreted as stronger effects of metformin treatment. For comparison of the PD parameters, we confirmed whether the data had a normal distribution through a normality test. Then, the paired *t* test was used for G_{\max} and AUGC, and the Wilcoxon signed rank test was used for HOMA-IR, with significance determined at the level of 0.05. Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).

2.4. Analysis of the gut microbiome

Stool samples were collected from all the recruited subjects for metagenomic sequencing. The samples were mixed using a 3 M sample mixer, dispensed into Eppendorf tubes and frozen at -70°C until analysis.

DNA was extracted from the stool samples using the PowerSoil® DNA Isolation Kit, and amplification of the 16S rRNA gene was conducted using the 16S V3-V4 primers. Normalization and pooling of the final product were performed using PicoGreen. The size of the libraries was verified using TapeStation DNA ScreenTape D1000 (Agilent), and sequencing was performed using the MiSeq™ platform (Illumina, San Diego, USA) [13]. Taxonomic profiling was performed using a module of MicrobiomeAnalyst for marker data profiling [14].

The alpha diversity (within-sample diversity) is presented as the Shannon index, and the Kruskal-Wallis test was performed for comparisons between periods. The beta diversity (between-sample diversity) is presented on a principal coordinate analysis (PCoA) plot, and Bray-Curtis dissimilarity was evaluated by permutational multivariate analysis of variance (PERMANOVA). Significantly different genera between periods were identified by linear discriminant analysis (LDA) effect size (LEfSe) analysis, and the data were subjected to total sum normalization. This treatment yielded a relative proportional value for each feature by dividing each count of each feature by the size of the total library, which eliminated bias related to different sequencing depths. The cutoffs for the false discovery rate (FDR)-adjusted *p*-value and log LDA scores were 0.05 and 2.0, respectively. The change induced in the gut microbiome by metformin administration was identified through comparison between baseline and postmetformin periods.

2.5. Chemicals

The fatty acid methyl ester mixture (FAME) used for the relative retention time index and the authentic standards used

for the identification of significant metabolic markers were purchased from Sigma-Aldrich (St. Louis, MO, USA). The extraction solvents used for sample preparation, such as isopropanol, acetonitrile, and water (HPLC grade), were obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Pyridine, methoxyamine hydrochloride (MeOX), and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) were used for derivatization and purchased from Sigma-Aldrich.

2.6. Sample preparation for untargeted metabolomics

All the samples were prepared using a protocol from a previous study with minor modifications [15]. Frozen plasma, urine, and stool samples were thawed on ice, and quality control (QC) samples, made by pooling equal volumes (100 μL of the 1st extracted solution) of each sample, were used to validate the stability of the analytical performance and perform data filtering. For preparation of the plasma and urine samples, a 50 μL sample was extracted using 1 mL of N_2 -degassed 1st extraction solution (3:3:2, acetonitrile:isopropanol: H_2O). For preparation of the stool sample, the 1st extraction solution was spiked into the stool sample at a sample mass to solution volume ratio of 50 mg of stool sample to 1 mL of the 1st extraction solution. Then, the samples were mixed for 15 min and centrifuged for 10 min at 18,945 RCF and 4°C . Four hundred microliters of the supernatant was dried using a SpeedVac for 6 h at 45°C and 5.1 vacuum pressure. The dried samples were re-extracted with 400 μL of N_2 -degassed 2nd extraction solution (1:1, acetonitrile: H_2O). Then, the extracted samples were redried using a SpeedVac for 8 h under the same conditions used in the first extraction step. The dried samples were derivatized with methoxyamine (20 mg/mL in pyridine) at 30°C for 90 min and subsequently trimethylsilylated with a mixture of fatty acid methyl ester (used for the retention time index) in *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide at 70°C for 45 min. Finally, 1 μL of the prepared samples was split-injected into an Agilent 7890 series gas chromatography system (Agilent, Santa Clara, CA) coupled to a time-of-flight mass spectrometer (LecoCorp., St. Joseph, MI, USA) (GC-TOFMS) for untargeted metabolomics analysis.

2.7. Metabolomic data analysis

Chroma TOF version 4.72 (LECO Corporation, MI, USA) was used for peak extraction, peak alignment, peak deconvolution, and peak identification. Data processing and multivariate analysis were performed using MetaboAnalyst 4.0 [16]. Detected metabolic features with greater than 50% missing values were removed, and then, the metabolic features were filtered out according to a relative standard deviation of greater than 30% in the QC samples. The filtered metabolic features were normalized by sum, and Pareto scaling was applied for multivariate analysis. The metabolic markers were selected using a *t* test with a *p*-value cutoff value of less than 0.05. Pearson correlation and Spearman correlation analyses were performed after the normality test. Statistical analysis and correlation analysis were performed in GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).

2.8. Identification of metabolic markers

For metabolic marker identification, the online HMDB database (<https://hmdb.ca/>) and three commercially available libraries (NIST, LECO-Fiehn Rtx5, and Wiley 9) were used. After matching the mass spectra of the markers with the libraries, authentic standards were analyzed to compare the spectra. Then, the retention times of the markers and the standards were compared by calculating the relative retention index [15]. A network diagram was generated by using MetaMapp [17] and Cytoscape (version 3.5) [18].

2.9. Correlation analysis

Pearson correlation and Spearman correlation analyses were performed after the normality test. Spearman correlation analysis was performed between the relative abundance of the microbiome and metabolic markers and between the abundance of the microbiome and PD parameters (ΔG_{\max} , $\Delta AUGC$, and $\Delta HOMA-IR$). Pearson correlation analysis was performed between the relative abundance of metabolic markers and PD parameters. Correlation analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA), and the p-value cutoff was 0.05. The absolute values of the PD parameters were used in the correlation analysis.

3. Results

3.1. Glucose parameters and PD parameters represented the hypoglycemic effect of metformin

We evaluated the PD effects of metformin according to glucose parameters, such as the values of G_{\max} , AUGC, and HOMA-IR. The serum glucose concentrations after the OGTT at baseline and postmetformin periods are presented in [Supplementary Fig. 2](#). The mean \pm SD values of G_{\max} were 169.1 ± 19.68 mg/dL at baseline and 138.1 ± 15.43 mg/dL during the postmetformin period. The AUGC values of the baseline and postmetformin periods were 287.2 ± 36.29 h·mg/dL and 235.8 ± 26.17 h·mg/dL, respectively. The G_{\max} and AUGC values were significantly decreased after metformin administration. However, the HOMA-IR value did not show a statistically significant change (p-value = $2.71E-06$ for G_{\max} ; $4.74E-06$ for AUGC; 0.0539 for HOMA-IR) ([Supplementary Table 1](#)). The absolute values of the PD parameters, including ΔG_{\max} , $\Delta AUGC$, and $\Delta HOMA-IR$, are listed in [Supplementary Table 1](#).

3.2. Administration of metformin changed the composition of the gut microbiome

We analyzed the difference in the relative abundance of the gut microbiome at the genus level between baseline (before metformin administration) and postmetformin (after the last metformin administration). The relative abundances were changed after administration of metformin ([Fig. 1a](#)). The alpha diversity, which represented the bacterial species diversity in the samples, was significantly increased in the postmetformin period (Kruskal-Wallis test, p-value = 0.043

([Fig. 1b](#)). A PCoA plot showed significantly higher beta diversity at the postmetformin period than at baseline (PERMANOVA, p-value < 0.004), indicating a more heterogeneous species composition at the postmetformin period than at baseline ([Fig. 1c](#)). LEfSe analysis, for determining both statistical and biological relevance, was used for microbial biomarker discovery (FDR-adjusted p-value < 0.05, linear discriminant analysis (LDA) score greater than 2.0). As a result, four bacterial genera were significantly changed between the two periods. The relative abundances of *Intestiniibacter*, *Clostridium*, and *Romboutsia* tended to decrease in the postmetformin period compared to those at baseline, whereas the abundance of *Escherichia* tended to increase in the postmetformin period ([Fig. 1d](#), [Supplementary Table 2](#)).

3.3. Hypoglycemic effect of metformin was related to gut microbial changes

To confirm whether the hypoglycemic effect of metformin was correlated with the microbiome, we investigated the correlation between the PD parameters of metformin and the microbiome ([Supplementary Fig. 3](#)). We found that *Firmicutes* was negatively correlated with the ΔG_{\max} and $\Delta AUGC$ values. Conversely, *Proteobacteria* was positively correlated with the parameters ([Supplementary Fig. 3a](#)). Furthermore, we analyzed correlations at the genus level and identified that the ΔG_{\max} and $\Delta AUGC$ values were positively correlated with *Escherichia*. However, the PD parameters were negatively correlated with *Intestiniibacter*, *Clostridium*, and *Romboutsia* ([Supplementary Fig. 3b](#)).

3.4. Administration of metformin altered urine, plasma, and stool metabolites

We next assessed metabolites that changed after administration of metformin as key drivers between the hypoglycemic effect and microbiome. Untargeted metabolomic profiling of urine, stool, and plasma samples was performed to identify differential metabolites between baseline and postmetformin periods. A total of 1255, 1463, and 796 metabolic features were detected in urine, stool, and plasma samples, respectively, and the data were processed using MetaboAnalyst 4.0 [16]. After data processing, 170 urine, 482 stool, and 411 plasma metabolic features were used for further analysis. The reliability of the analytical performance and the quality of the data were validated by using QC samples that were tightly clustered in a plot of the principal component analysis (PCA) score derived from the urine, stool, and plasma metabolomes ([Supplementary Fig. 4](#)). We identified 25 urine, 10 stool, and 4 plasma metabolites that significantly changed in relative abundance between the two periods ([Fig. 2](#)), and of these metabolites, 21 urine, 2 stool, and 3 plasma metabolites were increased during the postmetformin period. All urinary and plasma amino acids were increased during the postmetformin period. Most urinary carbohydrates and stool carbohydrates were decreased ([Supplementary Table 3](#)). Fatty acids, such as palmitoleic acid, a common metabolite in plasma and stool samples, were decreased during the postmetformin period. Of the significantly changed metabolites, we identified 13 urine, 4 stool, and 1 plasma microbial metabolite using the

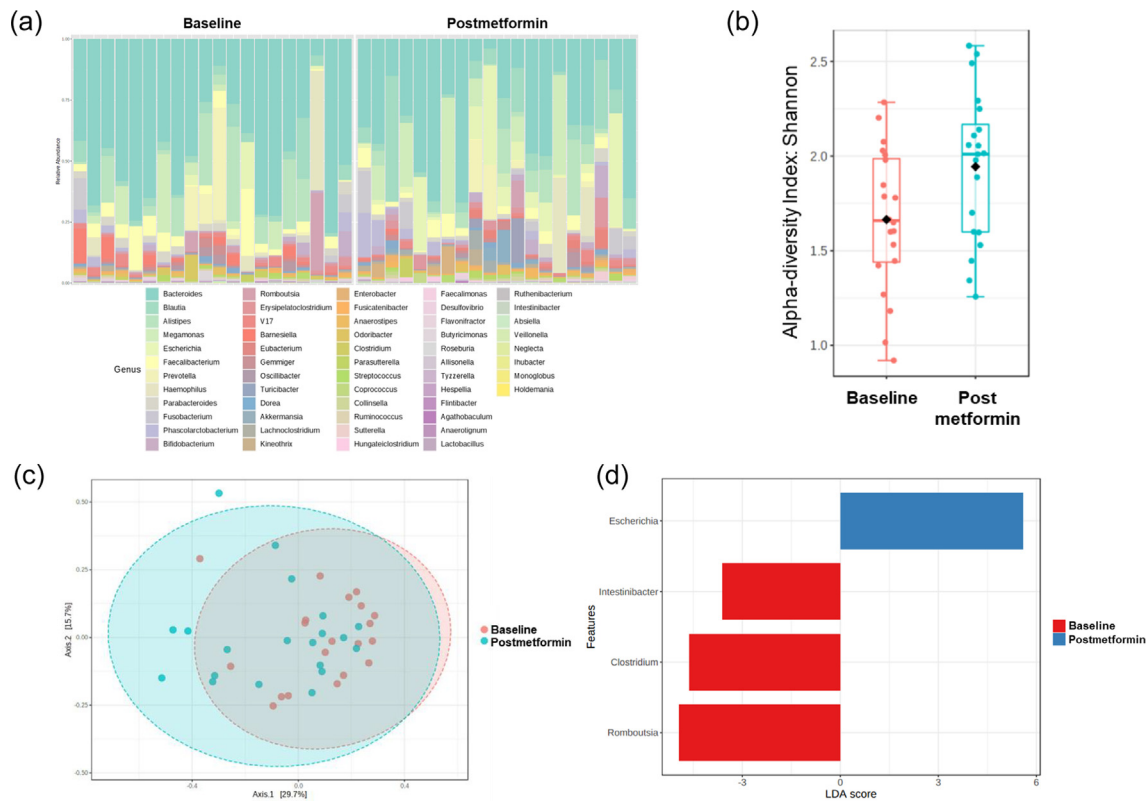


Fig. 1 – Metformin treatment promotes changes in the composition of the gut microbiota. (a) Relative abundances of recognized bacterial genera observed in the targeted metagenomics sequence data between baseline and postmetformin. (b) Alpha and beta diversity plots to visualize the difference in the microbiota structure between the baseline and postmetformin periods. Alpha diversity measures with the most common indices, and (c) PCoA plots showing beta diversity, which represents the dissimilarity of samples or groups. (d) Bacteria differentially represented between baseline and postmetformin identified by linear discriminant analysis coupled with effect size (LEfSe). LEfSe analysis, which emphasizes both statistical and biological relevance, was used for microbial biomarker discovery (FDR adjusted p-value < 0.05, LDA score greater than 2.0). Histogram showing the genera of bacteria that were more abundant at baseline (red color) or postmetformin (blue color) ranked by the linear discriminant analysis (LDA) score. The LDA score indicates the effect size and ranking of each differentially abundant genus. Orange: baseline (control), Blue: postmetformin. Box plots showing medians as well as the lower and upper quartiles. Each dot represents an individual sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

method of identification. Most microbial metabolites were decreased during the postmetformin period, as listed in Supplementary Table 3.

3.5. Changes in carbohydrates, branched-chain amino acids, and fatty acids were correlated with gut microbiota

To investigate functional changes in the gut microbiome using metabolites as mediators, we performed correlation analysis between gut microbiota and metabolites whose relative abundance significantly changed during the postmetformin period. We found that carbohydrates, amino acids, hydroxy acids, and fatty acids were correlated with the microbiome (Fig. 3). Among the correlations of urinary metabolites and microbiota, *Intestinibacter* was positively correlated with carbohydrates. *Intestinibacter* and *Clostridium* were positively correlated with amino acids (Fig. 3a). D-galactose, glyceric acid, glycolic acid, and 2,4-dihydroxybutyric acid were positively correlated with *Intestinibacter*, *Clostridium*, and *Rombout-*

sia. In contrast, hypoxanthine and hippuric acid were negatively correlated with *Intestinibacter*, *Clostridium*, and *Romboutsia*. In the correlation of stool metabolites and microbiota, essential amino acids such as phenylalanine and L-isoleucine were negatively correlated with *Intestinibacter*, *Clostridium*, and *Romboutsia*. The three genera were positively correlated with carbohydrates, fatty acids, hydroxy acids, and L-serine, which are classified as nonessential amino acids (Fig. 3b). For plasma markers, amino acids were positively correlated with the genus, and palmitoleic acid was positively correlated with *Escherichia* (Fig. 3c).

3.6. Changed metabolites showed correlations with the hypoglycemic effect

We performed a correlation analysis to determine whether changes in the PD parameters of metformin correlated with changes in metabolites (Fig. 4). The PD parameters were positively correlated with urinary metabolites except for β -

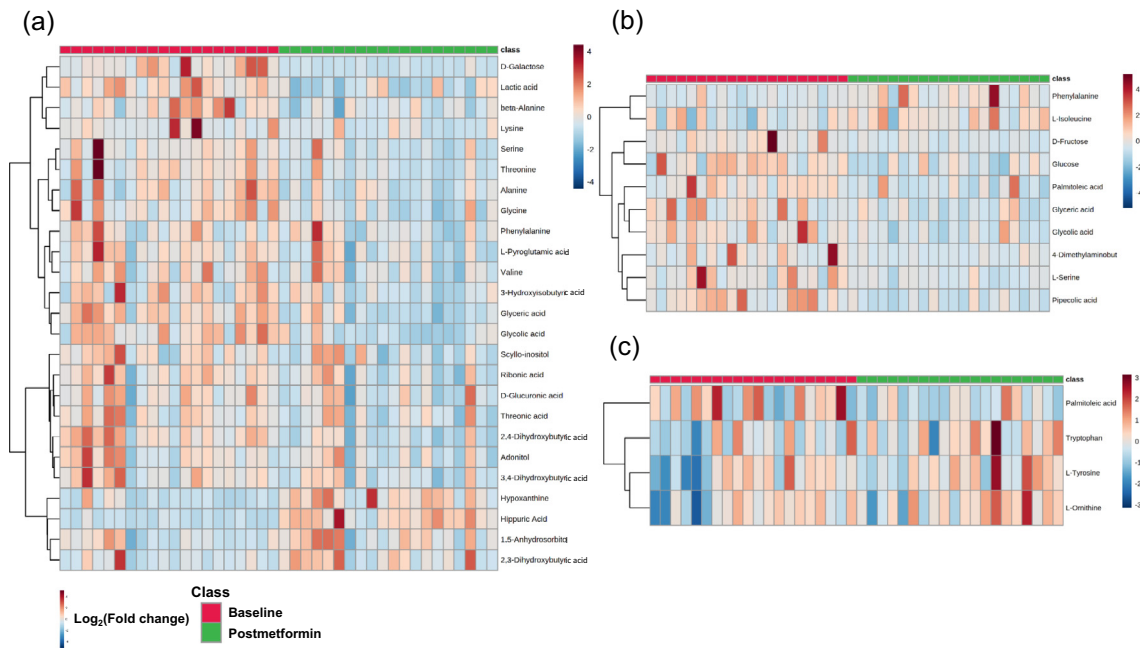


Fig. 2 – Heatmap representing significantly different metabolites between the baseline and postmetformin periods. Heatmap visualization of (a) urinary metabolites, (b) fecal metabolites, and (c) plasma metabolites. Metabolites were selected by p-value < 0.05. Each colored cell represents a log₂-fold change of metabolite, and the comparison type of fold change is postmetformin/baseline.

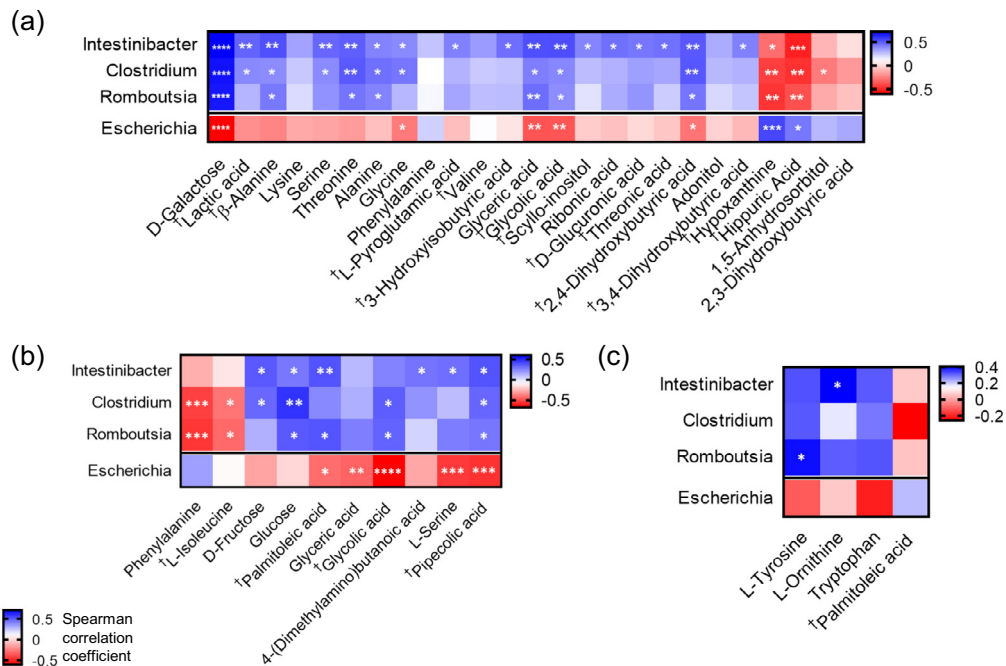


Fig. 3 – Association between microbial genus abundance and metabolite abundance. Heatmap showing the Spearman correlation coefficient between the relative abundance of individual microbial genera and the relative abundance of (a) urinary metabolites, (b) fecal metabolites, and (c) plasma metabolites. The y-axis represents the microbial genus, and the x-axis represents metabolic biomarkers. The intensity of the colors represents the degree of association between the relative abundance of individual genera and the relative abundance of metabolites as measured by Spearman’s correlations. White asterisks denote a significant correlation. †microbial metabolites. *p-value < 0.05, **p-value < 0.005, *p-value < 0.001, ****p-value < 0.0001.**

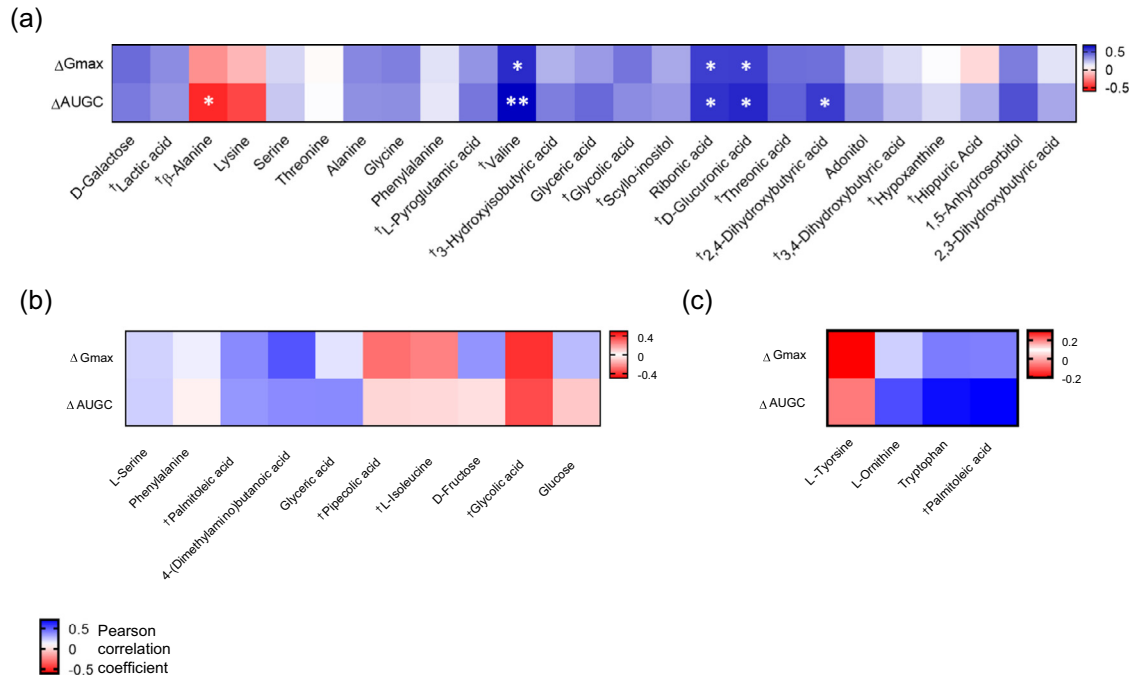


Fig. 4 – Pearson correlation analysis of the pharmacodynamic parameters of postmetformin and metabolites. Pearson correlation analysis was performed to investigate the association between pharmacodynamic parameters (ΔG_{\max} , $\Delta AUGC$) and (a) urinary metabolites, (b) fecal metabolites, and (c) plasma metabolites. The y-axis represents pharmacodynamic parameters, and the x-axis represents metabolites. Each square shows the correlation coefficient value. Blue squares indicate a positive correlation, and red squares indicate a negative correlation between the pharmacodynamic parameters and metabolite abundances. White asterisks denote a significant correlation. †microbial metabolites. *p-value < 0.05, **p-value < 0.005. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

alanine and lysine (Fig. 4a). Amino acids of stool metabolic markers were weakly positively correlated with the PD parameters, and carbohydrates were negatively correlated with the PD parameters (Fig. 4b). Plasma metabolic markers were positively correlated with the PD parameters except for L-tyrosine (Fig. 4c).

3.7. Changed metabolites during the postmetformin period were involved in gluconeogenesis, amino acid metabolism, and carbohydrate metabolism

We found that changed metabolites were correlated with microbiome and hypoglycemic effects. Thus, we performed a metabolite set enrichment analysis to explore the potential pathways, including the metabolites (Supplementary Fig. 5). As a result, numerous pathways, including those involved in gluconeogenesis and amino acid and carbohydrate metabolism, were affected by metformin. Moreover, we conducted pathway mapping analysis to identify correlated networks between metabolites. In the network of urinary metabolites, carbohydrate metabolism and serine-glycine metabolism were affected by administration of metformin (Fig. 5(a)). In addition, microbial metabolites were included in branched-chain amino acid metabolism and gluconeogenesis. These networks were related to the microbiota (Fig. 5(b)).

3.8. Safety

Safety was evaluated in the 20 subjects administered metformin at least once. There were 15 adverse events (AEs) after administration of metformin. Of these AEs, 10 were gastrointestinal disorders. One case of diarrhea and one case of vomiting were evaluated as moderate AEs, and one case of vomiting was evaluated as a severe AE. All other AEs were mild.

4. Discussion

In this study, we investigated the effects of changes in microbial composition and metabolites on the hypoglycemic effect associated with metformin administration by using plasma, urine, and stool samples from healthy adult males through multiomics, including metagenomic and metabolomic approaches. The subjects taking metformin showed significant decreases in G_{\max} and AUGC values, with a nonsignificant but trending decrease in the value of HOMA-IR. These results were supported by studies of metformin treatment in T2D patients unable to control blood glucose due to insulin resistance, preferentially regulating hepatic glucose output by inhibiting gluconeogenesis rather than controlling insulin levels [19,20].

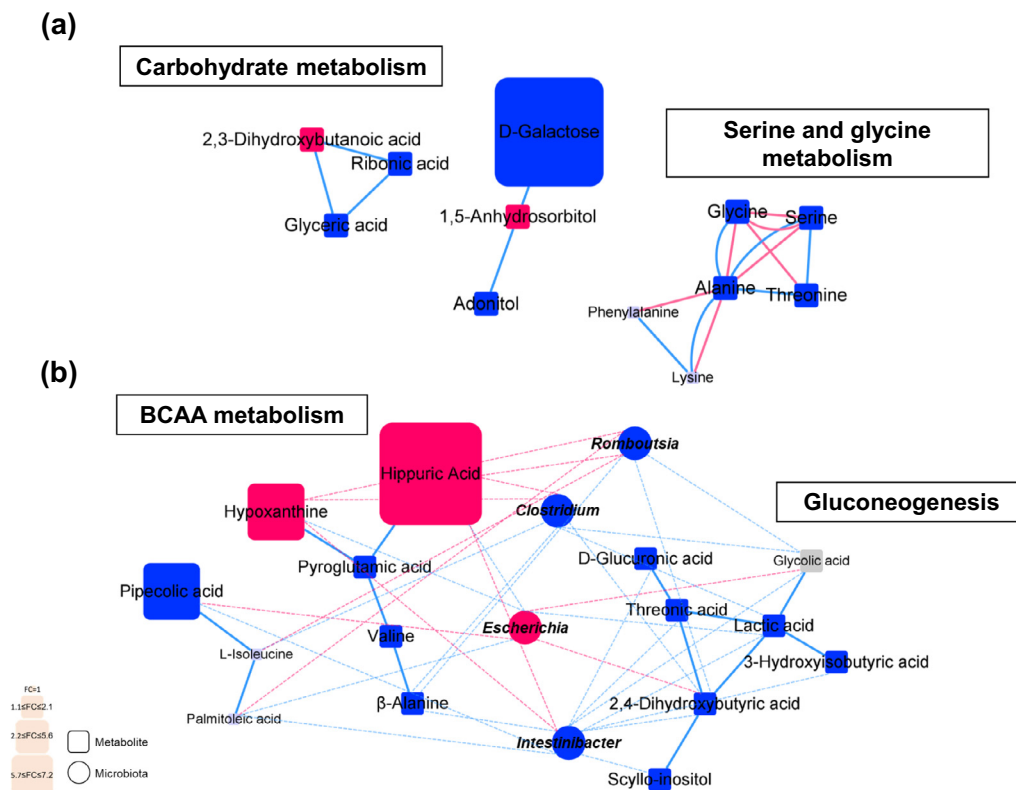


Fig. 5 – Metabolic correlation network diagram. (a) A fully connected network of metabolites detected in the urine samples. (b) Urinary and stool microbial metabolite correlation network diagram. The metabolites and microbiome are shown in color: red represents an increase, blue represents a decrease in the postmetformin period, and gray represents no changes. The size of the nodes represents the fold change of metabolites. The blue solid line represents chemical relationships, and the red solid line represents biochemical relationships. The red dashed line denotes a negative correlation, and the blue dashed line denotes a positive correlation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In addition, by performing 16S rRNA sequencing of stool samples, we examined the changes in microbial composition after multiple administrations of metformin. We observed changes in various microbiome, including *Akkermansia*, *Escherichia*, *Intestinibacter*, *Clostridium*, and *Romboutsia*, a finding that is in agreement with results reported in previous studies [1,11,21]. The changes in *Akkermansia* were observed after taking metformin but were not significant. The change in the relative abundance of *Escherichia* is assumed to be indirectly affected by bacteria-bacteria interactions or other physiological alterations [1]. The growth of *Firmicutes*, including *Intestinibacter*, *Clostridium*, and *Romboutsia*, was impeded by metformin [11,21]. In addition, a decrease in *Firmicutes* has been reported to decrease insulin resistance and other factors that lead to the development of T2D [22].

To investigate whether the changed gut microbiota is correlated with the hypoglycemic effect of metformin, we performed correlation analysis. As a result, *Escherichia* was positively correlated with the hypoglycemic effect. *Escherichia* could use glucose as a carbon source in the β -alanine pathway [23], and the increase in *Escherichia* was found to be related to improved glucose homeostasis by the regulation of metabolism, such as carbon uptake, catabolism, and energy and redox production [11,24]. In fact, rats that underwent Roux-en-Y gastric bypass (RYGB) surgery to treat obesity had

increased *Escherichia* and decreased glucose levels. Similarly, mice that underwent ileal interposition (IT) surgery to treat T2D had the same results [25,26]. Therefore, an increase in the relative abundance of *Escherichia* after administration of metformin may contribute to improving the hyperglycemic effect. In contrast, *Intestinibacter*, *Clostridium*, and *Romboutsia*, belonging to *Firmicutes*, were negatively related to the hypoglycemic effect. *Firmicutes* can generate surplus energy from carbohydrates by fermenting unabsorbed carbohydrates, and the accumulation of surplus energy can cause obesity and T2D [27]. In an animal study, *Firmicutes* were increased in *ob/ob* mice, and body fat mass and energy harvesting ability were increased in germ-free mice transplanted with *ob/ob* microbiomes, such as *Firmicutes* [28]. Thus, hyperglycemia may be improved as the relative abundance of *Firmicutes* decreases after administration of metformin.

We used global metabolomic approaches to explore the underlying pathway of the hypoglycemic effect. Global metabolomic analysis showed changes in metabolic signatures, including amino acid, carbohydrate, and fatty acid metabolism. In particular, amino acids, hippuric acid, glyceric acid, galactose, and palmitoleic acid were largely changed after administration of metformin. Hippuric acid, largely increased after administration of metformin, is a normal urinary component derived from the degradation of phenols and aromatic

amino acids by microbiota belonging to *Clostridium* sp. [29]. The level of hippuric acid, a metabolite derived from the gut microbiome, decreased in obese patients and increased approximately 30-fold in patients who underwent RYGB surgery [30] and was also associated with impaired glucose tolerance [31]. Additionally, the decrease was reduced in T2D patients after treatment with antidiabetic drugs, which is correlated with a protective effect on gut microbiota metabolism [32]. The intermediates of energy metabolism were decreased after administration of metformin (Supplementary Fig. 6). In previous studies, urinary TCA intermediates were elevated in *db/db* mice [33], and myo-inositol, the bioconverted form of scyllo-inositol, is known to be elevated in T2D patients [34]. The high level of myo-inositol is due to competitive inhibition with glucose in renal tubular transport, and the level was decreased after administration of antidiabetic drugs. Thus, both the levels of myo-inositol and scyllo-inositol are expected to be good indicators of T2D treatment by antidiabetic drugs. Fatty acids, including palmitoleic acid, were increased in gestational diabetes mellitus patients. In particular, palmitoleic acid, produced by desaturation of palmitic acid, promotes gluconeogenesis [35]. In our study, the changes in these metabolites may affect the hypoglycemic effect of metformin.

In terms of the comprehensive metabolic effects on hypoglycemia, amino acid metabolism, fatty acid β -oxidation, and BCAA metabolism were important metabolic pathways (Fig. 5, Supplementary Fig. 6). First, amino acid metabolism, such as serine-glycine metabolism, influences signaling associated with obesity and insulin resistance [36]. In particular, mammalian target of rapamycin complex (mTORC), which has been implicated in specific human pathologies, including obesity, T2D, and cancer, is affected by amino acid metabolism [37,38]. Metformin inhibits mTORC1, which reduces ATP production and activates AMPK [39], but amino acids stimulate mTORC1 signaling by activating a family of GTPases [40]. Therefore, we presumed that a decrease in amino acids could affect mTORC1 signaling, thereby lowering blood glucose levels through the regulation of AMPK.

Moreover, AMPK activation can also affect fatty acid β -oxidation [41]. Fatty acid β -oxidation, the first step of fatty acid catabolism, is an energy production process. In T2D patients, fatty acid β -oxidation was decreased and associated with insulin resistance by impaired β -cell function [42]. Under this condition, more fatty acids were metabolized to more diacylglycerols, which inhibited the interaction between insulin and glucose transporter type 4 (GLUT4) [43]. Peroxisome proliferator-activated receptor-gamma (PPAR- γ) is a nuclear receptor that regulates fatty acid metabolism and glucose metabolism [44], and it is known to be activated by metformin [45]. The activation of AMPK by metformin induces PPAR- γ activity, which plays an important role in the transcriptional control of mitochondrial fatty acid β -oxidation by upregulating the expression of genes involved in fatty acid β -oxidation [41,45].

BCAA metabolism could be affected by *Escherichia*, which contributes to an increase in BCAA biosynthesis and a reduction in BCAA transport into bacterial cells [46]. However, the role of BCAA is still controversial. On the one hand, insulin resistance is related to increased levels of BCAAs [46], but

on the other hand, glucose homeostasis and insulin sensitivity are improved in mice fed a diet enriched in leucine [47]. We confirmed that the hypoglycemic effect is influenced by BCAAs and *Escherichia*.

In the present study, we used paired samples to reduce the effect of interindividual variations, a common issue in previous studies exploring the effect of metformin on the human gut microbiome [1,11,48,49]. Additionally, to reduce the effect of dietary intake on the human gut microbiome, the subjects were hospitalized and fed the same diet prior to starting metformin administration. Thus, the design of our study enabled us to decrease the effect of confounding factors that have an impact on the human gut microbiome. In addition, this study, for the first time, revealed the underlying pathway of the hypoglycemic effect of metformin through metagenomic and metabolomic approaches utilizing plasma, urine, and stool samples from healthy subjects. However, this study has some limitations. First, the proposed pathway of the hypoglycemic effect has not been substantiated by further mechanistic studies and has been presented only as a correlation. Therefore, the results of this study need to be verified through additional mechanistic studies. Second, we conducted a clinical trial in Korean adults who were provided a normal diet, not a high-fat/high-fiber diet, to reduce the effect of diet on the results. Nevertheless, the human gut microbiota is affected by genetic and environmental factors, including diet, medications, and stress [50], and varies across different ethnic groups. Thus, further evaluation is needed in different ethnic groups with different diets.

5. Conclusion

Our study indicates that specific changes in metabolites may affect the hypoglycemic effect through both signaling related to AMPK activation and pathways correlated to the microbiome. In particular, amino acid metabolism and energy metabolism were related mainly to hypoglycemic effects, and among them, BCAA metabolism and gluconeogenesis were associated with microbial changes. However, further studies combining metabolomics and metagenomics are essential to identify the effects of the gut microbiome and metabolites on metformin. Future studies will be of great help in elucidating the mechanism of metformin.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Author contributions to manuscript

Eunwoo Kim, Andrew Hyounjin Kim, Seunghwan Lee, Kyung-Sang Yu, In-Jin Jang, Jae-Yong Chung, and Joo-Youn Cho designed the research; Yujin Lee, Andrew Hyounjin

Kim, and Eunwoo Kim performed the research; Yujin Lee and Eunwoo Kim analyzed the data; Yujin Lee wrote the manuscript; and Eunwoo Kim, Andrew Hyoungjin Kim, SeungHwan Lee, Kyung-Sang Yu, In-Jin Jang, Jae-Yong Chung, and Joo-Youn Cho revised the manuscript.

Availability of data and materials

The metabolomics data are available in the electronic [Supplementary Material](#) and at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website [Project ID: PR001107]. The 16S rRNA gene sequences were submitted to GenBank under the accession numbers MW768153-MW768700.

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Ethics approval and consent to participate

The human study was approved by the institutional review board of Seoul National University Bundang Hospital (B-1809-492-003) and Korea Ministry of Food and Drug Safety (ClinicalTrials.gov Identifier: NCT03809260).

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.diabres.2021.108985>.

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