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The gut metagenome harbors metabolic and antibiotic resistance signatures of moderate-to-severe asthma

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Abstract

Asthma is a common allergic airway disease that has been associated with the development of the human microbiome early in life. Both the composition and function of the infant gut microbiota have been linked to asthma risk, but functional alterations in the gut microbiota of older patients with established asthma remain an important knowledge gap. Here, we performed whole metagenomic shotgun sequencing of 95 stool samples from a cross-sectional cohort of 59 healthy and 36 subjects with moderate-to-severe asthma to characterize the metagenomes of gut microbiota in adults and children 6 years and older. Mapping of functional orthologs revealed that asthma contributes to 2.9% of the variation in metagenomic content even when accounting for other important clinical demographics. Differential abundance analysis showed an enrichment of long-chain fatty acid (LCFA) metabolism pathways, which have been previously implicated in airway smooth muscle and immune responses in asthma. We also observed increased richness of antibiotic resistance genes (ARGs) in people with asthma. Several differentially abundant ARGs in the asthma cohort encode resistance to macrolide antibiotics, which are often prescribed to patients with asthma. Lastly, we found that ARG and virulence factor (VF) richness in the microbiome were correlated in both cohorts. ARG and VF pairs co-occurred in both cohorts suggesting that virulence and antibiotic resistance traits are coselected and maintained in the fecal microbiota of people with asthma. Overall, our results show functional alterations via LCFA biosynthetic genes and increases in antibiotic resistance genes in the gut microbiota of subjects with moderate-to-severe asthma and could have implications for asthma management and treatment.

Introduction

Asthma is a common respiratory disease characterized by symptoms of airway obstruction including wheeze, cough, and shortness of breath. In most cases, asthma onsets in early childhood with the development of sensitization to environmental allergens. Ongoing environmental exposures lead to airway inflammation and ultimately result in asthma symptoms manifesting within the first few years of life. Recent findings support the notion that asthma develops in association with the human gut microbiome composition early in life (Arrieta et al. 2015, Hufnagl et al. 2020). This finding is supported by 16S rRNA sequencing surveys demonstrating that alterations in the gut microbiota precede asthma development within the first few months of life (Arrieta et al. 2015, Fujimura et al. 2016).

Early childhood gut microbial communities have been proposed to contribute to asthma by several mechanisms. Epoxide hydrolases encoded by enterococci and other gut bacteria produce the lipokine 12,13-diHOME that predisposes toward atopic sensitization and asthma (Fujimura et al. 2016, Levan et al. 2019). Similarly, short-chain fatty acids (SCFAs), produced by the metabolism of dietary fibers by diverse members of the gut microbiota, are thought to protect from asthma through their effect on the host G-protein coupled receptor GPR41, shaping immune cell differentiation in the lungs, and ameliorating allergic airway inflammation (Trompette et al. 2014, Arrieta et al. 2015, Zaiss et al. 2015, Cait et al. 2018, Roduit et al. 2018).

In addition to microbially encoded metabolic features, carriage of antibiotic resistance genes (ARGs) within the gut microbiota, termed the resistome, has been associated with asthma risk. In infants, microbial signatures associated with the development of asthma are also associated with increased richness of ARGs in the gut microbiome (Li et al. 2021). These differences in ARG carriage were found to be driven primarily by *Escherichia coli*, which is a common colonizer in the first days of life (Li et al. 2021). These findings are important in understanding the origins of asthma since antibiotic exposure correlates both to the number of ARGs within the gut microbiome (Ramirez et al. 2020) and the later development of asthma and other allergic diseases (McKeever et al. 2002, Kozyrskyj et al. 2007, Hoskin-Parr et al. 2013). This association between antibiotic exposure and asthma is supported by animal models that found antibiotic treatment worsens allergic airway inflammation (Russell et al. 2013, Yang et al. 2019, Borbet et al. 2022).

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While there is an abundance of data supporting the idea that asthma susceptibility is associated with features of the gut microbiota in early childhood, the potential effect of gut microbial functions on asthma later in life remains an important knowledge gap. Since asthma often begins in infancy when the gut microbiota composition is highly unstable, disease-causing microbial functions may not persist into older children and adults. Nevertheless, the gut microbiota in older individuals could underlie the variable manifestations of asthma (Wenzel 2012) and may hold valuable prognostic and therapeutic significance.

Asthma-associated differences in later childhood and adult gut microbial communities have already been noted in several reports. Studies in preschool-aged children have noted distinct taxonomic composition of gut microbial communities in subjects with asthma compared to healthy controls (Hufnagl et al. 2020). These differences are reported to include reductions in *Akkermansia muciniphila* (Michalovich et al. 2019), *Faecalibacterium prausnitzii* (Wang et al. 2018) as well as *Roseburia* species (Chiu et al. 2019). Functional characterization of microbial communities by whole metagenomic sequencing from an older population of women with asthma (Wang et al. 2018) has shown that pathways related to lipid and amino acid metabolism, as well as carbohydrate utilization were enriched compared to healthy controls. In contrast, microbial pathways involved in the production of SCFAs, like butyrate, were enriched in the healthy cohort of the same study (Wang et al. 2018). These findings are supported by a complementary study designed to test the effect of probiotic supplementation on asthma that found an association of improved asthma symptoms with SCFA biosynthesis as well as tryptophan metabolism pathways in the adult gut microbiota (Liu et al. 2021).

Here, we describe an analysis of whole metagenomic sequencing data from a cohort of 36 subjects with physician-diagnosed, moderate–severe asthma along with a matched cohort of 59 healthy controls. This study tests the hypothesis that the gut metagenome harbors signatures of asthma after the disease has been established. Our results identify global differences in metagenomic functions between the asthma and healthy cohorts and reveal an enrichment in the asthma cohort for long-chain fatty acid (LCFA) biosynthesis pathways. We also find increased richness of ARGs associated with asthma and co-occurrence of ARGs with known bacterial virulence factors (VFs), suggesting a potential relationship between antibiotic exposure and pathogen colonization in people with asthma.

Materials and methods

MARS study population

The Microbiome and Asthma Research Study (MARS) consisted of 104 subjects from the St Louis, MO United States area that are either healthy or had physician-diagnosed moderate-to-severe asthma. This study included an adult cohort (ages 18–40 years) and pediatric cohort (ages 6–10 years). As described in previous manuscripts (Jaeger et al. 2020, Wilson et al. 2023), nine patients were disqualified or did not donate stool samples. The remaining 95 patients donated stool samples either at home or at the recruitment visit and were evaluated with a clinical questionnaire to gather relevant metadata. Stool samples were kept at −20°C and delivered within 24 hours to the study site, Kau Lab at Washington University School of Medicine, where they were stored at −80°C for no more than 3 years until processing for DNA isolation. All recruitment, follow up, and sample acquisition occurred between November 2015 and December 2017. This study was approved by the Washington University Institutional Review Board (IRB# 201412035). Identifying information was accessible only to the study principal investigator (A.L.K.) and study coordinators while the study was ongoing, but was not accessible to other members of the study team. Written informed consent documents were obtained from all MARS subjects or their legal guardians.

Fecal DNA isolation

Frozen human stool samples were pulverized in liquid nitrogen using a pestle and mortar. We then homogenized the stool in a mixture of phenol, chloroform, and isoamyl alcohol with a bead beater using sterilized zirconium and steel beads as previously described (Kau et al. 2015) to extract crude DNA. We purified the fecal DNA with a 96-well QIAGEN PCR Clean up kit and quantitated by measuring the absorbance at 260/280 nm. Sample DNA concentrations were normalized to 0.5 ng/ml. Neither depletion of human DNA sequence nor enrichment of microbial or viral DNA was performed. No experimental quantification like a spike-in were used.

Whole metagenomic sequencing of fecal communities

To generate fecal metagenomic sequencing data, we adapter-ligated libraries by tagmentation using an adaptation of the Nextera Library Prep kit (Illumina, catalog number FC-121–1030/1031) (Baym et al. 2015). Individual libraries were then purified with AMPure XP SPRI beads, quantitated using Quant-iT (Invitrogen, catalog number Q33130), and then combined in an equimolar ratio. We confirmed that each library was adequately represented in the combined library by preliminary sequencing on a MiSeq instrument at the Washington University in St. Louis Center for Genome Sciences to assess the evenness of the library. Once the quality of the library was assured, we sequenced the combined library on a NovaSeq 6000 S4 with 2 × 150 bp chemistry to achieve an average of 3.4 Giga-base-pairs (Gb) per sample. NovaSeq services and data demultiplexing were performed by the Genome Technology Access Center at the McDonnell Genome Institute (St Louis, MO). All samples were tagmented simultaneously and sequenced on the same run to avoid batch effects.

Processing of sequencing data

Metagenomic raw demultiplexed reads were then processed to (1) remove spurious human sequences (human reference database was hg37dec_v0.1.1), (2) remove low quality sequences, and (3) trim remaining adapter content using Kneaddata v. 0.10.0 (huttenhower.sph.harvard.edu/kneaddata) bypassing the tandem repeat finder step ("- -bypass-trf"). FastQC (fastqc v0.11.7) and MultiQC (multiqc v1.2) with default settings were used to create quality reports and visualize processing steps. See Figure S1(A) and Table S1 (Supporting Information) for number of reads dropped per processing step. After trimming and filtering, no samples had adaptor content, overrepresented sequences, or an average sequence quality score below Phred 24. Estimated metagenome coverage was calculated with Nonpareil (Rodriguez-R and Konstantinidis 2014, Rodriguez-R et al. 2018) (version 3.4.1) via the online querying tool at http://enve-omics.ce.gatech.edu/nonpareil/submit.

Read-based metagenome profiling

To obtain functional information about the metagenomic contents of fecal samples, we processed samples using HUMAnN (Beghini et al. 2021) v3.0.0 on filtered reads with default param-

eters. The marker gene database used by HUMAnN to identify taxonomic identities was ChocoPhlAn v201901b and the protein database used by HUMAnN to identify functions was the UniRef90 full database v201901b. Alpha diversity analysis of Uniref90 genes and two-sample tests of KEGG orthologs were performed on respective genes that were present (> 0 copies per million) in at least 16 out of 95 samples, which was the lowest prevalence cutoff that would allow for Bonferroni corrected Wilcoxon *P*-values below .0001. HUMAnN was used to determine the abundance of metagenomic pathways by mapping UniRef90 genes to the MetaCyc database. We performed differential abundance analysis using the Wilcoxon 2-sample tests on pathways that had a minimum of 10% prevalence.

To identify ARGs present in the fecal metagenomes of MARS stools, we used ShortBRED-identify (Kaminski et al. 2012) (v0.9.4) with the Comprehensive Antibiotic Resistance Database (CARD; Alcock et al. 2019) (downloaded 2021–07-05 16:10:04.04555) and Virulence Factor Database (Chen et al. 2005) (downloaded Friday July 16 10:06:01 2021). ShortBRED-Quantify was run on the filtered reads with default parameters. ARGs or VFs that had an abundance greater than zero in less than 7 out of 95 samples were excluded from downstream analyses. This prevalence cutoff was determined using the binomial distribution to maintain a 95% confidence that enrichment was not due to random chance (using stats:: binom in R). In the analyses that compared VF profiles to ARG profiles, any gene with the same name was excluded from the list of antibiotic resistance and considered a VF only, to prevent spurious results due to cocorrelations. Only one gene matched this criterion: *ugd* (UDP-glucose 6-dehydrogenase).

Microbial composition was determined with MetaPhlAn 3.0 (Beghini et al. 2021), which is included in the HUMAnN pipeline described (Beghini et al. 2021). MaasLin (Mallick et al. 2021) (Maaslin2_1.5.1) was used in R to find taxa of any taxonomic level that correlated with asthma by setting asthma as a fixed effect and setting age group and race as random effects.

For PERMANOVA analyses, BMI class refers to two stratifications: Nonobese (underweight, healthy, or overweight) and obese determined for adults by BMI cutoffs and for pediatric patients by BMI-for-age percentile as defined by the Centers for Disease Control and Prevention (see cdc.gov/healthyweight/assessing/bmi/childrens_bmi/ about_childrens_bmi.html). Self-reported race was used as a covariate in our PERMANOVAs since precise variables that would better describe the many facets of racism and health disparities were absent (see the section "Study limitations"). Subject-reported race was represented in our models as a dichotomous variable of either "Caucasian" or "Non-Caucasian", with 92% of the latter population having reported as "Black or African–American" and the remaining 8% as "Other". We chose to combine self-reported "Black or African–American" and "Other" populations into a single category of "Non-Caucasian" because there were insufficient numbers of reported "Other" to power a robust analysis of this group but we still wanted to account for potential health disparities associated with non-Caucasian races (Generate Health 2017).

Metagenome assemblies

Filtered reads were assembled into contigs using spades (Bankevich et al. 2012) (v3.14.0) with the "meta" flag and k-mers lengths as follows: -k 21,33,55,77. The resulting scaffolds achieved an average N50 of 3525 +/− 178 bp, an average L50 of 7192 +/− 372, and an average total length of 136.8 +/− 4.5 Mbp as measured by QUAST (v 4.5) (Gurevich et al. 2013, Mikheenko et al. 2016) (see Table S1, Supporting Information). Determination of *ermF* location was performed by aligning the 801-bp coding sequence of *ermF* from CARD (Alcock et al. 2019) to all scaffolds. Scaffolds containing BLAST hits with 98% identity or higher to the full-length CARD *ermF* sequence were further annotated by Prokka (v1.14.5) to find open reading frames and annotate them. Manual BLAST was used to annotate "hypothetical protein" open reading frames for the contexts of *ermF* hits.

Statistics and reproducibility

R version 3.6.3 was used for all analyses downstream of HUMAnN and ShortBRED, and for data visualization. Wilcoxon tests with false discovery rate multiple testing correction or Type II ANOVAs were used to determine statistically significant differences with the car:: Anova package in R. PERMANOVAs were performed in R using the vegan:: adonis package with default settings and 100 000 iterations. The following symbols were used to designate significance: * $P < .05$, ** $P < .01$, and *** $P < .001$ and the following for q-values (FDR-adjusted *P*-values): * $q < 0.2$ and ** $q < 0.05$.

Results

Whole metagenomic shotgun sequencing of fecal samples from adults and children with asthma and healthy controls

We performed whole metagenomic sequencing on fecal samples from subjects with allergic asthma and healthy controls taking part in the MARS, which we have previously described (Jaeger et al. 2020, Wilson et al. 2023). MARS participants were recruited from the St. Louis, Missouri area and included pediatric (6–10 years) and adult (18–40 years) age groups. All asthma cohort patients had a physician diagnosis of moderate-to-severe asthma, and history of allergic sensitization as evidenced by positive skin testing or serum specific-IgE to one or more common aeroallergens. In total, we analyzed 95 patient stool samples including 17 adults and 19 school-aged participants with asthma, and 40 adults and 19 school-aged participants without asthma.

NovaSeq S4 sequencing of our libraries yielded 1.69 billion paired-end reads translating to a total of approximately 500 Gb. After filtering for read quality, dropping host contaminants, and trimming adaptor content, we achieved 1.23 billion paired-end reads and an average 3.4 Gb per stool sample with a range of 0.4–9.9 Gb/sample (Figure S1A, Supporting Information). Neither host contamination nor sequencing depth differed between asthma and healthy cohorts (t-test $P = .2$ and 0.7; Table S1, Supporting Information). All samples achieved an estimated average metagenomic coverage of at 89% (range of 61%–98%) with the annotation-free redundancy-based metagenome coverage estimator, Nonpareil (Rodriguez-R and Konstantinidis 2014) (Figure S1B, Supporting Information). Further, estimated metagenome coverage was not different between the asthma and healthy cohorts, although we noted coverage was slightly reduced in the pediatric cohort (Figure S1B and Table S1, Supporting Information). We employed the read-based annotation pipeline, HUMAnN (Beghini et al. 2021) to determine the abundance of genes and functional pathways in the stool metagenomes. We found that the most abundant functional pathways (Figure S1C, Supporting Information) across all MARS participants are involved in essential processes of gut microbes such as starch degradation and glycolysis, demonstrating that our sequencing captured core functions of the

gut metagenome, as expected. Taken together, we concluded that our sequencing is of sufficient depth and quality to be used for further analyses.

Gut taxonomic composition differs between people with and without asthma

We first leveraged the clade marker annotation tool, MetaPhlAn (Beghini et al. 2021), to analyze the taxonomic composition of the study participants. We found dominate genera typical in gut microbiota communities including *Bacteroides* (phylum Bacteroidota) and *Faecalibacterium* (phylum Bacillota) (Figure S1D, Supporting Information). Simpson alpha diversity was slightly higher in the asthma cohort even when taking read depth and age group into account (Figure S1E, Supporting Information). Bray–Curtis dissimilarity (Figure S1F, Supporting Information) was shifted between the asthma and healthy cohorts ($P < .0004$, $R^2 = 0.029$) even when accounting for other covariates including age ($P < .001$, $R^2 = 0.032$), race ($P = .0006$, $R^2 = 0.026$), recent antibiotic usage ($P = .9$, $R^2 = 0.006$), read depth ($P = .2$, $R^2 = 0.013$), obesity ($P = .7$, $R^2 = 0.008$), sex ($P = .4$, $R^2 = 0.011$), and tobacco exposure ($P = .2$, $R^2 = 0.012$) by sequential PERMANOVA (Figure S1G, Supporting Information). There was also no significant interaction between asthma status and age group ($P = .8$, $R^2 = 0.007$), or between asthma status and recent antibiotic usage ($P = .6$, $R^2 = 0.009$) (Figure S1G, Supporting Information). To determine differentially abundant taxa, we tested the fixed effect of asthma along with the random effects of age group and race in a general linear model (Mallick et al. 2021) and found *Eubacterium rectale* and *Prevotella copri* were enriched in the healthy cohort (Figure S1H and Table S2, Supporting Information). All of these findings are consistent with 16S rRNA sequencing performed in a previous study (Wilson et al. 2023), which lent us further confidence that our sequencing data was suitable for functional profiling.

Fatty acid metabolism pathways are enriched in the gut metagenomes of people with asthma

Given that our samples had adequate coverage to capture expected taxonomic shifts, we started interrogating the differences in metagenomic functions of the gut microbiota attributable to asthma status. The alpha diversity of genes (UniRef90 clusters) was neither different between the asthma and healthy cohorts nor between the pediatric and adult cohorts, suggesting that our gene profiling reached a similar total number of genes in both cohorts (Fig. 1A). Using PERMANOVA, we noted that, even while accounting for significant covariates of age ($P < .001$, $R^2 = 0.029$), race ($P < .001$, $R^2 = 0.024$), and read depth ($P = .03$, $R^2 = 0.015$), asthma status also significantly impacted gut microbiome functional composition ($P = .008$, $R^2 = 0.017$; Fig. 1B and C). We note that age group's interaction term with asthma did not significantly contribute to the variance in beta diversity, suggesting that the influence of asthma and age on beta diversity is nonoverlapping. These findings support the idea that the gut metagenomic content of people with asthma is different than that of healthy individuals, even when accounting for other clinical sources of interpersonal gut microbiome variation.

We next considered which metagenomic functions and metabolic pathways may be involved in the differences between asthma and healthy cohorts. We first examined a list of specific metagenomic functions previously implicated in asthma, including genes related to histamine production, 12–13 diHOME biosynthesis, and tryptophan metabolism, but we were unable to identify a difference between cohorts (Figure S2A, Supporting Information). To identify pathways that differed between asthma and healthy subjects, we performed a Wilcoxon rank-sum test with a false discovery rate $q < 0.2$ on the relative abundance of all pathways annotated by the MetaCyc database that were above 10% prevalence within the population. Using these criteria, we found seven pathways that were enriched in asthma and one that was enriched in the healthy cohort out of 312 total pathways (Fig. 1D). To determine if these findings were robust to other analysis methods, we performed additional differential abundance approaches on the 312 MetaCyc pathways, including a Wilcoxon test on centered log-transformed counts and ALDEX2, both of which demonstrated that these pathways differed between healthy and asthma cohorts (see Table S3, Supporting Information). All differentially abundant pathways enriched in patients with asthma were involved in fatty acid synthesis, and included the production of oleate, palmitoleate, (5Z)-dodecenoate, 8-amino-7-oxononanoate, biotin, and octanoyl acyl-carrier protein, as well as saturated fatty acid elongation. In the healthy cohort, only a single L-lysine biosynthesis pathway was enriched.

Using taxonomically tiered functional mapping, we determined which taxa were driving the observed differences in asthma-associated pathways. For the L-lysine biosynthesis III pathway, which was more abundant in healthy subject, we found that it primarily originated from *Blautia obeum*; Figure S2B, Supporting Information). In the case of the asthma-enriched pathways, we found that *Bacteroides vulgatus* and *Alistipes finegoldii* account for the largest fraction of complete fatty acid biosynthesis pathways (Fig. 1E; Figure S3C, Supporting Information). However, the differential abundance of these asthma-associated pathways was probably not due solely to an enrichment of *B. vulgatus* or *A. finegoldii* in asthma stool since neither species was differentially abundant (maaslin2 q-value = 0.58 and 0.25, respectively; see Table S2, Supporting Information). Further, the majority of mapped pathways were not attributable to any single species and these unmapped pathway counts made up more of the overall pathway richness than *B. vulgatus* (Wilcoxon q-values < 0.05 for all seven pathways; see "Community" stratification in Figure S2C, Supporting Information). Taken together, these findings indicate that the differences may be either driven by community-level effort (i.e. distinct steps of the pathway are encoded across more than one species), or that current databases are insufficiently granular to identify the key taxa responsible for these differences.

We reviewed the enzymatic steps of each of the eight pathways represented in Fig. 1(D) and found that, of the 78 total reactions in these pathways, only 11 reactions were shared between two pathways (Figure S3, Supporting Information). The 8-amino-7-oxononanoate biosynthesis I pathway consists of the first 11 reactions of the larger biotin biosynthesis pathway and the latter only has four additional reaction steps past synthesizing 8-amino-7-oxonanoate to produce biotin. Additionally, the (5Z)-dodecenoate pathway can feed directly into the palmitoleate biosynthesis pathway, and that the octanoyl acyl carrier protein pathway shares an upstream substrate (acetoacetyl-acyl carrier protein) with the saturated fatty acid elongation pathway (Figure S3, Supporting Information). Together, our findings indicate that long chain fatty acid biosynthesis is differentially abundant in the asthma gut metagenome via related but largely nonredundant pathways.

Given the association between obesity with fatty acid metabolism (Brayner et al. 2021) as well as asthma (Scott et al. 2011, Wendell et al. 2014, Mizuta et al. 2019), we next wanted to determine whether obesity (which we define here as a BMI greater than 30 in adults or a BMI-for-age percentile of greater

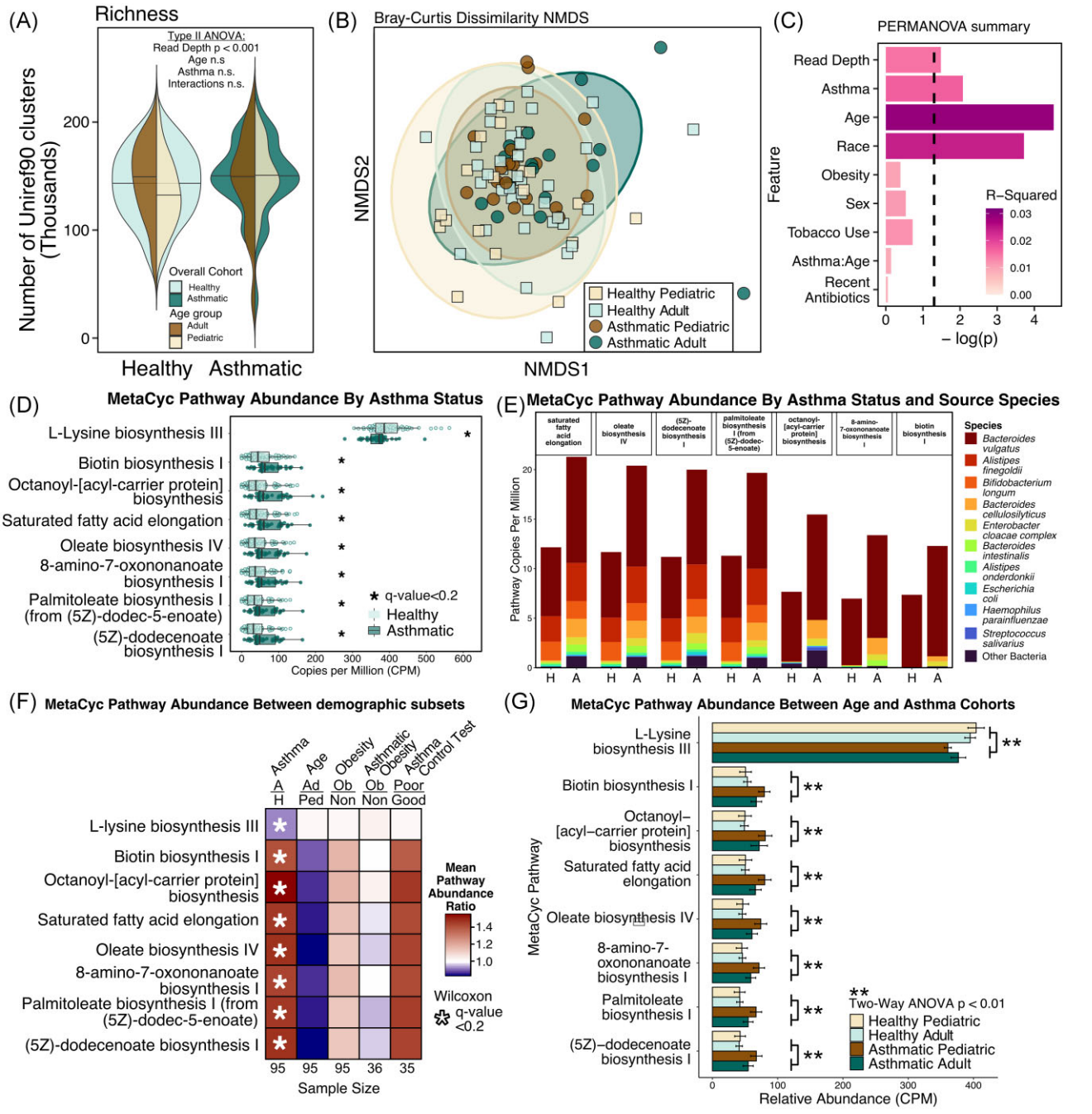


Figure 1. Gut metagenomes from individuals with asthma show increased genes encoding fatty acid metabolism. (A) Stacked violin plots of Uniref90 cluster richness (unique Uniref90 cluster with CPM > 0) grouped by either healthy and asthma cohort (background) or age (foreground). (B) Nonmetric multidimensional scaling plot of Bray–Curtis Dissimilarity distance between Uniref90 (copies per million) profiles. Axis 1 and 2 of five total are shown of an NMDS with stress value 0.09. (C) Sequential PERMANOVA of Bray–Curtis dissimilarities between Uniref90 profiles. Input order of terms to the test is identical to the order of the barplot from top to bottom. (D) Relative abundance of MetaCyc pathways that were differentially abundant given a Wilcoxon q-value below 0.2 (P-value after FDR correction). (E) Stacked bar plot of differentially abundant fatty acid metabolism pathways mapped to respective taxa by MetaPhlAn3.0/HUMAnN3.0, averaged within asthma or healthy cohorts. (F) Heatmap of MetaCyc pathway abundance ratios between groups in important clinical demographics: Asthma vs. Healthy, Adult vs. Pediatric, Obese vs. Non-Obese, and Well-Controlled Asthma vs. Poorly Controlled Asthma. Asterisk denotes a significant differential abundance ($*q < 0.2$) according to Wilcoxon tests controlled for multiple comparison testing within each demographic category. (G) Differentially abundant MetaCyc pathways plotted as four cohorts: asthma by age with respective Two-Way ANOVAs. Only statistically significant P-values shown.

than 95% in children) confounds the association of microbial fatty acid metabolism with asthma. We compared the abundance of the differentially abundant fatty acid pathways between all nonobese and obese patients and found no significant difference (Fig. 1F). Within the asthma cohort, there was similarly no statistically significant difference between the patients with and without obesity, suggesting that obesity is not a confounder for the difference we observed in fatty acid metabolism. To determine whether fatty acid metabolism is related to the intensity of asthma symptoms and their effect on everyday life

activities, we utilized a validated survey of asthma control (The Asthma Control Test; ACT) (Schatz et al. 2006). None of the fatty acid pathways were differentially abundant between patients with well-controlled and poorly controlled asthma (Fig. 1F). We tested if age group affects the differentially abundant metabolic pathways and found that these pathways were not differentially abundant between age groups alone (Fig. 1F). We also tested the impact of asthma and age as independent variables to differentially abundant metabolic pathways using a Two-way ANOVA. We found that, even while taking age into account, these pathways are differentially abundant between asthma and healthy cohorts, but are not different by age or an interaction between asthma and age (Fig. 1G; 2-Way ANOVA). Given that the effect of asthma status on differentially abundant metagenomic functions was distinct from that of age, we primarily focused our subsequent analyses on the asthma and healthy cohorts overall, combining age groups.

Richness of ARGs is increased in the gut metagenomes of people with asthma

Since people with asthma tend to be prescribed antibiotics frequently (Snyder et al. 2021) and oral antibiotic exposure is a risk factor for the acquisition of ARGs in the gut (Ramirez et al. 2020), we wanted to determine if the members of our asthma cohort were more likely to have received antibiotics. To test this, we counted how many subjects had taken a course of antibiotics within 1 year of their participation in the study. As part of the study design, participants could not take antibiotics in the month prior to fecal donation. The median time since antibiotic exposure for those who took antibiotics in the past 1–12 months was 6 months. We found that a greater proportion of the asthma cohort received antibiotics in the past year compared to that of healthy participants (42% of asthma cohort versus 15% of the healthy cohort, Fisher's test, $P = .011$; Fig. 2A). This finding represents evidence of increased antibiotic exposure amongst subjects with asthma in our study.

We next sought to characterize the gut antibiotic resistome in the asthma and healthy cohorts. To test if the increased antibiotic exposure in the asthma cohort was reflected in the gut resistome, we utilized the ShortBRED pipeline (Kaminski et al. 2012) to detect reads mapped to the CARD (Alcock et al. 2019). We first asked whether there were more ARGs in our asthma cohort by summarizing our dataset into richness (total number of unique ARGs detected per sample) and load (total sum of ARG RPKM per sample). We found that ARG richness was higher in people with asthma even when accounting for differences due to age ($P = .03$) and sequencing depth ($P = .09$), while ARG load was not different between asthma and healthy cohorts ($P = .4$) when accounting for age ($P < .001$) and read depth (0.002) (Fig. 2B). We note that *E. coli* was not differentially abundant between asthma and healthy cohorts ($P = .52$; Table S2, Supporting Information), so the richness increase we observe in the asthma cohort is not due solely to an increase in *E. coli* relative abundance. These results suggest that there are a higher number of unique ARGs, or a higher diversity, in asthma compared to healthy controls.

From our 95 stool samples, we detected 71 unique ARGs, comprising 32 antimicrobial resistance families, 29 drug classes, and seven mechanisms of resistance, with 26 ARGs (37% of the total) conferring multidrug resistance (Fig. 2C). Similar to previous studies of gut resistomes, we found that tetracycline resistance markers were the most commonly detected ARGs and inactivation is the most common mechanism of resistance followed by efflux pumps (Li et al. 2021) (Fig. 2C). Using the abundance data of each detected ARG, we determined that asthma ($P = .005$, $R^2 = .028$) and age ($P < .001$, $R^2 = 0.053$) were the strongest factors contributing to the variance in ARG beta diversity even when accounting for important technical and demographic covariates (Fig. 3A and B). We next wanted to ascertain to what degree the resistome profile was determined by microbial composition. We used a Procrustes analysis (Mardia et al. 1979) to compare compositional data generated from MetaPhlAn (Beghini et al. 2021) to the antibiotic resistome profile derived from ShortBRED and found that the microbiome composition correlated to the resistome profile (Fig. 3C, PROTEST corr = 0.627, *P*-value < .0001), indicating that ARG profiles are directly related to bacterial species composition.

Macrolide resistance markers are differentially abundant in asthma

To determine gut-associated ARGs that are differentially abundant between patients with and without asthma, we applied negative binomial tests to the abundance of all ARGs detected in at least seven samples. This prevalence cutoff was chosen because it is the minimum number of samples needed to detect a difference using a negative binomial distribution. We found that genes encoding resistance to macrolides (*ermF, ermB*, and *ermA*), vancomycin (*vanRO*), tetracycline (*tet(45)*), as well as multidrug efflux pumps (*smeB*, *mdtO*, and *oqxA*) were enriched in the asthma cohort (Fig. 4A; Table S4, Supporting Information). Prominent amongst these was the 23S rRNA methyltransferase *ermF*, which is typically encoded by *Bacteroides* species and confers resistance to macrolides.

Next, we explored the genomic context of *ermF* by assembling metagenomic sequencing reads into contigs with metaSPAdes (Bankevich et al. 2012) and annotating open reading frames with Prokka (Seemann 2014) and BLAST. We detected full-length *ermF* with 98% or higher identity in 53 out of 95 samples. Out of 53 contigs, the vast majority originated from members of the Bacteroidota, 75.4% originated from the *Bacteroides* genus and 60.3% of them were likely from *B. fragilis* based on the top BLAST homology. Of the contigs that encoded *ermF,* 68% occurred on scaffolds with at least one other open reading frame within 10 kilobases (Fig. 4B). We found that many *ermF genes* are colocated with genes associated with mobile genetic elements such as transposases, mobilization genes, and toxin/antitoxin systems, as well as with other ARGs like *btgA*, which encodes clindamycin resistance (Fig. 4B and C). This indicates that *ermF* occurs in multiple different genomic contexts within our cohort and suggests that its presence is not strictly due to propagation of a single *B. fragilis* strain.

People with asthma have a distinct set of coexisting pairs of ARGs and VFs in the gut metagenome

In our prior work on this same cohort of patients, we found that, compared to healthy subjects, a greater portion of asthma subjects were colonized with *B. fragilis* strains harboring the VF *B. fragilis* toxin (*bft*), which we showed has the potential to shape inflammation in the lung (Wilson et al. 2023). Given that our resistome analysis pointed to an enrichment of a *B. fragilis* ARG, we wanted to test whether the *ermF* gene is associated with *bft* in the asthma cohort. We found that metagenomes harboring both *ermF* and *bft* were more prevalent in individuals with asthma compared to those without (Fig. 4D). In our MARS samples, we did not find any instances where *bft* and *ermF* occurred on the same scaffold, so it remains unclear whether these two genes are encoded within

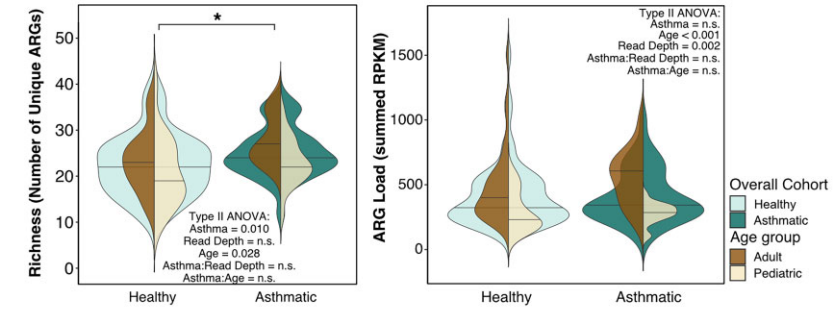
(A)

MARS Cohort	Antibiotics in the past year		Fisher's Exact test
	YES	NO	p
Asthmatic	15	21	0.011
Healthy	9	48	

(B)

Type II ANOVAs

	Richness	Load
Asthma	**	n.s.
Age Group	*	***
Read Depth	n.s.	**



(C)

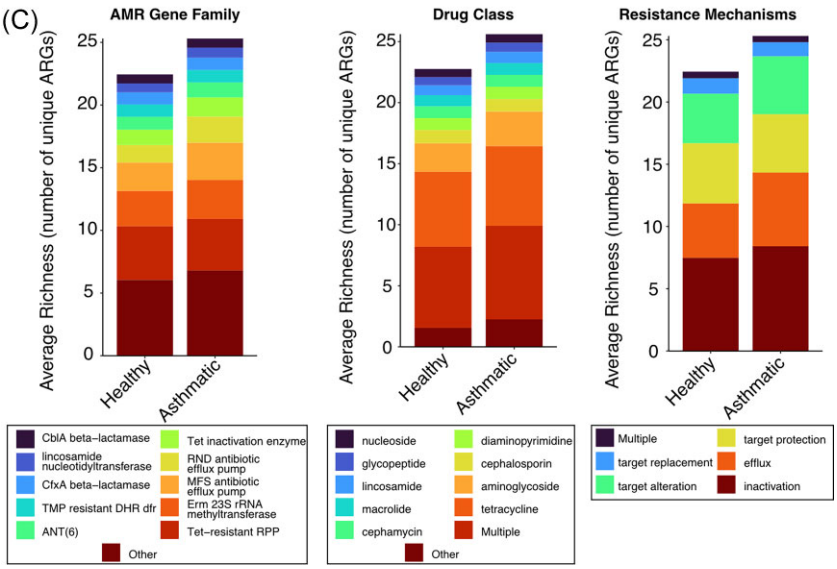


Figure 2. Gut metagenomes from individuals with asthma harbor an increased richness of ARGs. (A) Table describing short-term antibiotic usage in the MARS cohorts. (B) Overlapping violin plots of ARG richness and load by grouped by either healthy and asthma cohort (background) or age (foreground). (C) Stacked bar plots of average ARG richness painted by antimicrobial family (AMR), drug class to which the ARG confers resistance, and ARG resistance mechanism.

the same *B. fragilis* strain or within two separate strains. Nevertheless, the enrichment of *ermF* and *bft* in adults and older children with asthma could suggest that the intestinal habitat of individuals with asthma presents opportunities or niches for macrolide ARGs and VFs such as *bft*.

To explore the possibility that virulence traits and ARGs are linked in the gut microbiota, we characterized VF content of all samples using the Virulence Factor Database (Chen et al. 2005) and compared these data to the antibiotic resistome profiles. We did not find the same overall shift in the VF beta diversity between asthma and healthy that we observed with the resistomes (Figure S4A–C, Supporting Information), but we did find differentially abundant VFs belonging to capsule and peritrichous flagella VF families (Table S5, Supporting Information; q-values < 0.2). Further, we found that microbiota composition is highly correlated with VF profile (Figure S4D, Supporting Information; Protest correlation coefficient = 0.61, $P < .0001$). Given that microbiota composition strongly affects both VF and ARG content, we used a partial correlation between VF and ARG richness to test our hypothesis while removing the effect of total metagenomic content. We found a positive partial correlation between VF and ARG richness in both the asthma and healthy cohorts (Fig. 5A). Similarly, VF and resistome beta diversity profiles were also positively correlated (Fig. 5B, Protest correlation coefficient = 0.574, P = 1e-4). Together, our results suggest that these two microbial features, virulence and antibiotic resistance, are closely linked within the gut metagenome.

We next performed a co-occurrence analysis to uncover co-occurring VFs and antibiotic resistance traits that could be important in gut ecology. We found numerous co-occurring VF-ARG pairs in MARS gut metagenomes (Fig. 5C, $P < .05$). Several of these

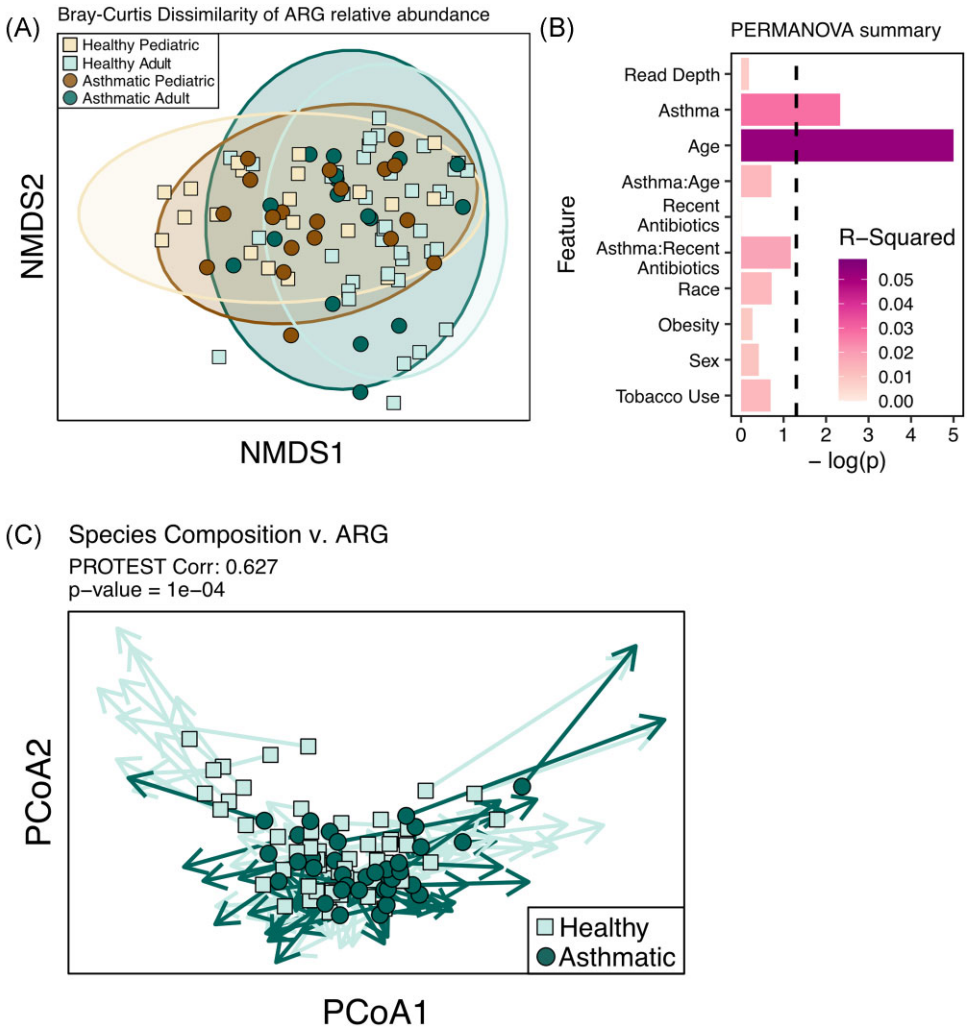


Figure 3. The gut antibiotic resistome is altered in asthma patients. (A) Nonmetric multidimensional scaling (NMDS) plot of antibiotic resistome with units in Bray–Curtis dissimilarity of total-sum scaled RPKM, labeled by asthma and age cohorts. Showing two axes out of five with stress value = 0.1. (B) Effect of demographic categories on antibiotic resistome data in A (sequential PERMANOVA). (C) Procrustes and PROTEST analysis between MetaPhlAn species-level Bray–Curtis dissimilarity distances and CARD ShortBRED Bray–Curtis dissimilarity distances. Arrows connect the two data points belonging to identical samples.

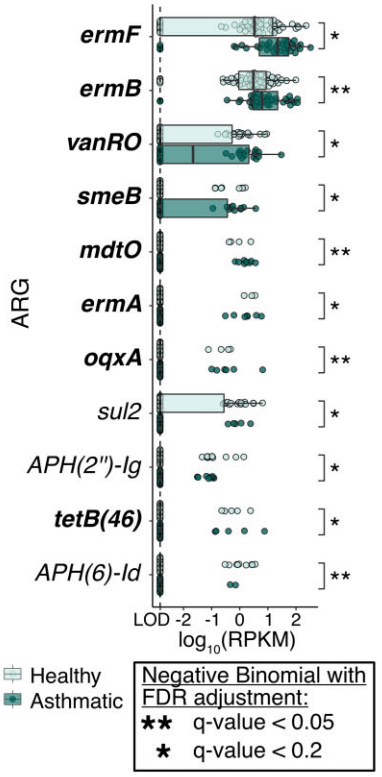
positively co-occurring pairs were shared between the two cohorts (yellow), suggesting that these relationships are not dependent on asthma status. In contrast, many pairs specifically co-occur in one cohort and may indicate microbial interactions important in asthma but not healthy gut metagenomes (Fig. 5C). In summary, we found that VF and ARG presence is linked in the gut metagenome and that people with asthma have a distinct set of co-occurring functions compared to healthy people.

While our co-occurrence analysis between VFs and ARGs demonstrated multiple examples of virulence and antibiotic resistance traits found in the same gut metagenome, this analysis does not indicate if these genes are present in a single organism. To obtain a more granular view of VF-ARG co-occurrence, we limited our analysis to look for VF-ARG pairs that could be encoded by the same species. This analysis showed that the asthma cohort had a greater number of ARGs ($P = .007$ and .01) and VFs ($P = .005$ and .09) annotated as coming from *Klebsiella pneumoniae* and *E. coli*, respectively (Figure S5A, Supproting Information). Individual co-occurrences attributable to each of these species are summarized in Figure S5(B) (Supproting Information) and show that *cepA*, encoding a beta-lactamase, and *chuU*, a VF involved in iron acquisition, are both putatively encoded by *E. coli* and co-occur in patients with asthma, suggesting that the metagenome-wide co-occurrence of CepA and Chu families observed in Fig. 5(C) may be due to enrichment within one or more *E. coli* strains harboring these VF/ARG pairs. Together, our co-occurrence analyses show that there appear to be multiple co-occurring VFs and ARGs in the gut metagenome and within putative individual species that are unique to the asthma cohort. The cohort-specific co-occurring VF-ARG pairs found here could serve as candidates for future studies of asthma gut microbiome ecology.

Discussion

In this study, we present an exploratory analysis of fecal whole metagenomic sequencing contrasting subjects with moderate-to-severe asthma to a group of healthy controls to identify disease-associated microbial genes with the strongest likelihood of affecting disease. Our sequencing and subsequent analyses revealed that the functional content of individuals with asthma differed significantly from that of healthy controls. We found an enrichment of functions associated with saturated and mono-

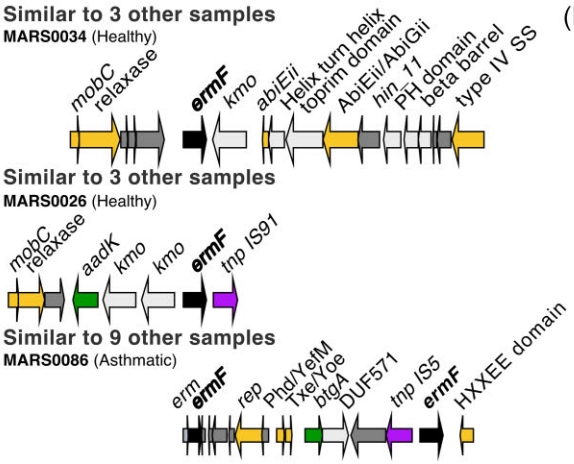
(A) Differentially abundant ARGs



(B) *ermF* Context Summary

Groups of ORFs	Prevalence % (count)	
Plasmid-related	55.6 (20)	
	Families (% contigs with plasmid-related gene)	
	Replication initiation (Rep_3)	60 (12)
	Toxin/antitoxin (Phd/YefM)	60 (12)
	Toxin/antitoxin (Txe/YoeB)	55 (11)
	Toxin/antitoxin (AbiEii/AbiGii)	30 (6)
	Mobilization relaxosome (*mobC*)	25 (5)
	DNA replication and repair (RecF)	25 (5)
	Relaxase/mobilization nuclease domain-containing protein	5 (1)
	Toxin-antitoxin system (AbiEi)	5 (1)
Transposase	47.2 (17)	
	Families (% Tnp-positive contigs)	
	IS5	52.9 (9)
	IS91	23.5 (4)
	IS30	11.8 (2)
	IS982	5.9 (1)
	tnp B. ovatus	5.9 (1)
	tnp Prevotella sp. MGM2	5.9 (1)
Antibiotic resistance	47.2 (17)	
	Families (% ARG-positive contigs)	
	clindamycin (*btgA*)	64.7 (11)
	streptomycin (*aadK*)	11.8 (2)
	tetracycline (*tetQ*)	11.8 (2)
	tetracycline (*tetO*)	5.9 (1)
	penicillin (*bla* OXA-10)	5.9 (1)

(C)



(D) MARS whole fecal metagenomes

	bft(+) *ermF*(+)	One or neither gene detected	Fisher's p-value
Asthmatic	7	29	0.004
Healthy	1	58	

Figure 4. Resistance gene *ermF* is differentially abundant in diverse genomic contexts of gut resistomes belonging to individuals with asthma. (A) Boxplots of ARG abundance by cohort on log-scale. Showing only ARGs present in at least 7 out of 95 samples and have *q*-values less than 0.2. A pseudocount of 0.0015 RPKM (designated as the limit of detection "LOD") was used for the negative binomial tests. Bolded genes are enriched in the asthma cohort while nonbolded are enriched in the healthy cohort. (B) Summary of *ermF* contexts on contigs from metagenomic assemblies that had at least one detectable open reading frame flanking the *ermF* within 10 kilobases. (C) Three representative *ermF* context maps generated in GeneSpy. (D) Count table of fecal metagenomes with codetection of *bft*+ and *ermF*+ vs. detection of one or neither of *ermF* and *bft*, split by donor asthma status. Fisher's Exact two-sided *P*-value shown.

unsaturated fatty acids, including oleate, palmitoleate, 5(Z)-dodecenoate, biotin, 8-amino-oxononanoate, saturated fatty acid elongation, and octanoyl acyl carrier protein pathways. Currently, the functional significance of gut bacterial synthesis of these LCFA to asthma has not been well defined. Excess LCFAs, usually studied in the context of dietary fat intake, have been associated with metabolic diseases including diabetes, obesity, and atherosclerosis risk (Mizuta et al. 2019) but is also linked to asthma risk in adults (Nagel and Linseisen 2005, Scott et al. 2011, Wendell et al. 2014, Mizuta et al. 2019). Increasing recognition that obesity predisposes to asthma has motivated investigation of the impact of fatty acids on airway biology and has shown that LCFA signaling through free fatty acid receptor 1 (FFAR1, also called GPR40) induces airway smooth muscle cell contraction and proliferation, both of which are important components of asthma pathophysiology (Mizuta et al. 2015, 2019). Notably, a study that sequenced airway microbes in children with cystic fibrosis implicated a similar list of LCFA production pathways during exacerbations,

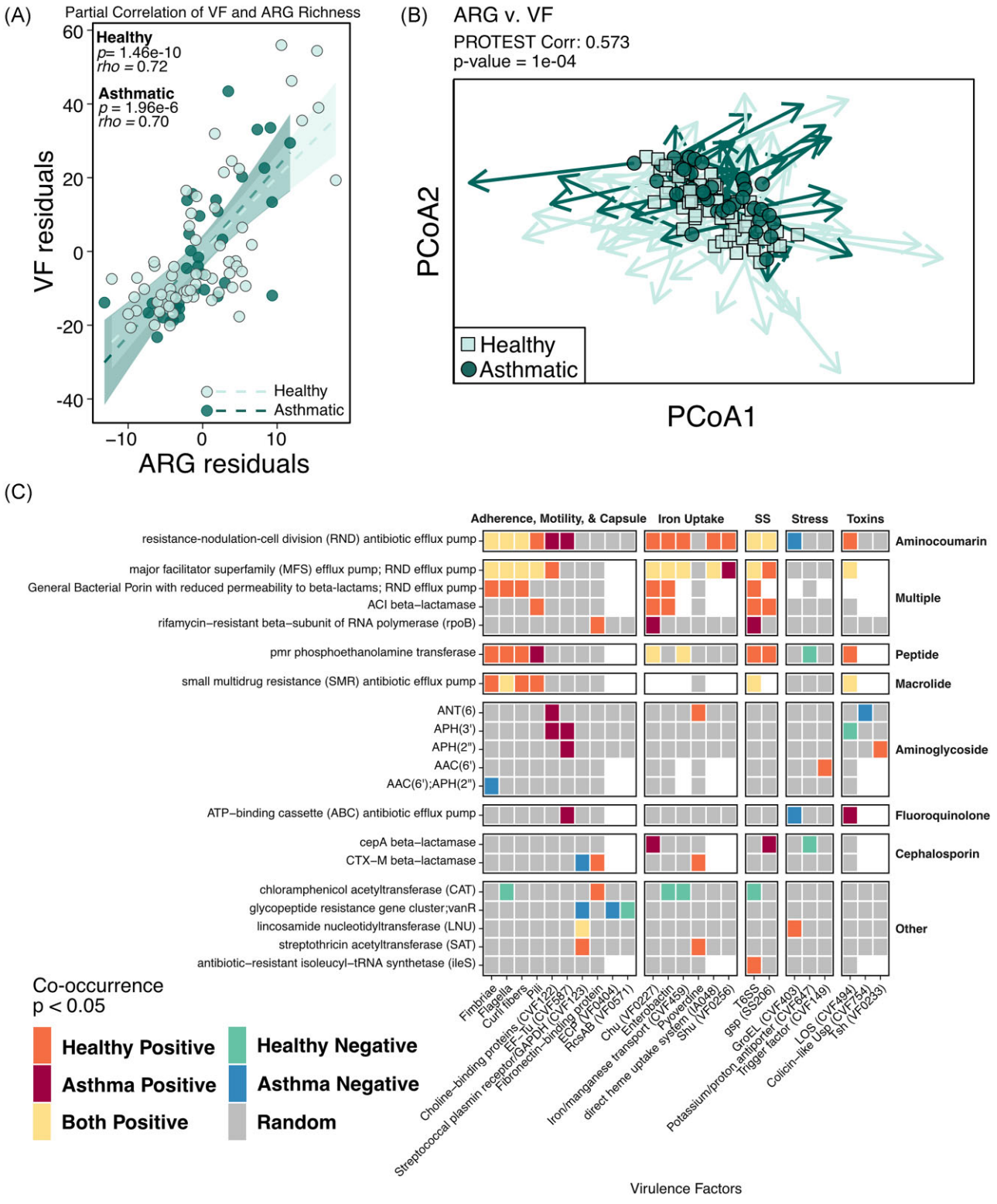


Figure 5. Asthma patients have unique sets of VF and ARG associations. (A) Partial correlations split by asthma status between VF richness and ARG richness after accounting for species richness. (B) Procrustes and PROTEST analysis between Bray–Curtis dissimilarity distances of VFs and CARD resistomes. Arrows connect the two data points belonging to identical samples. (C) Heatmap of statistically significant (co-occur R package $P < .05$) co-occurrence relationships between all VFs and ARGs. Legend labels indicate direction of co-occurrence and in which cohort(s) the respective effect was detected. The label "Random" marks pairs with no statistically significant co-occurrence. Blank squares were pairs filtered out due to a lack of observed co-occurrence.

suggesting that microbially produced LCFAs may influence airway physiology (Felton et al. 2021). To our knowledge, the potential for gut microbes to contribute to the amount of free fatty acids available to the lung has not yet been defined, however, LCFAs are readily absorbed into the circulation (Niot et al. 2009) and could plausibly reach the airways. Further, previous studies have shown the effect of SCFA (e.g. acetate, butyrate, and propionate) produced by gut microbes to directly alter lung inflammation via GPR41 (FFAR3) (Trompette et al. 2014, Zaiss et al. 2015). While our study did not find a direct enrichment of SCFA production pathways in the healthy cohort as has been previously reported (Wang et al. 2018), we did observe that lysine biosynthesis was enriched. Since lysine may serve as a precursor to the SCFA butyrate (Vital et al. 2014), SCFAs may still be more abundant in our healthy cohort but may be subject to transcriptional regulation that would not be detected by metagenomic DNA sequencing. Together, our metabolic pathway analyses of the gut metagenome demonstrate a positive association between LCFAs produced by gut microbes and asthma, in contrast to the negatively associated SCFAs.

In addition to metabolic alterations, analysis of the gut resistome demonstrated that subjects with asthma had a distinct ARG composition. In a recently published prospective gut metagenomic study of infants, asthma-associated taxonomic signatures were associated with a higher number of ARGs (Li et al. 2021). These differences in the resistome were largely driven by a single species of bacteria, *E. coli*, and reveals that acquisition of ARGs in subjects with asthma may begin in early childhood and could affect asthma development. In our study of older subjects with established asthma, we similarly found a higher richness of ARGs that is associated with asthma in both school-aged children and adults, supporting the idea that increased ARG carriage may persist in people with asthma throughout life. Based on our resistome annotation, however, ARGs in our cohort were likely from a diverse assemblage of bacteria in contrast to what was observed in infants. This is likely due to differences in gut dynamics between age groups. The infant microbiome is heavily shaped by limited available niches in the developing gut, which favor transient, facultative anaerobes like *E. coli* (Li et al. 2021), whereas the gut resistome in older subjects reflects selective pressures experienced over a lifetime. One important consequence of increased richness of ARGs in people with asthma is that it may promote persistence of some bacterial strains (Yassour et al. 2016, Schwartz et al. 2020) and contribute to the taxonomic differences in the gut microbiota between asthma and healthy people (Hufnagl et al. 2020, Wilson et al. 2023). However, taxonomic differences observed in our cohort do not entirely explain the increased ARG prevalence amongst people with asthma. We found that *ermF*, a gene primarily found in *B. fragilis*, was enriched in patients with asthma even though the taxonomic abundance of *B. fragilis* was not significantly increased. This finding suggests that differences in composition alone do not explain the enrichment of ARGs within the asthma cohort.

While asthma was among the important factors accounting for a significant amount of the variance in ARG beta diversity, we found that recent antibiotic exposure (within the past year) was not. Notably, no participant in our cohort received a course of antibiotics in the month prior to fecal sampling since this could have confounded our analyses on asthma-associated microbial community changes. Previous studies have shown that the gut microbiota recovers in approximately a month after perturbation from antibiotics in healthy adults (Palleja et al. 2018). We interpret these findings to mean that recent exposure (within 1–12 months) to antibiotics does not drastically change the resistome, whereas repeated exposures over time may be more important for driving the population-wide shifts we observed in our cohort (Schwartz et al. 2020).

Of the ARGs found to be enriched within asthma resistomes, the ARG *ermF*, as well as other markers encoding resistance to macrolide antibiotics, were especially prominent amongst the asthma cohort. While we did not collect data on the antibiotic drug classes, number of courses and their duration, or the reason for prescription of antibiotics, our subjects received, it is likely that our asthma population has been exposed to macrolides. Macrolide antibiotics, including clarithromycin and azithromycin, are commonly prescribed for upper and lower airway infections which disproportionately affect people with asthma (Juhn 2014). This class of antibiotics, particularly azithromycin, have been a focus of special concern for driving antibiotic resistance due to their frequent usage and pharmacological properties (Doan et al. 2019a, b, Malhotra-Kumar et al. 2007). Nevertheless, azithromycin has been noted to have beneficial effects in asthma, and some (Gibson et al. 2017), but not all (Brusselle et al. 2013), studies suggest that azithromycin may prevent exacerbations in patients with asthma. Given the interest in azithromycin as a treatment modality in asthma, there will be an urgent need for additional studies to determine the robustness of the association between asthma and macrolide ARG differential abundance in the gut to inform parameters for antibiotic selection and prescription in people with asthma.

Additional exploration of the gut metagenomes revealed potential coselection in people with asthma for ARGs and VFs. Untargeted analysis of gut resistomes revealed multiple examples of VF and ARG co-occurrence as well as positive correlations between ARG and VF richness in people with and without asthma. Our findings are consistent with previous reports that found correlations between VF richness and ARG richness, as well as VF-ARG cooccurrence relationships in both gut metagenomes (Escudeiro et al. 2019) and human-associated bacterial genomes (Pan et al. 2020). Our findings also add to these studies by demonstrating that, while the correlation between VF and ARG richness does not appear to be any stronger in the asthma cohort compared to the healthy cohort after taking gene richness into account, the two MARS cohorts do not have identical sets of statistically significant co-occurring VF–ARG pairs. These data suggest that people with asthma may be experiencing different selection pressures from that of healthy people, leading to accumulation of a distinct set of virulence and antibiotic determinants. Given that antibiotics induce gut inflammation through the disruption of the gut microbiota (Strati et al. 2021), and strains encoding VFs such as *bft* are known to thrive in an inflammatory environment (Casterline et al. 2017), one plausible model for the apparent accumulation of distinct VF–ARG pairs is that antibiotic treatment not only selects for ARGs (Ramirez et al. 2020, Schwartz et al. 2020), but simultaneously selects for VFs. Together with evidence that virulence determinants, such as *bft,* are associated with airway inflammation (Wilson et al. 2023), our model implies that heightened antibiotic treatment may contribute to the manifestations of asthma via coselection for VFs and ARGs. Considering that prenatal and early life antibiotic exposure is linked to asthma risk (McKeever et al. 2002, Strati et al. 2021), this model could be used to test whether the initial events driving VF and ARG co-occurrence start with the first vertical transmission events in very early life.

Study limitations

Our study has several limitations that constrain the scope of our claims. First, MARS is an exploratory, cross-sectional study with only a moderate number of subjects recruited from a single site,

which is less ideal for identifying disease-associated microbiome differences (Walter et al. 2020). As a result, our study had limited statistical power to detect less prevalent or abundant functions. Second, our study focused on school-aged and older subjects with moderate-to-severe asthma, and thus our findings may not be applicable to other younger populations or those with less severe disease. These population differences may explain why we were unable to identify statistically significant differences in microbial metabolic pathways identified from other studies including bile acid metabolism (Arrieta et al. 2015), epoxide hydrolases (Levan et al. 2019), histamine metabolism (Barcik et al. 2016, 2019), or tryptophan metabolism (Van der Leek et al. 2017, Licari et al. 2019) (Figure S2A, Supproting Information). Third, the factors driving the shift in gut bacterial metabolism to LCFA biosynthesis and whether gut microbiome enrichment of this pathway is sufficient to change the hosts' LCFA profile is not known. Collecting blood to interrogate host metabolism as well as dietary information at the time of fecal sample collection would have helped to disentangle the effects of diet on host and gut microbiota metabolism. Fourth, we lacked relevant subject information, such as diet, environment, infrastructure, stress level, and social relationships, needed to precisely disentangle the effects of social, environmental, and health disparities on the gut microbiome (De Wolfe et al. 2021). We recognize that our finding of subject-reported race as a statistically significant covariate in our analyses of the gut metagenome likely does not reflect a direct effect of race on biology (Cooper 2013). Rather, we interpret this finding as a proxy for the biological consequences of active systemic disparities associated with race (Cooper 2013). We included race in our models to account, albeit imperfectly, for the impact that multifaceted ecosocial factors underlying race are known to have on asthma and the microbiome (Findley et al. 2016, Fitzpatrick et al. 2019). Fifth, a record of the frequency and class of antibiotics administered to our participants would have allowed us to confirm whether macrolide administration associates with the enrichment of *ermF* in our asthma cohort and whether a higher diversity of antibiotic usage correlates with ARG richness. It is likely that antibiotic exposures accumulated throughout life contribute to the resistome, and a complete catalog of exposures is critical to determine patterns of antibiotic prescription most likely to account for the ARG associations to asthma found in this study. Lastly, as with all metagenomic sequencing studies, we are limited by annotation bias in existing databases. This is a concern for our VF and antibiotic resistance profiling especially, where we rely on the database to predict source species for ARGs and VFs. We also recognize that the databases we used for these two analyses are biased towards well-studied human pathogens rather than commensals or opportunistic pathogens. However, we note that other investigators have reported similar co-occurrence of ARGs and VFs (Escudeiro et al. 2019, Pan et al. 2020), and coselection of these features is biologically plausible.

Despite these constraints on the scope of our study, we provide evidence that there is an increased production of LCFA and an increased richness of ARGs encoded by the gut microbiota in people with asthma. These findings could have applications in the care of patients with asthma. If LCFA pathways are shown to play a causal role in airway inflammation in future studies, microbiota-directed therapeutics in the form of dietary interventions or probiotics, could be developed to modify gut microbial metabolism to protect against asthma. Additionally, our resistome findings add to the growing concern over antibiotic resistance in patients with asthma by suggesting that antibiotic administration may also contribute to gut carriage of VFs that can alter airway inflammation. Ultimately, our study shows that the gut microbiota of school-aged and older subjects with moderate-to-severe asthma harbor important functional alterations that could serve as a foundation for future studies investigating how gut microbial functions affect pulmonary diseases.

List of abbreviations

Antibiotic resistance gene (ARG), long-chain fatty acid (LFCA), short-chain fatty acid (SCFA), and virulence factor (VF).

Author contributions

Naomi G. Wilson contributed through conceptualization, data curation, formal analysis, investigation, methodology, software, visualization, and writing the manuscript. Ariel Hernandez-Leyva contributed through data curation, software, and editing the manuscript. Drew J. Schwartz contributed through editing the manuscript. Leonard B. Bacharier contributed through resources and editing the manuscript, and Andrew L. Kau contributed through conceptualization, data curation, funding acquisition, methodology, project administration, resources, supervision, and writing the manuscript.

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Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

Conflict of interest : None declared.

Data availability

The metagenomic sequencing dataset generated during the current study are available at European Nucleotide Archive (https://www.ebi.ac.uk/ena/browser/home) under the project accession number PRJEB59709. Demographic data needed to reproduce results can be found in this manuscript (Table S1, Supporting Information). A full record of all statistical analyses is included as a PDF document generated by knitr in R (Xie 2014) in Additional File 1. A STORMS (Strengthening The Organizing and Reporting of Microbiome Studies) checklist (Mirzayi et al. 2021) is available at doi: 10.5281/zenodo.7492635.

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