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Population-level Metagenomics Uncovers Distinct Effects of Multiple Medications on the Human Gut Microbiome

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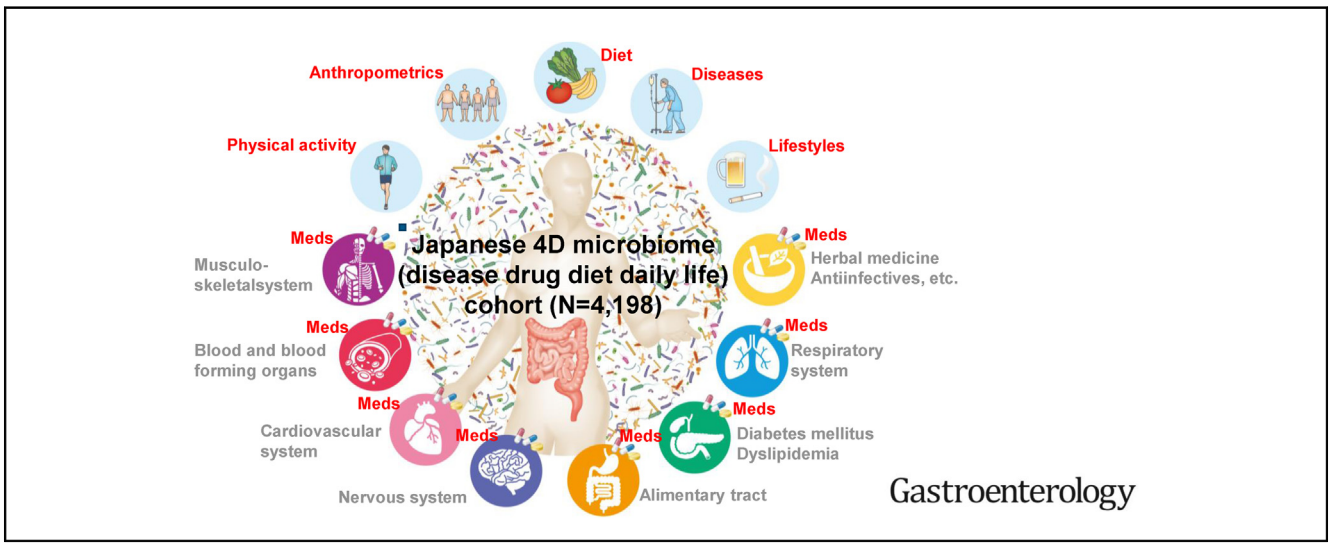

BACKGROUND & AIMS: Medication is a major determinant of human gut microbiome structure, and its overuse increases the risks of morbidity and mortality. However, effects of certain commonly prescribed drugs and multiple medications on the gut microbiome are still underinvestigated. **METHODS:** We performed shotgun metagenomic analysis of fecal samples from 4198 individuals in the Japanese 4D (Disease, Drug, Diet, Daily life) microbiome project. A total of 759 drugs were profiled, and other metadata, such as anthropometrics, lifestyles, diets, physical activities, and diseases, were prospectively collected. Second fecal samples were collected from 243 individuals to assess the effects of drug initiation and discontinuation on the microbiome. **RESULTS:** We found that numerous drugs across different treatment categories influence the microbiome; more than 70% of the drugs we profiled had not been examined before. Individuals exposed to multiple drugs, polypharmacy, showed distinct gut microbiome structures harboring significantly more abundant upper gastrointestinal species and several nosocomial pathobionts due to additive drug effects. Polypharmacy was also associated with microbial functions, including the reduction of short-chain fatty acid metabolism and increased bacterial stress responses. Even nonantibiotic

drugs were significantly correlated with an increased antimicrobial resistance potential through polypharmacy. Notably, a 2-time points dataset revealed the alteration and recovery of the microbiome in response to drug initiation and cessation, corroborating the observed drug-microbe associations in the cross-sectional cohort. **CONCLUSION:** Our large-scale metagenomics unravels extensive and disruptive impacts of individual and multiple drug exposures on the human gut microbiome, providing a drug-microbe catalog as a basis for a deeper understanding of the role of the microbiome in drug efficacy and toxicity.

Keywords: Drug Combination; Polypharmacy; Gut Microbiota; Pathobiont; Gut Resistome.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Even nonantibiotic drug use profoundly changes the gut microbiome. However, the effects of drug-drug interactions on the microbiome remain to be elucidated.

NEW FINDINGS

Shotgun metagenomics in the Japanese 4D ((Disease, Drug, Diet, Daily life) cohort (N = 4198) reveals substantial associations of multiple drugs with microbial species, functions, pathobionts, and antibiotic resistant genes. A 2-time points dataset indicates causal effects of observed drug-microbiome associations.

LIMITATIONS

No animal experimental models were performed to validate the detailed functions of the altered gut microbiome on host physiology.

IMPACT

This is the largest study to construct a catalog of drug-microbe associations, potentially opening new avenues to understand the mechanisms of adverse drug events caused by disruption of the gut microbiome.

Medication is one of the most influential factors for the human gut microbiome.[1,2] Recent cohort studies have revealed that both antibiotic and nonantibiotic drugs drastically change the structure of the microbial community in the gut.[1–4] The altered gut microbiome could become dysfunctional, induce inflammation, and increase susceptibility to pathogens[5,6]; thus, the interaction between the gut microbiome and drugs is profoundly involved in host physiology. However, despite recent efforts to evaluate the effects of drugs on the gut microbiome, the number of drugs analyzed in previous cohort studies[1–4] is still small (~50) in comparison with the number of drugs (~6000) registered with the Anatomical Therapeutic Chemical (ATC) Classification System in 2020.[7] Certain commonly prescribed drugs have not been extensively evaluated yet, including antidiabetic, antidyslipidemic, and antithrombotic drugs, as well as herbal medicines. The construction of a catalog that comprehensively maps drug-microbiome relationships would provide the basis for a deeper study of these interactions.

Importantly, the impact of the simultaneous or excessive administration of multiple drugs, referred to as polypharmacy,[8,9] on the gut microbiome is also poorly understood. Two previous studies have suggested the effects of polypharmacy, but there was little overlap between the results,[3,10] possibly because of the very limited number of drugs (~40) analyzed. In the United States, more than one-third of community-dwelling elderly people take 5 or more prescriptions.[11] Cumulative evidence indicates that the prevalence of polypharmacy is rising globally as the population ages, increasing the risks of morbidity and mortality through drug-drug interactions.[8,9] There is a need to evaluate the effects of individual drugs and their interactions on the human gut microbiome on a larger scale.

The large-scale clinical study is a powerful approach for comprehensive investigation of drug effects on the gut microbiome. An in vitro system is also useful for such assessment,[12] but it cannot evaluate the indirect effects of drugs influencing the gut microbiome by changing physiological conditions in the host, such as gastric pH, bile acid production, and intestinal motility. Although cross-sectional studies identify only associations, time-series data, in which samples are collected before and after drug intake or discontinuation in the same individuals, can reveal the dynamics of the gut microbiome. Therefore, in this study, we used large-scale cross-sectional data combined with a time-series (2-time points) dataset to investigate the broad effects of individual and multiple medications on the gut microbiome.

Methods

Study Design, Setting, and Participants

We conducted a prospective cross-sectional study from 4198 individuals participating in the Japanese 4D (Disease, Drug, Diet, Daily life) microbiome project, which commenced in January 2015 and is ongoing. The protocol for the Japanese 4D project was approved by the medical ethics committees of the Tokyo Medical University (Approval No.: T2019-0119), National Center for Global Health and Medicine (Approval No.: 1690), the University of Tokyo (Approval No.: 2019185NI), Waseda University (Approval No.: 2018-318), and the RIKEN Center for Integrative Medical Sciences (Approval No.: H30-7). Written informed consent was obtained before participation in the project. Metadata and fecal samples are prospectively collected from both healthy and diseased participants

*** Authors contributed equally.**

Abbreviations used in this paper: **4D, disease, drug, diet, daily life; AGI, alpha-glucosidase inhibitor; ARG, antibiotic resistance gene; ATC, Anatomical Therapeutic Chemical; GI, gastrointestinal; KO, Kyoto Encyclopedia of Genes and Genomes orthology; MO, Kyoto Encyclopedia of Genes and Genomes module; P-CAB, potassium-competitive acid blockers; PPI, proton pump inhibitor.**

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(Supplementary Methods). Second fecal samples were additionally collected from 243 participants at intervals of 2 weeks or more. This 2-time points analysis was approved by the medical ethics committee of the National Center for Global Health and Medicine (Approval Nos.: 1785 and 2083). Associations between the gut microbiome and drugs were evaluated using the 4198 cross-sectional dataset, and the effects of initiation and discontinuation of drugs on the microbiome were also investigated using the 2-time points dataset.

Metadata Collection

We collected metadata of anthropometrics, smoking, alcohol, dietary habits, physical activities, diseases, and medications using self-reported questionnaires, face-to-face interviews, and physicians' electronic medical records (Supplementary Table 1). Dietary habits were assessed using 23 questionnaire items on food intake habits, and the participants were specifically asked about typical eating patterns in the previous month, rated on a 7-point Likert scale (1, never or rarely; 2, 1–3 times/month; 3, 1–3 times/week; 4, 4–6 times/week; 5, once/day; 6, twice/day; and 7, 3 or more times/day).[13] Drug use was defined as oral or self-injected administration within the previous month. To verify that there were no omissions or discrepancies within the self-reported data, health professionals compared the questionnaire data with entries in the participants' medication pocketbooks (the "Okusuri-techo") made by pharmacists when filling prescriptions. The medication pocketbook serves as a personal health record, listing the owner's drug history, including brand names and prescription dates. Medications were classified into 5 different levels according to the World Health Organization's ATC classification system (1st–5th levels).[7] Polypharmacy is defined as the administration of several drugs at the same time, without consideration of drug type or efficacy,[8,9] and the number of drugs taken at the ATC 5th level was used for analysis. Past history or comorbidities were mainly evaluated from the electronic medical database, because self-reported disease data had low sensitivity for diagnosis compared with the data contained in a searchable collection of records into which physicians prospectively input information when people visit hospitals (Supplementary Table 2). A total of 759 drugs at the ATC 5th level and 51 diseases were evaluated (Supplementary Table 1).

Sample Collection

The participants collected fecal samples at home, and samples were refrigerated for up to 2 days before the hospital visit. As bowel-cleansing agents for colonoscopy have a profound effect on the gut microbiome and metabolome,[14] we avoided the collection of samples within 1 month after the administration of a bowel-cleansing agent. Cary-Blair medium-containing samples (Toyobo Co., Ltd., Tokyo, Japan) were used for gut microbiome analysis because the gut microbiome composition is more stable in Cary-Blair medium at 25°C or 4°C than in samples without a preservative, and these samples can potentially be used for bacterial isolation.[15] Health professionals checked that the amount of stool was sufficient for analysis (ie, larger than the size of the little finger).[13] As soon as patients arrived at the hospital, their fecal samples were frozen at −80 °C until DNA extraction.[13]

Metagenomic Analysis of the Fecal Samples

Bacterial DNA were prepared from the collected fecal samples as previously described,[16] with minor modifications (Supplementary Methods). Library preparation was performed using a NEBNext Ultra II DNA Library Prep Kit for Illumina according to the manufacturer's instructions, and 151 base pair end reads were sequenced by HiSeq X. Quality control and adapter trimming for the metagenomic reads was performed using Prinseq[17] and cutadapt,[18] respectively (Supplementary Table 3). Taxonomic profiles at the species and genus levels were obtained with the marker-gene–based approach using mOTUs2.[19]

The quality-controlled reads were assembled into contigs using MEGAHIT,[20] and genes on the contigs were predicted using Prodigal.[21] The predicted genes were clustered with a 95% identity threshold using Linclust,[22] and a nonredundant gene set (N = 9,901,170) was constructed. Functional annotations were performed using eggNOG-mapper based on the eggNOG orthology database.[23] In addition, the Comprehensive Antibiotic Resistance Database[24] was used to detect antibiotic resistance genes (ARGs) in the nonredundant gene set. For the quantification of these annotated genes, the metagenomic reads were mapped to the nonredundant genes using Bowtie2[25] with a $\geq$95% identity threshold, and the number of mapped reads for each gene was counted.

Statistical Analysis

Associations between the gut microbiome variation (Bray-Curtis dissimilarity) and metadata categories were assessed with stepwise redundant analysis using the ordiR2step function in the vegan package. Associations between the gut microbiome and metadata were analyzed by permutational analysis of variance using the adonis function. *P* values were adjusted with the Benjamini-Hochberg method.

To investigate associations between individual metadata and the microbial features (eg, taxonomies and gene functions) by adjusting for other possible confounding factors, we used multivariate regression analysis (Supplementary Methods), which is a straightforward statistical model to adjust for covariates.[26] First, univariate single-linear regression analysis was performed with the log10-transformed abundance of each microbial feature as an objective variable and each metadata factor as an explanatory variable. *P* values were adjusted with the Benjamini-Hochberg method by taking into account the total number of single-linear regression analyses (number of features multiplied by number of metadata). To exclude confounding factors, we then performed multiple-regression analysis for each feature, including all the significant (false discovery rate < 0.05) explanatory variables, as well as age, sex, and body mass index, in the model. We next performed variable selection based on Akaike's information criterion using the step function in the stats package. The remaining explanatory variables in the model with $P < .05$ were treated as significant for each feature. Diseases and drugs with fewer than 10 individuals were excluded from this analysis. In a 2-time points analysis, the paired Wilcoxon rank-sum test was used to assess changes in relative abundance of each microbial feature between the first and second samples.

All statistical analysis was conducted by R (version 3.6.1). For boxplots, boxes represent the interquartile range, and the

lines inside show the median. Whiskers denote the lowest and highest values within 1.5 times the interquartile range.

Additional detailed methods are provided in the online Supplementary Methods.

Results

Substantial Effects of a Wide Range of Drugs on the Gut Microbiome

We established the Japanese 4D (Disease, Drug, Diet, Daily life) microbiome cohort, in which we sequenced and analyzed stool samples from 4198 participants (59% men, mean age 66.4 years, Supplementary Table 1). Taxonomic and functional analyses of the metagenomic data identified a total of 284 genera, 1773 species, and 10,689 Kyoto Encyclopedia of Genes and Genomes orthologies (KOs) (Supplementary Figure 1). We exhaustively profiled patient drug use and assigned 759 common nonantibiotic and antibiotic drugs to the 5th and 4th levels as per the ATC classification system[7] (Supplementary Table 1). The mean number of drugs taken per individual was 5.1, and 87.0% (3,654/4,198) of the subjects took at least 1 drug. The most commonly prescribed drugs were proton pump inhibitors (PPIs), dihydropyridine derivatives, and hydroxy-methylglutaryl coenzyme reductase inhibitors. Our literature search revealed that 91.2% (272 of 298) of drugs at the ATC 5th and 70.4% (112 of 159) at the ATC 4th level have not been reported to be associated with the human gut microbiome (Supplementary Tables 4 and 5).

Among 844 metadata, consisting of anthropometrics, lifestyles, diets, physical activities, diseases, and medications (Supplementary Table 1), medications had the highest explanatory power for microbiome variation, explaining 10.4%, 5.0%, and 8.0% of the total variance in the gut microbial community at the genus, species, and KO levels, respectively (Figure 1*A*). Among drug categories that are based on anatomical or pharmacological groups, drugs for the alimentary tract, diabetes, and "anti-infectives for systemic use" had considerably higher covariance with the structure of the gut microbiome than other medication categories (Figure 1*B*). Even within drug categories, we found variations with large or small effects on the microbiome (Figure 1*D*). Specifically, drugs exhibiting large effects included PPIs and osmotic laxatives in alimentary tract drugs, alpha-glucosidase inhibitors (AGIs) in diabetes drugs, antivirals for HIV infection in anti-infective drugs, and platelet aggregation inhibitors (including aspirin) in antithrombotic drugs, whereas those exhibiting quite small effects included glucagon-like peptide-1 analogues, vitamin B12, testosterone 5-alpha-reductase inhibitors, and bisphosphonates (Supplementary Table 6). Consistent with the results of previous studies,[1–4] PPIs and osmotic laxatives showed relatively strong association with the microbiome (Figure 1*D*). Because our cohort was composed of relatively elderly individuals, we tested how age affects the drug-microbe associations. The result showed that the effect sizes of each drug on the microbiome were comparable between 2 age groups (Figure 1*C*), suggesting that the drug-microbe associations are robust regardless of age.

Multivariate regression analysis controlling for the effects of possible confounding variables revealed a large number of significant associations between each drug and gut microbes (Figure 2*A*, Supplementary Tables 6–11). Of the 298 drugs (ATC 5th level) taken by ≥10 individuals, 128 (43.0%) were independently associated with at least 1 genus (Supplementary Table 7), and 209 (70.1%) were associated with at least 1 species (Supplementary Table 9). Among the newly examined drugs in this study, several commonly prescribed drugs (ATC 4th level) also showed significant associations with microbial species, including contact laxatives, bulk-forming laxatives, dihydropyridine derivatives, angiotensin II antagonists, bisphosphonates, direct oral anticoagulants, vitamin preparations, and herbal medicines (Supplementary Tables 4 and 5). We further found that some commonly prescribed drugs, which may be used for a long time (eg, PPIs, osmotic laxatives, and 5-aminosalicylic acid), were consistently associated with the same species regardless of the duration of administration (Figure 2*B* and Supplementary Figure 2*A*), suggesting that the alteration of the gut microbiome persists during the treatment. For these 3 drugs, there was no significant difference in effects on the gut microbiome between low and high doses (Figure 2*C* and Supplementary Figure 2*B*).

Various drugs were also significantly associated with functional profiles of the gut microbiome based on KOs (Supplementary Tables 12–15). Among drugs showing the strongest associations, PPIs were positively associated with various amino acid transporters (eg, serine, glutamine, methionine, and lysine), whereas AGIs were positively associated with KOs related to sugar metabolism. These findings suggest that undigested nutrients found in the upper gastrointestinal (GI) tract due to PPI and AGI effects can reach the colon, where gut microbes metabolize them (Supplementary Note). Also, many genes for mucin degradation showed significant negative associations with PPIs, AGIs, amino acids, and derivatives (eg, hyaluronoglucosaminidase, alpha-L-fucosidase, and beta-galactosidase), but infrequently with other drugs (Supplementary Table 12), suggesting a possible association between increased metabolism of carbohydrates and amino acids and suppressed mucin turnover in the microbiome.[27]

To test how the mechanisms of action of drugs are involved in the changes to the gut microbiome, we examined and compared the effects of closely related drugs prescribed for the same disease. PPIs and potassium-competitive acid blockers (P-CABs), both of which inhibit gastric acid,[28] had significant associations with *Lactobacillus* and *Streptococcus* (Figure 2*A*). Within PPIs, esomeprazole, lansoprazole, omeprazole, and rabeprazole were all associated with the same genera (Supplementary Table 7). Contrarily, H2 receptor antagonists in the same category were associated with different genera, possibly because of the differences in the strength of acid pump inhibition activity between PPIs/P-CABs and H2 receptor antagonists[28] (Figure 2*A* and Supplementary Table 6). Similarly, those drugs with similar

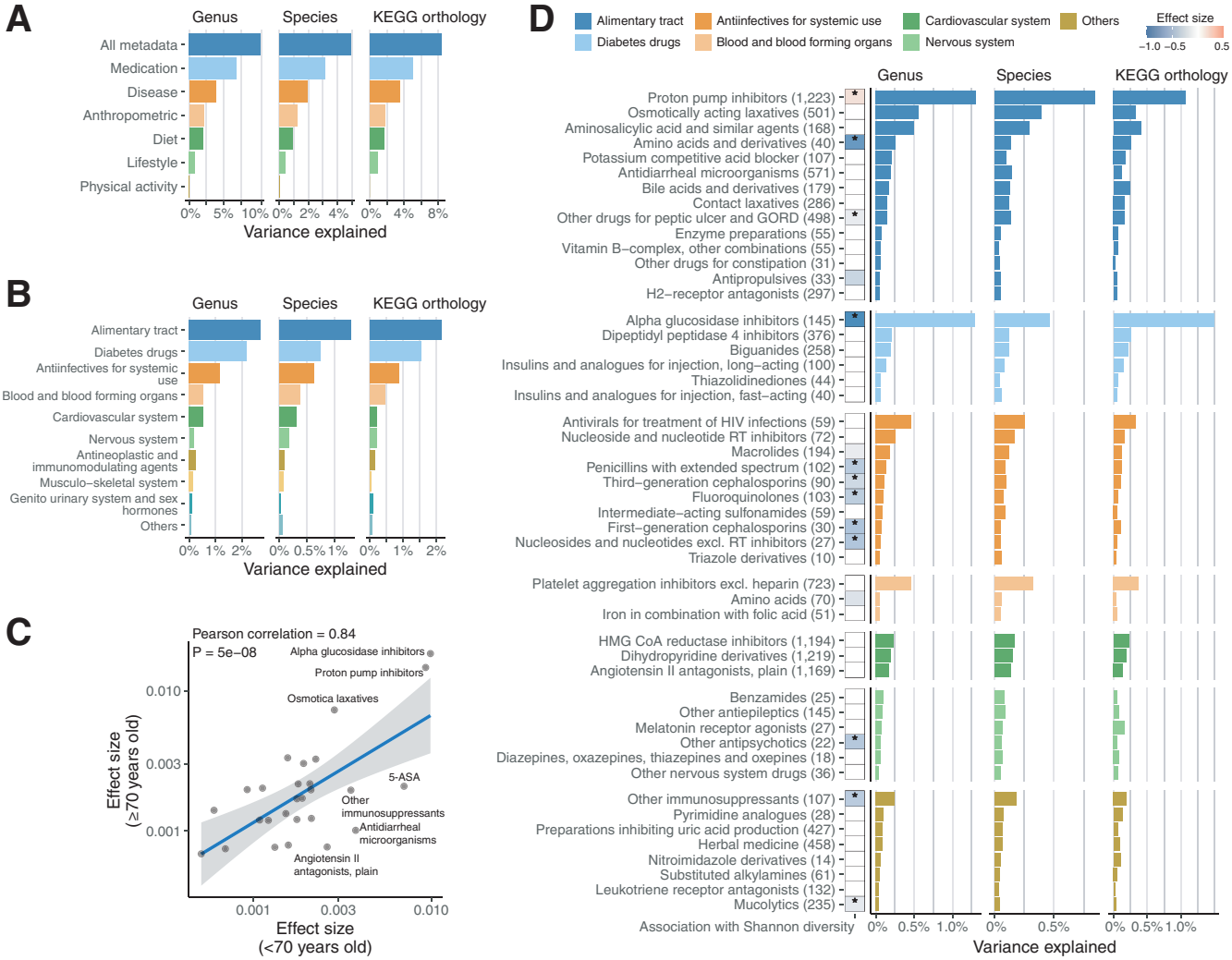


Figure 1. Medications strongly associate with the gut microbial diversity. (*A, B, D*) *Bars* showing the variance of the gut microbiome (the Bray-Curtis distance, N = 4198) explained by the metadata categories (*A*), medication categories at the ATC 1st level (*B*), and drugs at the ATC 4th level (*D*). Stepwise redundancy analysis was used for (*A*) and (*B*), and permutational analysis of variance was used for (*D*) to estimate the cumulative effect size for the microbiome profile at the genus, species, and KEGG orthology levels. The 50 drugs that were strongly associated with the gut microbiome and had at least 1 significant association with the genus (multivariate regression analysis) are shown in (*D*). Heatmap shows effect sizes of each drug on Shannon diversity of the microbiome. *Asterisks* denote significance ($P < .05$). No correction for multiple testing was performed here because only variables with false discovery rate <0.05 in the univariate regression analysis were included in the multivariate regression analysis. *Numbers in parentheses* indicate the number of individuals using the drug. (*C*) Comparison of effect sizes of each drug on the microbiome between individuals ≥70 years old and those <70 years old. 5-ASA, 5-aminosalicylic acid; GORD, gastroesophageal reflux disease; HIV, human immunodeficiency virus; KEGG, Kyoto Encyclopedia of Genes and Genomes; RT, reverse transcriptase.

mechanisms (osmotic laxatives and chloride channel activator) had similar effects on the gut microbiome compared with those with different mechanisms (bulk-forming laxatives and contact laxatives) (Supplementary Note). These data suggest that drug-induced changes in the gut microbiome partly depend on differences in the drugs' effects on the host's physiology.

Because even nonantibiotic treatment (eg, PPIs) could be correlated with an increase in pathogenic microbes in the gut,[29] we next explored associations between various drugs and possible pathobionts. Multivariate analysis identified that 26.8% (40 of 149) of nonantibiotic drugs and 70.0% (7 of 10) of antibiotics at the ATC 4th level were positively associated with at least 1 pathobiont that potentially causes serious nosocomial infections[30] (Figure 2*A*, Supplementary Tables 10 and 11). PPIs were frequently associated with pathobionts such as *Enterococcus faecalis, Klebsiella oxytoca*, and *Streptococcus pneumoniae*. In addition, other drugs such as bile acids and derivatives, melatonin receptor agonists, other nervous system drugs, and dipeptidyl peptidase-IV inhibitors showed significant positive associations with several pathobionts (Figure 2*A*).

To test whether diseases confound the drug-microbe associations identified previously, we examined the effects of 8 commonly used drugs (>100 users with at least 3 significant associations with genera) and compared the gut

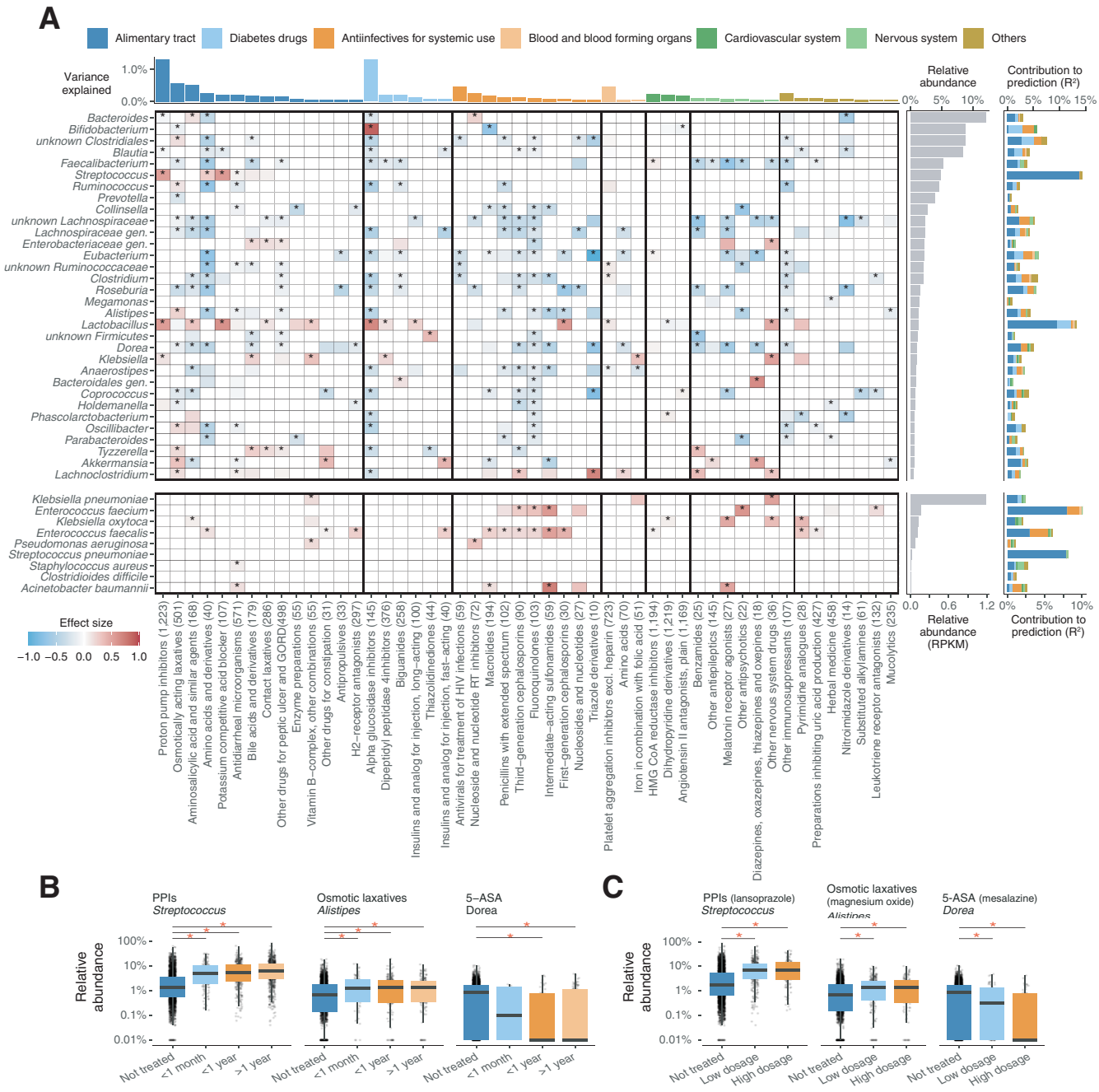


Figure 2. Medications are strongly associated with the gut microbial species and pathobionts. (*A*) Heatmap showing the associations between the top 50 drugs at the ATC 4th level and the top 32 genera (average relative abundance >0.5%) and the 9 pathobionts determined by multivariate regression analysis. The 50 drugs that were significantly associated with at least 1 genus are shown. *Asterisks* denote significance ($P < .05$). No correction for multiple testing was performed here because only variables with false discovery rate <0.05 in the univariate regression analysis were included in the multivariate regression analysis. *Numbers in parentheses* indicate the number of individuals using the drug. The *top bar* represents the coefficient of determination of the drugs assessed by permutational analysis of variance. The *gray bar* represents the average relative abundance of each genus in the 4198 individuals. The *right bar* shows the coefficient of determination of each medication category in the regression model. (*B*) Association between the duration of drug use (x-axes) with genus abundance (y-axes). (*C*) Association between the dosage of drug use with genus abundance. Comparison between nonusers and users (duration and dosage) was conducted by multivariate regression analysis. High and low dosages were defined based on the median volume of dosage. 5-ASA, 5-aminosalicylic acid; GORD, gastro-oesophageal reflux disease; HIV, human immunodeficiency virus; RT, reverse transcriptase.

microbiomes between patients receiving these drugs and patients treatment-naïve for each of 5 common diseases (diabetes, dyslipidemia, hypertension, metabolic syndrome, and thromboembolism). The results showed that most of the drug-microbe associations were consistent across the diseases (Supplementary Figure 3), suggesting that drug-microbe associations were not greatly affected by disease status.

Disentangling the Confounding Effects of Medication on Disease-Microbe Associations

Although drug treatment could confound disease-microbe associations,[31] the confounding effects have not been studied systematically in a large-scale cohort. To explore this, we investigated associations between microbes and 35 diseases in the cohort by multivariate analysis adjusted with and without drug information (Supplementary Figure 4). The multivariate regression model without considering drugs yielded 1.9 times more significant associations, on average, than the model accounting for drugs (Figure 3). In total, 11 diseases, such as inflammatory bowel disease, diabetes, and depression, showed significantly more associations with microbes in the model without drugs than with drugs, suggesting that many of these significant associations were confounded by drugs. For example, consistent with a previous study, we found a significant association between diabetes and *Lactobacillus* without adjustment for drugs[32] (Supplementary Figure 4); however, this association disappeared when we adjusted for antidiabetic drugs. In contrast, antidiabetic drugs such as dipeptidyl peptidase-IV and AGIs were independently associated with *Lactobacillus*, suggesting that these antidiabetic drugs confounded the diabetes-*Lactobacillus* associations (Figure 3). Similarly, we observed a significant negative association between depression and *Eubacterium* without adjustment for drugs, but this association disappeared when we adjusted for the antidepressant drug, diazepine. Indeed, the antibacterial effect of diazepine (asenapine) on *Eubacterium* was observed in in vitro experiments in a previous study.[12] Other significant associations between diseases and microbes also disappeared when we adjusted for drugs, such as inflammatory bowel disease, *Akkermansia*; HIV, *Streptococcus*; and chronic hepatitis, *Alistipes*. These results showed extensive effects of drugs on disease-microbe associations, highlighting the importance of controlling for the effects of drugs to detect true microbe associations with a disease.

Effects of Combination Use of Drugs on the Gut Microbiome

In our cohort, 3303 (78.7%) individuals were taking 2 or more drugs (Supplementary Table 16), allowing an exploration of the effects of drug-drug interactions on the gut microbiome. While single use of PPIs or AGIs significantly increased the relative abundances of *Lactobacillus/Streptococcus* and *Lactobacillus/Bifidobacterium*, respectively (Figure 2*A*), use of both drugs concurrently increased the relative abundances of *Bifidobacterium*, *Lactobacillus,* and *Streptococcus* (Figure 4*A*), indicating additive drug effects on the gut microbiome. Similar effects were observed for the combinations of PPIs and platelet aggregation inhibitors, of PPIs and osmotic laxatives, of PPIs and antidiarrheal microorganisms, and of osmotic laxatives and bile acids and derivatives (Figure 4*B–D*, and Supplementary Figure 5). Moreover, we found that drug pairs in which each drug was weakly associated with the gut microbiome showed a weak additive effect (Supplementary Figure 5).

Effects of Multidrug Exposure on the Gut Microbiome

The prevalence of polypharmacy is increasing worldwide in rapidly aging societies, potentially increasing the risks of morbidity and mortality through drug-drug interactions.[8,9] Using multivariate analysis, we investigated how the number of drugs administered influenced the microbiome and found that it showed a slight but significant negative correlation with the Shannon index (Figure 5*A* and *B*). Furthermore, the number of drugs was the third strongest factor associated with gut microbiome variation, following age and sex (Supplementary Figure 6*A*). The polypharmacy-associated changes were largely attributable to significant negative associations with species in Firmicutes, such as *Roseburia*, unknown Lachnospiraceae, and *Dorea*, and to positive associations with several species of *Streptococcus* and *Lactobacillus* predominant in the upper GI tract[33,34] (Figure 5*C* and *E*, Supplementary Figure 6*B*, and Supplementary Tables 17 and 18). As many of these species overlapped with those associated with PPIs, we excluded PPI users from the analysis, and confirmed the consistent associations, suggesting that polypharmacy-induced alteration was not driven by PPI users (Supplementary Figure 7*A*). The robustness of the associations was further verified in several subgroups stratified by age and sex (Supplementary Figure 7*B–E*). The number of drugs taken was also positively associated with abundance and prevalence of several pathobionts (Figure 5*D* and Supplementary Figure 6*C* and *D*).

The number of drugs taken had significant associations with microbial functions (Supplementary Tables 19 and 20), including positive associations with Kyoto Encyclopedia of Genes and Genomes modules (MOs) for C5 isoprenoid biosynthesis (M00095), cationic antimicrobial peptide resistance (M00725), and the osmoprotectant transport system (M00209) (Figure 5*F*), some of which could be involved in bacterial stress response.[35,36] Pathway enrichment analysis showed that the number of drugs taken was positively associated with various transporters in the phosphotransferase system and with ATP-binding cassette transporters, and negatively associated with porphyrin and chlorophyll metabolism and with biosynthesis of amino acids (Figure 5*G*). Contrarily, the number of drugs was negatively associated with KOs for the biosynthesis of amino acids (K01089: histidinol-phosphatase, K01755; argininosuccinate lyase, and K01915; glutamine synthetase), and with a KO for butyrate biosynthesis (K00929: butyrate

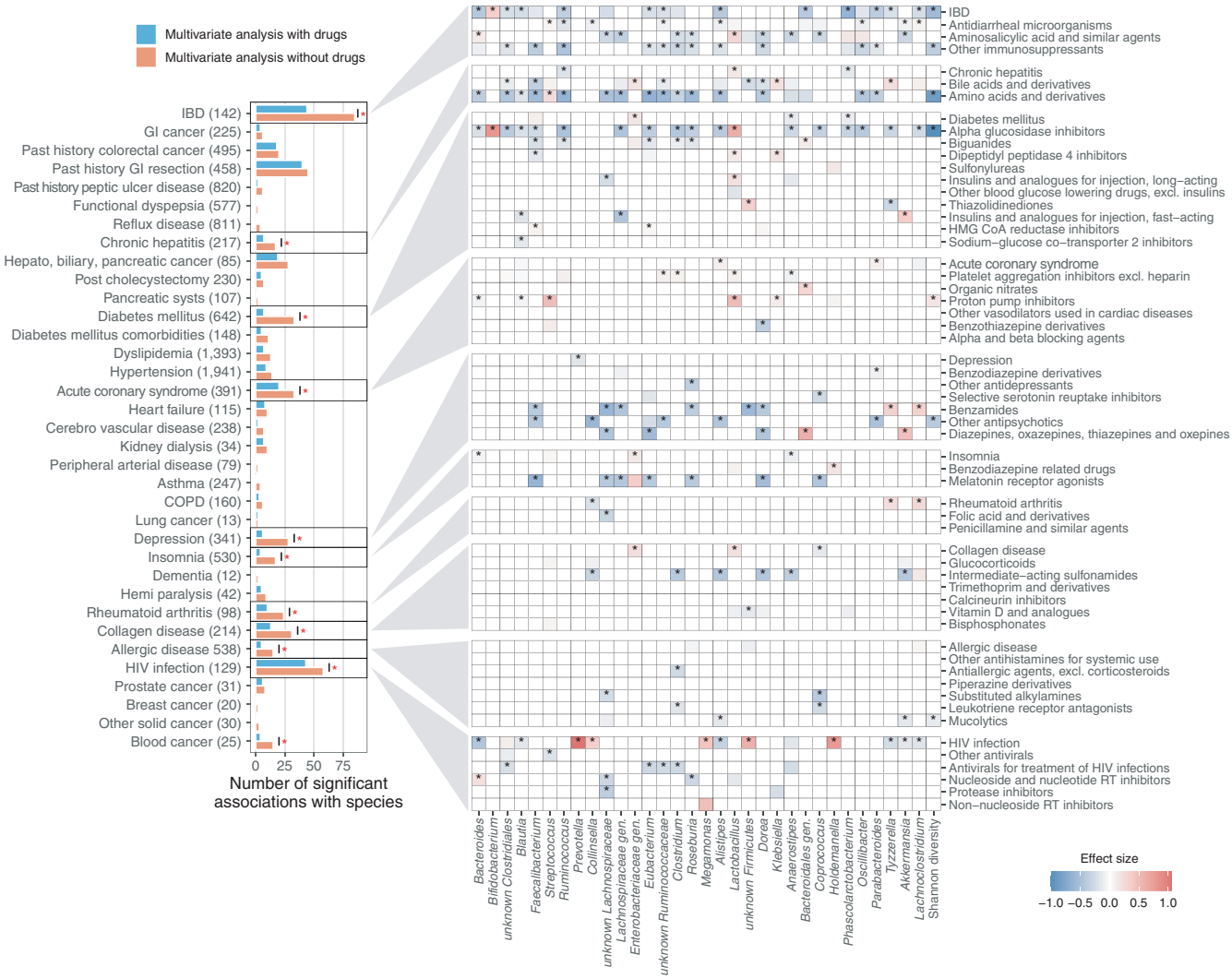


Figure 3. Disentangling effects of diseases and drugs on the gut microbiome. Associations between diseases and each microbe were evaluated with multivariate regression models with (*blue bar*) and without (*orange bar*) drug information. The *left bar plot* shows the number of significant associations with the species detected by each model ($P < .05$). No correction for multiple testing was performed here because only variables with false discovery rate <0.05 in the univariate regression analysis were included in the multivariate regression analysis. *Asterisks* represent significant differences in the number of associations detected between the models ($P < .05$, Fisher's exact test). Thirty-five current and past diseases in the cohort were included in this analysis. The heatmap shows the associations of the 10 diseases and drugs for the disease treatments with the gut microbiome. The 10 diseases that showed more significant associations with the gut microbiome in the model without drug information are selected. Blood cancer was excluded from the heatmap because no specific drugs for the disease were included in this cohort. COPD, chronic obstructive pulmonary disease; HIV, human immunodeficiency virus; IBD, inflammatory bowel disease.

kinase), which was consistent with the significant depletion of butyrate-producing *Clostridia* (Figure 5*E*). These results suggest a substantial impact of polypharmacy on the functional profile of the gut microbiome.

Given the significant positive correlations between the number of drugs taken and underlying diseases (Figure 5*H*), we examined the confounding effects of the number of diseases in polypharmacy-microbe associations. Multivariate analysis revealed that the numbers of drugs and active diseases had distinct effects on the gut microbiome (Figure 5*I*); for example, the number of drugs retained significant associations with *Streptococcus* and *Lactobacillus*, whereas the number of active diseases was significantly associated with *Klebsiella, Streptococcus, Lactobacillus,* and *Oscillibacter*. The data in general suggest that the polypharmacy-microbe associations identified previously are mostly independent of the number of diseases.

Dynamics of the Gut Microbiome in Response to Drug Initiation and Discontinuation

To further examine effects of drug intake and cessation, we analyzed 486 fecal samples collected at 2 points from 243 individuals, of whom some individuals started or discontinued drug intake after the first sampling (Supplementary Table 21). Comparative analysis across the

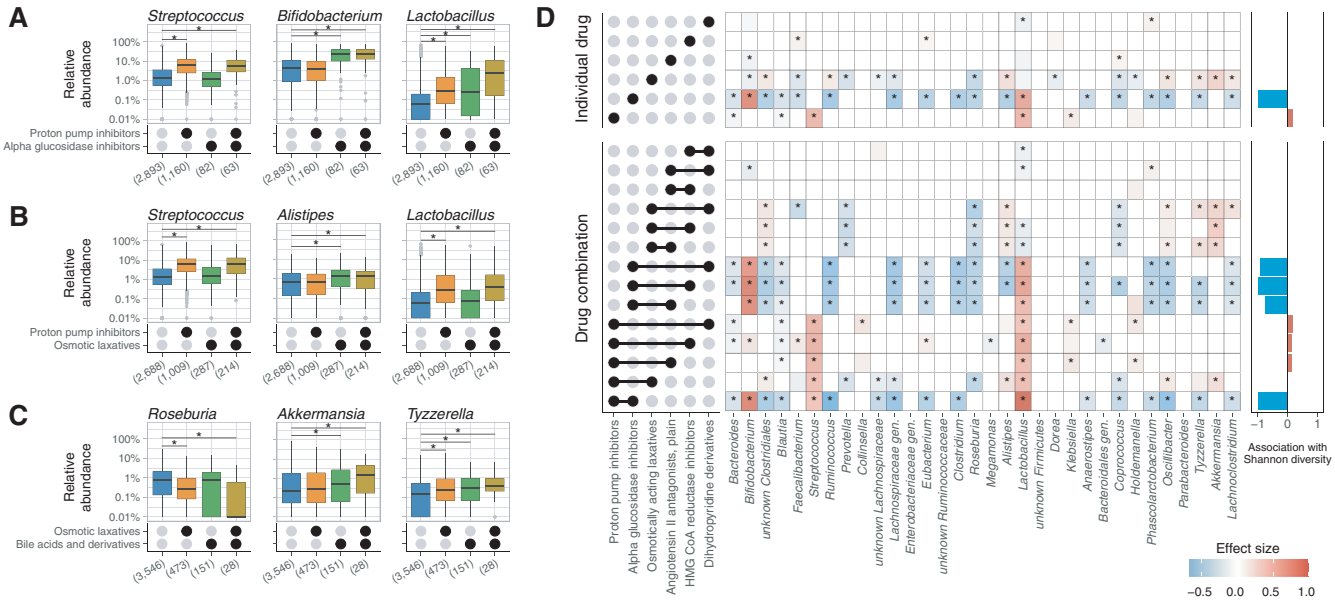


Figure 4. Additive effect of drugs on the human gut microbiome. (A–C) Significant associations between drug pairs and the human gut microbiome. *Box plots* showing the relative abundances of selected genera. *Black circles* below the plots denote the drug taken. Numbers in parentheses indicate the number of individuals taking the drug or drug pair. *Asterisks* indicate significant associations of the drug or drug pair, as determined by multivariate regression analysis ($P < .05$). No correction for multiple testing was performed here because only variables with false discovery rate <0.05 in the univariate regression analysis were included in the multivariate regression analysis. (*D*) Associations between drug combinations at the ATC 4th level and the gut microbiome. Heatmap represents the effect sizes of the drugs obtained from multivariate regression analysis. *Red* and *blue* indicate positive and negative associations, respectively. *Asterisks* denote significant associations ($P < .05$). *Black circles* indicate the drug taken, and *2 black circles connected by a line* show a combination. The 6 upper rows show the results for single drug use, and the 15 lower rows show the results for combinations. The *bar on the right* shows significant associations with the Shannon index. Analysis included the 3 drugs (eg, PPIs, AGIs, and osmotic laxatives) that showed the strongest associations with the gut microbiome (Figure 1*D*) and the 3 most prevalent drugs (eg, angiotensin II antagonists plain, hydroxy-methyl-glutaryl coenzyme reductase inhibitors, and dihydropyridine derivatives) at the ATC 4th level (Supplementary Table 1). The pair of AGIs and osmotic laxatives was excluded from this analysis because of the substantially smaller number of individuals taking this pair (n = 13) than the other pairs (n > 50).

time points revealed that starting PPI therapy drastically changed the microbiome (Figure 6*A*). The altered microbiome showed significant increases in *Streptococcus* and *Lactobacillus* (generalized fold change [gFC] = 0.43 and 0.46, respectively, Figure 6*B*, Supplementary Methods), consistent with the cross-sectional analysis (Figure 2*A*). Notably, the 2 genera were significantly decreased, and were restored almost to the baseline level after discontinuing PPI intake (gFC = −0.38 and −0.57, respectively, Figure 6*B*). Moreover, several pathobionts were significantly enriched after PPI intake, whereas they were depleted after cessation (Figure 6*C*). These results indicate that PPIs are a causative factor that alters the gut microbiome, and that the altered microbiome has the resilience to return to its original state after cessation of the drug. Similarly, a comparison of samples taken before and after osmotic laxatives and P-CAB administration showed a significant increase in *Ruminococcus* and *Streptococcus*, respectively (gFC = 0.37 and 0.47, respectively, Supplementary Figure 8*A* and *B*), validating the alteration observed in the cross-sectional analysis (Figure 2*A*).

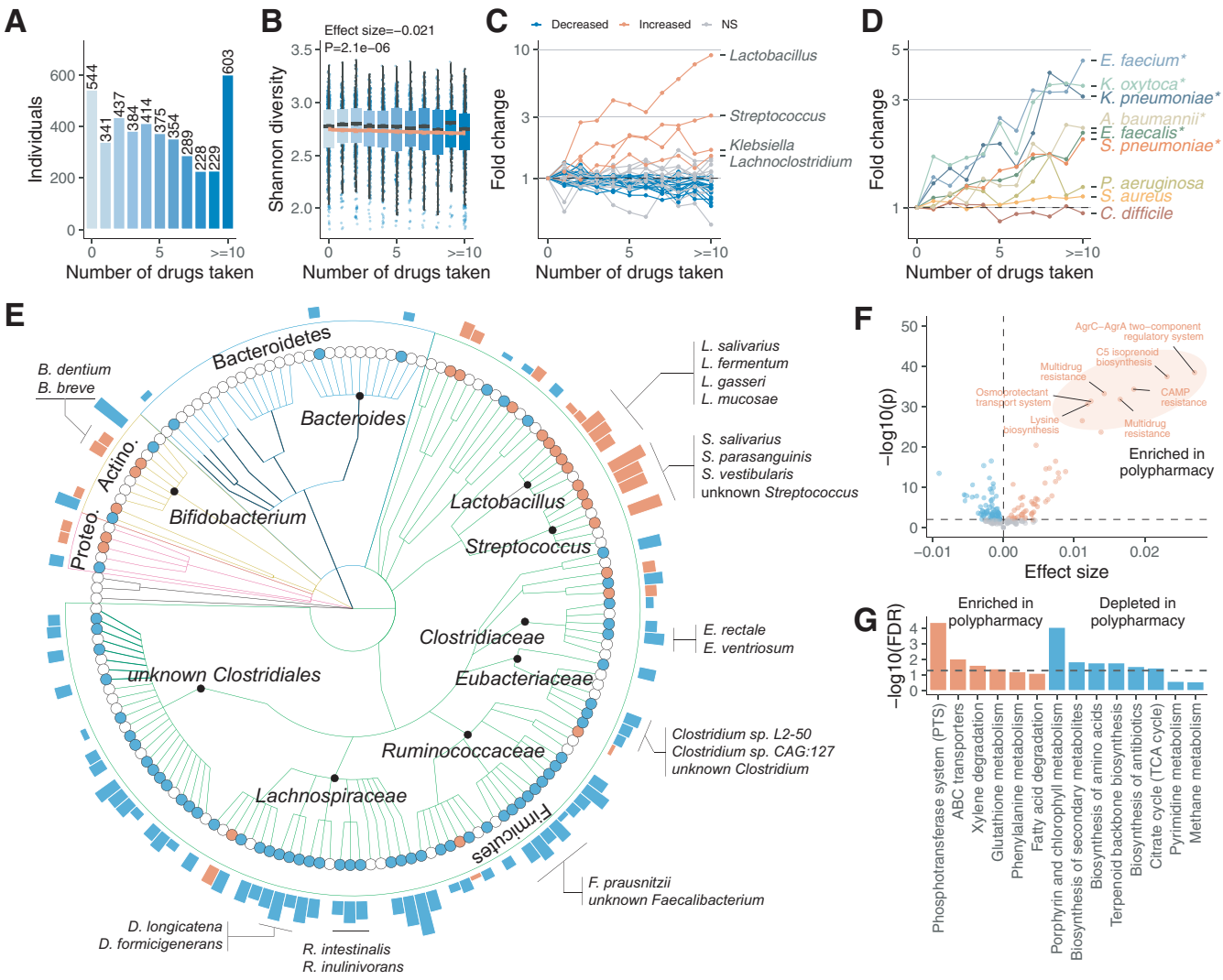
Focusing on the number of drugs taken, we found significant enrichments of *Streptococcus* and *Lactobacillus* in the second samples of individuals who increased the number of drugs taken after the first sampling (gFC = 0.22 and 0.20, respectively, Figure 6*D*). Contrarily, the 2 genera were slightly but significantly depleted in the second samples of individuals who decreased the number of drugs taken (gFC = −0.25 and −0.13, respectively, Figure 6*D*). As PPI intake also increases *Streptococcus* and *Lactobacillus* abundances, we excluded PPI users at either time point from the analysis, and found that the changes in the 2 genera remained consistent (Supplementary Figure 8*C*). In addition to these 2 genera, several species, such as *Ruminococcus gnavus*, *Enterobacter* sp, and unknown *Clostridiales* (Figure 6*E*), and functions such as C5 isoprenoid biosynthesis (M00095), cationic antimicrobial peptide resistance (M00725), and multidrug resistance (M00717), were significantly associated with increased or decreased drug intake (Figure 6*E*). Overall, these results uncovered the dynamics of the microbiome in response to the initiation and discontinuation of individual drugs and multiple drugs, validating the drug-microbe associations in the cross-sectional analysis (Figures 2 and 5).

Increase in ARGs by Nonantibiotic Drugs and Polypharmacy

Recent in vitro and clinical studies have reported that the abundance of ARGs in the gut microbiome was positively

correlated with some nonantibiotic drugs as well as antibiotic use.[3,12] To explore this further using our dataset, we investigated associations between drug intake and ARGs (Supplementary Tables 22–25). The most abundant ARGs in our dataset were those against tetracyclines and macrolides and those of the major facilitator superfamily and the resistance-nodulation-cell division superfamily of efflux pumps (Figure 7*A*, Supplementary Figure 9*A* and *B*). Redundancy analysis revealed that medication was the strongest factor influencing the gut resistome, explaining the largest variance in the ARG profile in the gut (the gut resistome; Figure 7*B*). Among the medication categories, "anti-infectives for systemic use" (eg, antibiotics) accounted for the largest variation in the gut resistome (Figure 7*C*), even though it was only the third most important category for explaining the variation in the taxonomic and functional profiles of the microbiome, following alimentary tract and diabetes drugs (Figure 1*B*).

Multivariate analysis revealed that 49.7% (74 of 149) of nonantibiotics and 70.0% (7 of 10) of antibiotics at the ATC



4th level were significantly associated with at least one ARG (Supplementary Table 22). In addition to antibiotics, nonantibiotic drugs, such as osmotic laxatives, bile acids and derivatives, nervous system drugs, and melatonin receptor agonists, showed significant positive associations with ARGs (Figure 7*A* and Supplementary Table 22). Similar findings were reported in a recent clinical study,[3] exemplified by positive associations among tetA, tetB, and mel with PPI intake, and mdtC, emrY, and PmrF with metformin intake (Supplementary Tables 24 and 25). We also found a significant positive association between the number of drugs taken and the total abundance of ARGs (Supplementary Tables 26 and 27), particularly with those against aminoglycosides and β-lactams, including several efflux pumps, such as the major facilitator superfamily and ATP-binding cassette transporters (Figure 7*E* and Supplementary Figure 9*C*). This significant positive association was also observed regardless of age (Figure 7*F*). Collectively, these results show that a wide range of nonantibiotic drugs are associated with the alteration of the human gut resistome.

Discussion

In the Japanese 4D (Disease, Drug, Diet, Daily life) cohort study, we have unveiled extensive effects of individual and multiple drugs (polypharmacy) on the gut microbiome, using both a large-scale metagenomic dataset collected from 4198 deeply phenotyped individuals and a 2-time points dataset collected before and after taking drugs. The strength of this study is that we assessed 759 drug types, a substantially larger number than those of previous studies.[1–4] More than 70% drugs we profiled had not previously been examined before, which enabled us to reveal numerous previously unknown associations for individual drugs and multiple drugs that previous studies could not cover (Figure 1).

Our results provided several novel insights regarding drug-microbiome interactions. First, we revealed various drugs with relatively large effects on the microbiome, as well as drugs with small effects, not only among drug categories but also within categories (Figures 1 and 2). For example, large effects were associated with PPIs, AGIs, and osmotic laxatives, some of which were consistent with previous studies,[1–4] whereas those with quite small effects included glucagon-like peptide-1 analogues, vitamin B12, and bisphosphonates (Supplementary Table 6). Considering that drugs with large effects substantially change gastrointestinal physiologies and nutrient profiles, their large effects on the microbiome could be caused by such physiological changes in the gut. We also revealed both similarities and dissimilarities of drug-microbe associations even among closely related drugs (Figure 2), some of which (eg, acid suppressants and laxatives) were explained by drug mechanisms of action. This result also suggests that these drugs affect the gut microbiome indirectly through enacting changes in the host's physiology, rather than directly contacting and inhibiting growth of microbes in the gut. Second, we found that even nonantibiotic drugs were significantly associated with pathobionts that are known to be responsible for nosocomial infections (Figure 2), which increase morbidity and mortality of patients.[30] PPIs were one of the drug types that showed strongest positive associations with these pathobionts.[29] Given the positive correlations of the pathogens with various nonantibiotic drugs with different mechanisms of action, reduced colonization resistance of the gut microbiome might be partly responsible for this result.[6,37] Because nosocomial infections present serious health and economic burdens, elucidating the mechanisms of the interaction will be necessary in future studies. Third, we demonstrated that drugs substantially confound the disease-microbe associations (Figure 3). This is, to the best of our knowledge, the first comprehensive assessment including a wide range of various drugs and diseases, and it substantially expands knowledge for confounding effects of drugs shown in previous small-scale studies.[31] Numerous disease-microbe associations were not significant when drug treatment was taken into account, underscoring the need to control for the drug treatment in order to detect true disease-microbe associations.

Figure 5. Significant alteration of the gut microbiome of individuals taking multiple drugs. (*A*) The number of individuals according to the number of drugs taken in this cohort (N = 4198 in total). Individuals taking more than 10 drugs were grouped into the category ≥10. (*B*) Association between the number of drugs taken and Shannon diversity. (*C, D*) Association between number of drugs taken and the abundance of the top 32 abundant genera (average relative abundance > 0.5%) and 9 pathobionts. Each *line* represents fold changes of their abundances compared with the average abundance in individuals without any drugs. *Orange* and *blue* colors show significant increases and decreases, respectively (*C*). *Asterisks* after the species name represent significant positive associations ($P < .05$) determined by multivariate regression analysis (*D*). No correction for multiple testing was performed here because only variables with false discovery rate <0.05 in the univariate regression analysis were included in the multivariate regression analysis. (*E*) Association between polypharmacy and species-level taxonomy. Phylogenetic tree represents the taxonomy of the 158 species whose average relative abundance was >0.1% in the cohort. From the center out, *circles* in the tree represent phylum, class, order, family, genus, and species-level taxonomies. *Orange* and *blue circles* and *bars* outside the tree represent significant positive and negative associations with the number of drugs taken, respectively. The bar height shows the effect sizes. (*F*) Association between the number of drugs taken and MOs assessed by multivariate regression analysis. (*G*) Pathway enrichment analysis for the significantly associated KOs. *Orange* and *blue bars* represent enrichment of the pathways in positively and negatively associated KOs with the number of drugs taken. Pathways with $P < .05$ are shown (Fisher's exact test). (*H*) Positive correlation between the number of active diseases and drug intake for the individuals in this cohort. (*I*) Heatmap showing the associations between the genera and number of drugs taken and active diseases, obtained by multivariate regression analysis controlling for other metadata. *Asterisks* show statistical significance ($P < .05$).

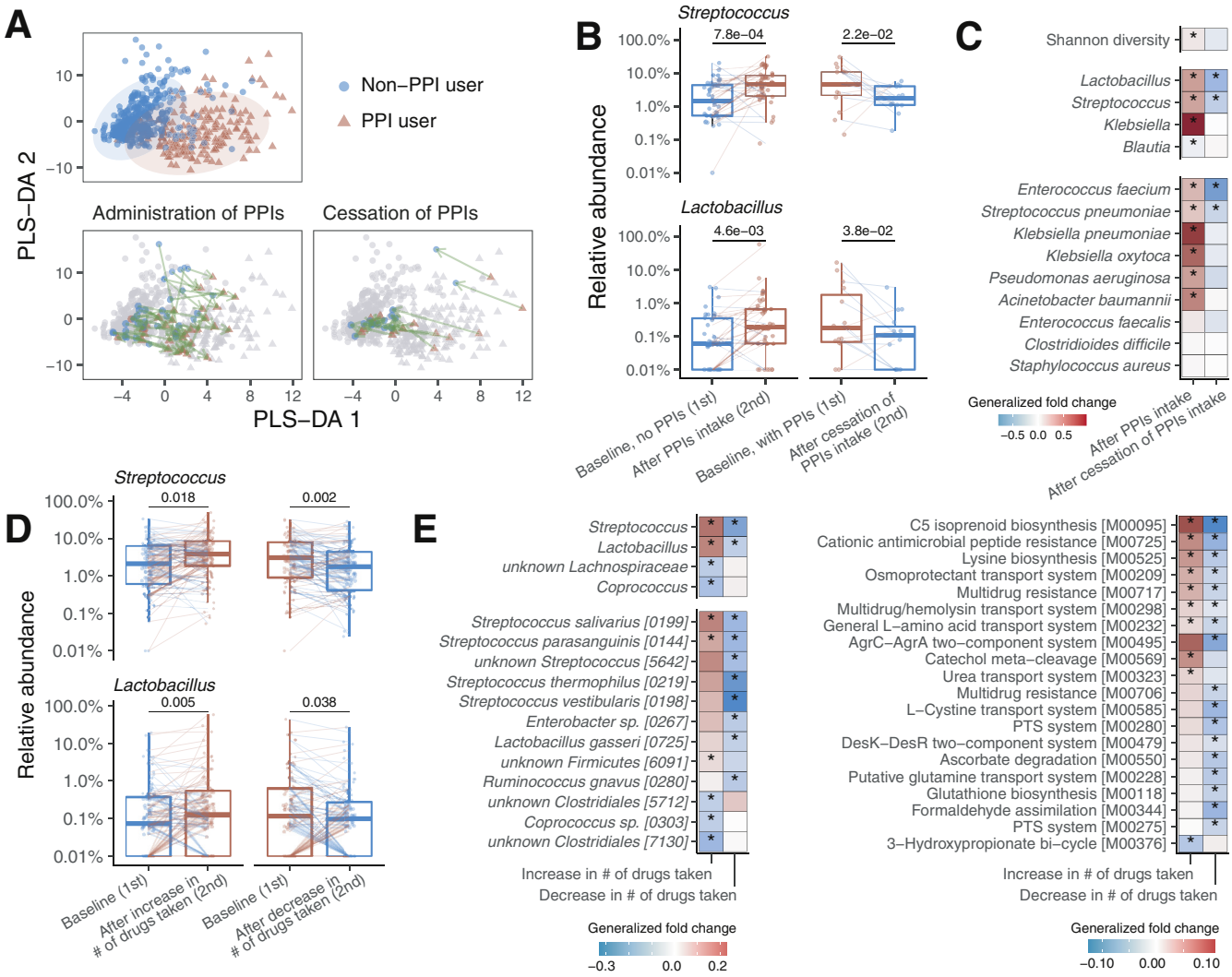


Figure 6. A 2-time points analysis of the gut microbiome after drug initiation and discontinuation. (*A–C*) Alteration of the microbial composition of individuals who started taking PPIs (n = 34) and stopped PPI intake (n = 15) after the first sampling. (*A*) Scatter plot of the 2-time points samples collected from the same individuals at the 2-time points (n = 243). *Red* and *blue colors* represent samples with and without PPI treatments at the time of sampling, respectively. The x- and y-axes show scores obtained from partial least squares-discriminant analysis to separate the microbial profiles of PPI users and nonusers. *Green arrows* show changes in the microbial profiles of each individual after taking PPIs or stopping PPI intake. (*B*) *Boxplots* showing changes of relative abundance of *Streptococcus* and *Lactobacillus* after taking PPIs or stopping PPI intake. Samples from the same individuals are connected by lines between the boxplots, in which *red* or *blue* coloring represents an increase or decrease, respectively. (*C*) *Heatmap* showing generalized fold changes of Shannon diversity and abundance of microbial taxonomies after taking PPIs or stopping PPI intake. *Asterisks* represent *P* values $< .05$. (*D, E*) Alteration of the microbial composition of individuals who increased (n = 102) or decreased (n = 90) the number of drugs taken after the first sampling. (*D*) *Boxplots* showing changes of relative abundances of *Streptococcus* and *Lactobacillus* after increasing or decreasing the number of drugs taken. (*E*) Heatmap showing generalized fold changes in abundances of genera, species, and MOs after increasing or decreasing the number of drugs taken. *Asterisks* represent *P* values $< .05$ (paired Wilcoxon rank-sum test). PTS, phosphotransferase system.

Intriguingly, our results showed strong effects of multiple drug use on the gut microbiome (Figure 5), potentially through additive effects of each drug taken (Figure 4). The gut microbiome altered by polypharmacy was characterized by a significant increase in *Streptococcus* and *Lactobacillus* spp and depletion of Shannon index and short-chain fatty acid producers (Figure 5). The significant decrease in genes for butyrate production may shift the gut into an anaerobic and oxidative environment that favors the growth of certain facultative anaerobes, including pathobionts (Figure 5*D*).[38] Given the diversity and variation of pharmacological effects on host physiologies, such as altering the GI tract environment (eg, pH and transit time)[39] or the intestinal integrity and permeability,[40] or by directly inhibiting bacterial growth,[12] the effects of individual drugs are likely to affect the gut microbiome cumulatively. Several studies have reported that microbial dysbiosis induced by drugs leads to various adverse events like diarrhea, enteric infections, and

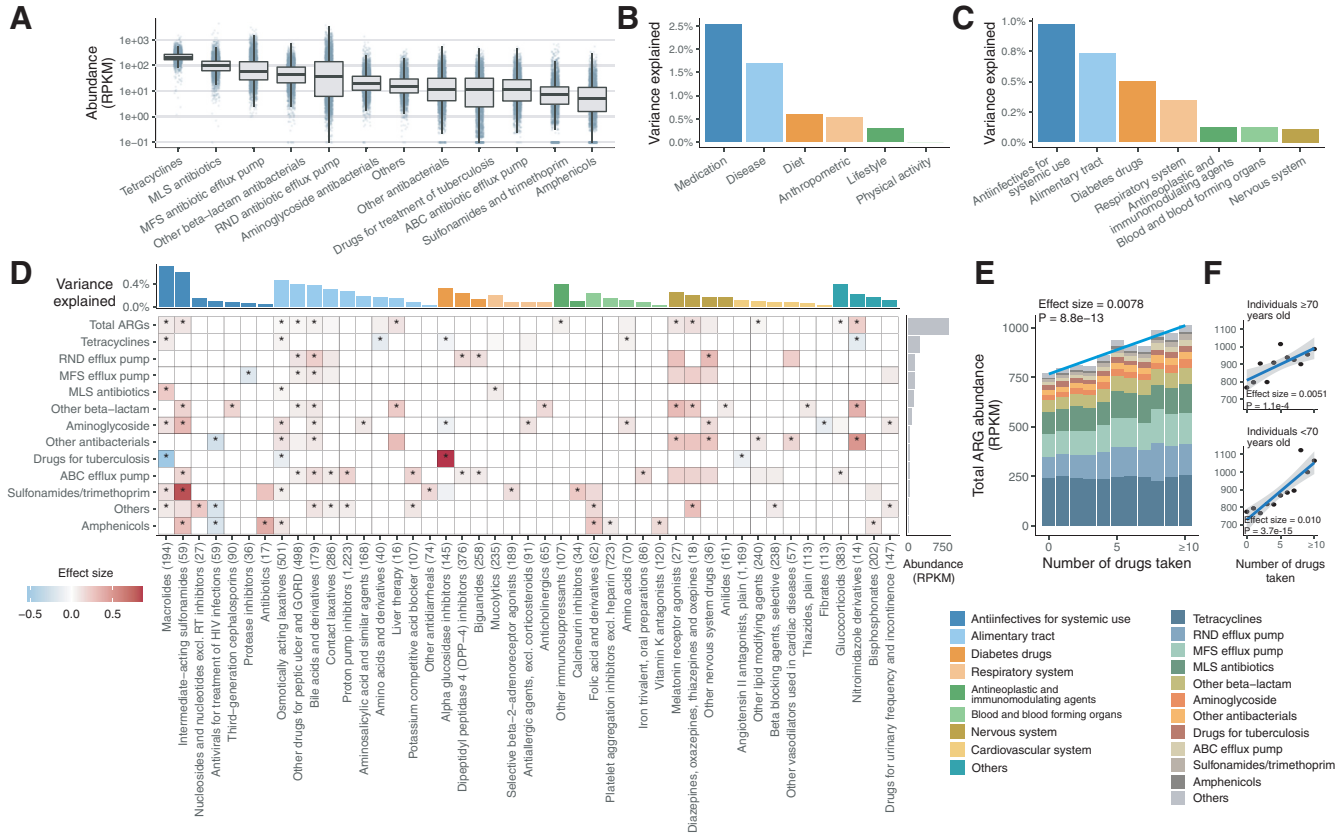


Figure 7. Extensive effects of medications on the human gut resistome. (*A*) *Barplot* showing the variation of abundances of ARGs in the human gut microbiome (N = 4198). (*B, C*) *Bars* showing the variance of the gut resistome explained by metadata categories (*B*) and by medication categories at the ATC 1st level (*C*). Stepwise redundancy analysis was performed to estimate the cumulative effect sizes for each category. (*D*) Significant associations between ARGs and drugs at the ATC 4th level. *Heatmap* shows the effect sizes of each drug on the gut resistome. *Asterisks* denote statistical significance ($P < .05$) obtained from multivariate regression analysis. *Numbers in parentheses* indicate the number of individuals taking the drug. (*E*) Significant positive correlations between the total abundance of ARGs and the number of drugs taken. *Bar plot* shows the average abundance of ARGs in each group of individuals. The *blue line* in the plot indicates the regression line. (*F*) Scatter plots represent associations between the number of drugs taken and average ARG abundance in individuals ≥70 years old and those <70 years old. HIV, human immunodeficiency virus; MFS, major facilitator superfamily; MLS, macrolide, lincosamide and streptogramin; RND, resistance-nodulation-division; RPKM, reads per kilobase million; RT, reverse transcriptase.

GI mucosal injury.[41] Considering the rapid increase of polypharmacy worldwide,[8] further studies will be needed to investigate the consequences of polypharmacy-induced dysbiosis.[8,9]

Notably, the 2-time points dataset collected from the same individuals before and after drug treatments revealed the dynamics of gut microbiome alteration and recovery due to initiation and cessation of individual drugs (PPIs, P-CAB, and osmotic laxatives) and of multiple drugs (Figure 6). These results highlight the importance of reducing the number of patient prescriptions as a way to restore the gut microbiome, which potentially contributes to reduction of medical costs and adverse events resulting from overprescription. In fact, up to 70% of PPI administration was reported as overprescription,[42] whereas 37.5% of prescriptions in people aged ≥65 years were potentially inappropriate medications, to be used with caution using the Beers criteria.[43]

In addition to antibiotic drugs, our metagenomic data revealed strong associations of human-targeted drugs with the human gut resistome (Figure 7). Particularly, the number of drugs taken was positively correlated with the total abundance of ARGs (Figure 7*E*). Previous studies have shown that human-targeted drugs could reach the gut and cause stress to bacteria and that bacterial resistance mechanisms partly overlap with those for antibiotics.[12,44] Although we cannot exclude the possibility that previous exposure to antibiotics, which we could not fully assess, may have a long-term effect on the gut microbiome, our results imply that even nonantibiotic drugs select ARGs in the human gut that provide competitive advantages to the bacteria. To demonstrate the causal relationships, other animal experimental models will be necessary in the future.

Although multivariate analysis accounting for potential confounders was a strength in this study, there are some information biases. For example, the dietary questionnaire includes recall bias because the subject is asked to recall information from a month ago. Some disease information is collected from electronic medical records, which may have an observer bias from the collector.

In summary, this large-scale cross-sectional dataset, coordinated with the prospective 2-time points dataset, comprehensively assessing metadata and metagenomic data, reveals extensive effects of individual drug and multidrug exposures on the ecology of the gut microbiome, providing emphasis to the matter of reducing overprescription. We anticipate that the drug-microbe associations identified in this study could serve as a catalog, forming a basis for future pharmacomicrobiomics to improve therapeutic effectiveness and reduce adverse drug events that may result from modifications to the gut microbiome.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2022.06.070.

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Suguru Nishijima, PhD (Conceptualization: Equal; Formal analysis: Lead; Methodology: Lead; Software: Lead; Supervision: Lead; Visualization: Lead; Writing – original draft: Lead; Writing – review & editing: Lead).

Tohru Miyoshi-Akiyama, PhD (Formal analysis: Lead; Methodology: Lead).

Yasushi Kojima, MD, PhD (Data curation: Supporting; Funding acquisition: Lead; Writing – original draft: Supporting; Writing – review & editing: Supporting).

Moto Kimura, PhD (Funding acquisition: Supporting; Writing – original draft: Supporting; Writing – review & editing: Supporting).

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Masahira Hattori, PhD (Conceptualization: Supporting; Formal analysis: Supporting; Methodology: Supporting; Supervision: Supporting; Writing – original draft: Supporting).

Conflict of Interest
The authors disclose no conflicts.

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Update

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Correction

Nagata N, Nishijima S, Miyoshi-Akiyama T, et al. Population-level Metagenomics Uncovers Distinct Effects of Multiple Medications on the Human Gut Microbiome. Gastroenterology 2022;163:1038–1052.

In the above article, there was an error in Figure 2A where some significant associations were missing due to a bug in the code to create the figure. The corrected figure is shown below.

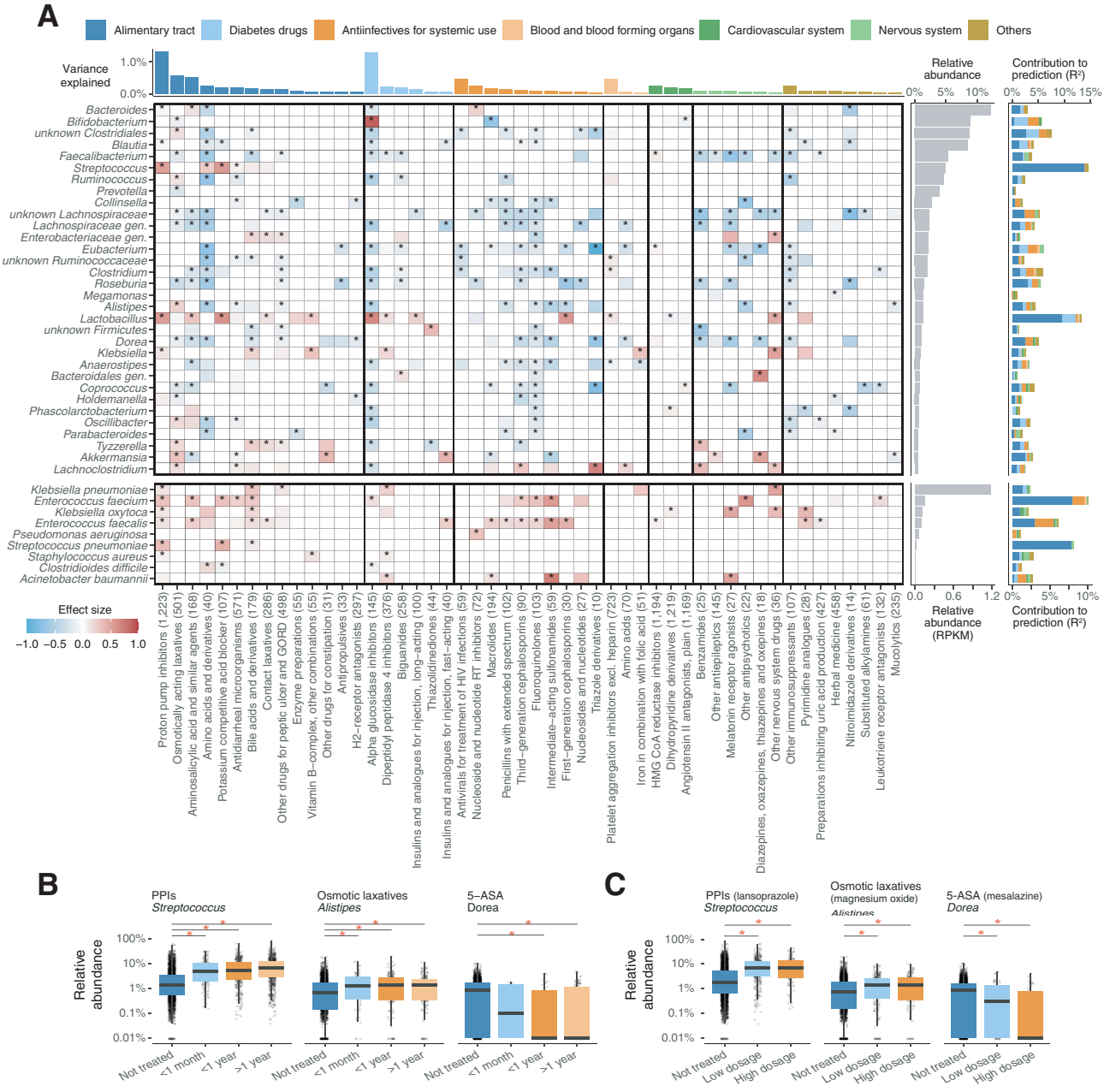

Figure 2.