HLA alleles associated with risk of ankylosing spondylitis and rheumatoid arthritis influence the gut microbiome

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ABSTRACT

Objectives. HLA alleles affect susceptibility to more than 100 diseases, but the mechanisms to account for these genotype-disease associations are largely unknown. HLA-alleles strongly influence predisposition to ankylosing spondylitis (AS) and rheumatoid arthritis (RA). Both AS and RA patients have discrete intestinal and faecal microbiome signatures. Whether these changes are cause or consequence of the diseases themselves is unclear. To distinguish these possibilities, we examine the effect of *HLA-B27* and *HLA-DRB1* RA-risk alleles on the composition of the intestinal microbiome in healthy individuals.

Methods. 568 samples from 6 intestinal sites were collected from 107 otherwise healthy unrelated subjects and stool samples from 696 twin pairs from the TwinsUK cohort. Microbiome profiling was performed using sequencing of the 16S rRNA bacterial marker gene. All patients were genotyped using the Illumina CoreExome SNP microarray, and HLA genotypes were imputed from these data.

Results. Association was observed between *HLA-B27* genotype, and RA-risk *HLA-DRB1* alleles, and overall microbial composition (P=0.0002 and P=0.00001 respectively). These associations were replicated in the TwinsUK cohort stool samples (P=0.023 and P=0.033 respectively).

Conclusions. This study shows that the changes in intestinal microbiome composition seen in AS and RA are at least partially due to effects of *HLA-B27* and –*DRB1* on the gut microbiome. These findings support the hypothesis that HLA alleles operate to cause or increase the risk of these diseases through interaction with the intestinal microbiome, and suggest that therapies targeting the microbiome may be effective in their prevention and/or treatment.

Keywords

Ankylosing spondylitis, rheumatoid arthritis, microbiome.

INTRODUCTION

HLA molecules affect susceptibility to many diseases, but in the majority of cases the mechanism by which HLA molecules predispose to disease remains a mystery. The risks of developing both ankylosing spondylitis (AS) and rheumatoid arthritis are primarily driven by genetic effects, with heritability >90% (1, 2) for AS, and 53-68% for RA (3, 4). In both diseases HLA alleles are the major susceptibility factors, with AS being strongly associated with *HLA-B27*, and RA with *HLA-DRB1* 'shared-epitope' (SE) alleles.

Particularly in AS, there is strong evidence of a role for gut disease in disease pathogenesis. Up to an estimated 70% of AS patients have either clinical or subclinical gut disease, suggesting that intestinal inflammation may play a role in disease pathogenesis (5, 6). Increased gut permeability has been demonstrated in both AS patients and their first-degree relatives compared with unrelated healthy controls (7-11). Crohn's disease (CD) is closely related to AS with a similar prevalence and high heritability. The two commonly co-occur with an estimated ~5% of AS patients developing CD, and 4-10% of CD patients developing AS (12, 13). Strong co-familiality (14), and the extensive sharing of genetic factors between AS and inflammatory bowel disease (IBD) (15, 16) suggests that they have a shared aetiopathogenesis. This is consistent with the hypothesis that increased 'leakiness' of the gut, and potentially the capability of HLA-B27 to present relevant microbial antigens, may alter the gut microbial composition and increase gut mucosal microbial dissemination, and hence drive inflammation in the disease (17, 18). Reduced expression of the intestinal barrier protein zonulin and histopathological evidence of gut wall invasion by bacteria in AS further support this hypothesis (19).

Using 16S rRNA community profiling we have previously demonstrated that AS cases have a discrete intestinal microbial signature in the terminal ileum (TI) compared with healthy controls (HC) ($P<0.001$) (20), a finding that has subsequently been confirmed by other studies (21, 22). We have also demonstrated that dysbiosis is an early feature of disease in *HLA-B27* transgenic rats, preceding the

103 onset of clinical disease in the gut or joints (23). Similarly, RA cases have also been shown to have gut
104 dysbiosis (24, 25), and animal models of RA such as collagen-induced arthritis have been shown to be
105 influenced by the gut microbiome (26, 27). In these studies it is difficult to distinguish between effects
106 of the immunological processes going on in the intestinal wall in cases, and the effects of treatments
107 on the intestinal microbiome, from potential effects of the gut microbiome on the disease.
108
109 The role of the host genetics in shaping intestinal microbial community composition in humans is
110 unclear. In animal models, host gene deletions have been shown to result in shifts in microbiota
111 composition (28). In addition, a recent quantitative trait locus mapping study in an inter-cross murine
112 model, linked specific genetic polymorphisms with microbial abundances (29). Large scale studies in
113 twins (n=1126 twin pairs) have demonstrated that of 945 widely shared taxa, 8.8% showed significant
114 heritability, with some taxa having heritability of >40% (e.g. family *Christensenellaceae*, heritability
115 42%) (30).
116
117 Further studies are needed into whether the changes in intestinal microbial composition are due to
118 host genetics, and how this affects the overall function of the gut microbiome in cases, including how
119 the microbiome then goes on to shape the immune response and influence inflammation. In AS,
120 given the strong association of *HLA-B27*, the hypothesis has been raised that *HLA-B27* induces AS by
121 effects on the gut microbiome, in turn driving spondyloarthritis and inducing immunological processes
122 such as IL-23 production (31, 32). Further experiments comparing the intestinal microbiome of *HLA-*
123 *B27* negative and positive patients would shed light of the influence of *HLA-B27* on overall intestinal
124 microbiome composition, particularly given the work in *HLA-B27* transgenic rats showing that *HLA-*
125 *B27* was associated with altered ileal, caecal, colonic and fecal microbiota (23, 33, 34). Similar theories
126 have been proposed with regard to interaction between the gut microbiome and the immunological
127 processes that drive RA (reviewed in (35)).
128

129 In this study we investigated if AS and RA-associated HLA alleles influence the gut microbiome in
130 healthy individuals, to support the hypothesis that they influence the risk of developing AS and RA
131 through effects on the gut microbiome.
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133

134 **METHODS**

135 **Human subjects**

136 A total of 107 subjects, aged 40-75, predominately Caucasian (~90%), typically following an
137 omnivorous diet (~95%) and were undergoing routine colorectal cancer screening at Oregon Health &
138 Science University's Center for Health and Healing were included in this study. Individuals were
139 excluded if they had a personal history of inflammatory bowel disease or colon cancer, prior bowel or
140 intestinal surgery or were pregnant. All subjects underwent a standard polyethylene glycol bowel prep
141 the day prior to their colonoscopy procedure. During the procedure, biopsies were collected for
142 research purposes from the terminal ileum or other tissue sites as indicated. Subjects were instructed
143 to collect a stool sample on a sterile swab at home, just prior to starting their bowel prep procedure.
144 Stool samples were brought to the colonoscopy appointment at room temperature. All samples
145 (biopsies and fecal swabs) were placed at 4°C in the clinic and transported to the lab within 2 hours of
146 the colonoscopy procedure, where they were snap frozen and stored at -80°C prior to processing.
147 Patient samples were obtained over a 24-month period.

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149 Ethical approval for this study was obtained from the Oregon Health & Science University Institutional
150 Review Board. Written informed consent was obtained from all subjects. This study was performed
151 subject to all applicable U.S. Federal and State regulations.

152

153 **TwinsUK**

154 All work involving human subjects was approved by the Cornell University IRB (Protocol ID
155 1108002388). Matched genotyped and stool samples were collected from 1392 twins. Genotyping,
156 16S rRNA amplicon sequencing, filtering and analysis were performed as described in Goodrich *et al.,*
157 2014 (36).

158

159

160 **16S rRNA amplicon sequencing and analysis**

161 568 stool and biopsy samples across 107 individuals were extracted and amplified for the bacterial
162 marker gene 16S rRNA as previously described (20). Samples were demultiplexed and filtered for
163 quality using the online platform BaseSpace (http://basespace.illumina.com). Paired end reads were
164 joined, quality filtered and analysed using Quantitative Insights Into Microbial Ecology (QIIME) v1.9.1
165 (37). Operational taxonomy units (OTU) were picked against a closed reference and taxonomy was
166 assigned using the Greengenes database (gg_13_8) (38), clustered at 97% similarity by UCLUST (39)
167 and low abundance OTUs were removed (<0.01%).

168

169 Multidimensional data visualisation was conducted using a sparse partial least squares discriminant
170 analysis (sPLSDA) on centered log ratio transformed data, as implemented in R as part of the MixOmics
171 package v6.3.1 (40). Association of the microbial composition with metadata of interest was
172 conducted using a PERMANOVA test as part of vegan v2.4-5 (41) on arcsine square root transformed
173 data at species level, taking into account individual identity where multiple sites per individual were
174 co-analysed, as well as the sources of covariation such as BMI and gender. Alpha diversity was
175 calculated at species level using the rarefy function as implemented in vegan v2.4-5 and differences
176 were evaluated using a Wilcoxon rank-sum test. The metagenome functional content was predicted
177 using PICRUSt v1.1.3 (42) and the resulting predictions were mapped to KEGG pathways using
178 HUMAnN2 v0.11.1 (43) Differential abundance of bacterial taxa and KEGG pathways were tested for
179 significance using MaAsLin v0.0.5 (44).

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186 **Genotyping**

187 DNA was extracted from mucosal biopsies and stool samples, and genotyped using Illumina
188 CoreExome SNP microarrays according to standard protocols. Bead intensity data were processed
189 and normalized for each sample, and genotypes called using Genome Studio (Illumina). We imputed
190 *HLA-B* genotypes using SNP2HLA (45), as previously reported (46). The distribution of *HLA-B27* and
191 *HLA-DRB1* RA-risk, -protective and –neutral subtypes is available in Supplementary Table 1.

192

193 **RESULTS**

194 16S rRNA profiling and SNP array genotyping was successfully completed for 107 individuals (61
195 female, 46 male) involving a total of 564 biopsy samples (see Table 1).

196

197 We studied the effect of BMI, gender and sampling site on the gut microbiome to identify relevant
198 covariates for analysis of AS-associated genes and their association with the gut microbiome.
199 Considering sample site, striking differences were observed, particularly between the stool samples
200 and mucosal samples (Figure 1A, P<0.0001). Excluding stool samples, marked difference was still
201 observed between sites (P<0.0001), but it can be observed that this is mainly driven by differences of
202 the ileal samples from the colonic mucosal samples (left and right colon, cecum, rectum), which largely
203 clustered together (Figure 1B).

204

205 Stool samples are much more convenient to obtain than ileal or colonic mucosal samples, which
206 require an endoscopic procedure for collection. Given the prior evidence of primarily ileal
207 inflammation in AS (5), we were interested in the relationship between the ileal and stool microbiome.
208 In this comparison marked differences were observed between sites, though with some overlap seen
209 on the sPLSDA plot (Supplementary Figure 1, P<0.0001).

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Site	Total	Female	Male	HLA-B27 Negative	HLA-B27 Positive	HLA-DRB1 Risk Genotype	HLA-DRB1 Protective Genotype	HLA-DRB1 Neutral Genotype
Cecum	103	59	44	93	10	34	8	47
Ileum	90	51	39	80	10	36	8	45
Left Colon	100	57	43	90	10	33	7	47
Rectum	91	53	38	81	10	33	7	41
Right Colon	97	57	40	87	10	33	8	45
Stool	83	46	37	73	10	29	8	36

Table 1: Number of samples and *HLA-B27* and *HLA-DRB1* shared epitope allele status by site. Note that different subjects had different numbers of samples obtained, and at no individual site did all subjects have samples obtained.

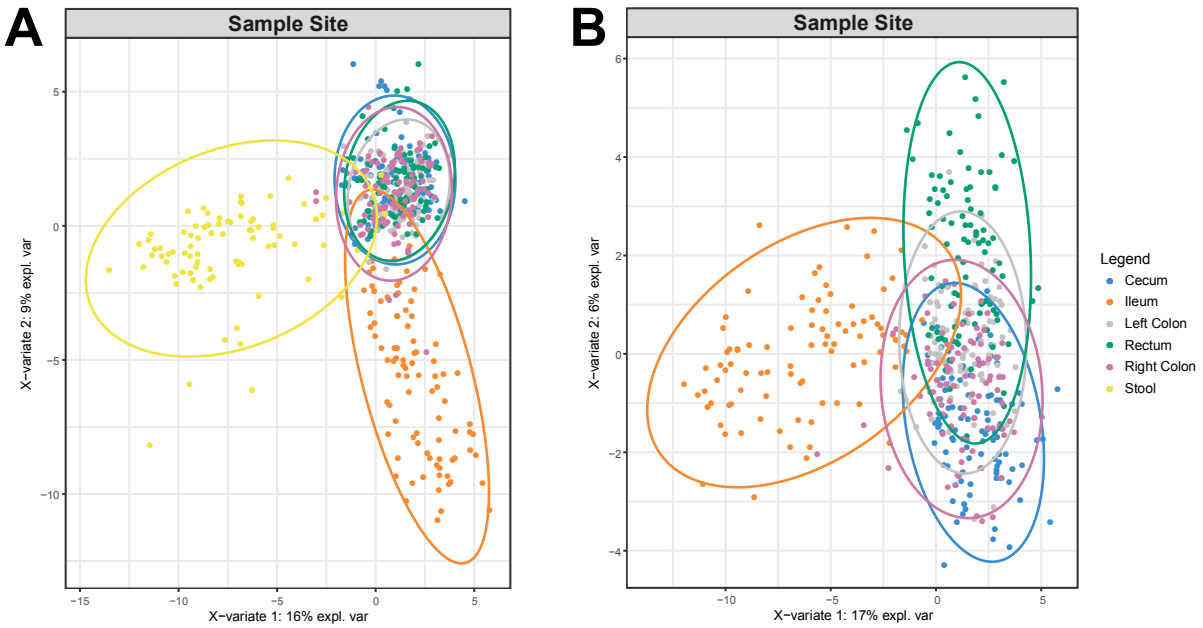

Figure 1: sPLSDA comparing the microbiome composition at various sample sites, showing **A.** marked difference of stool/luminal site compared with all other sites, which are mucosal, and **B.** in the absence of stool samples, the ileal site remains distinct from colonic sites.

Several studies have noted an increase (47), decrease (20, 21) or no change (48) in alpha diversity metrics for AS cases, and an increase (22) or decrease (49) in alpha diversity for RA cases. In the current study, calculation of rarefied species richness revealed that carriage of *HLA-B27* and *HLA-DRB1* alleles was not associated with differences in alpha diversity, except for stool samples for which carriage of *HLA-DRB1* RA-risk alleles was associated an increased alpha diversity across both cohorts (Figure 2).

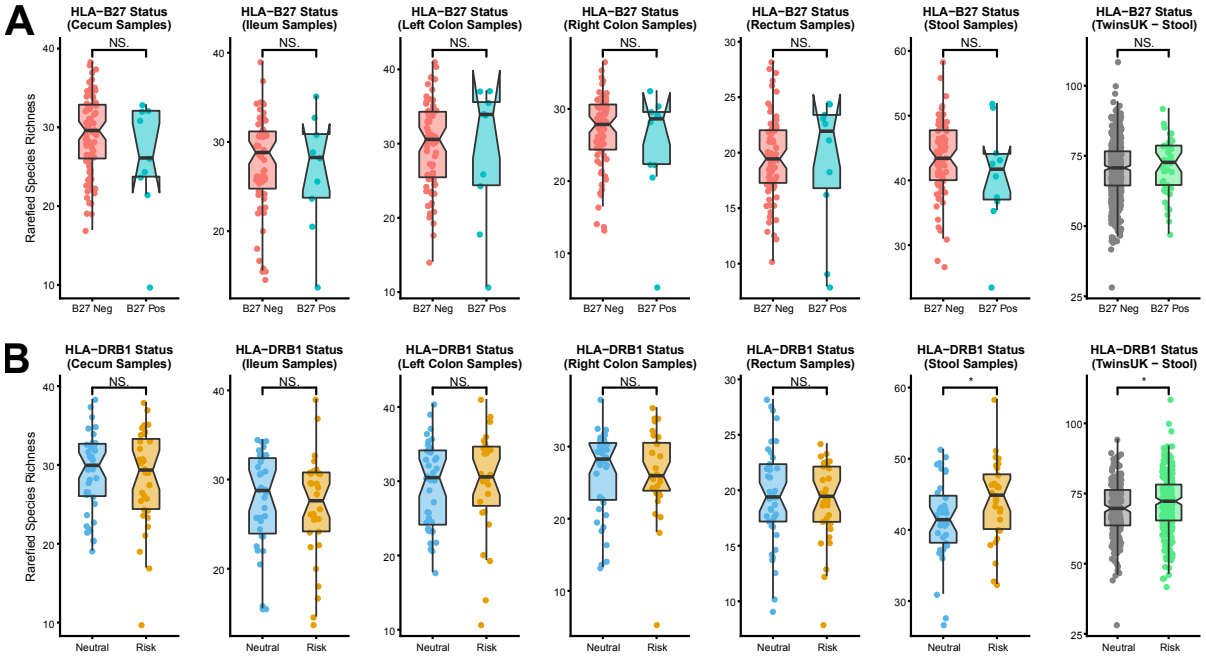

Figure 2: Alpha diversity across each sampling site, and in the TwinsUK cohort **A.** Alpha diversity according *HLA-B27* status. **B.** Alpha diversity according to *HLA-DRB1* status, revealing increased alpha diversity in stool samples of both cohorts.

Considering beta diversity via sPLSDA and PERMANOVA, significant association of BMI category was seen with microbiome composition (P=0.0022)(Supplementary Figure 2A). This appears to be driven particularly by the difference of underweight individuals (BMI<18.5) compared with other BMI categories. Removing underweight samples from the analysis, a non-significant trend of association of BMI category with microbiome composition is seen (P=0.078)(Supplementary Figure 2B), consistent with previous reports.

260 Given the marked gender biases in RA and AS, and evidence in mice that gender related hormonal
261 differences are associated with differences in the intestinal microbiome (53, 54), we sought to
262 evaluate the influence of gender on the microbiome in this cohort. Whilst substantial overlap
263 between males and females was evident (Supplementary Figure 3), significant difference between
264 genders in microbiome composition was observed (considering all sites, P=0.0004). Considering
265 indicator species, a significant reduction in carriage of *Prevotella* genus in males was observed
266 (P=0.005).

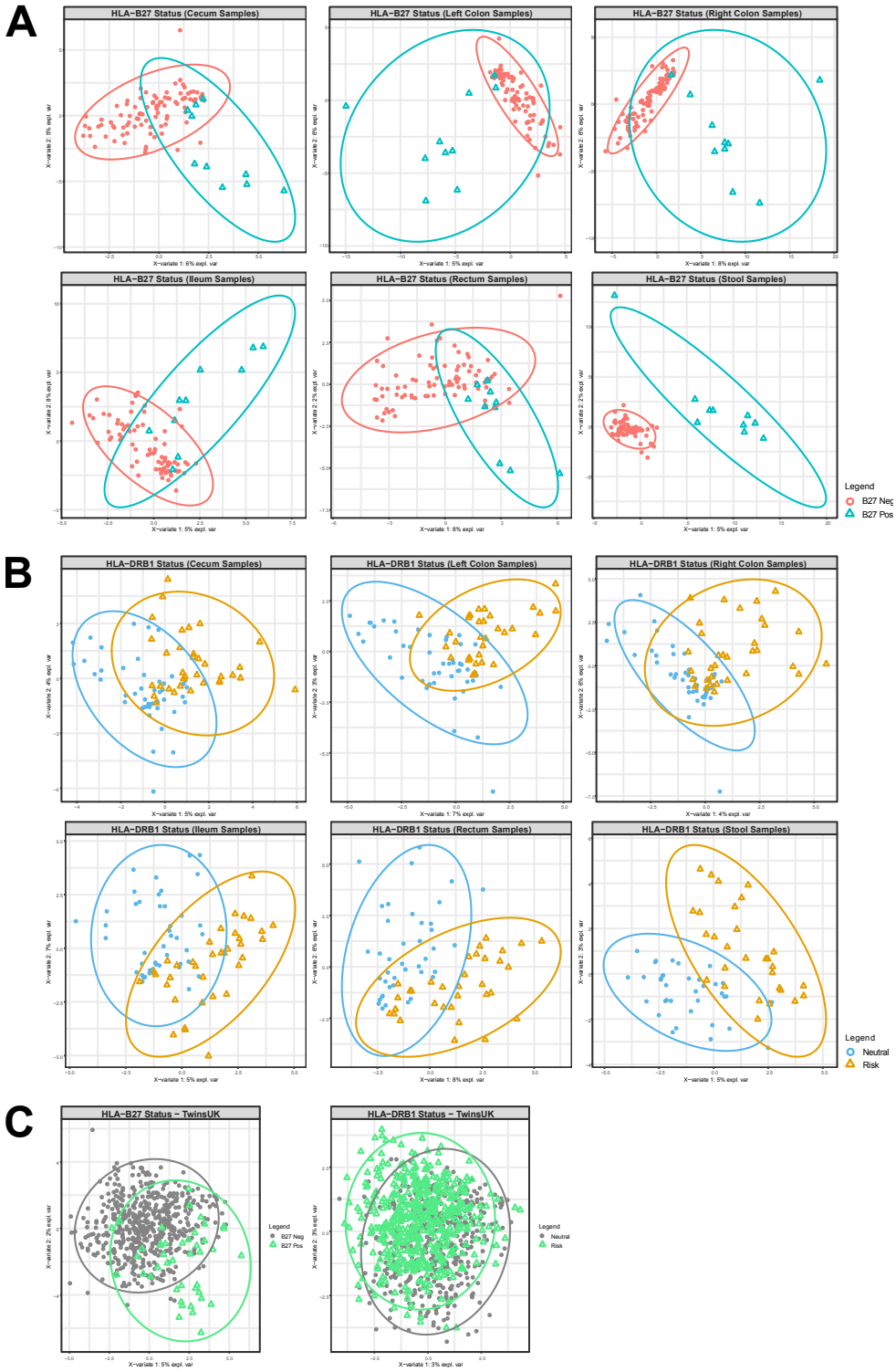

Figure 3: A. sPLSDA comparing the microbiome composition of *HLA-B27* positive and negative individuals across each sampling site. Considering all sampling sites and accounting for repeated sampling, significant differentiation of the microbiome was observed (PERMANOVA P=0.002). **B.** sPLSDA comparing individuals carrying the *HLA-DRB1* RA-risk and -neutral genotypes across each sampling site. Considering all sites and accounting for repeated sampling, significant differentiation of the microbiome was observed (PERMANOVA P=0.0001). **C.** sPLSDA plot comparing *HLA-B27* positive and negative twins (one twin randomly selected from each twin pair, PERMANOVA P=0.023), and *HLA-DRB1* risk and neutral genotypes (one twin randomly selected from each twin pair, PERMANOVA P=0.033).

277

278 We tested whether HLA-B alleles associated with AS were also associated with gut microbial profiles.
279 The association of HLA-B alleles with AS is complex, with risk associations observed with *HLA-B27, -*
280 *B13, -B40, -B47* and *–B51*, and protective associations with *HLA-B7* and *–B57* (55). Of these, only *HLA-*
281 *B27* showed statistically significant association with microbiome profile across both cohorts.
282 Differences in the microbiome composition were more pronounced when comparing risk-associated
283 alleles to protective alleles. For example, when focusing on a subset of data (ileal samples), marginal
284 differentiation for *–B27* (P=0.16) and no differentiation for *–B7* (P=0.61) was observed, potentially
285 highlighting sample size constraints. However, direct comparison of *–B27* to *–B7* revealed significant
286 differentiation (P=0.008).

287

288 *HLA-B27*-positive subjects exhibited reduced carriage (P<0.05) of *Bacterioides ovatus* across multiple
289 sites (ileum, cecum, left colon, right colon and stool), as well as *Blautia obeum* (left colon and right
290 colon) and *Dorea formicigenerans* (rectum and stool). Increased carriage of a *Roseburia* species was
291 observed across multiple sites (left colon, right colon, rectum and stool) and family *Neisseriaceae*
292 (cecum and ileum). For subjects with RA-risk *HLA-DRB1* alleles, numerous taxonomic groups were
293 enriched across multiple sites, notably a *Lachnospiraceae* species (ileum, cecum, left colon, right colon
294 and rectum), a *Clostridiaceae* species (left colon, right colon, rectum and stool) *Bifidobacterium*
295 *longum* (cecum, right colon and rectum), amongst many others. Enrichment of *Ruminococcus gnavus*
296 was also observed in the ileum of subjects carrying risk alleles. A full list of differently abundant taxa
297 according to *HLA-B27* and *HLA-DRB1* status are available in Supplementary Tables 2 and 3,
298 respectively. Interestingly, when accounting for false discovery rate, no single taxa was significantly
299 associated with the investigated genotypes, indicating that community-level differences detectable
300 via PERMANOVA may be driven by subtle changes in a high number of taxa, as opposed to marked
301 changes in a select few.

302

303 Considering the inferred metabolic profiles for *HLA-B27* positive and negative subjects, we observed
304 significant differences (P<0.05) across multiple sites for numerous KEGG pathways (Supplementary
305 Table 4). Examples include flagellar assembly (ileum, cecum, left colon, right colon and rectum),
306 alanine metabolism (cecum, ileum, left colon, and right colon), lysine biosynthesis (left and right colon)
307 and degradation (ileum, rectum and stool), secondary bile acid biosynthesis (ileum and stool) and
308 bacterial invasion of epithelial cells (ileum). For the RA-risk alleles (*HLA-DRB1*), numerous differences
309 in KEGG pathways were observed (Supplementary Table 5). Examples include thiamine metabolism,
310 the citric acid cycle, lipopolysaccharide biosynthesis, biosynthesis of ansamycins, and bacterial
311 chemotaxis, all of which were differentially abundant across every intestinal site.

312 **DISCUSSION**

313 In this study we have demonstrated for the first time that in the absence of disease or treatment, *HLA-*
314 *B27* and *HLA-DRB1* have significant effects on the gut microbiome in humans. This extends previous
315 demonstrations that AS and RA are characterized by intestinal dysbiosis by confirming that this is at
316 least in part due to the effects of the major genetic risk factors for AS and RA, *HLA-B27* and *HLA-DRB1*
317 *risk* alleles, respectively.

318

319 We demonstrate a clear distinction in microbiome profile between luminal stool samples and mucosal
320 samples, as well as between mucosal samples from different intestinal sites. Of particular note,
321 marked difference was observed between ileal and stool samples. These findings contrast a previous
322 smaller study, which may not have observed a difference between ileal and colonic biopsies due to
323 sample size considerations (48). Many studies of the influence of gut microbiome focus on stool
324 samples, as they are easier to obtain than mucosal samples. The existence of gut inflammation,
325 particularly involving the ileum, in AS cases has been well documented. Therefore, our findings
326 suggest that studies of the microbiome in AS and RA, particularly where the aim is to identify the key
327 indicator species driving or protecting from the disease, should use samples that reflect the ileal
328 microbiome. As the microbiome profile of stool samples do not closely correlate with the ileal
329 microbiome, they would not appear to be an optimal sample to study, although studying IgA coated
330 bacteria isolated from stool samples may prove more informative (56).

331

332 Following our initial study, three further studies have now reported on the difference in gut microbial
333 composition in AS cases and controls. Tito et al (48) in a study of 27 spondyloarthritis patients (i.e.
334 not necessarily AS) and 15 healthy controls using 16S rRNA profiling report association of carriage of
335 *Dialister* in ileal or colonic mucosal biopsies with disease activity assessed by the self-reported
336 questionnaire the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), and Ankylosing
337 Spondylitis Disease Activity Score (ASDAS). We did not observe *Dialister* in our study and therefore

338 cannot comment on whether it is associated with *HLA-B27* carriage. Tito et al did not observe
339 association of the gut microbiome with *HLA-B27* carriage, but the sample size, particularly in healthy
340 controls, was too small to exclude other than a large effect. Wen et al used shotgun sequencing of
341 stool samples from in 97 Chinese AS cases and 114 healthy controls, and reported significant dysbiosis
342 in the AS cases (21). Breban et al (22) used 16S rRNA profiling of the stool microbiome to study 87 +-
343 patients with axial spondyloarthritis (42 with AS), 69 healthy controls and 28 rheumatoid arthritis
344 patients. They also report evidence of intestinal dysbiosis in the spondyloarthritis patients, and report
345 correlation of *Ruminococcus gnavus* carriage with BASDAI. Whilst we did not observe an association
346 with the carriage of *HLA-B27*, *Ruminococcus gnavus* was noted to be enriched in the ileum of
347 individuals carrying the *HLA-DRB1* RA-risk alleles (Supplementary Table 3). In a comparison of *HLA-*
348 *B27* positive and negative siblings (n=22 and 21 respectively), no difference in microbial composition
349 was noted overall, but *HLA-B27* positive siblings had increased carriage of the *Microcaccaceae* family
350 (including the species *Rothia mucilaginosa* within it), several *Blautia* and *Ruminococcus* species, and
351 of *Egerthella lenta*. They also observed a reduced carriage of *Bifidobacterium* and *Odoribacter* species.
352 Of these we also see reduction in *Blautia obeum*. Although we did not find dysbiotic changes that
353 were shared with these specific taxa, we note the enrichment of genera within the Lachospiraceae-
354 Ruminococcaceae grouping in *HLA-B27* carriers was a shared feature of our studies; *Roseburia* and
355 *Ruminococcus* by Breban et al (22) and *Roseburia, Blautia, Dorea* and *Oscillospira* in our current study.
356 These bacteria are known to be enriched within the intestinal mucosa (57), and are plausibly more
357 immunogenic as a result (58). The differences observed between these studies may relate to
358 analytical differences such as handling of covariates, disease definition, sample site studied, ethnicity
359 and diet, and the different methods employed to profile the microbiome. Our study also confirms the
360 significant effect of gender and BMI category on gut microbial profiles, suggesting that future studies
361 should control for these covariates.
362
363 HLA molecules affect susceptibility to many diseases, most of which are immunologically mediated. In

364 almost all instances, the mechanism that accounts for that predisposition is not known. The
365 microbiome has now been implicated in a long list of diseases, many of which are immunologically
366 mediated. Our studies suggest that HLA molecules could be important factors that contribute to the
367 heterogeneity of the microbiome and operate at least partially through this mechanism in the
368 pathogenesis of many different diseases, not just AS and RA. Consistent with this hypothesis, in celiac
369 disease carriage of *HLA-DQ2* has been shown to influence the gut microbiome in infants as yet
370 unaffected but at high risk through family history of the disease (59).
371
372 The hypothesized metabolic changes imbued by dysbiosis in our current work are of interest in light
373 of a recent study by our group in the *HLA-B27* transgenic rat model of spondyloarthritis (60). We
374 observe a number of *HLA-B27* dependent metabolic changes in this model that include enrichment of
375 bile acid metabolism, lysine metabolism, fatty acid metabolism and tryptophan metabolism. All of
376 these pathways were predicted to be enriched in *HLA-B27* positive individuals in our current study.
377 Importantly, *HLA-B27*-dependent dysbiosis can be observed prior to the onset of disease in this model.
378 Thus, our human and rat studies support the hypothesis that *HLA-B27* dependent dysbiosis is a
379 preceding event in AS pathogenesis and may not merely be secondary to disease.
380
381 In conclusion, this study demonstrates that *HLA-B27* and RA-associated *HLA-DRB1* allele carriage in
382 humans influences the gut microbiome. In association with the replicated demonstration of intestinal
383 changes in microbiome in AS, this is consistent with disease models in which HLA molecules interact
384 with the gut microbiome to cause disease. Different models as to how this may occur include effects
385 of *HLA-B27* to favour a more inflammatory gut microbiome, increased invasiveness of the gut mucosa
386 in *HLA-B27* carriers, and/or aberrant immunological responses to bacteria in *HLA-B27* carriers. Similar
387 hypotheses may explain the role of *HLA-DRB1* in driving the immunopathogenesis of RA. Whichever
388 of these models is correct, the data presented here support further research in this field, including
389 into whether manipulation of the gut microbiome may be therapeutic in AS or RA, or even potentially

390 capable of preventing disease in at risk subjects.

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