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

Naomi G. Wilson[1], Ariel Hernandez-Leyva[1], Drew J. Schwartz[2], Leonard B. Bacharier[3], Andrew L. Kau[1,*]

[1] Division of Allergy and Immunology, Department of Medicine and Center for Women's Infectious Disease Research, Washington University School of Medicine, St. Louis, MO, 63110, USA

[2] Division of Infectious Diseases, Department of Pediatrics and Center for Women's Infectious Disease Research, Washington University School of Medicine, St. Louis, MO, 63110, USA

[3] Division of Allergy, Immunology and Pulmonary Medicine, Department of Pediatrics, Monroe Carell Jr Children's Hospital at Vanderbilt University Medical Center, Nashville, TN 37232, USA

* Correspondence: akau@wustl.edu

Abstract

Asthma is a common allergic airway disease that develops in association with 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 59 healthy and 36 subjects with moderate-to-severe asthma to characterize the metagenomes of gut microbiota in children and adults 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. One differentially abundant ARG was a macrolide resistance marker, *ermF*, which significantly co-occurred with the *Bacteroides fragilis* toxin, suggesting a possible relationship between enterotoxigenic *B. fragilis*, antibiotic resistance, and asthma. Lastly, we found multiple virulence factor (VF) and ARG pairs that co-occurred in both cohorts suggesting that virulence and antibiotic resistance traits are co-selected 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.

Importance:

Asthma is an airway disease that affects the everyday lives of millions of people and accounts for approximately 1.5 million emergency room visits yearly in the US(1) Both antibiotic usage and gut microbiota dysbiosis have been linked to the development of asthma, however, little is known about the specific gut microbial functions associated with asthma, particularly in older populations. In this study, we characterize the gut microbiota of school-aged children and adults with moderate-to-severe asthma to uncover asthma-associated microbial functions that may contribute to disease features. We find that people with asthma have an increase in gut microbial genes associated with long-chain fatty acid metabolism as well as an accumulation of antibiotic resistance genes, both of which may have practical consequences for monitoring and treatment of asthma.

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(2, 3). 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(2, 4).

63 Early childhood gut microbial communities have been proposed to contribute to
64 asthma by several mechanisms. Epoxide hydrolases encoded by enterococci and other
65 gut bacteria produce the lipokine 12,13-diHOME that predisposes towards atopic
66 sensitization and asthma(4, 5). Similarly, short-chain fatty acids (SCFAs), produced by
67 the metabolism of dietary fibers by diverse members of the gut microbiota, are thought
68 to protect from asthma through their effect on the host G-protein coupled receptor
69 GPR41, shaping immune cell differentiation in the lungs, and ameliorating allergic
70 airway inflammation(2, 6–9).
71 In addition to microbially-encoded metabolic features, carriage of antibiotic
72 resistance genes (ARGs) within the gut microbiota, termed the resistome, has been
73 associated with asthma risk. In infants, microbial signatures associated with the
74 development of asthma are also associated with increased richness of ARGs in the gut
75 microbiome(10). These differences in ARG carriage were found to be driven primarily by
76 *E. coli*, which is a common colonizer in the first days of life(10). These findings are
77 important in understanding the origins of asthma since antibiotic exposure correlates
78 both to the number of ARGs within the gut microbiome(11) and the later development of
79 asthma and other allergic diseases(12–14). This association between antibiotic
80 exposure and asthma is supported by animal models that found antibiotic treatment
81 worsens allergic airway inflammation (AAI)(15–17).
82 While there is an abundance of data supporting the idea that asthma
83 susceptibility is associated with features of the gut microbiota in early childhood, the
84 potential effect of gut microbial functions on asthma later in life remains an important
85 knowledge gap. Since asthma often begins in infancy when the gut microbiota

86 composition is highly unstable, disease-causing microbial functions may not persist into
87 older children and adults. Nevertheless, the gut microbiota in older individuals could
88 underlie the variable manifestations of asthma(18) and may hold valuable prognostic
89 and therapeutic significance.

90 Asthma-associated differences in later childhood and adult gut microbial
91 communities have already been noted in several reports. Studies in preschool-aged
92 children have noted distinct taxonomic composition of gut microbial communities in
93 asthmatic subjects compared to healthy controls(3). These differences are reported to
94 include reductions in *Akkermansia muciniphila*(19), *Faecalibacterium prausnitzii*(20) as
95 well as *Roseburia* species(21). Functional characterization of microbial communities by
96 whole metagenomic sequencing from an older population of asthmatic women(20) has
97 shown that pathways related to lipid and amino acid metabolism, as well as
98 carbohydrate utilization were enriched in asthmatics. In contrast, microbial pathways
99 involved in the production of SCFAs, like butyrate, were enriched in the healthy cohort
100 of the same study(20). These findings are supported by a complementary study
101 designed to test the effect of probiotic supplementation on asthma that found an
102 association of improved asthma symptoms with SCFA biosynthesis as well as
103 tryptophan metabolism pathways in the adult gut microbiota(22).

104 Here, we describe an analysis of whole metagenomic sequencing data from a
105 cohort of 36 subjects with physician-diagnosed, moderate-severe asthma along with a
106 matched cohort of 59 healthy controls. This study tests the hypothesis that the gut
107 metagenome harbors signatures of asthma later in life. Our results identify global
108 differences in metagenomic functions between healthy and asthmatic subjects and

109 reveal an enrichment in long-chain fatty acid biosynthetic pathways. We also find an
110 increased richness of ARGs in asthmatics and co-occurrence of ARGs with known
111 bacterial virulence factors, suggesting a potential relationship between antibiotic
112 exposure and pathogen colonization in asthmatics.
113
114 **Results**
115 **Whole metagenomic shotgun sequencing of fecal samples from adults and**
116 **children with asthma and healthy controls**
117 We performed whole metagenomic sequencing on fecal samples from asthmatic
118 subjects and healthy controls taking part in the Microbiome & Asthma Research Study
119 (MARS), which we have previously described(23, 24). MARS participants were recruited
120 from the St. Louis, Missouri area and included pediatric (6-10 years) and adult (18-40
121 years) age groups. All asthma cohort patients had a physician diagnosis of moderate-to-
122 severe asthma, and history of allergic sensitization as evidenced by positive skin testing
123 or serum specific-IgE to one or more common aeroallergens. In total, we analyzed 95
124 patient stool samples including 17 adults and 19 school-aged participants with asthma,
125 and 40 adults and 19 school-aged participants without asthma.
126 NovaSeq S4 sequencing of our libraries yielded 1.69 billion paired-end reads
127 translating to a total of approximately 500 Gigabases (Gb). After filtering for read quality,
128 dropping host contaminants, and trimming adaptor content, we achieved 1.23 billion
129 paired-end reads and an average 3.4 Gb per stool sample with a range of 0.4-9.9
130 Gb/sample (Figure S1A). Neither host contamination nor sequencing depth differed
131 between asthma and healthy cohorts (t-test p=0.2 and 0.7, Table S1). All samples

132 achieved an estimated average metagenomic coverage of at 89% (range of 61-98%)
133 with the annotation-free redundancy-based metagenome coverage estimator,
134 Nonpareil(25) (Figure S1B). Further, estimated metagenome coverage was not different
135 between the asthma and healthy cohorts, although we noted coverage was slightly
136 reduced in the pediatric cohort (Figure SB, Table S1). We employed the read-based
137 annotation pipeline, HUMAnN(26) to determine the abundance of genes and functional
138 pathways in the stool metagenomes. We found that the most abundant functional
139 pathways (Figure S1C) across all MARS participants are involved in essential
140 processes of gut microbes such as starch degradation and glycolysis, demonstrating
141 that our sequencing captured core functions of the gut metagenome, as expected.
142 Taken together, we concluded that our sequencing is of sufficient depth and quality to
143 be used for further analyses.
144

145 **Gut taxonomic composition differs between people with and without asthma**

146 We first leveraged the clade marker annotation tool, MetaPhlAn(26), to analyze
147 the taxonomic composition of the study participants. We found dominate genera typical
148 in gut microbiota communities including *Bacteroides* (phylum Bacteroidota) and
149 *Faecalibacterium* (phylum Bacillota) (Figure S1D). Simpson alpha diversity was slightly
150 higher in the asthma cohort even when taking read depth and age group into account
151 (Figure S1E). Bray-Curtis dissimilarity (Figure 1F) was shifted between the asthma and
152 healthy cohorts ($p<0.0004$, $R^2=0.029$) even when accounting for other covariates
153 including age ($p<0.001$, $R^2=0.032$), race ($p=0.0006$, $R^2=0.026$), recent antibiotic usage
154 ($p=0.9$, $R^2=0.006$), read depth ($p=0.2$, $R^2=0.013$), obesity ($p=0.7$, $R^2=0.008$), sex ($p=0.4$,

155 R^2=0.011), and tobacco exposure (p=0.2, R^2=0.012) by sequential PERMANOVA
156 (Figure 1G). There was also no significant interaction between asthma status and age
157 group (p=0.8, R^2=0.007), or between asthma status and recent antibiotic usage (p=0.6,
158 R^2=0.009) (Figure S1G). To determine differentially abundant taxa, we tested the fixed
159 effect of asthma along with the random effects of age group and race in a general linear
160 model(27) and found *Eubacterium rectale* and *Prevotella copri* were enriched in the
161 healthy cohort (Figure S1H, Table S2). All of these findings are consistent with 16S
162 rRNA sequencing performed in a previous study(23) which lent us further confidence
163 that our sequencing data was suitable for functional profiling.
164

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

167 Given that our samples had adequate coverage to capture expected taxonomic
168 shifts, we started interrogating the differences in metagenomic functions of the gut
169 microbiota attributable to asthma status. The alpha diversity of genes (UniRef90
170 clusters) was neither different between the asthma and healthy cohorts nor between the
171 pediatric and adult cohorts, suggesting that our gene profiling reached a similar total
172 number of genes in both cohorts (Figure 1A). Using PERMANOVA, we noted that, even
173 while accounting for significant covariates of age (p<0.001, R^2=0.029), race (p<0.001,
174 R^2=0.024), and read depth (p=0.03, R^2=0.015), asthma status also significantly
175 impacted gut microbiome functional composition (p=0.008, R^2=0.017; Figure 1B, C). We
176 note that age group's interaction term with asthma did not significantly contribute to the
177 variance in beta diversity, suggesting that the influence of asthma and age on beta

178 diversity is non-overlapping. These findings support the idea that the gut metagenomic
179 content of people with asthma is different than that of healthy individuals, even when
180 accounting for other clinical sources of interpersonal gut microbiome variation.

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

200 Using taxonomically tiered functional mapping, we determined which taxa were

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

217 We reviewed the enzymatic steps of each of the eight pathways represented in
218 Figure 1D and found that, of the 78 total reactions in these pathways, only 11 reactions
219 were shared between 2 pathways (Figure S3). The 8-amino-7-oxononanoate
220 biosynthesis I pathway consists of the first 11 reactions of the larger biotin biosynthesis
221 pathway and the latter only has four additional reaction steps past synthesizing 8-
222 amino-7-oxonanoate to produce biotin. Additionally, the (5Z)-dodecenoate pathway can
223 feed directly into the palmitoleate biosynthesis pathway, and that the octanoyl acyl

224 carrier protein pathway shares an upstream substrate (acetoacetyl-acyl carrier protein)
225 with the saturated fatty acid elongation pathway (Figure S3). Together, our findings
226 indicate that long chain fatty acid biosynthesis is differentially abundant in the asthma
227 gut metagenome via related but largely non-redundant pathways.

228 Given the association between obesity with fatty acid metabolism(28) as well as
229 asthma(29–31), we next wanted to determine whether obesity (which we define here as
230 a BMI greater than 30 in adults or a BMI-for-age percentile of greater than 95% in
231 children) confounds the association of microbial fatty acid metabolism with asthma. We
232 compared the abundance of the differentially abundant fatty acid pathways between all
233 non-obese and obese patients and found no significant difference (Figure 1F). Within
234 the asthma cohort, there was similarly no statistically significant difference between the
235 asthmatic obese and asthmatic non-obese patients, suggesting that obesity is not a
236 confounder for the difference we observed in fatty acid metabolism. To determine
237 whether fatty acid metabolism is related to the intensity of asthma symptoms and their
238 effect on everyday life activities, we utilized a validated survey of asthma control (The
239 Asthma Control Test; ACT)(32). None of the fatty acid pathways were differentially
240 abundant between well-controlled and poorly-controlled asthmatics (Figure 1F). We
241 tested if age group affects the differentially abundant metabolic pathways and found that
242 these pathways were not differentially abundant between age groups alone (Figure 1F).
243 We also tested the impact of asthma and age as independent variables to differentially
244 abundant metabolic pathways using a Two-way ANOVA. We found that, even while
245 taking age into account, these pathways are differentially abundant between asthma
246 and healthy cohorts, but are not different by age or an interaction between asthma and

247 age (Figure 1G, 2-Way ANOVA). Given that the effect of asthma status on differentially
248 abundant metagenomic functions was distinct from that of age, we primarily focused our
249 subsequent analyses on the asthma and healthy cohorts overall, combining age groups.
250

251 **Richness of antibiotic resistance genes is increased in the gut metagenomes of**
252 **people with asthma**

253 Since people with asthma tend to be prescribed antibiotics frequently(33) and
254 oral antibiotic exposure is a risk factor for the acquisition of ARGs in the gut(11), we
255 wanted to determine if the members of our asthma cohort were more likely to have
256 received antibiotics. To test this, we counted how many subjects had taken a course of
257 antibiotics within one year of their participation in the study. As part of the study design,
258 participants could not take antibiotics in the month prior to fecal donation. We found that
259 a greater proportion of the asthma cohort received antibiotics in the past year compared
260 to that of healthy participants (42% of asthma cohort versus 15% of the healthy cohort,
261 Fisher's test, p=0.011, Figure 2A). This finding represents evidence of increased
262 antibiotic exposure amongst subjects with asthma in our study.

263 We next sought to characterize the gut antibiotic resistome in the asthma and
264 healthy cohorts. To test if the increased antibiotic exposure in the asthma cohort was
265 reflected in the gut resistome, we utilized the ShortBRED pipeline(34) to detect reads
266 mapped to the Comprehensive Antibiotic Resistance Database (CARD)(35). We first
267 asked whether there were more ARGs in our asthma cohort by summarizing our dataset
268 into richness (Total number of unique ARGs detected per sample) and load (Total sum
269 of ARG RPKM per sample). We found that ARG richness was higher in people with

270 asthma even when accounting for differences due to age (p=0.03) and sequencing
271 depth (p=0.09 while ARG load was not different between asthma and healthy cohorts
272 (p=0.4) when accounting for age (p<0.001) and read depth (0.002) (Figure 2B). We note
273 that *E coli* was not differentially abundant between asthma and healthy cohorts (p=0.52,
274 Table S2), so the richness increase we observe in the asthma cohort is not due solely to
275 an increase in *E. coli* relative abundance. These results suggest that there are a higher
276 number of unique ARGs, or a higher diversity, in asthma compared to healthy controls.
277 From our 95 stool samples, we detected 71 unique ARGs, comprising 32
278 antimicrobial resistance families, 29 drug classes, and 7 mechanisms of resistance, with
279 26 ARGs (37% of the total) conferring multi-drug resistance (Figure 2C). Similar to
280 previous studies of gut resistomes, we found that tetracycline resistance markers were
281 the most commonly detected ARGs and inactivation is the most common mechanism of
282 resistance followed by efflux pumps(10) (Figure 2C). Using the abundance data of each
283 detected ARG, we determined that asthma (p=0.005, R^2=0.028) and age (p<0.001,
284 R^2=0.053) were the strongest factors contributing to the variance in ARG beta diversity
285 even when accounting for important technical and demographic covariates (Figure 3A
286 and 3B). We next wanted to ascertain to what degree the resistome profile was
287 determined by microbial composition. We used a Procrustes analysis(36) to compare
288 compositional data generated from MetaPhlAn(26) to the antibiotic resistome profile
289 derived from ShortBRED and found that the microbiome composition correlated to the
290 resistome profile (Figure 3C, PROTEST corr = 0.627, p-value < 0.0001), indicating that
291 ARG profiles are directly related to bacterial species composition.
292

293 **Macrolide resistance markers are differentially abundant in asthma**

294 To determine ARGs that are differentially abundant between asthmatic and
295 healthy gut metagenomes, we applied negative binomial tests to the abundance of all
296 ARGs detected in at least 7 samples. This prevalence cutoff was chose because it is
297 the minimum number of samples needed to detect a difference using a negative
298 binomial distribution. We found that genes encoding resistance to macrolides (*ermF,*
299 *ermB* and *ermA*), vancomycin (*vanRO*), tetracycline (*tet(45)*), as well as multi-drug
300 efflux pumps (*smeB, mdtO*, and *oqxA*) were enriched in the asthma cohort (Figure 4A,
301 Table S4). Prominent amongst these was the 23S rRNA methyltransferase *ermF*, which
302 is typically encoded by *Bacteroides* species and confers resistance to macrolides.

303 Next, we explored the genomic context of *ermF* by assembling metagenomic
304 sequencing reads into contigs with metaSPAdes(37) and annotating open reading
305 frames with Prokka(38) and BLAST. We detected full-length *ermF* with 98% or higher
306 identity in 53 out of 95 samples. Out of 53 contigs, the vast majority originated from
307 members of the Bacteroidota, 75.4% originated from the *Bacteroides* genus and 60.3%
308 of them were likely from *B. fragilis* based on the top BLAST homology. Of the contigs
309 that encoded *ermF,* 68% occurred on scaffolds with at least one other open reading
310 frame within ten kilobases (Figure 4B). We found that many *ermF genes* are co-located
311 with genes associated with mobile genetic elements such as transposases, mobilization
312 genes, and toxin/antitoxin systems, as well as with other ARGs like *btgA* which encodes
313 clindamycin resistance (Figure 4B,C). This indicates that *ermF* occurs in multiple
314 different genomic contexts within our cohort and suggests that its presence is not strictly
315 due to propagation of a single *B. fragilis* strain.

316

317 **People with asthma have a distinct set of co-existing pairs of antibiotic resistance**
318 **genes and virulence factors in the gut metagenome**

319 In our prior work on this same cohort of patients, we found that, compared to
320 healthy subjects, a greater portion of asthma subjects were colonized with *B. fragilis*
321 strains harboring the virulence factor *B. fragilis* toxin (*bft*), which we showed has the
322 potential to shape inflammation in the lung(23). Given that our resistome analysis
323 pointed to an enrichment of a *B. fragilis* ARG, we wanted to test whether the *ermF* gene
324 is co-selected with *bft.* When only taking metagenomes encoding *ermF* into account, we
325 observed an enrichment of *bft* prevalence in the asthma cohort (Figure 4D, p=0.009). In
326 contrast, among metagenomes with no detectable *ermF,* there is no enrichment of *bft* in
327 the asthma cohort (Figure 4D, p=0.39). When reviewing the entire MARS population, we
328 found no statistically significant co-occurrence of *ermF* with *bft* (Figure 4E; one-tailed
329 Fishers test p>0.05) and this was consistent with healthy gut metagenomes from the
330 Human Microbiome Project (Figure 4E, Fisher's test p=0.56). However, in our MARS
331 samples, we did not find any instances where *bft* and *ermF* occurred on the same
332 scaffold, so it remains unclear whether these two genes are encoded within the same *B.*
333 *fragilis* strain or within two separate strains. Nevertheless, these results suggest that the
334 environment supporting the gut microbiota of asthmatic individuals presents
335 opportunities or niches for *ermF* and *bft* to co-occur.

336 To explore the possibility that virulence traits and ARGs are linked in the gut
337 microbiota, we characterized virulence factor (VF) content of all samples using the
338 Virulence Factor Database(39) and compared these data to the antibiotic resistome

339 profiles. We did not find the same overall shift in the virulence factor beta diversity
340 between asthma and healthy that we observed with the resistomes (Figure S4A-C), but
341 we did find differentially abundant VFs belonging to capsule and peritrichous flagella VF
342 families (Table S5, q values<0.2). Further, we found that microbiota composition is
343 highly correlated with virulence factor profile (Figure S4D, Protest correlation
344 coefficient=0.61, p<0.0001). Given that microbiota composition strongly affects both VF
345 and ARG content, we used a partial correlation between VF and ARG richness to test
346 our hypothesis while removing the effect of total metagenomic content. We found a
347 positive partial correlation between VF and ARG richness in both the asthma and
348 healthy cohorts (Figure 5A). Similarly, virulence factor and resistome beta diversity
349 profiles were also positively correlated (Figure 5B, Protest correlation coefficient=0.574,
350 p=1e-4). Together, our results suggest that these two microbial features, virulence and
351 antibiotic resistance, are closely linked within the gut metagenome.
352 We next performed a co-occurrence analysis to uncover other linked virulence
353 and antibiotic resistance traits that could be important in gut ecology. We found
354 numerous co-occurring VF-ARG pairs in MARS gut metagenomes (Figure 5C, p<0.05).
355 Several of these positively co-occurring pairs were shared between the two cohorts
356 (yellow), suggesting that these relationships are not dependent on asthma status. In
357 contrast, many pairs specifically co-occur in one cohort and may indicate microbial
358 interactions important in asthma but not healthy gut metagenomes (Figure 5C). In
359 summary, we found that VF and ARG presence is linked in the gut metagenome and
360 that people with asthma have a distinct set of co-occurring functions compared to
361 healthy people.

362 While our co-occurrence analysis between VFs and ARGs demonstrated multiple
363 examples of virulence and antibiotic resistance traits found in the same gut
364 metagenome, this analysis does not indicate if these genes are present in a single
365 organism. To obtain a more granular view of VF-ARG co-occurrence, we limited our
366 analysis to look for VF-ARG pairs that could be encoded by the same species. This
367 analysis showed that the asthma cohort had a greater number of ARGs (p=0.007 and
368 0.01) and VFs (p=0.005 and 0.09) annotated as coming from *Klebsiella pneumoniae*
369 and *Escherichia coli*, respectively (Figure S5A). Individual co-occurrences attributable to
370 each of these species are summarized in Figure S5B and show that *cepA*, encoding a
371 beta-lactamase, and *chuU*, a VF involved in iron acquisition, are both putatively
372 encoded by *E. coli* and co-occur in asthmatics, suggesting that the metagenome-wide
373 co-occurrence of CepA and Chu families observed in Figure 5C may be due to
374 enrichment within one or more *E. coli* strains harboring these VF/ARG pairs. Together,
375 our co-occurrence analyses show that there appear to be multiple co-occurring VFs and
376 ARGs, similar to *B. fragilis*-encoded *bft* and *ermF*, in the gut metagenome and within
377 putative individual species that could be important for asthma. The cohort-specific co-
378 occurring VF-ARG pairs found here could serve as candidates for future studies of
379 asthma gut microbiome ecology.
380

381 **Discussion**

382 In this study, we present an exploratory analysis of fecal whole metagenomic
383 sequencing contrasting subjects with moderate-to-severe asthma to a group of healthy
384 controls to identify disease-associated microbial genes with the strongest likelihood of

385 affecting disease. Our sequencing and subsequent analyses revealed that the
386 functional content of individuals with asthma differed significantly from that of healthy
387 controls. We found an enrichment of functions associated with saturated and mono-
388 unsaturated fatty acids, including oleate, palmitoleate, 5(Z)-dodecenoate, biotin, 8-
389 amino-oxononanoate, saturated fatty acid elongation, and octanoyl acyl carrier protein
390 pathways. Currently, the functional significance of gut bacterial synthesis of these long-
391 chain fatty acids (LCFA) to asthma has not been well defined. Excess LCFAs, usually
392 studied in the context of dietary fat intake, have been associated with metabolic
393 diseases including diabetes, obesity, and atherosclerosis risk(30) but is also linked to
394 asthma risk in adults(29–31, 40). Increasing recognition that obesity predisposes to
395 asthma has motivated investigation of the impact of fatty acids on airway biology and
396 has shown that LCFA signaling through free fatty acid receptor 1 (FFAR1, also called
397 GPR40) induces airway smooth muscle cell contraction and proliferation, both of which
398 are important components of asthma pathophysiology(30, 41). Notably, a study that
399 sequenced airway microbes in children with cystic fibrosis implicated a similar list of
400 LCFA production pathways during exacerbations, suggesting that microbially produced
401 LCFAs may influence airway physiology(42). To our knowledge, the potential for gut
402 microbes to contribute to the amount of free fatty acids available to the lung has not yet
403 been defined, however, LCFAs are readily absorbed into the circulation(43) and could
404 plausibly reach the airways. Further, previous studies have shown the effect of SCFA
405 (e.g. acetate, butyrate, propionate) produced by gut microbes to directly alter lung
406 inflammation via GPR41 (FFAR3)(8, 9). While our study did not find a direct enrichment
407 of SCFA production pathways in the healthy cohort as has been previously

408 reported(20), we did observe that lysine biosynthesis was enriched. Since lysine may
409 serve as a precursor to the SCFA butyrate(44), SCFAs may still be more abundant in
410 our healthy cohort but may be subject to transcriptional regulation that would not be
411 detected by metagenomic DNA sequencing. Together, our metabolic pathway analyses
412 of the gut metagenome demonstrate a positive association between LCFAs produced
413 by gut microbes and asthma, in contrast to the negatively associated SCFAs.

414 In addition to metabolic alterations, analysis of the gut resistome demonstrated
415 that subjects with asthma had a distinct ARG composition. In a recently published
416 prospective gut metagenomic study of infants, asthma-associated taxonomic signatures
417 were associated with a higher number of ARGs(10). These differences in the resistome
418 were largely driven by a single species of bacteria, *E. coli*, and reveals that acquisition
419 of ARGs in subjects with asthma may begin in early childhood and could affect asthma
420 development. In our study of older subjects with established asthma, we similarly found
421 a higher richness of ARGs that is associated with asthma in both school-aged children
422 and adults, supporting the idea that increased ARG carriage may persist in people with
423 asthma throughout life. Based on our resistome annotation, however, ARGs in our
424 cohort were likely from a diverse assemblage of bacteria in contrast to what was
425 observed in infants. This is likely due to differences in gut dynamics between age
426 groups. The infant microbiome is heavily shaped by limited available niches in the
427 developing gut, which favor transient, facultative anaerobes like *E. coli* (10), whereas
428 the gut resistome in older subjects reflects selective pressures experienced over a
429 lifetime. One important consequence of increased richness of ARGs in people with
430 asthma is that it may promote persistence of some bacterial strains(45, 46) and

431 contribute to the taxonomic differences in the gut microbiota between asthma and
432 healthy people(3, 23).
433 While asthma was among the important factors accounting for a significant
434 amount of the variance in ARG beta diversity, we found that recent antibiotic exposure
435 (within the past year) was not. Notably, no participant in our cohort received a course of
436 antibiotics in the month prior to fecal sampling since this could have confounded our
437 analyses on asthma-associated microbial community changes. Previous studies have
438 shown that the gut microbiota recovers in approximately a month after perturbation from
439 antibiotics in healthy adults(47). We interpret these findings to mean that recent
440 exposure (within 1 - 12 months) to antibiotics does not drastically change the resistome,
441 whereas repeated exposures over time may be more important for driving the
442 population-wide shifts we observed in our cohort(46).
443 Of the ARGs found to be enriched within asthmatic resistomes, the ARG *ermF*,
444 encoding resistance to macrolide antibiotics, was especially prominent amongst the
445 asthmatic cohort. While we did not collect data on the antibiotic drug classes, number of
446 courses and their duration, or the reason for prescription of antibiotics, our subjects
447 received, it is likely that our asthma population has been exposed to macrolides.
448 Macrolide antibiotics, including clarithromycin and azithromycin, are commonly
449 prescribed for upper and lower airway infections which disproportionately affect people
450 with asthma(48). This class of antibiotics, particularly azithromycin, have been a focus
451 of special concern for driving antibiotic resistance due to their frequent usage and
452 pharmacological properties(49–51). Nevertheless, azithromycin has been noted to have
453 beneficial effects in asthma, and some(52), but not all(53), studies suggest that

454 azithromycin may prevent exacerbations in asthmatics. Given the interest in
455 azithromycin as a treatment modality in asthma, there will be an urgent need for
456 additional studies to determine the robustness of the association between asthma and
457 macrolide ARG accumulation in the gut to inform parameters for antibiotic selection and
458 prescription in people with asthma.

459 Additional exploration of the gut metagenomes revealed potential co-selection in
460 people with asthma for *B. fragilis* genes *ermF* and *bft* (*B. fragilis* toxin), the latter of
461 which is more prevalent in fecal samples from the asthma compared to healthy
462 cohort(23). Untargeted analysis of gut resistomes revealed multiple examples of
463 virulence factor and ARG co-occurrence as well as positive correlations between ARG
464 and VF richness in people with and without asthma**.** Our findings are consistent with
465 previous reports that found correlations between VFs and ARG richness and VF-ARG
466 cooccurrence relationships in both gut metagenomes(54) and human-associated
467 bacterial genomes(55). Our findings also add to these studies by demonstrating that,
468 while the correlation between VF and ARG richness does not appear to be any stronger
469 in the asthma cohort after taking gene richness into account, the two MARS cohorts do
470 not have identical sets of statistically significant co-occurring VF-ARG pairs. These data
471 suggest that people with asthma may be experiencing different selection pressures from
472 that of healthy people, leading to accumulation of a distinct set of virulence and
473 antibiotic determinants. Given that antibiotics induce gut inflammation through the
474 disruption of the gut microbiota(56), and strains encoding virulence factors such as *bft*
475 are known to thrive in an inflammatory environment(57), one plausible model for the
476 apparent accumulation of distinct VF-ARG pairs is that antibiotic treatment not only

477 selects for ARGs(11, 46), but simultaneously selects for VFs. Together with evidence
478 that virulence determinants, such as *bft,* are associated with airway inflammation(23),
479 our model implies that heightened antibiotic treatment may contribute to the
480 manifestations of asthma via co-selection for VFs and ARGs. Considering that prenatal
481 and early life antibiotic exposure is linked to asthma risk(13, 56), this model could be
482 used to test whether the initial events driving VF and ARG co-occurrence start with the
483 first vertical transmission events in very early life.

484 Our study has several limitations that constrain the scope of our claims. First,
485 MARS is an exploratory, cross-sectional study with only a moderate number of subjects
486 recruited from a single site, which is less ideal for identifying disease-associated
487 microbiome differences(58). As a result, our study had limited statistical power to detect
488 less prevalent or abundant functions. Second, our study focused on school-aged and
489 older subjects with moderate-to-severe asthma, and thus our findings may not be
490 applicable to other younger populations or those with less severe disease. These
491 population differences may explain why we were unable to identify statistically
492 significant differences in microbial metabolic pathways identified from other studies
493 including bile acid metabolism(2), epoxide hydrolases(5), histamine metabolism(59, 60),
494 or tryptophan metabolism(61, 62) (Figure S2A). Third, the factors driving the shift in gut
495 bacterial metabolism to LCFA biosynthesis and whether gut microbiome enrichment of
496 this pathway is sufficient to change the hosts' LCFA profile is not known. Collecting
497 blood to interrogate host metabolism as well as dietary information at the time of fecal
498 sample collection would have helped to disentangle the effects of diet on host and gut
499 microbiota metabolism. Fourth, a record of the frequency and class of antibiotics

500 administered to our participants would have allowed us to confirm whether macrolide
501 administration associates with the enrichment of *ermF* in our asthma cohort and
502 whether a higher diversity of antibiotic usage correlates with ARG richness. It is likely
503 that antibiotic exposures accumulated throughout life contribute to the resistome, and a
504 complete catalog of exposures is critical to determine patterns of antibiotic prescription
505 most likely to account for the ARG associations to asthma found in this study. Fifth, as
506 with all metagenomic sequencing studies, we are limited by annotation bias in existing
507 databases. This is a concern for our virulence factor and antibiotic resistance profiling
508 especially, where we rely on the database to predict source species for ARGs and VFs.
509 We also recognize that the databases we used for these two analyses are biased
510 towards well-studied human pathogens rather than commensals or opportunistic
511 pathogens. However, we note that other investigators have reported similar co-
512 occurrence of ARGs and VFs(54, 55), and co-selection of these features is biologically
513 plausible.

514 Despite these constraints on the scope of our study, we provide evidence that
515 there is an increased production of LCFA and an increased richness of ARGs encoded
516 by the gut microbiota in people with asthma. These findings could have applications in
517 the care of patients with asthma. If LCFA pathways are shown to play a causal role in
518 airway inflammation in future studies, microbiota-directed therapeutics in the form of
519 dietary interventions or probiotics, could be developed to modify gut microbial
520 metabolism to protect against asthma. Additionally, our resistome findings add to the
521 growing concern over antibiotic resistance in patients with asthma by suggesting that
522 antibiotic administration may also contribute to gut carriage of virulence factors that can

523 alter airway inflammation. Ultimately, our study shows that the gut microbiota of school-
524 aged and older subjects with moderate-to-severe asthma harbor important functional
525 alterations that could serve as a foundation for future studies investigating how gut
526 microbial functions affect pulmonary diseases.

527 **Author Contributions**

528 N.G.W. and A.L.K. conceptualized the work. L.B.B. and A.L.K. planned the clinical
529 study. N.G.W., A.H-L., D.J.S., and A.L.K. analyzed the data and drafted the manuscript.
530 All authors interpreted the data and contributed to revising the manuscript.

531

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543

544 **Declaration of Interests**

545 The authors have no competing interest to declare.

546 **Methods**

547 **MARS Study Population**

548 The Microbiome and Asthma Research Study (MARS) consisted of 104 subjects
549 from the St Louis, MO USA area that are either healthy or had physician-diagnosed
550 moderate-to-severe asthma. This study included an adult cohort (ages 18-40 years) and
551 pediatric cohort (ages 6-10 years). As described in previous manuscripts(23, 24), 9
552 patients were disqualified or did not donate stool samples. The remaining 95 patients
553 donated stool samples either at home or at the recruitment visit and were evaluated with
554 a clinical questionnaire to gather relevant metadata. Stool samples were kept at -20°C
555 and delivered within 24 hours to the study site, Kau Lab at Washington University
556 School of Medicine, where they were stored at -80°C for no more than three years until
557 processing for DNA isolation. This study was approved by the Washington University
558 Institutional Review Board (IRB# 201412035). Written informed consent documents
559 were obtained from all MARS subjects or their legal guardians. All recruitment, follow
560 up, and sample acquisition occurred between November 2015 and December 2017.

561

562 **Fecal DNA Isolation**

563 Frozen human stool samples were pulverized in liquid nitrogen using a pestle
564 and mortar. We then homogenized the stool in a mixture of phenol, chloroform, and
565 isoamyl alcohol with a bead beater using sterilized zirconium and steel beads as
566 previously described(63) to extract crude DNA. We purified the fecal DNA with a 96-well
567 QIAGEN PCR Clean up kit and quantitated by measuring the absorbance at 260/280
568 nm. Sample DNA concentrations were normalized to 0.5 ng/mL. Neither depletion of

569 human DNA sequence nor enrichment of microbial or viral DNA was performed. No
570 experimental quantification like a spike-in were used.
571
572 **Whole Metagenomic Sequencing of Fecal Communities**
573 To generate fecal metagenomic sequencing data, we adapter-ligated libraries by
574 tagmentation using an adaptation of the Nextera Library Prep kit (Illumina, cat. No. FC-
575 121-1030/1031)(64). Individual libraries were then purified with AMPure XP SPRI
576 beads, quantitated using Quant-iT (Invitrogen, cat. Q33130), and then combined in an
577 equimolar ratio. We confirmed that each library was adequately represented in the
578 combined library by preliminary sequencing on a MiSeq instrument at the Washington
579 University in St. Louis Center for Genome Sciences to assess the evenness of the
580 library. Once the quality of the library was assured, we sequenced the combined library
581 on a NovaSeq 6000 S4 with 2x150 bp chemistry to achieve an average of 3.4 Giga-
582 base-pairs (Gb) per sample. NovaSeq services and data demultiplexing were performed
583 by the Genome Technology Access Center at the McDonnell Genome Institute (St
584 Louis, MO). All samples were tagmented simultaneously and sequenced on the same
585 run to avoid batch effects.
586
587 **Processing of sequencing data**
588 Metagenomic raw demultiplexed reads were then processed to (1) remove
589 spurious human sequences (human reference database was hg37dec_v0.1.1), (2)
590 remove low quality sequences, and (3) trim remaining adapter content using Kneaddata
591 v. 0.10.0 (huttenhower.sph.harvard.edu/kneaddata) bypassing the tandem repeat finder

592 step (“- -bypass-trf"). FastQC (fastqc v0.11.7) and MultiQC (multiqc v1.2) with default
593 settings were used to create quality reports and visualize processing steps. See Figure
594 S1A and Table S1 for number of reads dropped per processing step. After trimming and
595 filtering, no samples had adaptor content, overrepresented sequences, or an average
596 sequence quality score below Phred 24. Estimated metagenome coverage was
597 calculated with Nonpareil(25, 65) (version 3.4.1) via the online querying tool at
598 http://enve-omics.ce.gatech.edu/nonpareil/submit.
599
600 **Read-based metagenome profiling**
601 To obtain functional information about the metagenomic contents of fecal
602 samples, we processed samples using HUMAnN(26) v3.0.0 on filtered reads with
603 default parameters. The marker gene database used by HUMAnN to identify taxonomic
604 identities was ChocoPhlAn v201901b and the protein database used by HUMAnN to
605 identify functions was the UniRef90 full database v201901b. Alpha diversity analysis of
606 Uniref90 genes and two-sample tests of KEGG orthologs were performed on respective
607 genes that were present (>0 copies per million) in at least 16 out of 95 samples, which
608 was the lowest prevalence cutoff that would allow for Bonferroni corrected Wilcoxon p-
609 values below 0.0001. HUMAnN was used to determine the abundance of metagenomic
610 pathways by mapping UniRef90 genes to the MetaCyc database. We performed
611 differential abundance analysis using the Wilcoxon 2-sample tests on pathways that had
612 a minimum of 10% prevalence.
613 To identify antibiotic resistance genes present in the fecal metagenomes of
614 MARS stools, we used ShortBRED-identify(34) (v0.9.4) with the Comprehensive

615 Antibiotic Resistance Database(35) (downloaded 2021-07-05 16:10:04.04555) and
616 Virulence Factor Database(39) (downloaded Fri Jul 16 10:06:01 2021). ShortBRED-
617 Quantify was run on the filtered reads with default parameters. ARGs or VFs that had
618 an abundance greater than zero in less than 7 out of 95 samples were excluded from
619 downstream analyses. This prevalence cutoff was determined using the binomial
620 distribution to maintain a 95% confidence that enrichment was not due to random
621 chance (using stats::binom in R). In the analyses that compared virulence factor profiles
622 to antibiotic resistance gene profiles, any gene with the same name was excluded from
623 the list of antibiotic resistance and considered a virulence factor only, to prevent
624 spurious results due to co-correlations. Only one gene matched this criterion: *ugd*
625 (UDP-glucose 6-dehydrogenase).
626 Microbial composition was determined with MetaPhlAn 3.0(26) which is included
627 in the HUMAnN pipeline described(26). MaasLin(27) (Maaslin2_1.5.1) was used in R to
628 find taxa of any taxonomic level that correlated with asthma by setting asthma as a fixed
629 effect and setting age group and race as random effects.
630 For PERMANOVA analyses, BMI class refers to two stratifications: Non-obese
631 (underweight, healthy, or overweight) and obese determined for adults by BMI cutoffs
632 and for pediatric patients by BMI-for-age percentile as defined by the Centers for
633 Disease Control and Prevention (see
634 cdc.gov/healthyweight/assessing/bmi/childrens_bmi/about_childrens_bmi.html). Race
635 was reported by the subject and split into the two categories of Caucasian and non-
636 Caucasian.
637

638 **Metagenome Assemblies**

639 Filtered reads were assembled into contigs using spades(37) (v3.14.0) with the
640 "meta" flag and k-mers lengths as follows: -k 21,33,55,77. The resulting scaffolds
641 achieved an average N50 of 3525 +/- 178 bp, an average L50 of 7192 +/- 372 and an
642 average total length of 136.8 +/- 4.5 Mbp as measured by QUAST (v 4.5) (66, 67) (see
643 Table S1). Determination of *ermF* location was performed by aligning the 801-bp coding
644 sequence of *ermF* from CARD(35) to all scaffolds. Scaffolds containing BLAST hits with
645 98% identity or higher to the full-length CARD *ermF* sequence were further annotated
646 by Prokka (v1.14.5) to find open reading frames and annotate them. Manual BLAST
647 was used to annotate "hypothetical protein" open reading frames for the contexts of
648 *ermF* hits.

649

650 **Statistics and Reproducibility**

651 R version 3.6.3 was used for all analyses downstream of HUMAnN and
652 ShortBRED, and for data visualization. Wilcoxon tests with false discovery rate multiple
653 testing correction or Type II ANOVAs were used to determine statistically significant
654 differences with the car::Anova package in R. PERMANOVAs were performed in R
655 using the vegan::adonis package with default settings and 100,000 iterations. The
656 following symbols were used to designate significance: * $p < 0.05$, ** $p < 0.01$, *** $p <$
657 0.001 and the following for q values (FDR-adjusted p-values): * $q < 0.2$, ** $q < 0.05$.

658

659 **Data Availability**

660 All sequencing data available at European Nucleotide Archive

661 (https://www.ebi.ac.uk/ena/browser/home) under project accession number
662 PRJEB56741. Demographic data can be found in this manuscript (Table S1) and
663 previous articles about the MARS study(23, 24). A STORMS (Strengthening The
664 Organizing and Reporting of Microbiome Studies) checklist(68) is available at doi:
665 10.5281/zenodo.7492635.

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924

Figures

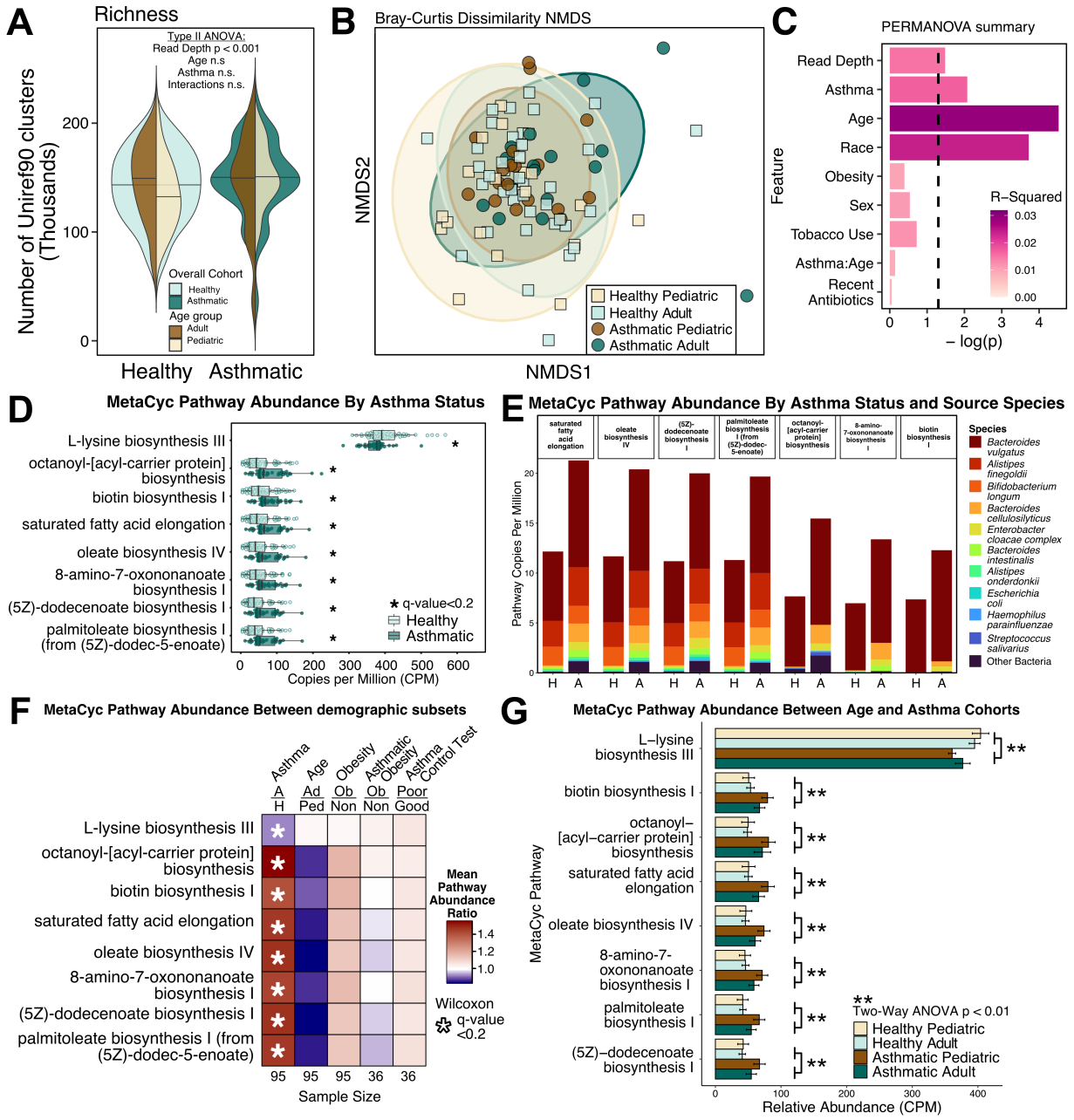


Figure 1: Gut metagenomes from individuals with asthma show increased genes encoding fatty acid metabolism. A) Stacked violin plots of Uniref90 cluster richness (unique Uniref90 cluster with CPM>0) grouped by either healthy and asthma cohort (blue green colors in background) or age (brown colors in foreground. **B)** Non-metric 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 Asthmatics vs. Poorly-Controlled Asthmatics. 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.

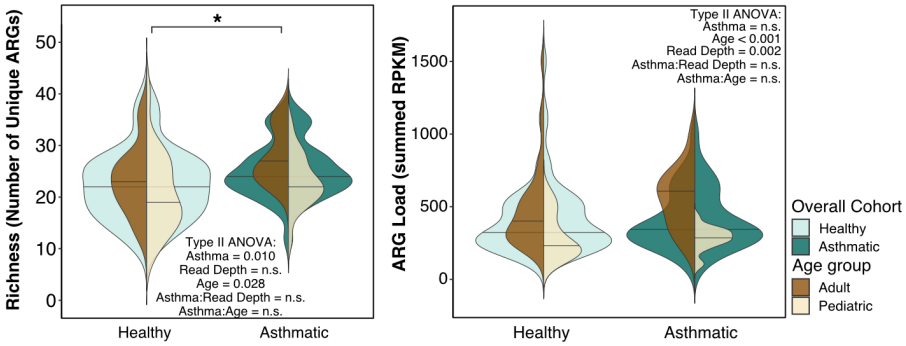
A

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.	**



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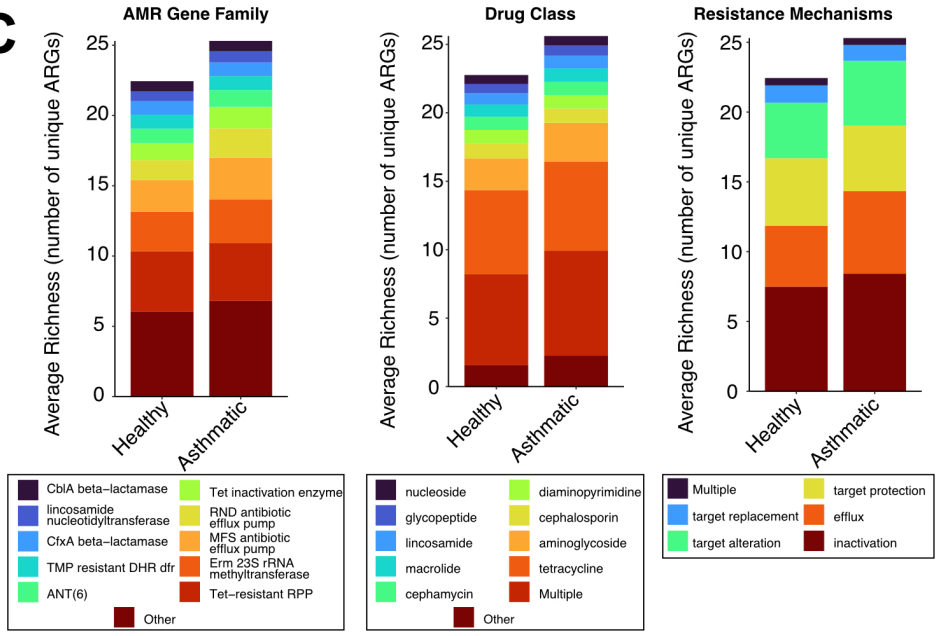

Figure 2: Gut metagenomes from individuals with asthma harbor an increased richness of antibiotic resistance genes. A) Table describing short-term antibiotic usage in the MARS cohorts. **B)** Overlapping violin plots of ARG richness and load by grouped by either healthy and asthma cohort (blue green colors in background) or age (brown colors in 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.

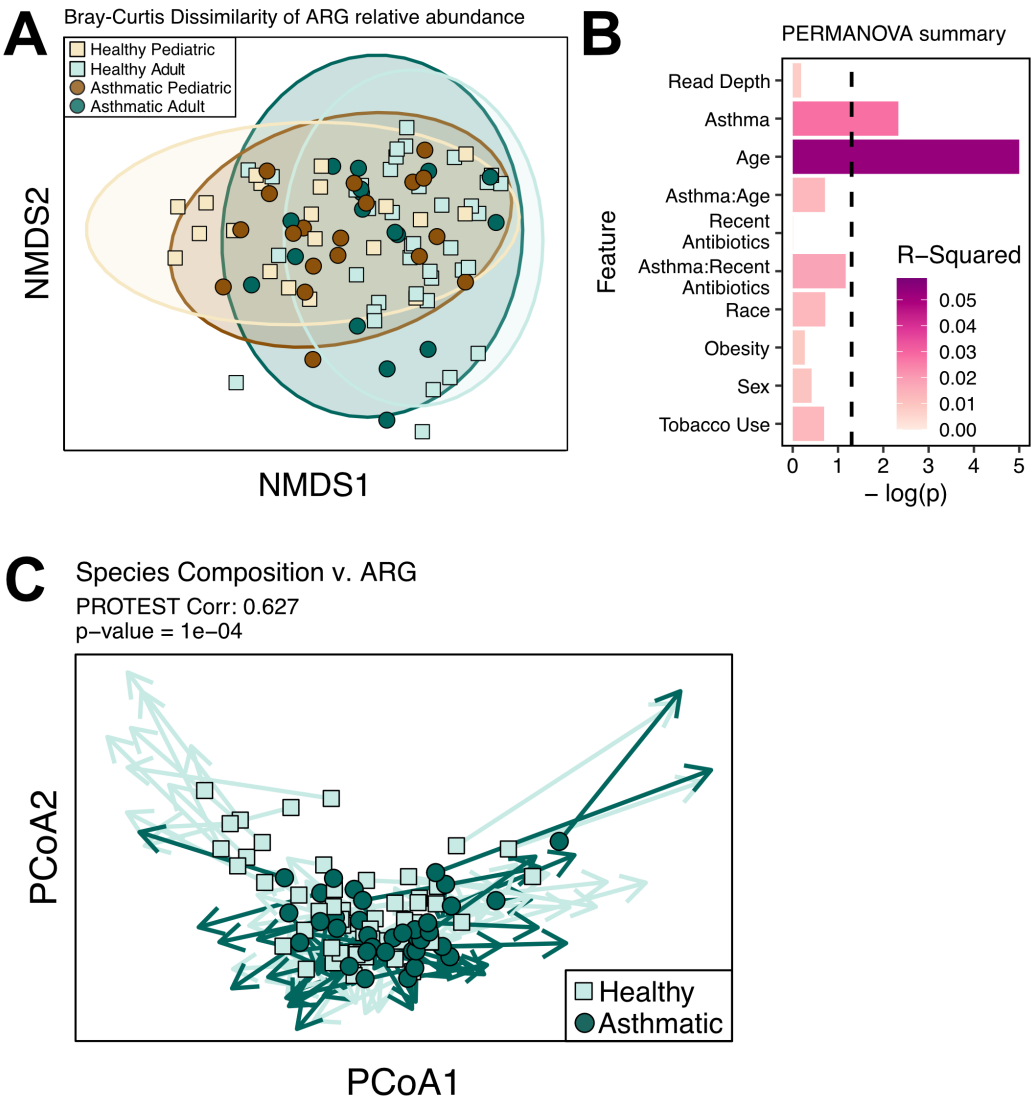

Figure 3: The gut antibiotic resistome is altered in asthma patients. A) Non-metric Multidimensional Scaling (NMDS) plot of antibiotic resistome with units in Bray-Curtis dissimilarity of total-sum scaled RPKM, labeled by asthma and age cohorts. Showing two axes out of five with stress value=0.1. **B)** 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.

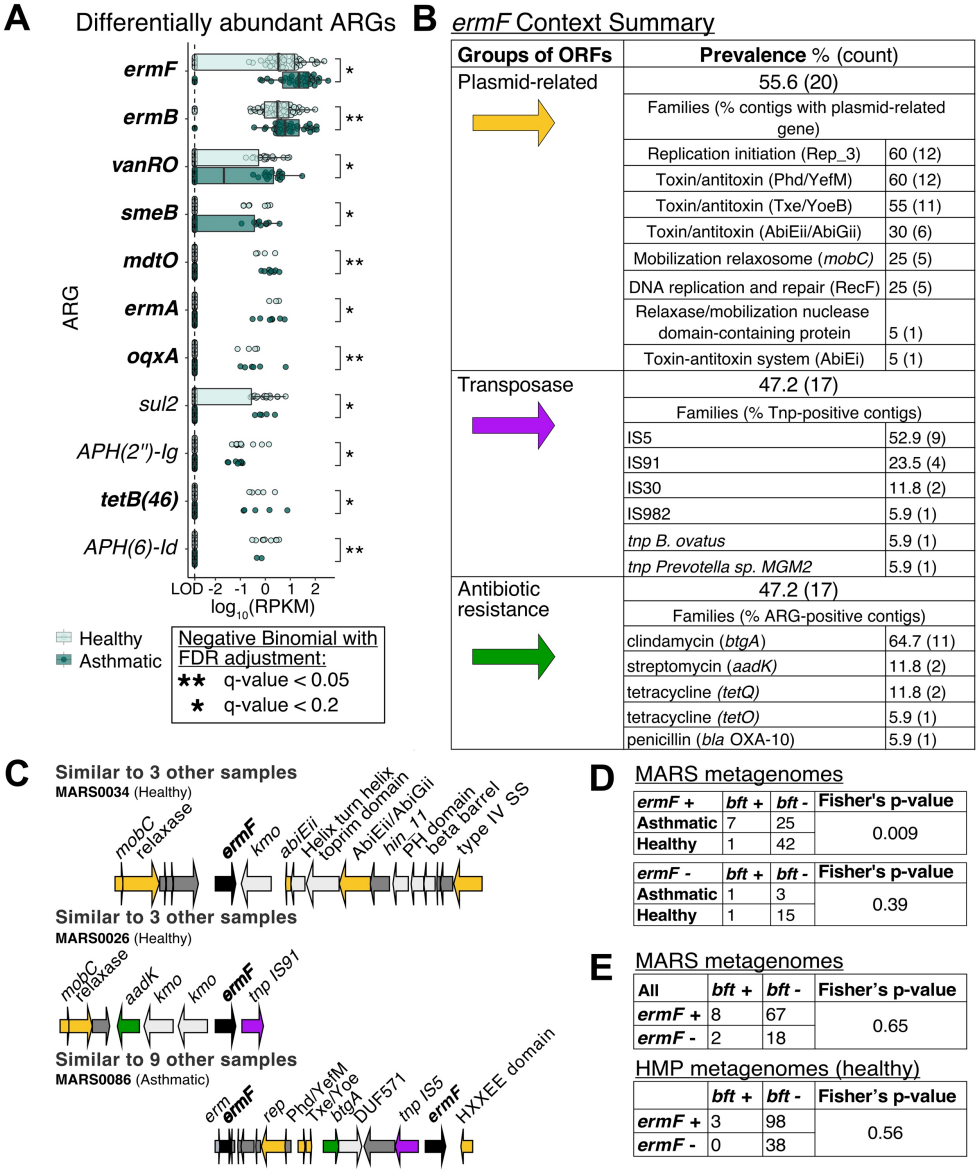

Figure 4: **Resistance gene *ermF* is differentially abundant in diverse genomic contexts of gut resistomes belonging to individuals with asthma. A)** Boxplots of antibiotic resistance gene (ARG) abundance by cohort on log-scale. Showing only ARGs present in at least 7 out of 95 samples and have q-values 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 non-bolded 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 tables of *ermF*+ (top) and *ermF*- (bottom) MARS fecal samples split by *bft* presence and asthma status. Both tables showing two-sided p-value. **E)** Count tables of metagenomes (top from MARS and bottom from Human Microbiome Project) split by the presence of *B. fragilis* toxin (*bft*) and *ermF*. Top: one-sided p-value shown; bottom: two-sided p-value shown.

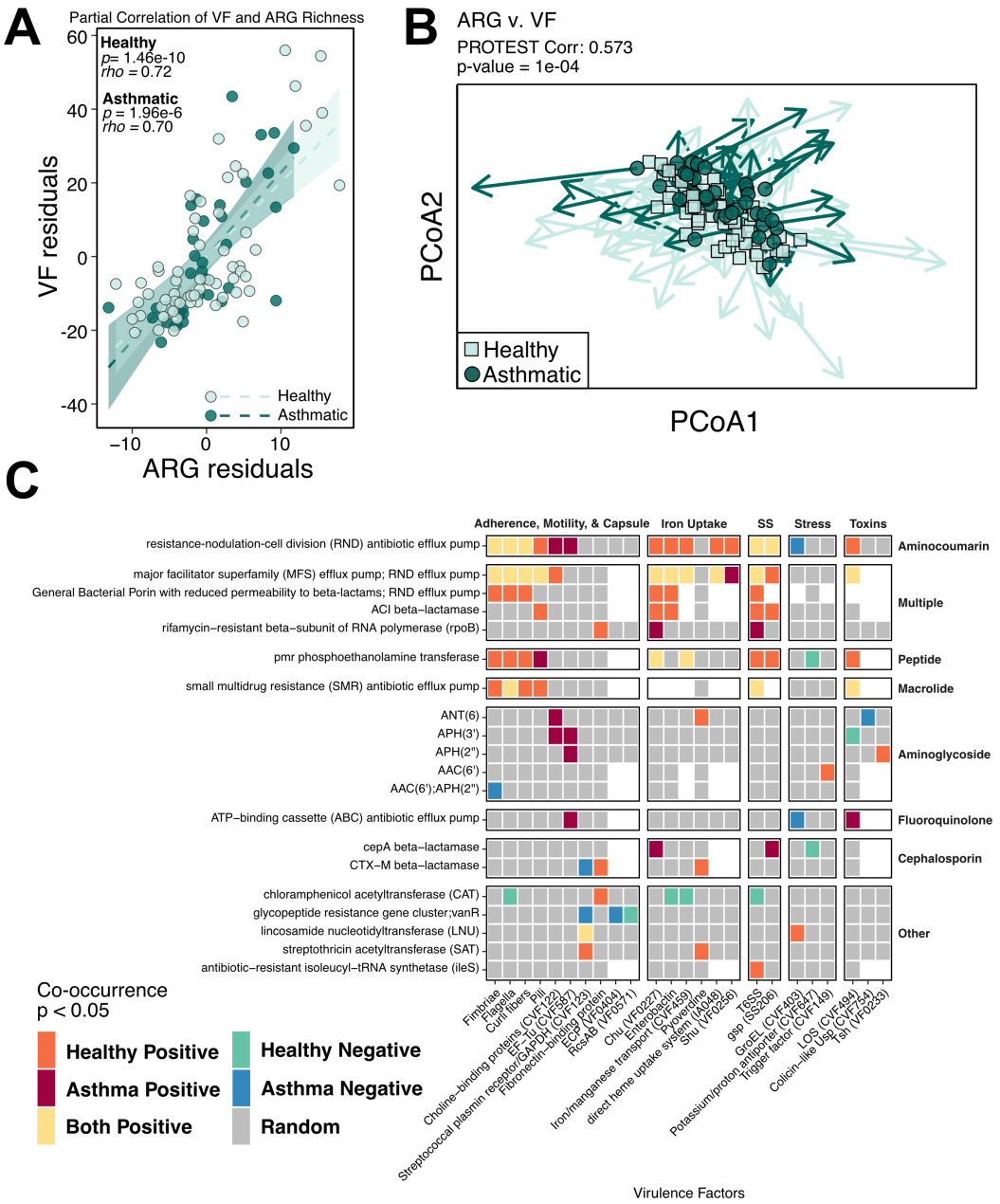

Figure 5: **Asthma patients have unique sets of virulence factor and antibiotic resistance gene associations. A)** Partial correlations split by asthma status between virulence factor richness and ARG richness after accounting for species richness. **B)** Procrustes and PROTEST analysis between Bray-Curtis dissimilarity distances of virulence factors and CARD resistomes. Arrows connect the two data points belonging to identical samples. **C)** Heatmap of statistically significant (cooccur R package p<0.05) co-occurrence relationships between all VFs and ARGs. Colors indicate direction of co-occurrence and in which cohort(s) the respective effect was detected. Grey squares mark pairs with no statistically significant co-occurrence. White squares were pairs filtered out due to a lack of observed co-occurrence.

991 **Supplemental Figure and Data Table Captions**
992
993 **Figures:**
994 **Figure S1: MARS whole metagenomic shotgun sequencing captures essential**
995 **functions and taxonomic shifts of the asthma gut microbiota. A)** Summary of select
996 sequencing statistics from NovaSeq shotgun metagenomic sequencing and subsequent
997 filtering steps. **B)** Boxplot of redundancy-based estimated metagenome coverage (%)
998 as calculated by running the forward reads through the Nonpareil tool. Split into asthma
999 and age group and Two-way Type II ANOVA results shown. **C)** Bar plot of MetaCyc
1000 pathway copies per million (CPM) in all MARS samples annotated by HUMANnN
1001 pipeline, with horizontal length representing mean and bars the standard error. For all
1002 panels: N= 20 healthy children, 39 healthy adults, 19 asthmatic children, 17 asthmatic
1003 adults. **D)** Relative abundance stacked barplots of top abundant bacterial genera split
1004 by age group and asthma cohort. **E)** Simpson alpha diversity boxplots split by asthma
1005 and age group cohorts (2-Way Type II ANOVA). **F)** NMDS of Bray-Curtis Dissimilarity of
1006 species-level relative abundance grouped by age and asthma. **G)** Sequential
1007 PERMANOVA to test effect of demographics on beta diversity. Terms were input into
1008 the test as ordered from top to bottom of barplot. Dotted vertical line represents a p
1009 value of 0.05. Color scale is mapped to the R^2 value. **H)** Arcsine transformed relative
1010 abundance boxplots of differentially abundant species as determined by Maaslin2 with
1011 age group and race modeled as random effects. For all panels: N= 20 healthy children,
1012 39 healthy adults, 19 asthmatic children, 17 asthmatic adults.
1013
1014 **Figure S2: KEGG orthologs, KEGG pathways, and differentially abundant**
1015 **MetaCyc fatty acid pathways. A)** Relative abundance of KEGG orthologs previously
1016 implicated in asthma. Copies per million (CPM) are counts normalized by gene size and
1017 read depth, then total-sum-scaled to one million. **B-C)** Stacked bar plots of differentially
1018 abundant pathways mapped to respective taxa including "Community" bin which
1019 accounts for the remaining reads that mapped to the pathway but not to any single
1020 species by MetaPhlAn3.0/HUMAnN3.0, averaged within asthma or healthy cohorts. **B)**
1021 L-lysine biosynthesis III pathway. Only top 13 taxa shown in addition to Community
1022 category. **C)** Seven fatty acid metabolism pathways differentially abundant in the
1023 asthma cohort. Only top 9 taxa shown in addition to Community category. Stars
1024 represent a q value < 0.05 of Wilcoxon tests between the Community pathway richness
1025 and *B. vulgatus*-encoded pathway richness. For all panels: N= 59 healthy, 36 asthmatic
1026 individuals.
1027
1028 **Figure S3: Pathway collage for differentially abundant MetaCyc pathways.** "PWY-
1029 6519: 8-amino-7-oxononanoate biosynthesis I" is completely overlapping with "BIOTIN-
1030 BIOSYNTHESIS-PWY: biotin biosynthesis I" and its steps are highlighted in blue text.
1031 Pathway collage made on MetaCyc browser tool.
1032
1033 **Figure S4: Gut virulence factor ecology shifts with age group but not asthma**
1034 **cohort. A)** Total-sum scaled RPKM Bray-Curtis Dissimilarity Non-metric
1035 Multidimensional Scaling (NMDS) plot labeled by asthma and age cohorts. Showing two
1036 axes out of 5 with stress value=0.09. **B)** Effect of demographic categories on virulence

1037 factor profile in A (by sequential PERMANOVA, input terms ordered from top to bottom
1038 of barplot). **C)** Stacked violin plots of virulence factor alpha diversity grouped by eithter
1039 healthy and asthma cohort (blue green colors in background) or age (brown colors in
1040 foreground). Two-Way ANOVA results shown in table above plot. **D)** Procrustes plot
1041 and PROTEST analysis between virulence factor profile Bray-Curtis dissimilarity
1042 distances and Metaphlan species relative abundance Bray-Curtis dissimilarity
1043 distances. Arrows connect the two data points belonging to identical samples. For all
1044 panels: N= 20 healthy children, 39 healthy adults, 19 asthmatic children, 17 asthmatic
1045 adults.
1046
1047 **Figure S5: Asthma-associated ARG richness and ARG-VF co-occurrence**
1048 **relationships are observed within *K. pneumoniae* and *E. coli.* A)** Richness bar plots
1049 between antibiotic resistance genes (ARGs) and virulence factors (VFs) grouped by
1050 asthma status. **B)** Heatmap of the co-occurrence of each VF/ARG pair colored by the
1051 direction in which (positively or negatively co-occurring) and the cohort for which
1052 (asthma vs. healthy) the pair had a p-value less than 0.05 via R cooccur function. Blank
1053 squares were pairs filtered out due to a lack of observed co-occurrence. SS: secretion
1054 systems. (N=36 Healthy, 59 Asthmatic)
1055
1056 **Tables:**
1057 Table S1: Fecal shotgun metagenomics filtering and assembly summary statistics
1058 Table S2: Maaslin 2 Analysis of Metaphlan Community Composition
1059 Table S3: Comparison of MetaCyc Pathway Differential Abundance Analyses
1060 Table S4: Negative Binomial tests of antibiotic resistance gene abundance (RPKM)
1061 between healthy and asthma cohorts
1062 Table S5: Negative Binomial tests of virulence factor abundance (RPKM) between
1063 healthy and asthma cohorts