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Metformin-induced changes in the gut microbiome and plasma metabolome are associated with cognition in men

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ABSTRACT

Background: An altered gut microbiome characterized by reduced abundance of butyrate producing bacteria and reduced gene richness is associated with type 2 diabetes (T2D). An important complication of T2D is increased risk of cognitive impairment and dementia. The biguanide metformin is a commonly prescribed medication for the control of T2D and metformin treatment has been associated with a significant reduction in the risk of dementia and improved cognition, particularly in people with T2D.

Aim: To investigate the associations of metformin use with cognition exploring potential mechanisms by analyzing the gut microbiome and plasma metabolome using shotgun metagenomics and HPLC-ESI-MS/MS, respectively.

Abbreviations: BMI, Body mass index; MRI, Magnetic resonance imaging; PVF, Phonemic verbal fluency; SVF, Semantic verbal fluency; MEIFLO, The Aging Imageomics Study and the CAIBERHJT_MEIFLO-Metformin and Intestinal Microflora Study; MBT-TDFR, The Memory Binding Test-Total Delayed Free Recall; MBT-TFR, The Memory Binding Test-Total Free Recall; SDMT, The Symbol Digit Modalities Test; TDS, Total Digits Span; T2D, Type 2 diabetes.

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Methods: We explored two independent cohorts: an observational study (Aging Imageomics) and a phase IV, randomized, double-blind, parallel-group, randomized pilot study (MEIFLO). From the two studies, we analyzed four study groups: (1) individuals with no documented medical history or medical treatment ($n = 172$); (2) people with long-term T2D on metformin monotherapy ($n = 134$); (3) people with long-term T2D treated with oral hypoglycemic agents other than metformin ($n = 45$); (4) a newly diagnosed T2D subjects on metformin monotherapy ($n = 22$). Analyses were also performed stratifying by sex.
Results: Several bacterial species belonging to the Proteobacteria (*Escherichia coli*) and Verrucomicrobia (*Akkermansia muciniphila*) phyla were positively associated with metformin treatment, while bacterial species belonging to the Firmicutes phylum (*Romboutsia timonensis*, *Romboutsia ilealis*) were negatively associated. Due to the consistent increase in *A. muciniphila* and decrease in *R.ilealis* in people with T2D subjects treated with metformin, we investigated the association between this ratio and cognition. In the entire cohort of metformin-treated T2D subjects, the *A.muciniphila/R.ilealis* ratio was not significantly associated with cognitive test scores. However, after stratifying by sex, the *A.muciniphila/R. ilealis* ratio was significantly and positively associated with higher memory scores and improved memory in men.
Metformin treatment was associated with an enrichment of microbial pathways involved in the TCA cycle, and butanoate, arginine, and proline metabolism in both cohorts. The bacterial genes involved in arginine metabolism, especially in production of glutamate (*astA, astB, astC, astD, astE, putA*), were enriched following metformin intake. In agreement, in the metabolomics analysis, metformin treatment was strongly associated with the amino acid proline, a metabolite involved in the metabolism of glutamate.
Conclusions: The beneficial effects of metformin may be mediated by changes in the composition of the gut microbiota and microbial-host-derived co-metabolites.

1. Introduction

Over the past decade, type 2 diabetes (T2D) has been linked to an altered gut microbiota [1]. People with T2D had a decrease in the Firmicutes phylum and an increase in Bacteroidetes and Proteobacteria compared to controls [1,2]. Butyrate-producing bacteria have reduced abundance in T2D including *Faecalibacterium prausnitzii*, and *Roseburia intestinalis* [1,2]. In contrast, *Lactobacillus* spp., *Clostridium ramosum*, and *Desulfovibrio sp. 3_1_syn3* are more abundant in individuals with T2D [2]. These changes in the gut microbiota could contribute to developing insulin resistance [3], creating a vicious cycle. On the other hand, insulin resistance and elevated circulating glucose levels are associated with impairments in attention, executive function [4], and memory [5,6]. People with T2D are 60 % more likely to develop dementia [7] and a higher incidence of cognitive impairment is also associated with T2D [8].

Metformin is widely used to treat T2D [9]. According to some studies, the gut, rather than the liver, is the primary target of metformin. This is consistent with the fact that intravenous metformin has no glucose-lowering effect [10,11]. Furthermore, metformin is associated with altered fecal metagenomes in humans and with microbial-dependent anti-aging effects in *Caenorhabditis elegans* [12]. Both *Bifidobacterium adolescentis* and *Akkermansia muciniphila* showed increased growth when metformin was added to pure cultures [13]. Metformin also increased the abundance of beneficial bacteria such as *Lactobacillus* and *A. muciniphila* in insulin-resistant mice fed a high-fat diet [14]. Thus, the impact of metformin on host physiology appears to be influenced by its interaction with the gut microbiota.

Regarding cognition, metformin reduced scopolamine-induced cognitive dysfunction in male Wistar rats. Short-term working memory and spatial learning impairments improved after metformin treatment [15]. Metformin has also been linked to enhanced spatial learning, improved coordination during running tasks, and decreased memory deficits in mice [16]. In people with T2D, metformin use was associated with improved memory, language, and executive function, [9].

Due to the beneficial effects, the interest in the potential effects of metformin on cognition and gut microbiota in humans has increased over the past decade. To our knowledge, no studies have investigated the interplay among metformin, gut microbiota, and cognition in humans. To explore this crosstalk, we first investigated the metagenomic and metabolomic profiles associated with metformin treatment. Then, we examined how the composition of the gut microbiota was associated with cognitive functions. Finally, we investigated how metformin was linked to cognition.

2. Subjects and methods

2.1. Subjects

We worked with two independent studies: the Aging Imageomics Study and the CAIBERHJT_MEIFLO-Metformin and Intestinal Microflora Study (MEIFLO). People with subjective memory complaints, mild cognitive impairment, or dementia were not included in the studies.

The Aging Imageomics Study ($n = 1030$) was an observational study in which subjects from the province of Girona (Spain) were enrolled. Participants were drawn from two independent cohorts: The Maturity and Satisfactory Aging in Girona study (MESGI50 study) and the Improving interMediAte RisK management study (MARK study). Data collection was between 14 November 2017 and 19 June 2019 at the facilities of the Dr. Josep Trueta University Hospital. The Ethics Committee of the Dr. Josep Trueta University Hospital approved the study protocol, and all participants gave written informed consent (Project Code SLT002-16/00250). Selection criteria were age $\geq$ 50 years, living in the community, no infection or antibiotic usage in the previous 15 days, and no contraindications to magnetic resonance imaging (MRI). Participants were visited twice. A clinical history, physical examination, dietary assessment, MRI, and neuropsychological assessment of the participants were performed. Samples of blood, urine, and feces were collected. Full details of the study protocol can be found elsewhere [17].

From the entire Aging Imageomics cohort, we consecutively selected three study groups. The first group consisted of individuals with no documented medical history or medical treatment ($n = 172$). The second group included people with long-term T2D (more than one year) on metformin monotherapy ($n = 134$). The last group included people with long-term T2D treated with oral hypoglycemic agents other than metformin ($n = 45$). We excluded from this group people treated with another biguanide or with a combination of metformin and other hypoglycemic agents, such as metformin and sitagliptin. Oral hypoglycemic agents used as treatment in this group included: sulfonylureas (gliclazide ($n = 8$), glimepiride ($n = 2$)), dipeptidyl peptidase-4 inhibitors (sitagliptin (n = 2), linagliptin (n = 2)), sodium-glucose cotransporter-2 inhibitors (dapagliflozin ($n = 1$), repaglinide ($n = 3$), and combination of drugs ($n = 27$). The consort diagram showing recruitment numbers and flows can be found in the supplementary material.

MEIFLO was a phase IV, randomized, double-blind, parallel-group,

randomized pilot study in which $n = 40$ T2D subjects from the province of Girona were enrolled. The subjects were randomly assigned to either the metformin group ($n = 22$) or the placebo group ($n = 18$) using a computational random generator called Aleator. From the entire cohort, we selected the group treated with metformin (n = 22). Metformin treatment started with an initial dose of 425 mg per day, which was gradually increased to 1700 mg during the first week. A clinical history, physical examination, and dietary assessment of the participants were performed. Fecal and plasma samples were collected at baseline, at two months, and at four months after treatment.

Inclusion criteria included: age between 18 and 65 years; T2D diagnosed within the previous 6 months based on the American Diabetes Association diagnostic criteria; absence of other systemic, and metabolic diseases; no infection in the previous month; absence of diet or drugs that could interfere with glucose homeostasis, such as antibiotics in the past three months; and glycated hemoglobin levels below 9 %. Exclusion criteria included: systemic diseases, including malignancy; clinical evidence of hemoglobinopathies or anemia; drug consumption; alcohol abuse >80 g/d for men and > 40 g/d for women; history of ischemic heart disease in the past six months; acute or chronic inflammatory or infectious diseases; and inability to understand the nature, scope, and possible consequences of the study.

The Ethics Committee of the Dr. Josep Trueta University Hospital approved the study protocol, and all participants gave written informed consent. Full clinical trial registration is available on the EU Clinical Trials Register (EudraCT number 2010-022394-34).

2.2. Laboratory parameters

Bioimpedance and *ad hoc* questionnaires were used to obtain anthropometric data and clinical history. Fasting plasma glucose, lipid profile, serum creatinine, serum urate, hemoglobin, and serum ferritin levels were determined by standard laboratory methods using an analyzer (Cobas 8000 c702, Roche Diagnostics, Basel, Switzerland). Glycated hemoglobin was measured by high-performance liquid chromatography (ADAMA1c HA-8180V, ARKRAY, Kyoto, Japan).

2.3. Neuropsychological assessment (aging Imageomics cohort)

Neurocognitive tests were employed to evaluate four distinct cognitive domains: attention, executive function, memory, and language. Cognitive tests were presented as adjusted normalized data. Further details of the neuropsychological protocol can be found in the supplementary material.

2.4. Extraction of fecal genomic DNA and whole-genome shotgun sequencing

The protocol for extraction of fecal genomic DNA and whole-genome shotgun sequencing can be found in the supplementary material.

2.5. Metabolomics analyses (aging Imageomics cohort)

2.5.1. HPCL-ESI-MS/MS metabolomics analyses

The protocol for non-targeted metabolomics analysis can be found in the supplementary material.

2.6. Statistical analysis

Normal distribution and homogeneity of variances were tested. Results are presented as numbers and frequencies for categorical variables, means and standard deviations for normally distributed continuous variables, and medians and interquartile ranges for non-normally distributed continuous variables. R Statistics was used for these statistical analyses. The statistics are presented in the form of figures and legends. From the entire Aging Imageomics cohort, we extracted three distinct study groups. Analyses were performed comparing people with T2D treated with metformin with the other two groups (healthy people and people with T2D not treated with metformin). We also studied a group of newly diagnosed T2D subjects ($n = 22$) on metformin monotherapy. In this group, we compared baseline (participants not yet taking metformin) with participants taking metformin for four months. Analyses were also performed on all subjects and then stratified by sex.

2.6.1. Metagenomics statistical analysis

The identification of metformin-associated microbial species was performed using the microbiome compositional analysis methodology with bias correction (ANCOM-BC) [18]. ANCOM-BC considers the bias resulting from different sampling fractions between samples by incorporating a sample-specific offset into a linear regression model derived from the observed data. To account for the compositional nature of metagenomics datasets, the linear regression model in log scale is equivalent to a log-ratio transformation, with the offset term functioning as a bias correction. All models were adjusted for age, sex, and body mass index (BMI). Sequential goodness of fit (SGoF) was used to correct *p*-values for multiple comparisons. SGoF methods increase their statistical power as the number of tests increases, differing from FDR methods, which decrease their statistical power as the number of tests increases. SGoF has been demonstrated to outperform FDR in settings with high numbers of tests and small sample sizes, such as large metagenomic datasets [19]. Statistical significance was fixed at p adjusted (padj) < 0.1. For principal component analysis, raw counts were transformed using a centered log-ratio (clr) transformation as implemented in the "microViz" R package. Pathway overrepresentation analyses were performed by mapping KEGG orthologs to KEGG pathways using the R package "ClusterProflier" ("enrichKEGG" function). A hypergeometric test was employed to determine the significance of the pathway, and a Storey procedure (q-values) was applied to correct for multiple testing. Statistical significance was set at Padj < 0.1.

To calculate the *Akkermansia muciniphila / Romboutsia ilealis* ratio in metformin-treated T2D subjects, the raw read count data of these bacteria were first centered log ratio-transformed using the R package "ALDEx2" ("aldex.clr" function). The *A. muciniphila/R. ilealis* ratio was then constructed from the clr-normalized data. The association between this ratio and cognition (Total Digits Span (TDS), Phonemic verbal fluency (PVF), Semantic verbal fluency (SVF), Symbol Digit Modalities Test (SDMT), the Memory Binding Test (Total Free Recall (MBT-TFR) and Total Delayed Free Recall (MBT-TDFR)) was examined by partial Spearman correlation, controlling the model for sex, BMI, age, and years of education. Wilcoxon's signed rank test was used to compare cognitive performance within the two T2D groups, and the results were presented using box plots.

2.6.2. Machine learning analyses (metabolomics)

Probabilistic quotient normalization was used to normalize the metabolomics data. The metabolomics data was then analyzed using machine learning methods. To determine the plasma metabolites associated with metformin treatment, we used an all-relevant machine-learning variable selection strategy using multiple random forests as implemented in the Boruta algorithm [20]. We adjusted for age, sex, and BMI in all models. We ran the Boruta algorithm with 500 iterations, a confidence level of 0.005 for Bonferroni adjusted *p*-values, and 5000 trees to grow the forest. To improve the interpretability of the models, the exact computation of Shapley Additive explanations (SHAP) values was used, which exploits the internal structure of random forest models. SHAP calculates each metabolite's contribution to the predicted response [21]. SHAP values were calculated and plotted using R packages "treeshap" and "SHAPforXGBoost". Violin plots were used to present the proline levels in the Aging Imageomics cohort according to T2D treatment (Metformin or other oral hypoglycemic agents). Red dots represent the mean. Overall significance was assessed using a Kruskal-Wallis test and between groups significance using a Wilcoxon test.

3. Results

3.1. Metformin treatment is associated with a specific microbial ecosystem (Aging Imageomics)

The clinical characteristics of the study groups are shown in (Table 1). We first assessed the relationships between microbial composition and metformin treatment by comparing metformin-treated T2D subjects ($n = 134$) with healthy subjects ($n = 172$) in a discovery cohort (Aging Imageomics). First, we applied principal component analysis to the clr-transformed data to reveal global patterns of variance in the microbial profiles and functions between the three study groups. The most notable differences were found at the level of functionality, comparing metformin-treated T2D subjects with healthy subjects and T2D subjects treated with other hypoglycemic agents (Supplementary Material-Fig. S1).

We identified 81 (padj < 0.1) metformin-associated microbial species (Fig. 1A, Table S1). Metformin treatment in T2D subjects was associated with an increase in bacterial species belonging to the Proteobacteria (*Escherichia coli*, *Shigella sonnei, Klebsiella pneumoniae,* and *Desulfovibrio fairfieldensis*) and Firmicutes phyla (*Enterocloster bolteae*, *Enterocloster citroniae*, and *Enterocloster clostridioformis*) compared to healthy subjects. In addition, metformin treatment was associated with an increase in the viral species, *CrAssphage ZA*, *CrAssphage LMMB* and *Podoviridae_uc*. Conversely, we observed a decrease in bacterial species belonging to the Firmicutes phylum such as *Romboutsia timonensis*, *Romboutsia ilealis*, and *Roseburia faecis* in metformin-treated T2D subjects compared to healthy subjects (Fig. 1A, Table S1).

When we stratified this population by sex, we identified 11 (padj<0.1) metformin-associated microbial species in men after comparing men with T2D to healthy men (Fig. 1B, Table S2). Again, we observed an increase in bacterial species belonging to the Proteobacteria phylum (*Escherichia coli*, *Shigella sonnei, Klebsiella pneumoniae*) and a decrease in bacterial species belonging to the Firmicutes phylum (*Romboutsia timonensis*, *Roseburia faecis*) in men with T2D treated with metformin compared to healthy men. No increase in virus species was found (Fig. 1B, Table S2). We identified 27 (padj<0.1) microbial species in metformin-treated T2D women compared to healthy women (Fig. 1C, Table S3). Again, we found an increase in *Escherichia coli*, *Shigella sonnei, Klebsiella pneumoniae* and a decrease in *Romboutsia timonensis*, *Romboutsia ilealis*, and *Roseburia faecis* in women with T2D treated with metformin compared to healthy women. In metformin-treated women with T2D, we observed an increase in the viral species *CrAssphage ZA*, *CrAssphage LMMB*, and *Podoviridae_uc* compared to healthy women (Fig. 1C, Table S3).

To confirm whether the changes found in gut microbiota composition were due to T2D or metformin treatment, we compared subjects with T2D treated with metformin ($n = 134$) and T2D treated with other hypoglycemic agents ($n = 45$). We identified 18 (padj<0.1) differential microbial species (Fig. 1D, Table S4). Consistent with our previous findings, we observed an increase in *Escherichia coli* (Proteobacteria phylum) and a decrease in *Romboutsia timonensis*, and *Romboutsia ilealis* (Firmicutes phylum) in subjects with T2D treated with metformin compared to subjects with T2D treated with other hypoglycemic agents. We also found an increase in bacterial species belonging to the Verrucomicrobia phylum such as *Akkermansia muciniphila, Unclassified Akkermansia*, *Akkermansia sp.*, and *Unclassified Akkermansiaceae* in subjects with T2D treated with metformin compared to subjects with T2D treated with other hypoglycemic agents (Fig. 1D, Table S4). After sex stratification, we identified 7 metformin-associated microbial species in men and 24 in women (Fig. 1E, F, and Tables S5, S6).

Due to the consistent increase in *Akkermansia muciniphila* and decrease in *Romboutsia ilealis* in people with T2D treated with metformin, we aimed to investigate the association between their ratio and cognition. In the entire cohort of metformin-treated T2D subjects, the *A. muciniphila/R. ilealis* ratio was not significantly associated with any cognitive test scores (Supplementary Material-Fig. S2). However, sex stratification, the *A. muciniphila/R. ilealis* ratio was significantly and positively associated with MBT-TFR ($r = 0.42$, padj = 0.02) and MBT-TDFR ($r = 0.38$, padj = 0.03) scores in men. No significant results were found in women (Fig. 2). We also compared cognitive test scores between the two groups of people with T2D. No significant results were found for the overall cohort of people with T2D and women. At the same time, men with T2D treated with other hypoglycemic agents had higher TDS ($p = 0.04$), SDMT ($p = 0.001$), and SVF (p = 0.04) test scores than those treated with metformin (Supplementary Material-Fig. S3).

Table 1
Clinical and neuropsychological data of the Aging Imageomics cohort.

Characteristics	T2D subjects treated with metformin. (N = 134)	T2D patients not treated with metformin. (N = 45)	Healthy subjects (*N* = 172)
Age (years)	68.0 [62.5–72.5]	72.7 [67.3–74.6]	63.1 [58.5–68.4]
Women n (%)/ Men n (%)	(56) 41.8 % / (78) 58.2 %	(22) 48.9 % / (23) 51.1 %	82(47.7)/90(52.3)
Education (years)	8.0 [8.0–12.0]	8.0 [8.0–8.0]	12.0 [8.0–12.0]
BMI (kg/m2)	29.4 ± 4.6	29.4 ± 3.9	26.1 ± 4.0
Waist (cm)	101.9 ± 11.2	102.6 ± 10.8	94.8 ± 12.2
MBT-TFR (score)	11.0 [8.0–13]	11.0 [8.8–14.0]	13.0 [9.0–16.0]
MBT-TDFR (score)	10.0 [8.00–13.00]	10.0 [8.0–13.8]	12.0 [9.0–17.0]
PVF (score)	9.5 [8.0–11.0]	9.0 [8.0–12.0]	10.0 [8.0–11.0]
SVF (score)	8.0 [7.0–10.0]	9.0 [8.0–10.0]	10.0 [8.0–11.0]
SDMT (score)	10 [8.0–12.0]	11.0 [9.0–13.0]	11.0 [10.0–13.0]
TDS (score)	15 [12.2–18.8]	18.0 [13.0–21.0]	16.0 [13.0–20.5]
FPG (mg/dL)	143.0 [129.5–160.3]	141.5 [122.8–168.3]	95.0 [90.0–104.0]
HbA1c (%)	7.1 ± 1.0	7.2 ± 1.1	5.6 ± 0.4
Fasting Insulin (mU/L)	11.0 [7.7–14.8]	11.8 [7.7–18.4]	7.0 [5.2–10.0]
Serum creatinine (mg/dL)	0.85 [0.7–1.0]	0.87 [0.7–1.0]	0.79 [0.7–1.0]
Serum urate (mg/dL)	5.7 [4.9–6.4]	5.2 [4.5–5.9]	5.1 [4.3–6.0]
Total cholesterol (mg/dL)	182.4 ± 31.2	176.6 ± 29.64	206.1 ± 30.7
HDL-C (mg/dL)	47.0 [37.0–55.3]	47.5 [42.0–59.0]	55.0 [47.5–69.0]
LDL-C (mg/dL)	104.3 ± 26.6	100.2 ± 28.9	127.7.2 ± 27.6
Fasting triglycerides (mg/dL)	130.0 [94.8–176.5]	102.0 [86.3–165.0]	89.0 [70.5–120.0]
Serum ferritin (ng/ml)	89.0 [35.5–189.3]	84.5 [42.0–149.8]	139.0 [77.5–208.0]

Results are expressed as numbers and frequencies for categorical variables, mean and standard deviation (SD) for normal distributed continuous variables, and median and interquartile range [IQ] for non-normal distributed continuous variables. BMI, body mass index; PVF, phonemic Verbal Fluency; SVF, Semantic verbal fluency; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C low-density lipoprotein-cholesterol.

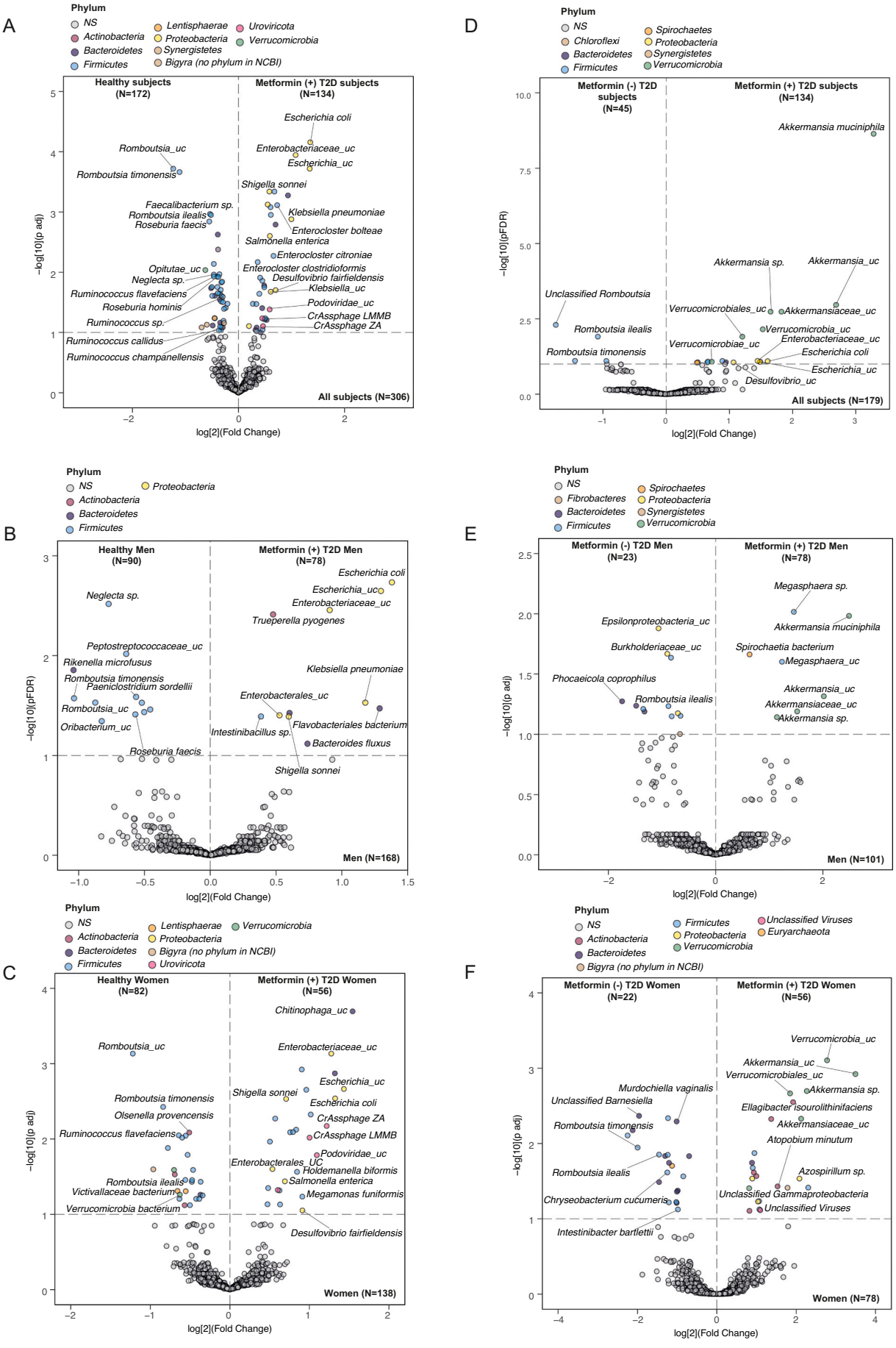

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Fig. 1. Gut microbiome profiles associated with metformin treatment. (A) Volcano plot of differential microbial abundance associated with metformin treatment between metformin-treated subjects and healthy subjects (Aging Imageomics cohort). (B) Volcano plot of differential microbial abundance associated with metformin treatment between metformin-treated men and healthy men. (C) Volcano plot of differential microbial abundance associated with metformin treatment between metformin-treated women and healthy women. (D) Volcano plot of differential microbial abundance associated with metformin treatment between T2D subjects treated with metformin and other oral hypoglycemic agents (Aging Imageomics cohort). (E) Volcano plot of differential microbial abundance associated with metformin treatment between T2D men treated with metformin and other oral hypoglycemic agents. (F) Volcano plot of differential microbial abundance associated with metformin treatment between T2D women treated with metformin and other oral hypoglycemic agents. Significant species were identified using the ANCOM-BC from shotgun metagenomics data adjusted for age, sex, and BMI. For each taxon, the log2 fold change associated with a unit change in metformin treatment and log10 *p*-values adjusted for multiple testing (padj) are plotted. Significantly different taxa are coloured according to phylum. Significance was set at padj<0.1. Metformin (+): T2D subjects' metformin-treated; Metformin (−): T2D subjects treated with other oral hypoglycemic agents.

3.2. Microbial functions linked to metformin treatment in the aging Imageomics cohort

To further assess the potential impact of the gut microbiome on metformin, we performed functional analyses by mapping reads to KEGG orthologues. We then identified microbial molecular functions associated with metformin treatment compared to other hypoglycemic treatments in T2D subjects after adjusting for age, sex, BMI, and years of education. We identified 1096 microbial functions (padj<0.1) that were enriched and 32 that were depleted by metformin treatment in the population with T2D (Fig. 3A, Table S7). In men with T2D, we identified 780 microbial functions that were enriched and 26 that were depleted by metformin treatment (Supplementary Material-Fig. S4, Table S8). In women, we identified 581 microbial functions that were enriched and 119 that were depleted by metformin treatment (Supplementary Material-Fig. S5, Table S9).

To obtain insights into the microbial pathways involved in this association, we performed a pathway overrepresentation analysis of KEGG orthologs. A significant over-representation of the pathways involved in the TCA cycle, and the metabolism of butanoate, arginine, and proline were found in the entire cohort of T2D subjects (Fig. 3B, Table S10). Pathways involved in butanoate, propanoate, and pyruvate metabolism were significantly enriched in men (Supplementary Material-Fig. S4, Table S11). In contrast, we found a significant enrichment of the pathway involved in the metabolism of butanoate, arginine, and proline in women, as well as a significant overrepresentation of the TCA cycle-related pathway (Supplementary Material-Fig. S5, Table S12). Additionally, a gene-concept network was performed to represent the linkage between significant KEGG orthologues involved in KEGG pathways associated with metformin treatment in the whole cohort (Fig. 3C), in men (Supplementary Material-Fig. S4) and in women (Supplementary Material-Fig. S5).

3.3. Microbial functions linked to metformin treatment in the MEIFLO cohort

We next aimed to validate the potential microbial functions associated with metformin treatment in a second independent cohort comparing the microbiome before and 4 months after metformin treatment in 22 newly diagnosed T2D patients. The clinical characteristics of the cohort is shown in (Table 2). A total of 8 men and 14 women matched for age, sex, BMI, and glycated hemoglobin were studied (Supplementary Material-Table 1).

We identified 360 microbial functions (padj<0.1) associated with metformin treatment in the whole cohort (Fig. 3D, Table S13), 274 in men, and 1096 in women (Supplementary Material-Fig. S6, Tables S14, S15). Consistent with our previous findings, a significant enrichment of the pathways involved in the TCA cycle and the butanoate, arginine, and proline metabolism was found in the entire cohort of T2D subjects after metformin treatment (Fig. 3E, Table S16). We found a significant enrichment of the pathway involved in the metabolism of pyruvate and propanoate in men (Supplementary Material-Fig. S6, Table S17). In

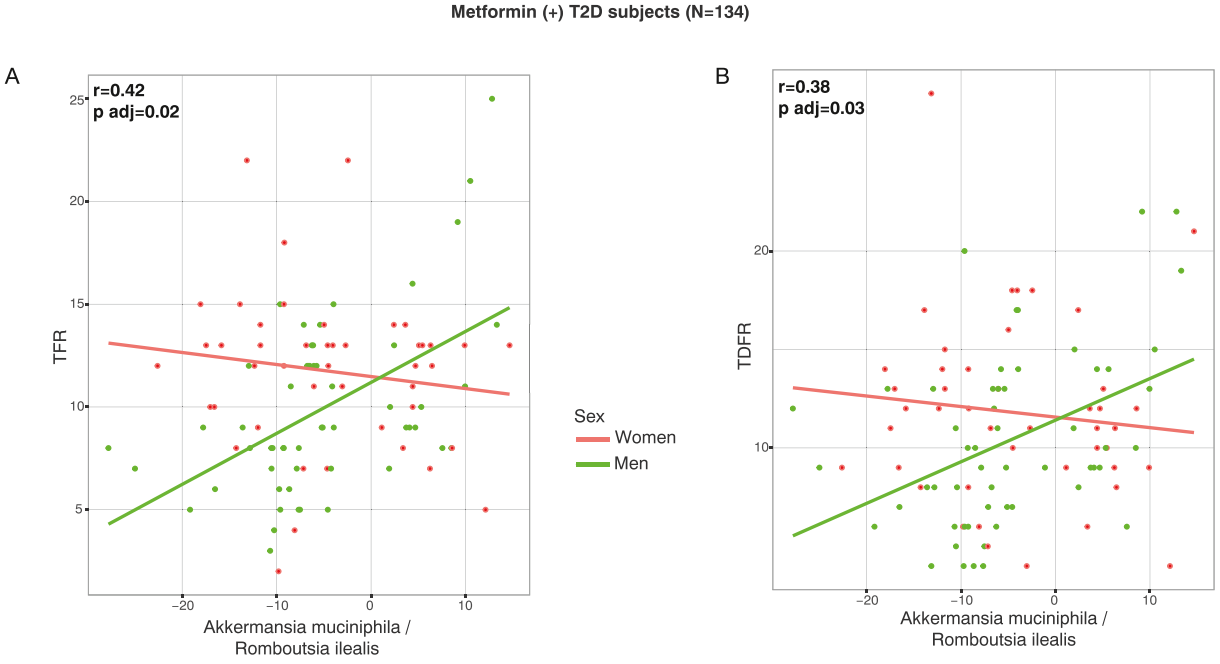
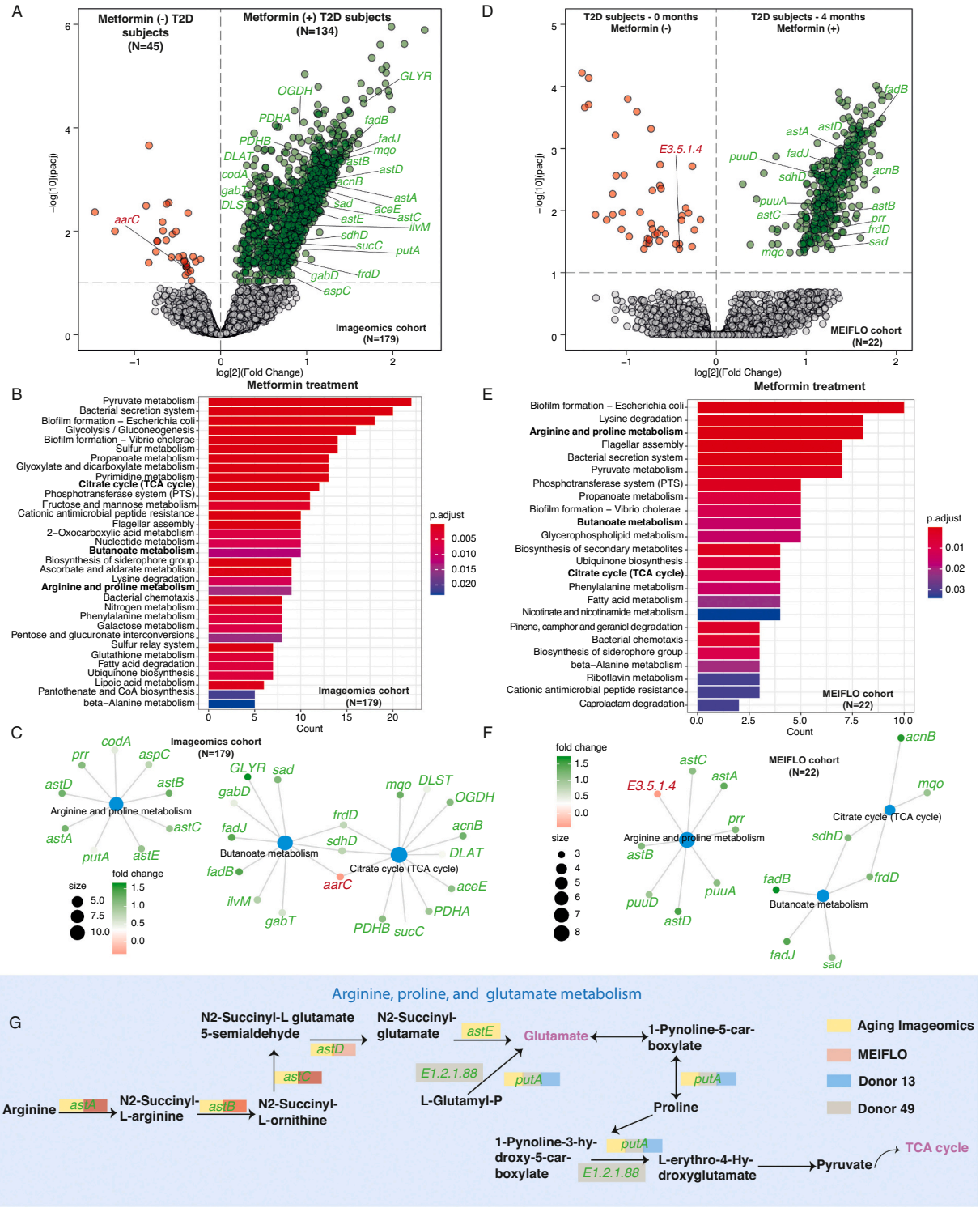


Fig. 2. Association between the *Akkermansia muciniphila/Romboutsia ilealis* ratio and cognitive scores by sex. Scatter plot showing the partial Spearman correlation between *Akkermansia muciniphila/Romboutsia ilealis ratio* and memory tests by sex, (A) MBT-TFR, and (B) MBT-TDFR. MBT-TFR, the Memory Binding Test-Total Free Recall; MBT-TDFR, the Memory Binding Test - Total Delayed Free Recall.



(caption on next page)

Fig. 3. Metformin treatment is associated with microbial molecular functions involved in glutamate metabolism. (A) Volcano plot of differential microbial gene abundance associated with the metformin treatment in Aging Imageomics cohort calculated by ANCOM-BC from shotgun metagenomics data adjusted for age, sex, and BMI (Aging Imageomics cohort). Microbial functions that are positively associated with metformin treatment are shown in green (upregulated), and those that are negatively associated are shown in red (downregulated). The log2 fold change associated with a unit change in metformin treatment and the log10 p-values adjusted for multiple testing (padj) are plotted for each taxon. Significance was set at padj<0.1. Only genes involved in the arginine, proline, and butanoate metabolism and the citrate cycle are labelled. (B) Barplot plot of enriched KEGG pathways (q-value <0.1) from significantly differentially expressed microbial molecular functions associated with metformin treatment in the Aging Imageomics cohort. (C) KEGG pathway enrichment network displaying the significant KEGG orthologues associated with metformin treatment and involved in the arginine, proline, and butanoate metabolism and the citrate cycle, coloured according to the fold change (Aging Imageomics cohort). (D) Volcano plot of differential microbial gene abundance associated with the metformin treatment in Aging Imageomics cohort calculated by ANCOM-BC from shotgun metagenomics data adjusted for age, sex, and BMI (MEIFLO cohort). Microbial functions that are positively associated with metformin treatment are shown in green (upregulated), and those that are negatively associated are shown in red (downregulated). The log2 fold change associated with a unit change in metformin treatment and the log10 p-values adjusted for multiple testing (padj) are plotted for each taxon. Significance was set at padj<0.1. Only genes involved in the arginine, proline, and butanoate metabolism and the citrate cycle are labelled. (E) Barplot plot of enriched KEGG pathways (q-value <0.1) from significantly differentially expressed microbial molecular functions associated with metformin treatment in the MEIFLO cohort. (F) KEGG pathway enrichment network displaying the significant KEGG orthologues associated with metformin treatment and involved in the arginine, proline, and butanoate metabolism and the citrate cycle, coloured according to the fold change (MEIFLO cohort). (G) Overview of the arginine, and proline pathway involved in glutamate metabolism. The microbial functions in the yellow, red, blue and grey boxes are those present in the Aging Imageomics cohort, MEIFLO cohort, donor 13 and 49 respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

women, we found a significant enrichment of the pathway involved in the longevity regulating pathways, among others (Supplementary Material-Fig. S6, Table S18). Additionally, a gene-concept network was performed to represent the linkage between significant KEGG orthologues involved in KEGG pathways associated with metformin treatment in the whole cohort (Fig. 3F). The most consistent results were found in the metabolism of arginine and proline, where enriched genes were involved in the synthesis of glutamate from arginine (*astA, astB, astC, astD, astE, putA, E.1.2.1.88*). Hence, a summary of the main microbial molecular functions associated with metformin treatment and involved in the arginine, proline, and glutamate metabolism is shown in Fig. 3G.

3.4. Metabolites of arginine and proline metabolism are associated with metformin treatment (aging Imageomics)

To further explore the microbiome functionally, we next performed metabolic profiling of plasma samples from T2D subjects treated with metformin ($n = 134$) and T2D subjects treated with other hypoglycemic agents ($n = 45$). Subsequently, metabolic signatures associated with metformin treatment were identified using a machine-learning variable selection strategy. We found that metformin treatment was strongly associated with proline (Fig. 4A, B, Table S19). Subjects taking metformin had significantly higher proline levels than those taking other oral hypoglycemic agents (Fig. 4C). When subjects were stratified by sex, metformin treatment was strongly associated with proline levels both in men (Fig. 4D, E) and women (Fig. 4F, F).

4. Discussion

Metformin is a widely used drug for the treatment of T2D. Recent human investigations suggest that metformin could alter the gut microbiome, and improve memory, semantic memory, and executive function [9]. To our knowledge, no studies have investigated the interplay among metformin, gut microbiota, and cognition in humans. Hence, we aimed to investigate this potential crosstalk. This study included two cohorts with slightly different inclusion criteria, but the findings could be replicated and thus ensure consistency of results.

In T2D subjects treated with metformin, we found an increase in *A. muciniphila* and a decrease *of R. ilealis* compared with people with T2D taking other hypoglycemic agents. Then, we assessed the association between the *A. muciniphila/R. ilealis* ratio and cognition. In men, this ratio was associated with higher memory scores and improved memory. Differences in sex hormones may explain these differences between men and women. Clinical and preclinical studies have shown that the gut microbiota is different in females and males [39]. In addition to differences in microbial diversity, studies in animals and humans showed a clear difference in the abundance of certain bacteria, which is higher in one sex than the other [39] while sex hormone levels have been shown to be directly influenced by the gut microbiota. There is also evidence that sex hormones can reciprocally affect the gut microbiome [39].

Consistent with our findings, human studies have described an increase in the abundance of *A. muciniphila* after metformin treatment [13,22,23]. In addition, the increase in *A. muciniphila* was positively associated with the number of mucin-producing goblet cells in metformin-treated mice [24]. Goblet cells may provide a barrier to lipopolysaccharides by increasing the thickness of the mucus layer

Table 2
Clinical data of the MEIFLO cohort.

Characteristics	Newly diagnosed T2D subjects at 0 months. ($N = 22$)	Newly diagnosed T2D subjects at 4 months. (N = 22)
Age (years)	53.5 [47.8–59.0]	–
Females n (%)/Males n (%)	(14) 37.5 % / (8) 62.5 %	(14) 37.5 % / (8) 62.5 %
BMI (kg/m^2)	36.4 ± 6.95	35.37 ± 6.79
Waist (cm)	111.4 ± 14.1	110.05 ± 12.05
FPG (mg/dL)	124.0 [110.25–137.00]	108.0 [103.5–115.5]
HbA1c (%)	6.6 [6.3–7.0]	6.0 [5.75–6.3]
Serum creatinine (mg/dL)	0.72 [0.67–0.92]	0.77 [0.7–0.9]
Serum urate (mg/dL)	6.05[4.65–6.85]	6.6 [4.8–7.6]
Total cholesterol (mg/dL)	211.5 [182.5–232.0]	196.0 [174.5–231.5]
HDL-C (mg/dL)	46.0 [36.25–57.75]	51.5 [37.0–63.0]
LDL-C (mg/dL)	129.0 [115.5–154.5]	119.0 [99.0–149.5]
Fasting triglycerides (mg/dL)	103.5[82.25–161.75]	116.0 [100.0–158.5]
Serum ferritin (ng/ml)	123.0 [52.5–254.75]	93.0 [45.30–180.0]

Results are expressed as numbers and frequencies for categorical variables, mean and standard deviation (SD) for normal distributed continuous variables, and median and interquartile range [IQ] for non-normal distributed continuous variables. BMI, body mass index; PVF, phonemic Verbal Fluency; SVF, Semantic verbal fluency; FPG, fasting plasma glucose; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C low-density lipoprotein-cholesterol.

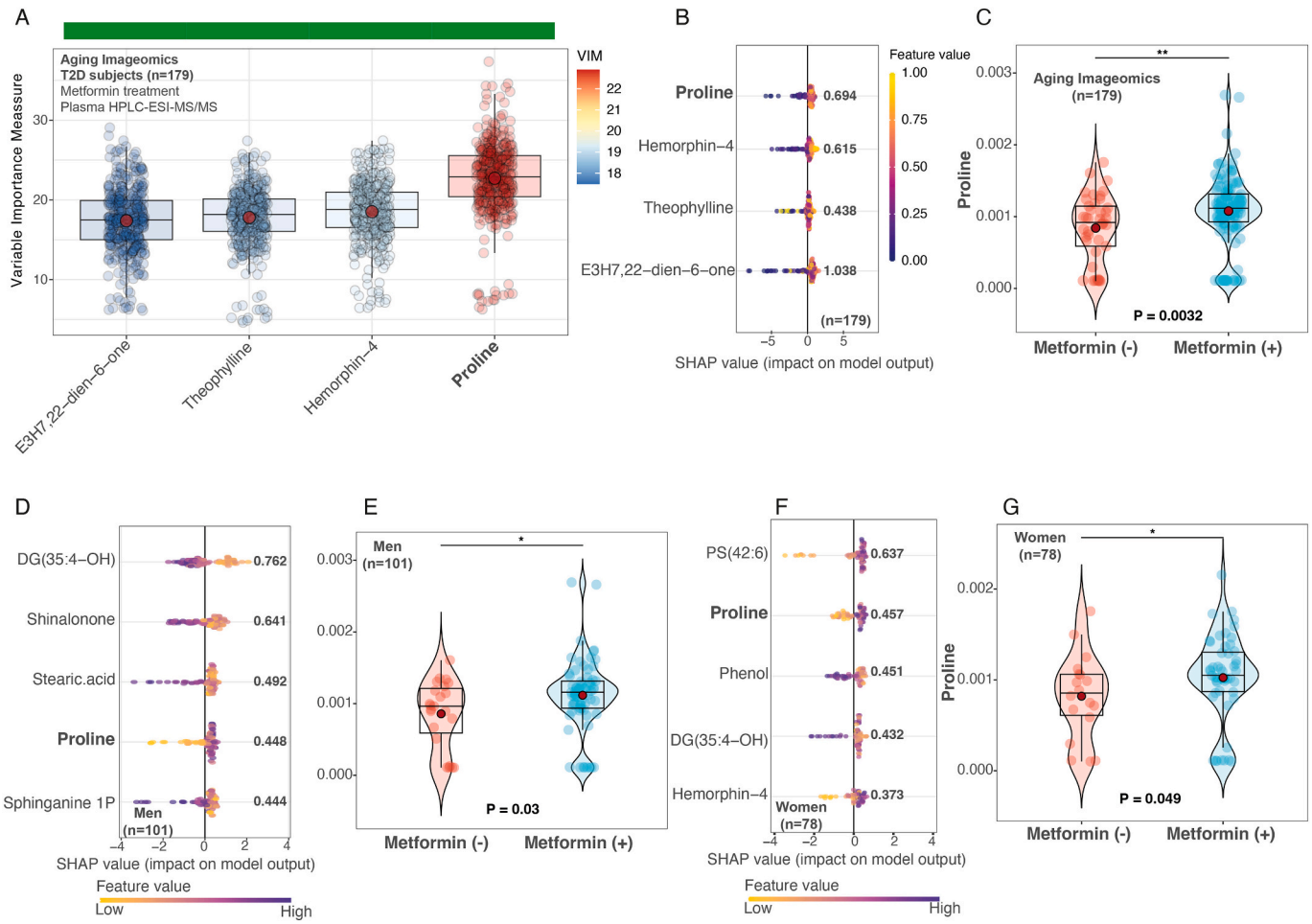


Fig. 4. Untargeted plasma metabolomics signatures associated with metformin treatment in the Aging Imageomics cohort. (A) Boxplot of the normalized variable importance measure for the metabolites associated with metformin treatment in all T2D patients ($n = 179$). Significant metabolites (confirmed) were identified using a machine learning variable selection strategy based on the application of multiple random forests as implemented in the Boruta algorithm. The Boruta algorithm was run with 500 iterations, a confidence level of 0.005 for Bonferroni adjusted p-values, and 5000 trees to grow the forest. The model was controlled for age, BMI, and sex. (B) SHAP summary of the metabolites associated with metformin treatment in all patients with T2D. Significant metabolites related to the glutamate pathway are highlighted in bold. Each dot represents a single sample. The x-axis represents the SHAP value. Bold indicates the overall importance of the final prediction (average absolute SHAP). Colors represent metabolite levels, from blue (low concentrations) to yellow (high concentrations). (C) Violin plots of the proline levels in the Aging Imageomics cohort according to the T2D treatment in all subjects (Metformin (−), T2D subjects treated with other oral hypoglycemic agents; Metformin (+), T2D subjects treated with metformin). Overall significance was assessed using the Kruskal-Wallis test. Significance between the T2D groups was assessed using the Wilcoxon test. Red dots represent the mean. $^{\#}p < 0.1$ $*p < 0.05$, $**p < 0.01$; $***p < 0.001$. (D) SHAP summary of the metabolites associated with metformin treatment in men with T2D ($n = 101$). Significant metabolites related to the glutamate pathway are highlighted in bold. Each dot represents a single sample. Bold indicates the overall importance of the final prediction (average absolute SHAP). Colors represent metabolite levels, from blue (low concentrations) to yellow (high concentrations). (E) Violin plots of the proline levels in the Aging Imageomics cohort according to the T2D treatment in men (Metformin (−), T2D men treated with other oral hypoglycemic agents; Metformin (+), T2D men treated with metformin). Overall significance was assessed using the Kruskal-Wallis test. Significance between the T2D groups was assessed using the Wilcoxon test. Red dots represent the mean. $^{\#}p < 0.1$ $*p < 0.05$, $**p < 0.01$; $***p < 0.001$. (F) SHAP summary of the metabolites associated with metformin treatment in women with T2D ($n = 78$). Significant metabolites related to the glutamate pathway are highlighted in bold. Each dot represents a single sample. Bold indicates the overall importance of the final prediction (average absolute SHAP). Colors represent metabolite levels, from blue (low concentrations) to yellow (high concentrations). (G) Violin plots of the proline levels in the Aging Imageomics cohort according to the T2D treatment in women (Metformin (-), T2D women treated with other oral hypoglycemic agents; Metformin (+), T2D women treated with metformin). Overall significance was assessed using the Kruskal-Wallis test. Significance between the T2D groups was assessed using the Wilcoxon test. Red dots represent the mean. $^{\#}p < 0.1$ $*p < 0.05$, $**p < 0.01$; $***p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[25–27]. In mice, *A. muciniphila* also had a negative association with low-grade inflammation, T2D, and insulin resistance [28]. Additionally, the increased abundance of *A. muciniphila* associated with metformin use correlated with reduced levels of the systemic inflammatory biomarker IL-6 in aged mice [29]. Systemic production of IL-6 has the potential to cross the blood-brain barrier and disrupt neurotransmission in key brain regions that regulate cognition, such as the hippocampus and prefrontal cortex [30,31]. Increases in *A. muciniphila* could partially restore the increased intestinal permeability caused by T2D and the high-fat diet. All the biological functions identified in *A. muciniphila* are consistent with improved cognition.

However, when we compared the two groups of people with T2D according to their cognitive performance, we found no differences or trends in the overall cohort or women. In men treated with other hypoglycemic agents, we found higher scores on tests of attention, working memory, and semantic memory compared with men treated with metformin. We believe that sample size could explain these findings on cognition. Metformin has been shown to reduce the incidence of dementia compared with other treatments in observational studies with larger populations [32]. Metformin treatment has also been associated

with improved memory and executive function [7]. Likewise, long-term metformin treatment has been shown to reduce vitamin B12 levels [33]. Vitamin B12 plays an important role in the reduction of homocysteine levels in the brain by participating in the synthesis of methionine from homocysteine. Elevated total plasma homocysteine levels can lead to central nervous system damage, cognitive impairment, dementia, and Alzheimer's disease [34]. Long-term treatment with metformin without monitoring vitamin B12 levels could, therefore, explain less favorable results when comparing metformin treatment with other oral hypoglycemic agents.

On the other hand, in T2D subjects treated with metformin, we found an increase in bacterial species belonging to the Proteobacteria phylum, particularly *Escherichia coli*, compared with healthy subjects and T2D subjects treated with other hypoglycemic agents. Consistent with our findings, in a cohort of newly diagnosed T2D patients, an increase in *E. coli* abundance was found at two and four months in metformin-treated T2D patients compared with placebo-treated T2D patients [13]. Another study found an increase *in Escherichia spp.* in subjects with T2D treated with metformin compared with subjects with T2D not treated with metformin [35]. Additionally, in healthy subjects treated with metformin an increase of these genera and species has also been described [36,37]. Finally, 18 healthy volunteers treated with metformin (2 × 850 mg) showed a significant increase in the abundance of the *Escherichia-Shigella* genera when comparing samples obtained seven days after treatment to the baseline [36]. This interaction between metformin and proteobacteria is counterintuitive. Indirect metformin treatment effects, including reduced intestinal lipid absorption and lipopolysaccharide-triggered local inflammation can provide a competitive advantage to *Escherichia* species possibly triggering a positive feedback loop further contributing to the observed taxonomic changes [35]. Growth of *E. coli* in an *in vitro* analysis was not affected directly by metformin, in contrast to other species like *Bifidobacterium adolescentis* [13]. This supports the hypothesis that metformin's effects on the abundance of *Escherichia species* is likely indirect and may result from altered bacteria-bacteria interactions or other physiological and/or environmental changes in the gut due to metformin treatment [13].

Changes in the gut microbiota, with a predominance of lipopolysaccharide-synthesizing bacteria such *as Escherichia*, are known to be associated with an increase in gut permeability. This in turn could lead to bacterial translocation into the systemic circulation and worsening of insulin resistance [38]. Furthermore, the increased abundance of *Escherichia/Shigella* genus was associated with elevated levels of pro-inflammatory cytokines, including IL-6, CXCL2, NLRP3, and IL-1β [39–42]. Hence, it appears paradoxical that an increase in *E. coli* abundance could lead to favorable effects. One possible hypothesis is an increase in the production of microbial agmatine by *E. coli* after metformin treatment [43]. In animal studies, agmatine reduced neuronal loss caused by excitotoxins or ischemia. Agmatine has also been reported to have anticonvulsant and antidepressant effects. Thus, agmatine seems to act as an endogenous neuromodulator of mental stress [44].

In the two cohorts of subjects with T2D, functional analyses identified an upregulated expression of microbial functions involved in arginine and proline metabolism (*astA, astB, astC, astD, astE, putA, E.1.2.1.88*) in metformin-treated patients. However, the bacterial functions with upregulated expression and associated with metformin treatment were implicated in synthesis of glutamate from arginine. Consistent with our results, in a gut simulator experiment using fecal samples from two participants (donor 13 and donor 49), metformin use was associated with an enrichment of microbial functions involved in arginine, proline and glutamate metabolism pathways [13]. Similarly, in the metabolomic analysis, we found that metformin treatment was strongly associated with proline, a metabolite derived from the microbial catabolism of glutamate. However, the proline catabolic pathway can potentially generate glutamate and GABA [45]. Glutamate, the primary neurotransmitter in the central nervous system, is essential for the normal functioning and development of the brain. It regulates communication among neurons and influences brain plasticity. Glutamate plays a key role in memory and learning [46,47]. Low levels of glutamate in the grey matter of the brain, have been associated with poorer performance on several cognitive tests [47]. In another study, fecal glutamate was associated with better performance on the Trail Making Test-A test, suggesting better visual and motor processing speed, and therefore better executive function [46]. Notably, *E. coli* strains are among the glutamate-GABA producers. In fact, *E. coli strains* with the ability to produce GABA from glutamate inhibited neurodegeneration in *C. elegans* models expressing a neurotoxic allele [48].

In conclusion, our results suggest that although metformin's exact mechanism of action remains elusive, some of its beneficial effects may be mediated by changes in the composition of the gut microbiota and microbial-host-derived co-metabolites. However, these results should be interpreted with caution. To provide understanding of potential causality and mechanism of action further animal studies as well as large human cohort studies with long-term follow-up are required.

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Ethics approval, consent to participate

The studies were approved by The Ethics Committee of the Dr. Josep Trueta University Hospital. All the participants provided informed consent.

CRediT authorship contribution statement

Marisel Rosell-Díaz: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Anna Petit-Gay:** Formal analysis. **Clàudia Molas-Prat:** Formal analysis. **Laura Gallardo-Nuell:** Formal analysis. **Lluís Ramió-Torrentà:** Supervision, Resources. **Josep Garre-Olmo:** Supervision, Resources. **Vicente Pérez-Brocal:** Resources. **Andrés Moya:** Resources. **Mariona Jové:** Resources. **Reinald Pamplona:** Resources. **Josep Puig:** Supervision, Resources. **Rafael Ramos:** Supervision, Resources. **Fredrik Bäckhed:** Writing – review & editing, Supervision. **Jordi Mayneris-Perxachs:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization. **José Manuel Fernández-Real:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization.

Declaration of competing interest

F.B. receives research support from Biogaia AB, is founder and shareholder of Implexion Pharma AB, Roxbiosens Inc., and on the scientific advisory board for Bactolife A/S. The other authors declare that

they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2024.155941.

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