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1 Multi-Method Characterisation of the Human Circulating Microbiome
2
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8
9 ## Abstract
10 The term microbiome describes the genetic material encoding the various microbial
11 populations that inhabit our body. Whilst colonisation of various body niches (e.g. the
12 gut) by dynamic communities of microorganisms is now universally accepted, the
13 existence of microbial populations in other "classically sterile" locations, including the
14 blood, is a relatively new concept. The presence of bacteria-specific DNA in the
15 blood has been reported in the literature for some time, yet the true origin of this is
16 still the subject of much deliberation. The aim of this study was to investigate the
17 phenomenon of a "blood microbiome" by providing a comprehensive description of
18 bacterially-derived nucleic acids using a range of complementary molecular and
19 classical microbiological techniques. For this purpose we utilised a set of plasma
20 samples from healthy subjects (n = 5) and asthmatic subjects (n = 5). DNA-level
21 analyses involved the amplification and sequencing of the 16S rRNA gene. RNA-
22 level analyses were based upon the *de novo* assembly of unmapped mRNA reads
23 and subsequent taxonomic identification. Molecular studies were complemented by
24 viability data from classical aerobic and anaerobic microbial culture experiments. At
25 the phylum level, the blood microbiome was predominated by Proteobacteria,
26 Actinobacteria, Firmicutes and Bacteroidetes. The key phyla detected were
27 consistent irrespective of molecular method (DNA vs RNA), and consistent with the
28 results of other published studies. *In silico* comparison of our data with that of the
29 Human Microbiome Project revealed that members of the blood microbiome were
30 most likely to have originated from the oral or skin communities. To our surprise,
31 aerobic and anaerobic cultures were positive in eight of out the ten donor samples
32 investigated, and we reflect upon their source. Our data provide further evidence of a
33 core blood microbiome, and provide insight into the potential source of the bacterial
34 DNA / RNA detected in the blood. Further, data reveal the importance of robust
35 experimental procedures, and identify areas for future consideration.
36
37
38

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39 **Background**

40 The term “microbiome” describes the genetic material encoding the various microbial
41 populations that inhabit our body. In contrast, the term “microbiota” refers to the
42 viable organisms that comprise these communities. Cell for cell, bacterial cells
43 outnumber our own cells by a factor of ten, and the bacterial microbiome encodes for
44 one hundred-fold more genes. The microbiome undertakes essential biological
45 processes and thus it is unsurprising that a number of disease states are associated
46 with changes in microbiome composition, termed “dysbiosis”. Whilst the colonisation
47 of specific body sites in contact with the external environment (such as the
48 gastrointestinal tract, skin and vagina) by microorganisms is both well-described and
49 universally accepted [1], the existence of microbial populations in other “classically
50 sterile” locations, including the blood, is a relatively new concept.
51
52 The presence of bacteria-specific DNA in the blood has been reported in the
53 literature for some time, yet the true origin of this is still the subject of much
54 deliberation. Mounting evidence supports the existence of a blood microbiome
55 (*specifically, the presence of bacterial genetic material*) in humans [2-11] and various
56 other species, including rodents, cats, chickens and cows [12-15]. This has primarily
57 been determined by amplification and sequencing of the bacterial 16S rRNA gene, or
58 via whole genome sequencing. Such studies report the existence of bacteria-derived
59 genetic material (DNA) within the circulation, but do not provide evidence for the
60 presence of viable organisms.
61
62 Convention tells us that the blood is sterile in health, and bacteraemia, even at 1 –
63 10 bacterial cells per millilitre whole blood, is potentially life threatening. Despite
64 this, several studies have presented evidence of bacteria or bacteria-like structures
65 within the circulation in the absence of overt disease. It should be noted however
66 that Martel and colleagues report that the bacteria-like particles often described
67 following a range of imaging techniques represent non-living membrane vesicles and
68 protein aggregates derived from the blood itself [16]. McLaughlin surveyed the blood of
69 25 healthy donors and observed the presence of pleomorphic bacteria using dark-
70 field microscopy, electron microscopy, polymerase chain reaction and fluorescent *in*
71 *situ* hybridisation, in all samples analysed [17]. Further, Potgieter and colleagues
72 described the presence of blood-cell associated bacteria in a range of blood
73 preparations using electron microscopic techniques [8]. Significantly, Damgaard and
74 colleagues found viable (*culturable*) bacteria in 62% of blood donations from donors
75 with no overt disease [18]. This finding is plausible given that various daily activities
76 including chewing, tooth brushing and flossing result in the translocation of oral
77 bacteria into the bloodstream [19-22], however one would expect such organisms to be
78 rapidly targeted and removed with by the immune system in healthy individuals. Kell
79 and colleagues provide a detailed hypothesis for the existence of the blood
80 microbiome [4,8] and suggest that it is likely composed of organisms (or parts thereof)
81 that translocate to the circulation from their usual place of habitation (classical niches
82 such as the gastrointestinal tract, oral cavity, skin, vagina) – a process termed
83 atopobiosis. They further describe how this could occur via well-described
84 physiological processes including dendritic cells processes, via micro-fold cells, and
85 in disease, via dysfunctional epithelial junctions. This explanation is supported by
86 studies demonstrating a correlation between gut microbiota dysbiosis and altered
87 microbial signatures detected in the blood [23-25], suggesting that the observed

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88 disease-associated blood microbiota is a consequence of increased bacterial
89 translocation across the gut barrier. Furthermore, characterisation of the microbial
90 populations in the coronary artery tissues by Lehtiniemi and colleagues [26] identified
91 known members of the oral microbiota, suggesting that bacteria had translocated
92 from the oral cavities into the bloodstream, potentially as a result of damage caused
93 by tooth brushing or by leakage across the mucosal surfaces.
94
95 Various disease states are associated with blood microbiome dysbiosis [2,6,24,25,27],
96 and these changes are likely reflective of dysbiosis at a distant site(s) with well-
97 characterised microbial communities, and the result of subsequent translocation.
98 Limited evidence also suggests that these changes may be disease-specific;
99 Alzheimer's disease for example, has been associated with the detection of mostly
100 coccus microbes, whilst Parkinson's disease has been associated with both coccus
101 and bacillus bacteria [8]. Such changes are of significant scientific and medical interest
102 as they offer opportunities for biomarker and therapeutic target development [25,27].
103
104 Due to the long-held belief that the bloodstream of healthy individuals is sterile and
105 since the blood is an unfavourable compartment for the microbes due to its
106 bacteriostatic and bactericidal components [1,13], it is of principal significance to
107 understand *whether* and *how* bacteria may persist in it. Accidental contamination
108 during the collection of blood and or during downstream experimental procedures
109 has been proposed as an alternative explanation for the existence of the blood
110 microbiome *per se*. We support this explanation for the detection of *viable* bacteria
111 within the bloodstream of healthy individuals, however, suggest that this
112 phenomenon does not adequately explain the existence of the blood microbiome
113 (*the presence of bacteria-derived nucleic acids*) when one considers the number of
114 studies that demonstrate significant and apparently disease-specific differences in
115 the composition of the blood microbiome. Moreover, examination of the bacterial
116 taxa reported in these studies reveal similar blood microbiota compositions across
117 the different studies, whereby Proteobacteria dominate (relative abundance values
118 typically ranging from 85%-90%), and Firmicutes, Actinobacteria, and Bacteroidetes
119 present to a lesser extent [2,7,25,28]. This suggests the existence of a core blood
120 microbiome profile that persists independent of study environment or analytical
121 methodology.
122
123 **Using a range of complementary molecular and classical molecular biology**
124 **techniques, here we characterise the human blood microbiome in unparalleled**
125 **detail; at the DNA level, we amplify and sequence the 16S rRNA gene, at the**
126 **RNA level, we assemble almost 500,000,000 unmapped mRNA reads and map**
127 **these to known taxa, and finally, we present viability data from classical**
128 **aerobic and anaerobic microbial culture experiments. Our experimental**
129 **approach is detailed in Figure 1.**
130

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131 **Materials and Methods**
132
133 **Donor Samples**
134 Atopic asthmatic individuals (n=5) with physician-diagnosed house dust mite allergy,
135 and gender and age-matched healthy control subjects (n=5) were recruited to the
136 study via SeraLabs Limited in accordance with the following criteria **(Table 1)**. Whole
137 blood was drawn, following alcohol cleansing of the skin surface, into EDTA
138 containing tubes and stored on ice prior to centrifugation at 1000×*g* to obtain the
139 plasma component. All samples were analysed anonymously, and the authors
140 obtained ethical approval (Keele University ERP3) and written informed consent to
141 utilise the samples for the research reported herein.
142
143 **Table 1** – Donor population characteristics
144

Patient Criteria
• Have a BMI < 30
• Be non-smokers
• Were diagnosed with atopic asthma during childhood
• Have severe/ poorly controlled symptoms
• Are not on current oral steroid treatment
• Must be allergic to house dust mite
• Must not have diabetes, COPD, or hypertension

145
146
147 **DNA-level: Meta-taxonomic Characterisation**
148 The circulating microbiome was investigated at the DNA level by amplification and
149 sequencing of the bacterial 16S ribosomal gene using oligonucleotide primers that
150 target variable region 4 [29] **(Table 2)**. Direct amplification of the V4 region was
151 performed using the Phusion Blood Direct kit (Thermofisher scientific) alongside a
152 negative control reaction (in which blood was replaced with molecular biology grade
153 water) that underwent the complete experimental procedure. Amplification was
154 performed in triplicate as 20µl reactions containing; 1.0 µl plasma, 10µl 2X Phusion
155 blood PCR buffer, 0.4µl Phusion Blood II DNA polymerase, 1.0 µl of each forward
156 and reverse oligonucleotide primer (10µM), and 6.6µl of UV-treated molecular
157 biology grade water. Cycling parameters were as follows; an initial 5 minutes
158 denaturation step at 98°C followed by 33 cycles of; denaturation (1 second at 98°C),
159 annealing (5 seconds at 55°C), and extension (15 seconds at 72°C), and a final
160 extension at 72°C for 7 minutes.
161
162 Amplicons resulting from triplicate reactions were combined and purified using the
163 MinElute PCR purification kit (Qiagen) prior to a further 7 cycles of PCR using

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164 AccuPrime *Pfx* SuperMix and a pair of V4 oligonucleotide primers we developed to
165 incorporate the Illumina XT adapter in preparation for sequencing (**Table 2**). Cycling
166 parameters were as follows; initial denaturation for 2 minutes at 95°C followed by 7
167 cycles of; denaturation (15 seconds at 95°C), annealing (30 seconds at 55°C), and
168 extension (25 seconds at 68°C), and a final extension at 68°C for 10 minutes. PCR
169 products were purified using the AMPure XP magnetic beads (Agencourt) at a ratio
170 of 0.8 beads to sample (v/v), eluted in 20μl molecular biology grade water, and
171 quantified using the Qubit 3.0 high-sensitivity DNA kit. Amplicons were barcoded
172 using the Nextera DNA library kit, multiplexed, and sequenced using the Ilumina
173 MiSeq system with a 250bp paired-end read metric. Bioinformatic analysis was
174 performed using QIIME[30] implemented as part of the Nephele 16S paired-end QIIME
175 pipeline using closed reference clustering against the SILVA database [31] at a
176 sequence identity of 99%. All other parameters remained as default.
177
178 **Table 2** – Oligonucleotide primer sequences

Primer Name	Sequence (5' – 3')	Length
V4_F	GTGCCAGCMGCCGCGGTAA	19
V4_R	GGACTACHVGGGTWTCTAAT	20
V4_XT_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGC CGCGGTAA	52
V4_XT_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVG GGTWTCTAAT	54

179
180
181 **RNA-level: De-novo Assembly of Unmapped RNA Reads**
182 For the purposes of increasing our understanding of the molecular processes that
183 are deregulated in patients with atopic asthma, we previously performed whole
184 transcriptome sequencing on RNA extracted from each donor plasma sample (data
185 currently unpublished). Here, we hypothesised that the non-mapping (likely non-
186 human) reads that often result from such analyses would represent microbial
187 community members that were present in the blood at the time of RNA extraction. To
188 this end, RNA reads were mapped to the *Homo sapiens* genome version hg38 using
189 Tophat with default parameters [32]. Reads failing to map to hg38 were identified from
190 the resulting BAM file and reads, in fastq format, extracted using bedtools
191 bamtofastq. In order to streamline our strategy, a single de novo transcriptome was
192 produced by concatenating all of the unmapped reads produced from the entire
193 study, and assembling these using Trinity [33] to form features representative of
194 candidate non-human genes. To increase computational efficiency, the total read
195 population was subsampled to a depth of 1M, 10M, 25M, and 100M, and the number
196 of reads that these features explained computed by building a bowtie2 index out of
197 each resulting transcriptome, and mapping the unmapped read population from each
198 sample back to it. The transcriptome assembly with the highest mapping rate was
199 used for further analysis as follows; (1) Abundance estimation – the transcriptome
200 was indexed for bowtie2 and the number of reads mapping to each feature
201 determined for each of our donor samples using RSEM [34]. (2) A matrix of expression
202 values was produced using the abundance_estimates_to_matrix.pl script packaged
203 with Trinity [35]. (3) Statistical analysis - a differential expression analysis was
204 conducted to identify candidate non-human genes that were significantly differentially
205 expressed between the disease and disease-free donors using edgeR [36]. (4)

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206 Identification - the taxonomic identity of each assembled feature was determined
207 using Kraken [37] and visualised using Pavian [38].

208 **Classical Culture: Bacterial Viability**

209 Classical microbiological culture, using a range of substrates, was carried out to
210 determine whether the human plasma samples contained any viable bacterial cells,
211 i.e. those capable of proliferation. For each sample, 250µl plasma was inoculated
212 into 9ml of brain heart infusion broth and incubated for 5 days at 37°C. For each
213 culture a negative control was generated, whereby 9ml of brain heart infusion broth
214 was inoculated with 250µl of ultra-violet sterilised distilled water and incubated for 5
215 days at 37°C. The inoculated broth was plated onto agar plates (Columbia agar + 5%
216 horse blood; CLED medium; A.R.I.A + horse blood) and incubated under either
217 aerobic (Columbia blood agar; CLED medium) or anaerobic (A.R.I.A agar) conditions
218 at 37°C for a minimum of three days. Bacterial growth was evaluated by sight, and a
219 selection of individual colonies from each plate were selected for identification by
220 total 16S gene amplification and Sanger sequencing.
221
222

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223 # Results and Discussion
224
225 ## Donor Characteristics
226 The donor population were all female and all "never smokers". The asthmatic
227 population were 39.6 years in age (range 19 – 52) with a mean body mass index of
228 24.4 (range 21.5 – 27.8) and all had physician-diagnosed atopic asthma resulting
229 from house dust mite allergy. The control population were 39.4 years in age (range
230 23 – 49) with a mean body mass index of 24.3 (range 21.0 – 26.4) and were
231 disease-free. There were no statistically significant differences in age (P = 0.98) or
232 BMI (P = 0.93) between the two groups **(Table 3)**.
233
234 **Table 3** – Donor Population Characteristics

Donor	Age (years)	BMI (kg/m^2)	Smoking History	Diagnosis
BRH1017873	50-60	27.8	Never	Atopic Asthma, HDM
BRH1017874	30-40	27.3	Never	Atopic Asthma, HDM
BRH1017875	40-50	23.3	Never	Atopic Asthma, HDM
BRH1017876	10-20	21.5	Never	Atopic Asthma, HDM
BRH1017877	40-50	22.3	Never	Atopic Asthma, HDM
BRH1017878	40-50	26.4	Never	Healthy
BRH1017879	20-30	21	Never	Healthy
BRH1017880	40-50	26.4	Never	Healthy
BRH1017881	40-50	24.9	Never	Healthy
BRH1017882	30-40	22.7	Never	Healthy

235
236 ## DNA-level Circulating Microbiome – Community Structure
237 The presence of bacterial DNA within the blood of our study cohort was evaluated by
238 amplification and sequencing of the 16S RNA gene variable region 4. A negative
239 experimental control sample mirrored our study samples through the entire
240 experimental procedure downstream of venepuncture. This involved using ultra-
241 violet treated distilled water in replacement of human blood during PCR amplification
242 of the 16S rRNA V4 region. The negative control PCR product was then submitted to
243 all downstream applications that the human blood underwent. This included bead-
244 based purification of the 16S rRNA V4 amplicons, agarose gel electrophoresis, XT-
245 tagging, library preparation and sequencing of the 16S rRNA V4 amplicons. Despite
246 this control reaction being PCR-negative, it was nevertheless submitted for
247 sequencing.
248
249 Using QIIME[30] implemented as part of Nephele, a total of 243, 853 sequencing reads
250 from the amplified V4 region passed quality assessment (mean = 24,385 reads per
251 sample; range = 10, 742 – 35, 701 reads). The results of our taxonomic classification
252 are shown in **(Figure 2 A-D)** at the phylum and genus levels.
253
254 Our negative control reaction generated a small number of reads that were identified
255 as the following genera; *Halomonas (6 reads)*, *Corynebacterium (64)*,
256 *Staphylococcus (24)*, *Ralstonia (1726)*, *Stenotrophomonas (460)*, *Pseudomonas*

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257 *(276)*, *Escherichia-Shigella (2420)* and *Ruminococcus* (405), but was
258 overwhelmingly composed of reads mapping to the genus *Serratia (18000).* These
259 genera have been reported previously as contaminants of next generation
260 sequencing experiments [39,40] but importantly here, were either distinct from the taxa
261 identified within our samples, or present at much lower levels.
262
263 At the phylum level, the blood microbiome was predominated by Proteobacteria
264 (88% of all bacterial DNA in the control population, and 80.9% in the asthmatic
265 population) followed by Actinobacteria (control = 7.8%, asthmatic = 7.1%),
266 Firmicutes (control = 3.5%, asthmatic = 9.2%) and Bacteroidetes (control = 0.1%,
267 asthmatic = 2.2%). These findings mirror previous studies [2,7,25,28] and further support
268 the notion of a core blood microbiome predominated by four key phyla.
269
270 At the genus level, our blood samples were predominated by the genus
271 *Achromobacter* [41] which accounted for 51.1% and 45.3% of the total bacterial DNA
272 detected in the control and asthma donors respectively. To a lesser extent, the blood
273 samples also comprised members of the *Pseudomonas* (12.8%, 7.5%) [7,9,18],
274 *Serratia** (0.9%, 11.6%) [42], *Sphingomonas* (3.8%, 5.1%) [7,25], *Staphylococcus* (5.5%,
275 2.8%) [7,18,43,44], *Corynebacterium* (3.2%, 5.5%) [7,9], and *Acinetobacter* (3.7%, 2.8%) [7]
276 genera. The genus *Serratia* was excluded from further analysis as the study samples
277 presented with less reads than did the corresponding negative control reaction and
278 thus it was considered a contaminant.
279
280 Whilst all genera found have been previously described in the blood (see
281 references), the predominance of *Achromobacter,* which is not classically associated
282 with the blood microbiome, warrants further consideration. Indeed, *Achromobacter*
283 has been detected abundantly in the lower respiratory tract of healthy mice [45],
284 humans (HPM airway dataset), and in various respiratory conditions [46,47].
285 Furthermore, no *Achromobacter* was detected in our experimental control reactions
286 suggesting that its presence is not the result of experimental contamination.
287
288 Although differential composition of the blood microbiome in response to pathology
289 was not the main focus of this study, we performed principal coordinates analysis
290 and despite the relatively small sample set, this revealed good separation between
291 the two treatment groups based upon their microbiome profile, suggesting that the
292 blood microbiome community was altered in the asthmatic subjects (**Figure 3**).
293
294

295 **DNA-level Circulating Microbiome – Likely Origins**

296 In accordance with our hypothesis that the blood microbiome exists as a
297 consequence of bacterial translocation from other microbiome niches within the
298 body, we compared the data generated herein with gastrointestinal tract, oral cavity,
299 and skin data made available by the Human Microbiome Project (HMP). In each
300 case, the HMP data and our own were combined, weighted UniFrac distances
301 calculated, and a principal coordinates analysis performed. **Figure 4** demonstrates
302 that the blood microbiome of our control and asthmatic donors clustered more
303 closely in PCoA space with the oral cavity and skin HMP data, than it did with the
304 gastrointestinal tract HMP data. This suggests that the blood microbiome community

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305 is perhaps more likely to result from the translocation of organisms from the oral
306 cavity and skin, than from organisms that colonise the gastrointestinal tract.
307
308 Various daily activities including chewing, tooth brushing and flossing have been
309 shown to result in the translocation of bacteria from the oral cavity into the
310 bloodstream [19-22]. Further, the skin has a distinct microbial community and is
311 susceptible to injury, and thus represents a large surface area through which such
312 organisms may translocate to the bloodstream. It is important here to consider
313 sources of contamination; venepuncture, the process through which the majority of
314 blood samples are obtained, is recognised as a cause of transient bacteraemia [48],
315 and despite the use of preventative measures such as alcohol cleansing of the skin
316 prior to breaking the surface, there remains the possibility that organisms entered the
317 sample from the skin via this route.
318
319 One important, but often overlooked limitation of DNA-based microbiome
320 characterisation is that DNA persists post-mortem, even in the presence of harsh
321 environmental conditions. From such analyses it is therefore impossible to confirm
322 whether an organism is present and viable, is present but non-viable, or whether the
323 organism has since left the environment in question yet it's DNA persists.
324
325
326

327 **RNA-level Circulating Microbiome – Community Structure**

328 We hypothesise that some of the non-mapping (likely non-human) reads that often
329 result from whole transcriptome analyses (RNA-seq) represent microbial community
330 members that were present (or parts thereof) in the blood at the time of RNA
331 extraction. Furthermore, given the unstable nature of extracellular circulatory RNA[49]
332 in addition to the presence of circulatory ribonucleases that actively degrade RNA [47],
333 , we suggest that the detection of bacterial RNA goes further towards confirming the
334 recent presence of these microbes within the blood when compared with DNA-based
335 approaches. From our previous studies of the circulating transcriptome of our donor
336 community, a total of 439,448,931 paired RNA reads failed to map to the human
337 genome. These reads were used for the following analyses as randomly subsampled
338 populations of 1 million, 10 million, 25 million and 100 million read pairs. Mapping the
339 total read population back to the subsampled populations allowed us to assess how
340 well each subsampled population approximated the starting (entire, ~ 440M read)
341 population. Data revealed only marginal improvements in whole community
342 representation as the subsampled population increased (1M, 10M, 25M, 100M
343 represented 65.05, 66.05, 66.81, 64.24% of the total read population). A subsampled
344 population of 25 million reads (25M) provided an acceptable balance between read
345 representation and computational efficiency and was therefore used for
346 transcriptome assembly. The transcriptome comprised 2050 candidate "non-human
347 genes" with a mean GC content of 53%. Ten-percent of these genes were greater
348 than 517bp, and over half were at least 263bp. Taxonomic identification of each
349 assembled feature was determined using Kraken [37] and this revealed that 729 of the
350 2050 features were of bacterial origin and 7 features were from archaea (pertaining
351 to the taxa Thermoplasmata, which has been previously associated with the human
352 microbiome [50]) (**Figure 5**). Although we identified 13 features of apparent viral origin,
353 we did not consider these in any further detail given they appeared to pertain to the

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354 Moloney murine leukemia virus, a commonly utilised reverse transcriptase used in
355 molecular procedures. It should be noted that the Kraken database does not include
356 fungi, and therefore this kingdom was not represented within our data.
357 At the phylum level, the whole transcriptome data was predominated by assembled
358 Proteobacteria sequences (379 sequences, 52%), followed by Firmicutes (143,
359 19.8%), Actinobacteria (112, 15.5%) and Bacteroidetes (35, 4.8%). In considering
360 the total number of reads mapping to each feature, 379.4M reads mapped to
361 bacterial features (out of a total of 395M reads). Of those reads mapping to bacterial-
362 derived sequences, Proteobacteria (74.9%, 47.0%; Control, Asthma) and Firmicutes
363 (19.5%, 48.0%) predominated with, Actinobacteria (0.01%, 0.04%) and
364 Bacteroidetes (0.05%, 0.008%) present to a much lesser extent. These findings
365 support our DNA-level phylum data and mirror previous studies [2,7,25,28].
366
367 At the genus level, the whole transcriptome data was predominated by the genera
368 *Paenibacillus* (17.8%, 44.6%; Control, Asthma reads)[51], *Escherichia* (11.8%, 13.1%)
369 [7,9,43,52], *Acinetobacter* (0.7%, 0.4%) [7], *Pseudomonas* (0.8%, 0.1%) [7,9,18,53] and
370 *Propionibacterium* (0.5%, 0.2%) [9,18,44,53]. With the exception of *Paenibacillus,* all
371 genera detected via *de novo* transcriptome assembly were also present in our DNA-
372 level data and all genera found have been previously described in the blood (see
373 references). The fact that *Paenibacillus* was present within our RNA-level analyses
374 yet absent from our DNA-level data led us to consider whether it could have been
375 introduced as a contaminant during the RNA extraction, library preparation or
376 sequencing procedures. Given that negative control reactions are not routinely
377 conducted for RNA-seq type applications, we were unable to experimentally confirm
378 this. However, we did identify from the literature that this genus has been reported as
379 a common reagent and laboratory contaminant, albeit at the DNA level [40].
380 Nevertheless there appeared to be little consistency in its presence within our
381 sample set (mean % of reads 39.5 ± 40.3%) and we noted a difference in abundance
382 between our experimental groups despite all preparation procedures being the
383 same. The exact source of this RNA thus remains open to speculation.
384
385

386 **Classical Culture – Presence of Viable Organisms**

387 The presence of viable, proliferating bacteria in the blood was assessed using
388 growth culture assays as previously described. Bacterial cultures were positive for
389 80% of blood samples assayed (8 out of 10 samples; 4 control blood samples and 4
390 asthma blood samples), whilst all negative control plates had no growth as expected.
391 These results are relatively consistent with previous studies, whereby 2-100% of
392 blood samples were positive for bacterial growth [18,54-57]. Unexpectedly, bacterial
393 growth was observed in aerobic conditions for all culture-positive blood samples, but
394 anaerobic growth was only observed for four of the culture-positive blood samples.
395 This is contradictory to previous studies, where bacterial growth from blood-cultures
396 has predominately been achieved using anaerobic conditions [18,55].
397
398 In all instances, bacterial growth was monoculture on microscopy and thus 16S
399 colony PCR (amplifying the entire 16S rRNA gene) and Sanger sequencing was
400 conducted on a three independent colonies per plate for identification purposes.
401 Bacteria were identified using Sanger sequencing followed by classification with
402 Kraken. Bacteria isolated from the aerobic cultures included the following genera;

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403 *Staphylococcus* (49 sequences), *Micrococcus* (12), *Kocuria* (6), *Corynebacterium* (6)
404 and *Propionibacterium* (1). Bacteria isolated from the anaerobic cultures were less
405 variable and included members from the facultatively anaerobic *Staphylococcus*
406 genus only (**Figure 6**). These genera belong to the phyla Actinobacteria
407 (*Corynebacterium*, *Kocuria*, *Micrococcus*) and Firmicutes (*Staphylococcus*) and were
408 all represented in our 16S DNA level data (**Figure 4**) and detected within our RNA
409 data. Individual sample data is presented in (**Table 4**). It is noteworthy that, with the
410 exception of *Kocuria*, all bacteria identified displayed some of the highest total
411 relative abundance scores in the 16S sequencing results; *Corynebacterium* (4.2%),
412 *Kocuria* (0.2%), *Micrococcus* (1.30%), and *Staphylococcus* at 4.3%.Due to the long-
413 held belief that the bloodstream of healthy individuals is sterile and since the blood is
414 an unfavourable compartment for the microbes due to its bacteriostatic and
415 bactericidal components; here we consider the likely source of these viable
416 organisms. The skin microbiome is dominated by members of the
417 genera *Corynebacterium*, *Micrococcus*, *Staphylococcus*, and *Propionibacterium*,
418 the proportions of which vary markedly between individuals [58]. Furthermore,
419 several studies report the presence of the genus *Kocuria* on the skin of humans
420 and other mammals [59-61]. We therefore suggest that the organisms detected
421 through our microbial culture experiments most likely originate from the skin.
422 Whilst transient bacteraemia due to a breach of the skin barrier is an accepted
423 occurrence, one would expect such organisms to be rapidly targeted and removed
424 by the immune system.
425
426 We therefore suggest that the viable organisms detected through classical
427 microbial culture analysis are the result of venepuncture contamination whereby
428 organisms from the skin are drawn into the vacutainer, contaminating the sample.
429 An alternative hypothesis suggests that these bacteria were present in the blood in
430 a dormant state (i.e. not contaminants), and were somehow revived following pre-
431 growth in brain heart infusion broth prior to plating (see Kell et al for a detailed
432 description of this hypothesis [8]), however, this hypothesis is still under intense
433 investigation.
434
435

436 **Table 4** – Identification of cultured organisms using 16S colony PCR and Sanger Sequencing. Culture Neg denotes no bacterial
437 growth on the substrates used.
438

Growth Condition	Control Samples					Asthma Samples			
	BRH1017878	BRH1017879	BRH1017880	BRH1017881	BRH1017882	BRH1017874	BRH1017875	BRH1017876	BRH1017877
Aerobic Growth	Kocuria *rhizophila*	Micrococcus *luteus*	Staphylococcus *haemolyticus* Staphylococcus *epidermidis* Propionibacterium *acnes*	Staphylococcus *haemolyticus*	Culture Neg	Staphylococcus *haemolyticus*	Culture Neg	Corynebacterium *halotolerans*	Staphylococcus *epidermidis*
Anaerobic Growth	Culture Neg	Culture Neg	Staphylococcus epidermidis	Staphylococcus hominis	Culture Neg	Culture Neg	Culture Neg	Culture Neg	Staphylococcus epidermidis

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Concluding Remarks

This study utilised a range of molecular and classical microbiology approaches to characterise the human blood microbiome in unparalleled detail. Our DNA and RNA-based studies revealed a diverse community of bacteria, the main members of which having been described in a range of other studies and therefore providing further evidence of a core blood microbiome. Although disease associated changes in the blood microbiome were not the focus of this study, the fact we identified such changes is encouraging and supports efforts to identify circulating microbiome signatures indicative of disease.

Whilst we attribute the finding of viable organisms in our plasma samples to venepuncture-associated contamination of the blood sample (and make recommendations to avoid this in future) and or the phenomena of dormancy, the presence of these viable organisms does not undermine our exciting molecular data that reports an abundance of bacteria-associated DNA and RNA within the blood, likely present due to translocation from classical microbiome niches (such as the gut, oral cavity and skin), and with the clear potential to be developed as a biomarker of microbiome status at distant anatomical sites.

On reflecting upon our experimental approach, we make the following recommendations for future studies;

(1) Significant attention should be paid to blood collection procedures as any contamination occurring at this stage impacts upon all downstream procedures. In addition to alcohol cleansing of the skin (as performed in this study), we recommend that the first volume drawn is diverted to a secondary tube, and analysed separately. This will allow investigation of the contribution that venepuncture-associated contamination makes, and allow robust analysis of the dormancy hypothesis.

(2) The inclusion of negative control reactions that are subject to the whole range of experimental procedures, including library preparation and sequencing, is absolutely essential.

(3) Where possible, RNA-seq studies used for unmapped read assembly should include negative control samples that are subject to all of the experimental procedures alongside the study samples. This will allow an appraisal of how significant reagent / laboratory procedure contamination is in these studies. Often the use of RNA-seq derived unmapped reads for microbiome characterisation is a “secondary use”, and this is simply not possible.

(4) Seminal studies are still required to satisfactorily investigate the phenomenon of bacterial translocation from well-characterised microbiome niches to the blood.

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491
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497
498
499 **Data Availability**
500 The sequencing data utilised in this project can be found in the Sequence Read
501 Archive, NIH, under the identifier SUB4654957.
502

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666 **Figure Legends**
667
668 **Figure 1** – Schematic representation of the multiple-method circulating microbiome
669 characterisation approach implemented herein. NB: Biomarker and mechanistic data are not
670 included within the scope of this publication and appear elsewhere.
671
672 **Figure 2** – Relative abundance of the most abundant taxa (>1%) as determined by
673 amplification and sequencing of the 16S rRNA gene variable region 4. Data are mean
674 abundance expressed as a percentage of the total bacterial sequence count. (A) Phylum-
675 level data grouped by condition, (B) Genus-level data grouped by condition, (C) Phylum-
676 level individual sample data, (D) Genus-level individual sample data.
677
678 **Figure 3 –** Principal coordinates analysis of weighted unifrac distances for control (blue) and
679 asthmatic (red) blood microbiome profiles. Each dot represents an individual sample, and
680 the microbiome of samples that appear more closely together are more similar.
681
682 **Figure 4** – Principal coordinates analysis of weighted unifrac distances between variable
683 region 4 16S sequencing data from our donors and the Human Microbiome Project (A) Gut,
684 (B) Oral Cavity and (C) Skin data. Each dot represents an individual sample, and the
685 microbiome of samples that appear more closely together are more similar. In each case,
686 our control donor samples appear in blue, and our asthmatic donor samples appear in red.
687 Further sample details are provided beneath each figure, and the number of datasets
688 representing each anatomic location is provided in brackets.
689
690 **Figure 5** – Taxonomic classification of each feature assembled from unmapped RNA
691 sequencing reads using Trinity and identified using Kraken. The numbers present by each
692 taxonomic classification refer to the number of features classified as such (e.g. 379
693 assembled features were identified as Proteobacteria). D – domain, P – phylum, F – family,
694 G – genus, S – species.
695
696 **Figure 6** – Taxonomic classification of total 16S data generated by colony PCR and Sanger
697 sequencing. The numbers present by each taxonomic classification indicate the number of
698 colonies that were identified with that identity. D – domain, P – phylum, F – family, G –
699 genus, S – species.
700
701