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DOI: 10.1016/j.chom.2020.06.022

Document Version Peer reviewed version

Link to publication record in King's Research Portal

Citation for published version (APA):

Adebayo, A. S., Ackermann, G., Bowyer, R. C. E., Wells, P. M., Humphreys, G., Knight, R., Spector, T. D., & Steves, C. J. (2020). The Urinary Tract Microbiome in Older Women Exhibits Host Genetic and Environmental Influences. *Cell Host and Microbe*, *28*(2), 298-305.e3. https://doi.org/10.1016/j.chom.2020.06.022

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| 1 | The urinary tract microbiome in older women exhibits host genetic and | | | | | |
|----------|---|--|--|--|--|--|
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| 10 | Summary | | | | | |
| 11 | The urinary microbiome is a relatively unexplored niche that varies with gender. | | | | | |
| 12 | Urinary microbes, especially in ageing populations, are associated with morbidity. We | | | | | |
| 13 | present a large-scale study exploring factors defining urinary microbiome | | | | | |
| 14 | composition in community-dwelling older adult women without clinically active | | | | | |
| 15 | infection. Using 1600 twins, we estimate the contribution of genetic and | | | | | |
| 16 | environmental factors to microbiome variation. The urinary microbiome is distinct | | | | | |
| 17 | from nearby sites and unrelated to stool microbiome with more Actinobacteria, | | | | | |
| 18 | Fusobacteria and Proteobacteria, but fewer Bacteroidetes, Firmicutes and | | | | | |
| 19 | Verrumicrobia. A quarter of variants had heritability estimates greater than 10% with | | | | | |
| 20 | most heritable microbes having potential clinical relevance, including Escherichia- | | | | | |
| 21 | Shigella linked to urinary tract infections. Age, menopausal status, prior UTI and host | | | | | |
| 22 | genetics were top factors defining the urobiome with increased microbial diversity | | | | | |
| 23 | tending to associate with older age. These findings highlight the distinct composition | | | | | |
| 24 | of the urinary microbiome and significant contributions of host genetics. | | | | | |
| 25 | | | | | | |
| 26 | Keywords: microbiome, genetics, urogenital tract, ageing | | | | | |
| 27 28 | Introduction | | | | | |
| 29 | The resident microbial community (microbiome) at different body sites continues to | | | | | |
| 30 | generate research interest, driven by evidence of a role in human physiology. The | | | | | |
| 31 | study of the urinary microbiome (urobiome) is much less established compared to the | | | | | |
| 32 | gut microbiome; perhaps due to the previous belief that the urine was sterile in the | | | | | |
| 33 | absence of a urinary tract infection. Recently, research has shown that this is not the | | | | | |
| 34 | case and that the urinary tract is in fact, another site with a microbiome, reflective of | | | | | |
| 35 | the microbes inhabiting the bladder and closely associated organs (Fouts et al., 2012; | | | | | |
| 36 | Wolfe et al., 2012; Whiteside et al.; 2015). A urinary microbiome has been found in | | | | | |
| 37 | in catheterized/aspirated urine (bladder microbiome) and voided urine, using both | | | | | |

- 38 enhanced quantitative cultures and DNA-based identification (16S marker
- 39 studies/metagenomics), and samples from different populations (e.g Khasriya et al.,
- 40 2013; Hilt et al., 2014; Wu et al., 2017; Adebayo et al., 2017; Kramer et al., 2018).

41 Studies to date have largely focused on differences in the urobiome in relation to 42 urinary tract conditions (Sihra et al., 2018; Wolfe & Brubaker, 2019) including 43 urinary infections (UTI). There is evidence for differences in the male and female 44 urinary microbiomes (Bajic et al. 2018; Pearce et al., 2014). Women are much more 45 likely to develop UTI, with a lifetime risk of up to 50% (Franco, 2005) compared to 46 12% for men (Lee & Neild, 2007). UTI is also the most common reason for antibiotic 47 treatment in adult women, which has implications for urinary and other microbiomes 48 and antimicrobial resistance. Early work has indicated that the non-infected state 49 microbiome may influence resilience to infection (Pearce et al., 2015; Thomas-White 50 et al., 2018). The present study is focused on understanding the major factors defining 51 the urobiome in community dwelling women without active infection, who are not 52 seeking clinical assessment.

53 Previous studies involving urinary/bladder microbiomes have involved relatively

small sample sizes (dozens or few hundreds of people) in hospital or clinic attending

patients. For instance, results from our literature search (Jan 2015 to September 2018)

56 included incontinence (Pearce et al., 2015, n=182); case-control studies on

57 elderly/non-elderly patients (Liu et al., 2017; n=100); urinary tract infections

58 (Moustafa et al., 2018; n=112,Price et al., 2016, n=150); cancer (Wang et al., 2017;

59 n=65); overactive bladder (Wu et al., 2017; Fok et al., 2018; n=55-126); chronic

60 kidney disease (Kramer et al, 2018; n=41); surgical transplant patients (Rani et al.,

61 2018, n=20); surgery (Thomas-White et al., 2018,n=104); and menopause (Curtiss et

62 al., 2018; n=78). Reinforcing this, a recent review (covering studies up to 2016)

63 carried out by Aragon et al. (2018) reported that the sample sizes in urinary

64 microbiome studies varied between 8 to 60 for healthy controls and 10-197 for cases.

65 Their report shows that many studies are commissioned on incontinence, bladder-

related and gynaecologic patients. Recently, Price et al (2019) have studied data from

67 224 patients who were free from known urinary tract conditions, bladder problems or

68 surgery.

69 Studies to date have not investigated the contribution of host factors, like age or 70 genetics to the "normal" urinary tract microbiome. Genetic factors have been shown 71 to influence the gut microbiome (Goodrich et al., 2014, Luca et al., 2018), although 72 environmental factors remain predominant (Rothchilds et al., 2018). The urine 73 microbiome has an additional complexity, that is that many samples may be below the 74 threshold for a detectable microbiome. Few studies to date have included study of 75 sequence negative samples; notably Pearce et al. (2015) reported that sequence-76 negative samples were comparable in many characteristics to sequence-positive ones 77 in incontinent individuals.

78 We aimed to characterize the host influence on the urinary tract microbiome in

women who are well. We analysed midstream urine samples from 1600 older females

80 in the TwinsUK cohort recruited from the community for research, who had no

81 apparent infection, and were not undergoing hospital treatment. We hypothesized that,

82 in an unselected average population, (1) the inherent core urinary bacterial

83 community could be defined (2) that the urobiome is influenced by host-specific

84 genetic and environmental factors, (3) that some host-specific factors may relate to

85 the microbial biomass in the urine.

86

87 Results

88 Urinary tract microbiome was distinct from proximal body sites and similar to other 89 urine samples.

90 Initially, we compared the overall composition of the urinary microbiome to other

body sites , applying a similar analytical pipeline to 4 datasets of women older than

92 45 years from published studies (Goodrich et al., 2014; Thomas-White et al., 2017;

93 Pearce et al., 2014; Yatsunenko et al. 2012) (Methods, Data S1). Species diversity

94 (Shannon index) was comparable in urine and the vaginal datasets and reduced

95 relative to the stool(Fig 1A). Stool samples in the vast majority ordinated separately

96 from urine samples, and the current study (urine3) was the most dissimilar to gut or

97 vagina samples of the urine studies sampled (Fig 1B, Data S1). Repeating these

diversity analyses with 100 randomly chosen samples each available for 3 datasets

showed similar results (SFig1A,B). There was no clear correlation in stool and urine

- 100 microbiome dissimilarity for paired stool and urine samples from TwinsUK, even
- 101 when obtained on the same day) (Mantel's $r \le 0.02$, $p \ge 0.1$) (Fig 1C-D, Data
- 102 S1,SFig1C). The urine studies also had some taxa differences (SFig1D).
- 103

104 General description of TwinsUK urinary tract microbiome

105 Urine samples from 1600 mainly postmenopausal women (mean age= 66.4) in the 106 TwinsUK cohort were analysed, revealing 10955 taxa variants from filtered 16S data. 107 Participant characteristics are shown in Table 1. There was a high level of variability 108 in particular taxa present in an individual, with only 245 (2.2%) variants occurring in 109 at least 5% of samples. The use of a compositionally-sensitive analysis improved the 110 ranking of some abundant taxa as compared to common non-compositional analysis 111 (SFig2A). To highlight potential intra-microbiome relationships, clusters of frequent (present in >20%), co-occuring proportionally-balanced species were predicted, 112 113 resulting in 61 clusters of common variants (hereafter referred to as the core taxa) 114 (Fig2A). There were more Actinobacteria, Fusobacteria and Proteobacteria, but fewer

115 Bacteroidetes, Firmicutes and Verrumicrobia in urine compared to gut microbiome

116 (SFig 2B).

117 Low read count (no reliably-detected microbiome (<2000 reads post-filtering)) (Data

118 S2) associated with slightly younger age and lower level of health deficit; specifically,

119 a ~20% increase in the chance of a detectable microbiome for unit increase in

- standardized age (p=0.0048, OR=1.21, CI=1.07 1.39) and ~14% increase for a unit
- 121 increase in standardized frailty index (OR=1.144,CI=1.01-1.30,p=0.0359). There was
- 122 no association between low read status and the number of previous Urinary Tract
- 123 Infections (UTIs), recent antibiotics usage, surgery episodes or number of childbirth
- episodes (parity). Amplicon concentrations associated with parity ($\beta = 1.89, p=0.0035$)
- 125 but not other demographics (Data S2).
- 126

127 Host genetics' influences variation of urine microbiome

- 128 Various measurements can be useful to detect host genetics effect. First, we used
- heritability, a quantitative measure of the contribution of additive genetics to
- 130 variability in a phenotype, in this case the microbiome data. This showed considerable
- and significant genetic contribution to variance in the first principal coordinate of
- 132 Bray-Curtis dissimilarities (PCo) (A=0.147) and 2nd PCo (A=0.356). When

accounting for phylogenetic closeness and dominant taxa using weighted unifrac

134 dissimilarities (which captured as much as 57% of the variation), heritability of the

135 first PC was estimated at 18% (A= 0.179, CI=0.05-0.415, p=0.003351; C=0.0049,

136 E=0.8164, n=760). Significant heritability was maintained when controlling for age,

history of UTI, menopause status and cohabitation (Data S3). Some clusters of

- 138 frequent, co-occuring balanced microbial species showed particularly high heritability
- 139 (Fig 2A).
- 140

141 Second, we used a form of family segregation by applying constrained principal 142 coordinates analysis on the Bray-Curtis dissimilarity, with the family identity as a 143 factor, and then compared dissimilarities in identical and non-identical twins. The 144 dissimilarity was lower for monozygotic twins (Fig 2B) (p=0.0022). The difference in 145 the dispersion within a twin-pair (Euclidean distances to the median) in the 146 unconstrained analysis of Bray-Curtis, was also lower for monozygotic pairs 147 (SFig3A) (p=0.027) (Data S3). The first PCo was also associated with family identity 148 (Kruskal-Wallis p=0.043). Third, we compared ancestral origin of participants. The 149 study population was primarily of British ancestry, with microbiome data available 150 for 1141 British, 27 non British white, 19 SouthEast Asian, and 9 others, and 151 therefore findings would need to be confirmed in other studies. We minimized 152 imbalanced groups' effect by subsampling (n=98-118) using 20-fold partition with 153 resampling of non-British groups, and bootstrapping Kruskal-Wallis statistic. The 154 second PCo of the microbiome diversity (Bray-Curtis) differed according to the 4 155 major ancestry present (1st PC; p=0.156; 2nd PC p=0.000143, bootstrap averaged p= 156 0.0081, percentileCI =(0.00003-0.1223),1000 replicates), as was the permanova test 157 of Bray-Curtis dissimilarity between the ancestry groups (Fig. 2C, Data S3). 158

Finally we analysed the heritability of the core microbiome. The relative abundance
of these common variants as a group were influenced by host genetics (heritability
23% (CI=8.77-33.7, C=1.66E-12,E=0.76). Almost a quarter (59 of 245) of variants

162 found in at least 5% of the participants had heritability estimates greater than 10%

163 (STable1, SFig3B), though in five of these confidence intervals span zero. Some of

164 the most heritable variants some sequence similarity (SFig4). One of such heritable

165 variants, *Lactobacillus iners* AB-1 showed phylogenetic relatedness with

166 Christenellaceae variant previously reported in the gut (SFig4). Another,

167 *Escherichia_Shigella* (A=0.165) is a potential culprit in urinary tract infection. Based

168 on this finding, we also tested the heritability of occurrence of prior urinary tract

169 infections, finding prior UTI to be significantly heritable (A=0.273(0.399 for high

170 recurrence), 95%CI=0.178 – 0.368, Data S3).

- 171 Taken together, these four different sets of analyses support the hypothesis that host
- 172 genetic factors influence the urinary tract microbiome.
- 173
- Host-related/environmental factors in urinary tract microbiome, especially age, have
 important effects

176 The potential effects of multiple factors were assessed (Fig 3). Age, diet, recent

antibiotic usage and overall health deficit were assessed in relation to the urobiome as

they are known 'host-specific' influencers of gut microbiome variation. Parity

179 (previous number of births) and surgical history (had previous surgery or not) were

assessed as host-related "environmental" factors as they could potentially alter

- structures in or proximal to the urinary tract. Previous history of UTI was alsoassessed.
- 183 With increasing age, there is overall increase in alpha diversity (Shannon) (Table 1),

184 which was robust to uneven sample sizes or exclusion of small number of participants

aged $<50 (0.10 \ge \beta \le 0.22, 0.00027 \le p \ge 0.0045)$. Age differed with beta diversity

186 estimates (p<0.001), and was a main influencer of the ordination patterns of samples

187 (Fig 3B). The core taxa and one-third (22) of the subclusters, differed with age

188 (1.92E-30≤FDR≤0.046).

189 Diet (Healthy Eating Index), health deficit (frailty index) and antibiotics usage did not

190 produce significant associations in alpha diversity (Shannon) but borderline

associations were found with changes in beta diversity (Bray-Curtis) (diet, p=0.052,

192 n=1004; recent antibiotics usage, p=0.041,n=992; health deficit, p=0.031, n=1139).

- 193 Parity trended toward an association with reduced alpha diversity (Shannon)
- 194 (p=0.058,n=1047), and was significantly associated with beta diversity (Bray-Curtis)
- 195 (p=0.026,n=1047); surgical history did not differ with Bray-Curtis or Shannon metrics
- 196 (n=540). Occurrence of UTI differed with alpha diversity (p=0.0027) and beta

197 diversity (p=0.001). Similar results were obtained using unweighted unifrac sample

198 distances or controlling for other factors.

199 The contribution to variance that could be attributed to all factors, including host

200 genetics was then examined (Fig 3A). For individuals with available data for genetic-

201 based kinship, microbiome data, and the phenotypes in the preceeding paragraph

202 (n=545), unique contribution was obtained from R^2 decomposition on microbiome

203 Bray-Curtis beta diversity estimates, in permanova models (1000 permutations)

204 controlling for other factors. The average for each factor was used after randomly

rearranging all factors 20 times. Age was the top contributor, followed by menopause

status, history of prior UTI and host genetics (Fig3A, Data S3).

207

208 <u>Metagenomes confirm overall 16S microbiome data variation</u>

209 In microbiome studies, metagenomes not only provide taxonomic information 210 comparable to 16S analyses, they also offer deep insights into metabolic pathways 211 and better species resolution. Using shotgun metagenome data for a subset of 178 212 individuals, we also examined how closely the overall patterns of the 16S data are 213 replicated in the metagenome data. The classified metagenome reads were 99.64% 214 Bacteria (Data S4) and a greater number of urine metagenomes (total and per 215 individual) were obtained than earlier reported in literature. Sample-sample variation or inter-sample distances in the microbiome data were highly correlated from 216 217 metagenome and 16S data (Bray dissimilarities, Mantel's r=0.799,p=0.001). Sixteen 218 of the top 20 abundant taxa using 16S are also within the top 20 of the metagenome 219 data. The core microbiome found in 16S data was largely recapitulated in the 220 metagenomics analysis; 27 of the 31 genera (87%) forming the core taxa using 16S 221 data were also replicated in the metagenome data. From this core, the total number of 222 species identifiable approximately doubled (125 vs 61 in total, 94 vs 53 in the 223 replicated genera) most likely to due to better species assignment. Given the choice of 224 the subset for shotgun sequencing (Methods), heritability values were inflated (STable 225 2). Considered together, the metagenomes largely mirror 16S data and consolidate 226 results on heritability.

227

228 Discussion

In this study, we used a relatively larger, unselected community-based study

230 population of women and sensitive approaches (amplicon sequence variants,

- 231 compositional clusters and environmental effect control in twin-pairs) to explore the
- 232 influence of host factors on the urinary tract microbiome. These approaches
- strengthen deductions made here, for instance that age is associated with increasing
- urinary tract alpha diversity, contrary to previous studies (e.g. Curtiss et al., 2018;
- 235 Kramer et al., 2018; Liu et al., 2017; Wang et al., 2017).

236 Urine and other body sites

237 The ordination patterns of the urine microbiomes support current thinking that the 238 urobiome is a distinct site, similar to the observations that bladder microbiome (urine 239 obtained directly by catheter) differ from vaginal or stool microbiome (Thomas-White 240 et al., 2018; Wolfe & Brubaker, 2019). Here, the more divergent of the urine studies 241 (Urine1 cohort) shared more vaginal taxa, involved patients with incontinence and 242 collection was wholly catheterized, though had smaller sample size. In a very small 243 minority of individuals where urine microbiome taxa appear closer to stool, this is 244 most likely due to phylogenetic or genome similarity in species (as no such closeness 245 occur with non-phylogenetic measures), rather than common demographics (Data S1). 246 In all, the current study show clear dissimilarities in stool and midstream urine for the 247 average unselected population.

248

249 Host-related factors and host genetics' contribution in urinary tract microbiome 250 Parity (childbirth episodes), previous UTI occurrence, recent antibiotics usage and 251 diet showed changes with urine microbiome diversity. Using heritability analysis, the 252 current study showed a considerable genetic influence in the microbiome of ageing 253 women, reaching almost a third of the variation, with the remainder of contribution 254 largely due to variance unique to individuals. Some clinically important genera such 255 as *Escherichia* (A=0.165) had variants with high heritability estimates, In addition, 256 Lactobacillus iners (A=0.177), a commonly found vaginal and bladder microbe, was 257 phylogenetically close to the heritable gut microbe Christenellaceae and heritable in 258 urine.

- 259 Previously, Rothschild and colleagues (2018) reported that environmental factors
- such as sharing household eclipse genetic influence in gut microbiome composition,
- while Goodrich and colleagues (2014) showed host genetics played roles in gut
- 262 microbiome patterns of twin-pairs. The current study, indicates significant
- 263 contributions of genetics to the pattern of urine microbial composition; and

- controlling for cohabitation (participants asked if they live together or close with their
- sibling) and other known factors in urine microbial variation, did not alter the
- estimated the significant contributions to the pattern. Other parameters from this study
- bolster the observation of genetic influence: (1) samples of a member in a twin-pair
- were not extracted or sequenced in the same batch as the other member, (2) there was
- lower intra-twin difference distance among monozygotic pairs, and (3) the second
- 270 PCo which had higher heritability than the first was the same PCo which differed
- along the lines of ethnic ancestry (though the proportion of white British was
- dominant). Thus we conclude that host genetics influenced variation in urinary
- 273 microbiome composition in this population of women.
- Relative to other factors, only age, menopause status and prior history of current UTI
 were greater than the influence of genetics. Incidental to our main purpose, we also
 report here that history of urinary tract infections itself has a significant heritability in
 humans as suggested in Scholes et al. (2000) using family records and in Norris et al.
 (2000) using dogs. The results here also show a projected shift in microbiome
 structure after five UTI episodes (Fig3E).
- 280

281 <u>Heritable urinary tract microbes</u>

282 While *Corynebacterium* variants were frequent among top core taxa and clusters with 283 high heritability, the patterns detected for Lactobacillus and Escherichia variants 284 deserve mention. Our study showed the Escherichia-Shigella taxon, renamed as such 285 to reflect the extreme sequence similarity of Escherichia coli and Shigella, was part of 286 the urinary tract microbiota in older women. In absence of diagnosed infections, the 287 current study shows that presence of this taxon is influenced by (1) host genetic make 288 up (its proportions had one of the top heritability estimates (A=0.17,CI=0.11-0.29) of 289 all frequent urine microbial variants); and (2) age. Price et al. (2019) also recently 290 reported that *Escherichia* urotype were more likely in older asymptomatic patients. 291 These findings may have implications in the mixed success of *E. coli* vaccine trials 292 (Huttner et al., 2017) and in diagnostics.

293

294 The current study has limitations. Questionnaire data, which is subject to accurate

recall and self-report by participants, was part of measures used in deriving variables

- such as UTI, diet and frailty. Another limitation may be the use of a single midstream
- 297 urine sample set from an individual, and as such, prior microbiome stability

- information is unknown. Clearly, further research is needed to confirm if the findingsalso relate to the male urinary microbiome.
- 300

301 To conclude, we report on the factors influencing composition of the urinary tract

- 302 microbiome in unselected community-dwelling adult women. The urinary
- 303 microbiome was distinct and apparently unrelated to stool microbiome. It shows a
- 304 significant contribution of host genetics. Key species known to be clinically relevant
- 305 were among the most heritable microbes. Age and menopausal status were the factors
- 306 with greatest influence on the urinary microbiome in women.
- 307

308 Author Contributions

- 309 Conceptualization: C.J.S, T.S. and A.S.A; Investigation: C.J.S., G.H., G.A., R.B.,
- 310 P.W. and R.K.; Methodology: C.J.S. and A.S.A.; Formal Analysis: C.J.S. and A.S.A;
- 311 Writing: A.S.A, C.J.S, G.H., T.S. and R.K.; Funding Acquisition: C.J.S. and T.S;
- 312 Supervision: C.J.S., T.S. and R.K.
- 313

314 Acknowledgement

315 We thank Prof Alan Wolfe and Roberto Limeira of Health Sciences Division, Loyola

- 316 University Chicago, United States for providing access to raw sequence data from two
- urine studies; the phenotype data team at TwinsUK; laboratory team at TwinsUK for
- 318 sample handling; and Rachel Horsfall, Marina Mora Ortiz, Mary NiLochlainn and
- 319 Max Freydin for discussions and comments on the manuscript. CS received research
- 320 funding through the Chronic Disease Research Foundation which receives funds from
- 321 the Denise Coates Foundation. We also thank all participants in TwinsUK who
- 322 altruistically donated their time and samples for this research (www.twinsuk.ac.uk).
- 323
- 324

325 **Declaration of Interests**

- 326 The authors declare no competing interests
- 327

328 Figure Legends

- 329 Fig 1. Urinary tract microbiome in older women is mostly distinct from proximal
- body sites and unrelated to stool microbiome. Alpha diversity plots were based on
- 331 Shannon index and beta diversity based on unweighted unifrac distances. (A) Alpha

332 diversity of urine microbiomes and other body sites. star symbol indicates 333 significance compared to TwinsUK midstream urine. (B) Dissimilarities in urine 334 microbiomes and other body sites. (C) Paired alpha diversity analysis of stool 335 and urine collected at same time point (D) Differences in paired stool and urine 336 microbiome from the same time point. 337 338 Fig 2. Host genetics considerably influences variation of urine microbiome. (A) 339 Heritability and interaction of core urinary tract microbes. Size of circles at each 340 subcluster and intensity of rectangular bars at the tips represent increasing heritability 341 of taxa. Neighbouring variants in a clade show co-abundance and clustering is not 342 phylogenetic. Taxa are annotated to indicate different variants. (B) Microbiome 343 dissimilarities within family of twin pairs. MZ-monozygotic; DZ-Dizygotic (C) 344 Microbiome principal coordinates with ancestral origin. Ellipses represent 95% 345 confidence interval. White British constitute>90% of individuals, and 346 bootstrap/permanova testing were used due to imbalanced sizes. 347 348 Fig 3. Top contributors to urinary microbiome variation. (A) Relative 349 contributions to urinary microbiome. Bars represents average R² for each variable, 350 controlled for the presence of other factors. Microbial variation was measured using Bray-Curtis dissimilarities. Genetic PC was derived from principal components of 351 352 SNP-based genetic kinship. (B) Trends in individual Shannon diversity with age 353 and prior number of UTI. (C-E) Microbiome dissimilarities with top factors: (C) 354 age (D) menopause (E) prior number of UTI. 355 356 357 358 359 **Tables**

360 Table 1. Summary of participants in TwinsUK urinary microbiome study

| Phenotype | Subcategory | α-D index | Ave. no of unique | No. of | Age |
|-------------------------|-------------|-----------------|-------------------|---------|-----------------------|
| category | | (mean±SD) | taxa(mean±SD) | samples | (mean±SD) |
| Participants | | 2.01±1.05 | 65.7±48.8 | 1600 | 66.7±8.3 |
| Previous UTI | 0 times | 2.14±1.0 | 66.1±43.1 | 393 | 67.6±8.2 ^s |
| occurrence ^s | 1-4 times | 2.02±1.04 | 67.5±51.0 | 719 | 65.9 ± 7.8 |
| | 5-9 times | 1.98 ± 1.03 | 65.4±45.2 | 208 | 66.3±8.3 |
| | 10times > | 1.79±1.17 | 60.0±53.9 | 201 | 65.7±8.3 |

| Age ^s | <50-54 | 1.56±0.76 | 45.9±32.2 | 117 | - |
|------------------|-----------|-------------------------|-------------------------|-----|------------------------|
| | 55-59 | 1.86±1.13 | 61.7±49.7 | 210 | - |
| | 60-64 | 2.00 ± 0.98 | 63.5±44.8 | 327 | - |
| | 65-69 | 2.04±1.03 | 66.0±49.8 | 409 | - |
| | 70-74 | 2.16±0.97 | 71.5±50.6 | 276 | - |
| | 75-79 | 2.26±1.12 | 74.5±50.1 | 170 | - |
| | 80-84 | 2.02±1.12 | 63.7±41.9 | 68 | - |
| | 85- | 1.73±1.42 | 71.7±62.3 | 23 | - |
| RecentAntibiotic | Yes | 1.97±1.20 ^{ns} | 70.0±53.0 ^{ns} | 47 | 68.3±8.0 ^{ns} |
| usage:3mths | No | 2.03±1.06 | 66.0±49.0 | 945 | 66.6±8.3 |
| Frailty | < 0.15 | 2.05±1.01 ^{ns} | 67.0+49.0 ^{ns} | 511 | 65.9±7.5 ^s |
| | 0.15-0.29 | 1.99 ± 1.05 | 64.8±49.0 | 834 | 66.1±8.0 |
| | 0.3-0.44 | 2.04±1.15 | 67.5 ± 48.0 | 227 | 68.4 ± 8.9 |
| | >0.45 | 1.75 ± 1.17 | 62.0±47.0 | 28 | 68.5 ± 8.2 |

361 Legend. α-D: Shannon H index of alpha diversity; No. of taxa refers to number of unique sequence variant per sample i.e. no of potential species. Diversity measures were calculated after subsampling to 2000. S/NS indicates
 363 statistical significance or not for tests of a phenotype as a continuous variable. Post-hoc pairwise comparisons
 364 showed no difference in alpha diversity for individuals aged 75years and older.

- 366 STAR Methods
- 367 <u>RESOURCE AVAILABILITY</u>
- 368 *Lead Contact*

365

- 369 Further information and requests for resources and data should be directed to and will
- be fulfilled by the Lead Contact, Claire Steves (claire.j.steves@kcl.ac.uk).
- 371 <u>Materials Availability</u>
- 372 This study did not generate new unique reagents
- 373 Data and Code Availability
- 374 Raw sequence data is available from EBI's European Nucleotide Archives with
- accession number ERP119822. Phenotype data is available on request from TwinsUK
- 376 data access committee at http://twinsuk.ac.uk/resources-for-researchers/access-our-
- 377 data.html. Scripts and codes used are available at github.com/waleadebayo/urobiome-
- 378 host-genetics
- 379

380 EXPERIMENTAL MODEL AND SUBJECT DETAILS

- 381 Cohort and Phenotypes
- 382 The TwinsUK cohort has been described elsewhere (Verdi et al. 2019). It comprises
- 383 over 14,000 volunteers in total over more than two decades, predominantly female
- 384 (>80%) and middle-aged (mean age 59). Data were collected with visits to the
- 385 Department of Twin Research and Genetic Epidemiology, King's College London,
- 386 resulting in biochemical, behavioral, dietary and socioeconomic cohort
- 387 characterization. Participants in the cohort are community dwelling twin pairs,

388 recruited without any specific clinical phenotype. The current study included 1600

- individuals, and various demographics were examined. Medical history
- 390 questionnaires were used to define age (from birth date), history of urinary tract

391 infections (UTIs), cohabitation (live together or close neighbourhood), antibiotic

392 usage, previous hysterectomy, previous oophorectomy, caesarian section and

393 menopause status. The frailty index, calculated from clinical, physiological and

394 mental domains (Livshits et al., 2017) was used as a measure of health deficit, and the

- Healthy Eating Index (Bowyer et al. 2018) based on food frequency questionnaires

396

397 <u>METHOD DETAILS</u>

used to assess diet.

398 <u>16S Microbiome Sequencing and Analysis</u>

399 Twin-pair samples were separated for processing. Extraction and Sequencing of 400 samples along with 128 negative controls was performed at the Knight Lab, 401 University of California San Diego using Earth Microbiome Project (EMP) protocols 402 (http://www.earthmicrobiome.org/protocols-and-standards/16s) with the Qiagen 403 MagAttract PowerSoil DNA kit. Amplicon PCR was performed on the V4 region of 404 the 16S rRNA gene using the primer pair 515f to 806r with Golay error-correcting 405 barcodes on the reverse primer. The barcoded amplicon pool was purified with the 406 MO BIO UltraClean PCR cleanup kit and sequenced on the Illumina MiSeq platform. 407 Sequence data were demultiplexed using the QIIME2 (Bolyen et al. 2019). Multilevel 408 quality filtering procedures and data analysis were applied to remove potential 409 contaminants (see below). Amplicon sequence variants (ASVs), were generated with 410 DADA2(Callahan et al, 2016), filtered and analysed as individual taxa. They were 411 also analysed as balances (Morton et al., 2017), essentially by forming clusters from 412 highly frequent variants (presence in >20% of individuals) which were transformed 413 compositionally, correlated and linked in an hierarchical fashion. ASVs are error-414 corrected sequences and offer better sequence resolution in taxonomy assignment, 415 which was done with Silva (v132) (Yilmaz et al., 2014). The current data was also 416 compared to those of previous microbiome studies with similar age-range of 417 participants after re-analysis of such data to produce ASVs (see below). Diversity 418 analysis was carried out with Shannon index, unifrac and Bray-Curtis metrics, and 419 permutational multivariate analysis of variance was used to test inter-sample

- 420 differences. Taxa counts were centred-log-ratio transformed after adding a
- 421 pseudocount (van de Boogart et al. 2019).
- 422 *Filtering and removal of possible contaminants*
- 423 Initially, common QC processes were followed such as artifact removal, chimera
- 424 checking, read-length trimming, and short-read discard. As low biomass microbiome
- tend to be influenced more by contaminants and cross-talk than high biomass sites
- 426 such as gut, more steps were utilised.
- 427 (a)Blank controls (n=105) were sequenced along with normal samples.
- 428 (b) Sequence variants (or potential taxa) were removed if the counts attributed to it in
- the blanks was more than 5% of the total counts for that taxa variant; OR if the
- 430 number of blanks in which a sequence variant occur is more than 10% of the total
- 431 number (blanks + actual samples).
- 432 (c) Sequence variants were removed if they significantly exhibit a pattern such that its
- 433 abundance was prevalent in blank controls that were sequenced along with normal
- 434 samples (e.g. the variant occurs in 50% of blanks but only in 10% of normal samples)
- 435 or a strong negative correlation (p<0.1) exist between the amplicon library
- 436 concentration and the number of reads generated for a sequence variant, as
- 437 implemented in Davis et al. (2018). Step (b) above was used to complement step c
- 438 which could not deal with this.
- 439 Subsequently, a sample with reads higher than 2000 was deemed to be reliably
- 440 detected. Setting cut-off at 2000 reads is based on the fact that
- 441 (1) It covers about 99.6% of diversity (Shannon) in rarefaction plots and 99.4%
- 442 coverage (Good's statistic)
- 443 (2) it was much higher than any number reads still present in any blanks after QC and
- 444 further filtering. i.e. after all QC steps, 30 of 105 blanks sequenced still contained
- some reads, 90% of these 30 blanks had less than 335 reads, the mean was 152.
- 446 Because the QC was rather rigorous, these reads are probably due to cross talk in
- 447 sequencer rather than contaminants.
- 448 We also briefly examined potential biological explanations for the occurrence of
- extremely-low DNA urine sample, apart from efficacy of technical protocols.
- 450 <u>Comparison of microbiome studies of similar age</u>
- 451 Raw sequence data used in Pearce et al. 2014 (Urine 1), Thomas-White et al. (2017)
- 452 (Urine 2), Puerto Rico and Plantanal study described as part of Yatsunenko et al. 2012
- 453 (Vaginal), Goodrich et al. 2014 (Gut), were obtained on request from authors or from

454 the EBI's ENA database. These studies, also using 16S V4 region, generally sampled 455 by requesting participants from the general population but some participants in the 456 urine studies were recruited based on specific phenotype of interest. Each sequence 457 set was analysed using the same bioinformatics pipeline described for the current 458 study, and each dataset was subsetted to include only women aged 45 years and above 459 to match current study. Re- analysis of these published datasets helped to avoid some 460 data-induced differences in alpha diversity and create a uniform platform for 461 comparison. Also, to minimise multi-study protocol variations and include as many 462 sample as possible, ASV counts were subsampled to 1000 reads in all studies and also 463 subsampled randomly to 100 subjects in each of two replicate sets (except Urine1 and 464 vaginal with smaller participants, n=57 and 11, respectively).

465 <u>Metagenome Analysis</u>

466 Shotgun metagenomic sequencing was carried out for 178 of the participants with 467 additional 14 blanks for quality control. The protocol involved 5ng DNA per reaction 468 quantified using a PicoGreen fluorescence assay. After fragmentation, end repair and 469 A-tailing, sequencing adapters and barcode indices are added following the iTru 470 adapter protocol. Unique error-correcting i7 and i5 indices were used after 471 purification, and indexed libraries were then purified again, quantified and 472 normalized, prior to sequencing on the Illumina HiSeq4000 platform. The approach 473 involved shallow shotgun methods (SHOGUN) (Hillman et al., 2018). This subset of 474 participants included equal numbers of dizygotic pairs and monozygotic twin pairs, as 475 well as equal numbers of twin pairs showing discordance and concordance in 16S 476 microbial diversity (pair-to-pair difference in diversity (Shannon index) greater than 477 3SD or lower than 1SD). This was expected to inflate heritability estimates. After 478 quality control filtering (with average $q \ge 30$), and mapped human reads' removal 479 (based on hg19) one sample was excluded, and the final data included 177 samples. 480 Potential contaminants, eleven species, were removed for presence in blanks and

- 481 constituting >2% (between 6% and 100%) of the abundance of that species. These
- 482 'contaminant' species included Mycobacterium_iranicum, Gordonia_paraffinivorans,
- 483 Staphylococcus_saprophyticus,
- 484 Delftia_acidovorans, Corynebacterium_matruchotii, Staphylococcus_capitis,
- 485 Acinetobacter_harbinensis, Corynebacterium_singulare, Cutibacterium_granulosum,
- 486 Acinetobacter_towneri, and Cutibacterium_acnes.
- 487

488 *Host genetics analyses*

489 Heritability was calculated using an ACE model in which the component of 490 phenotypes explained by genetics in twin pairs was estimated. Samples from co-twin 491 were separated into different batches for sample preparation and sequencing to 492 remove the shared technical environment related to batching. This further solidified 493 the deductions made from the analysis of the genetic effects. Discordance analysis, 494 quantitative differences in microbiome for pairs of monozygotic and dizygotic twins, 495 was approached using constrained principal coordinates as well as dispersions in 496 microbiome variance. Where constrained principal coordinates analysis was used, 497 microbiome data was ordinated with the family ID tested as a predictor, then the 498 dissimilarity within a family was then extracted to compare twin types. Analysis on 499 ethnic origin of participants was based on information obtained from questionnaires, 500 and to reduce the impact of the large difference in the group sizes, partitions were 501 created in which non-British groups were repeatedly sampled, before bootstrapped 502 Kruskal-Wallis statistic were estimated. Also, as a confirmation, permutation-based 503 testing were used for the original undivided data. To represent host genetic variation, 504 first principal component from genome-based kinship matrix data were obtained. 505 These analyses were carried out with plink1.9b, R base and R packages: vegan, mets, 506 car, phyloseq(see Resources Table).

507

508 QUANTIFICATION AND STATISTICAL ANALYSIS

509 All statistical details and tests can be found in Results and Method Details sections 510 following the contexts in which they were used. n represents number of individuals, 511 SD represents one standard deviation, confidence intervals were set at 95%, and 512 significance threshold was set at alpha less than 0.05. Throughout analysis, technical 513 covariates, including extraction kit lots, mastermix kit lot, batch, extraction and 514 sequencing processors, and depth/library sizes (sequence reads post-QC filtering) 515 were controlled for. Visualizations were generated using ade4, ggplot2, graphlan2, 516 decontam, FastTree and ggtree (see Resources Table). 517 518 STable1. Heritability of midstream urine microbiome abundance in paired 519 twins. Related to Figure 2.

521 STable2. Heritability of urine metagenomes from diversity-discordant and

522 diversity-concordant pairs of twins. Related to Figure 2.

523

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