

1 **Title: Fetal membrane bacterial load is increased in histologically confirmed inflammatory**  
2 **chorioamnionitis: A retrospective cohort study**

3 **Authors:** Ms Rochelle HOCKNEY MSc<sup>A</sup>, Dr Gareth J WARING MBChB MRCOG<sup>C</sup>, Dr Gillian  
4 TAYLOR PhD<sup>A</sup>, Professor Stephen P CUMMINGS PhD<sup>A</sup>, Professor Stephen C ROBSON MD  
5 MRCOG<sup>C</sup>, Dr Caroline H ORR PhD<sup>A</sup> and Dr Andrew NELSON PhD<sup>B</sup>.

6 <sup>A</sup> School of Health and Life Sciences, Teesside University, Middlesbrough, TS1 3BA, UK and  
7 National Horizons Centre, Teesside University, 38 John Dixon Lane, Darlington, DL1 1HG, UK.

8 <sup>B</sup> Faculty of Health and Life Sciences, Northumbria University, Newcastle, NE1 8ST, UK.

9 <sup>C</sup> Institute of Cellular Medicine, Newcastle University, Newcastle, NE2 4HH, UK.

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16 **Corresponding author contact information**

17 Name: Dr Caroline H ORR PhD

18 Email: c.orr@tees.ac.uk

19 Address: School of Health and Life Sciences, Teesside University, Middlesbrough, TS1 3BX  
20 and National Horizons Centre, Teesside University, 38 John Dixon Lane, Darlington, DL1  
21 1HG, UK.

22 Telephone number: 01642 342 553

23 Ms Rochelle Hockney r.hockney@tees.ac.uk

24 Dr Gareth Waring gareth.waring@nuth.nhs.uk

25 Dr Gillian Taylor g.taylor@tees.ac.uk

26 Professor Stephen P Cummings s.cummings@tees.ac.uk

27 Professor Stephen C Robson s.c.robson@newcastle.ac.uk

28 Dr Andrew Nelson andrew3.nelson@northumbria.ac.uk

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## 42 **Abstract**

### 43 **Introduction**

44 It is widely debated whether fetal membranes possess a genuine microbiome, and if  
45 bacterial presence and load is linked to inflammation. Chorioamnionitis is an inflammation  
46 of the fetal membranes. This research focussed on inflammatory diagnosed histological  
47 chorioamnionitis (HCA) and aims to determine whether the bacterial load in fetal  
48 membranes correlates to inflammatory response, including histological staging and  
49 inflammatory markers in HCA.

### 50 **Methods**

51 Fetal membrane samples were collected from patients with preterm spontaneous labour  
52 and histologically phenotyped chorioamnionitis (HCA; n=12), or preterm (n=6) and term  
53 labour without HCA (n=6). The bacterial profile of fetal membranes was analysed by  
54 sequencing the V4 region of the 16S rRNA gene. Bacterial load was determined using qPCR  
55 copy number/mg of tissue. The association between bacterial load and bacterial profile  
56 composition was assessed using correlation analysis.

### 57 **Results**

58 Bacterial load was significantly greater within HCA amnion ( $p=0.002$ ) and chorion ( $p=0.042$ ),  
59 compared to preterm birth without HCA. Increased bacterial load was positively correlated  
60 with increased histological staging ( $p=0.001$ ) and the expression of five inflammatory  
61 markers; IL8, TLR1, TLR2, LY96 and IRAK2 ( $p<0.050$ ). Bacterial profiles were significantly  
62 different between membranes with and without HCA in amnion ( $p=0.012$ ) and chorion  
63 ( $p=0.001$ ), but no differences between specific genera were detected.

### 64 **Discussion**

65 Inflammatory HCA is associated with infection and increased bacterial load in a dose  
66 response relationship. Bacterial load is positively correlated with HCA severity and the TLR  
67 signalling pathway. Further research should investigate the bacterial load threshold required  
68 to generate an inflammatory response in HCA.

69

70 **Short title:** Fetal membrane bacterial load is increased in HCA

71 **Highlights:**

- 72 - Increased bacterial load was significantly associated with inflammation
- 73 - Bacterial load is correlated with HCA severity in a dose dependent manner
- 74 - Bacterial load is correlated to the TLR signalling pathway
- 75 - Non-HCA samples and negative controls are not distinct in bacterial load.

76

77 **Keywords:** Histological chorioamnionitis; Placenta; Fetal membrane; Microbiome;

78 Inflammation; Bacterial load.

## 79 Introduction

80 Histological chorioamnionitis (HCA) is an inflammation of the fetal membranes [1], linked to  
81 adverse maternal and neonatal outcomes, including preterm birth [2], early onset sepsis and  
82 necrotising enterocolitis [3,4]. HCA incident rates are higher in preterm (15%) compared to  
83 term (5%) infants [5].

84 The origin of bacteria within the healthy fetal membrane microbiome is widely debated [6].

85 Conflicting studies have suggested that the placenta and fetal membranes are: (i) sterile  
86 [7,8,9], with any detection of bacteria linked to the mode of delivery [10]; (ii) typically

87 sterile, with any bacteria detected arising due to co-existent maternal conditions, such as  
88 periodontal disease [10,11], vaginal infection [12], or gestational diabetes [13]; (iii)

89 universally colonised with low abundant, non-pathogenic bacteria [14]. Although the  
90 existence of a unique microbiome in healthy membranes remains debated [6,14], the

91 healthy bacterial profile (composition and proportion of bacteria) is suggested to consist  
92 mainly of *Escherichia spp.* [14,15]. Alternatively, HCA membranes from preterm and term

93 labour have presented with *Ureaplasma spp.* in 59% and 60% of cases respectively [2],

94 suggesting any involvement is independent of gestation. Whilst other studies link HCA and  
95 inflammation with increased bacterial load (measurable quantity of bacteria) [16], with a

96 positive correlation between the load of *Prevotella spp.* and HCA severity [17]. Alternatively,

97 lower bacterial diversity has been implicated in preterm HCA membranes compared to

98 controls [15], with monomicrobial characteristics in 83% of HCA cases [2]. In contrast,

99 studies using shotgun and 16S rRNA gene sequencing have reported no distinct bacterial

100 profiles in HCA membranes [6].

101 Careful consideration is required when elucidating the microbiome of fetal membranes due  
102 to low biomass characteristics. It is stated that external bacterial contribution will occur  
103 from the use of commercial kits and reagents, especially in low biomass samples [18]. Thus  
104 comparison of samples to DNA extraction kit negative controls is required. However, within  
105 the placental and fetal membranes this may also originate from contributing vaginal or skin  
106 bacteria during delivery or labour [19,20].

107 Changes in inflammatory receptors and proinflammatory cytokines have been linked to  
108 HCA, including a two-fold increase in Toll-like Receptor 2 (TLR2)[21] and Interleukin 8  
109 (IL8)[22], suggesting the involvement of bacteria as pro-inflammatory agents. However, the  
110 increase in cytokines may be indicative of active labour rather than being specific to HCA  
111 [23]. Inflammatory biomarkers are routinely investigated for risk of preterm birth [24] and  
112 clinical chorioamnionitis [25], but not yet applied to monitoring the risk or prediction of  
113 HCA.

#### 114 **Aims and objectives**

115 Given HCA is a leading cause of preterm birth [26], research investigating the aetiology  
116 focused specifically on HCA is important. Although HCA and clinical chorioamnionitis  
117 overlap, the use of an established reproducible diagnostic criteria as a marker of fetal  
118 membrane infection ensure focus on HCA. This study aims to quantify the bacterial load,  
119 bacterial profile and diversity in fetal membranes to explore its relationship with the  
120 inflammatory response in HCA, including histological staging and inflammatory markers.

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## 123 **Methods**

### 124 **Tissue selection and preparation**

125 Samples of placenta and fetal membranes (amnion and chorion) were collected, stored and  
126 phenotyped histologically using the established histological criteria by an independent  
127 clinician. Full criteria are described in Waring *et al* (2015) [21]. The samples were utilised  
128 following informed consent for current research via a transfer agreement, with prior  
129 approval from Newcastle and North Tyneside 1 Research Ethics Committee (Ref:  
130 10/H0906/71).

131 Fetal membrane samples were collected from 24 patients. Following histological diagnosis  
132 of HCA, patients were prospectively assigned to spontaneous preterm birth with histological  
133 chorioamnionitis (PTB+HCA, n=12), spontaneous preterm birth without HCA (PTB-HCA, n=6)  
134 and spontaneous term birth without HCA (TB-HCA, n=6). Amnion and chorion were available  
135 for a subset of patients (PTB+HCA=8, PTB-HCA=5, TB-HCA=0). In the remainder, amnion  
136 (PTB+HCA=1, PTB-HCA=0, TB-HCA=0), or chorion only were processed (PTB+HCA=3, PTB-  
137 HCA=1, TB-HCA=6). Samples were processed in triplicate and prepared with nine DNA  
138 extraction kit negative controls. Negative controls were processed identical to samples, with  
139 dH<sub>2</sub>O replacing tissue samples.

140 HCA was defined by standardised criteria, at maternal stage 2 and above [27].

141 Subchorionitis was defined as inflammatory stage one [27]. Labour was defined as the  
142 presence of regular spontaneous uterine contractions with progressive cervical dilation  
143 leading to delivery. Term was defined as a gestational age of >37 weeks, term patients were  
144 excluded if presenting with histologically indicated chorioamnionitis. Preterm samples were  
145 collected from patients delivering with a singleton pregnancy, in spontaneous labour at <34

146 weeks gestation, due to the inverse relationship between HCA and gestation [5]. Further  
147 sampling methods are presented in Waring *et al* (2015) [21].

#### 148 **Genomic DNA extraction**

149 Total genomic DNA was extracted from samples (n=78) and negative controls (n=9) using  
150 QIAamp Fast DNA Tissue Kit (Qiagen) as per manufacturer protocol. NanoDrop 1000  
151 spectrophotometer (V3.8.1, Thermo Fisher) and agarose gel electrophoresis were used to  
152 assess yield, purity and quality of DNA prior to downstream analysis.

#### 153 **Quantitative PCR**

154 Plasmid standards (16S rRNA gene) were generated using *Escherichia coli* genomic DNA and  
155 amplified via 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R primers (5'-  
156 TACGGYTACCTTGTTACGACTT-3', Eurofins). PCR amplicons were purified (ExoSap-IT PCR  
157 clean up Kit; Thermo Fisher, Cat No:78201.1), before cloning into TOP10 competent *E. coli*  
158 cells (Thermo Fisher, Cat No:C404010) using PGEM-T Easy Vector System (Promega, Cat  
159 No:A1360). Plasmids were isolated using PureYield Plasmid MiniPrep (Promega, Cat  
160 No:A1223). A ten-fold serial dilution of pooled isolated plasmids was performed to create  
161 standard curves.

162 Absolute qPCR aimed to determine bacterial load within fetal membrane samples using  
163 BactQuant primers (F=5'-CCTACGGGDGGCWGCA-3' *E. coli* 341-356, R=5'-  
164 GGACTACHVGGGTMTCTAATC-3' *E. coli* 786-806) and probe ((6FAM) 5'-CAGCAGCCGCGGTA-  
165 3' (MGBNFQ) *E. coli* 518-532; Eurofins)[28]. Reactions contained 1µl sample DNA, 1.8µm  
166 forward and reverse primers, 225nM probe, 0.05µg/µl BSA, 4mM MgCl<sub>2</sub>, 1% formamide and  
167 1X TaqMan Fast Advanced Master Mix (Thermo Fisher, Cat No:4444557) in a total of 10µl.



168 Extracted DNA from samples and standards, plus controls of DNA extraction kit negatives  
169 and no template controls (NTC) were assayed in triplicate using CFX Connect Real Time  
170 System (Biorad, CFX Manager V3.1). BactQuant protocol was used [28], with an optimised  
171 annealing temperature of 55°C.

## 172 **Expression of inflammatory markers**

173 The expression of TLR signalling pathway components was undertaken by relative qPCR and  
174 has been reported previously [21]. Briefly, genes showing significant change in expression  
175 on signalling arrays were individually validated using qPCR. TaqMan GAPDH was selected as  
176 an endogenous control due to consistent results as a house-keeping gene in the signalling  
177 array study. Each assay was performed in triplicate. Findings indicated the involvement of  
178 TLRs in HCA, initiating this research into bacterial involvement in HCA.

## 179 **Microbiota analysis**

180 Sequencing of DNA samples and negative controls was performed by NU-OMICS  
181 (Northumbria University, UK) as described previously [29], with the universal 16S rRNA gene  
182 primer specific to the V4 region [30]. A sequencing negative control and ZymoBIOMICS  
183 mock microbial community standard were processed alongside samples.

184 Package DADA2 1.4 [31] and Bioconductor (Version 2)[32] were used to trim and filter  
185 MiSeq data with a q score of <30, to ensure consistent length and high-quality reads [32].  
186 Forward and reverse paired strands were merged and clustered into Amplicon Sequence  
187 Variants (ASVs)[33], with clusters differentiated by one nucleotide, for high resolution  
188 bacterial detection [33]. Chimeras were removed using remove BimeraDenovo, before

189 assigning taxonomy and constructing a phylogenetic tree using RDP14 reference database  
190 [34].

### 191 **Statistical analyses**

192 Patient characteristics were analysed using the package TableOne in R [35]. Outcomes were  
193 assessed between subgroups using Kruskal Wallis and Wilcoxon Rank-Sum, with categorical  
194 data analysed by Pearson's Chi-Squared or Fisher's Exact [35].

195 For the analysis of bacterial load, copy numbers of 16S rRNA gene/mg of tissue were  
196 calculated and  $\log_{10}$  transformed. Comparison between conditions were conducted using  
197 Kruskal Wallis followed by Pairwise Wilcoxon Rank-Sum and visualised with ggplot2 [35].

198 The correlation of bacterial load to histological staging or inflammatory marker fold change  
199 was performed using linear regression [36] and Spearman's Rho Bonferroni, respectively  
200 [35].

201 For bacterial abundance, PERMANOVA (GUniFrac) and Shannon Alpha Diversity were  
202 explored using Phyloseq [37]. Shannon Alpha diversity assesses local bacterial composition  
203 in a sample, determining variety and number of bacterial genera [38], with this method  
204 beneficial for low read count and low abundance samples [38]. Whereas beta diversity  
205 matrices (PERMANOVA GUniFrac) compare community level similarity across different  
206 samples and subgroups [39]. Further univariate analysis applied false discovery rate  
207 corrections (FDR). FDR controls for multiple comparisons and allows understanding of type  
208 one errors or false-positive results [39]. Comparison between conditions and the above  
209 findings were performed by Kruskal Wallis and Pairwise Wilcoxon Rank-Sum, before  
210 visualising with ggplot2 [35].

**211 Results**

212 Participant characteristics are shown in Table 1. No differences were identified between  
213 participants in the PTB+HCA and PTB-HCA subgroups other than HCA stage ( $p<0.001$ ) and  
214 grade ( $p=0.036$ ). Although the focus of this research was HCA, one patient with  
215 inflammatory diagnosed HCA also presented with clinical signs of chorioamnionitis.

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Characteristic	Preterm birth with HCA (PTB+HCA) (n=12)	Preterm birth without HCA (PTB-HCA) (n=6)	Term birth without HCA (TB-HCA) (n=6)	<i>p. value</i> PTB+HCA PTB-HCA TB-HCA	<i>p. value</i> PTB+HCA PTB-HCA
Gestational age (mean (SD))	29.6 (2.9)	29.7 (4.0)	40.4 (0.6)	<b>0.001</b>	0.779
Birthweight (mean (SD))	1387.0 (504.4)	1736.7 (402.3)	3250.0 (495.6)	<b>0.001</b>	0.291
Maternal age (mean (SD))	29.3 (8.0)	27.0 (5.5)	32.2 (6.0)	0.411	0.511
BMI (mean(SD))	22.0 (9.2)	22.5 (4.5)	22.3 (2.1)	0.515	0.580
Smoker	4.0 (33.3)	2.0 (33.3)	0.0	0.329	0.806
<u>Mode of delivery</u>					
Spontaneous vaginal	8.0 (66.7)	5.0 (83.3)	-	-	1.000
Caesarean section	4.0 (33.3)	1.0 (16.7)	-	-	
PPROM	9.0 (75.0)	3.0 (50.0)	-	-	0.330
Interval from PPRM to labour (mean(SD))	7.0 (3.2)	1.7 (0.6)	-	-	0.051
Previous preterm birth	5.0 (41.7)	1.0 (16.7)	-	-	0.600
Antibiotics	7.0 (58.3)	4.0 (66.7)	-	-	0.604
Antenatal corticosteroids	11.0 (91.7)	5.0 (83.3)	-	-	1.000
HCA Stage (mean (SD))	2.2 (0.4)	1.0 (0.0)	-	-	<b>&lt;0.001</b>

<b>HCA Grade (mean (SD))</b>	1.6 (0.5)	1.0 (0.0)	-	-	<b><i>0.036</i></b>
<b>Clinical cases of chorioamnionitis</b>	1.0 (8.3)	0 (0.0)	-	-	0.556

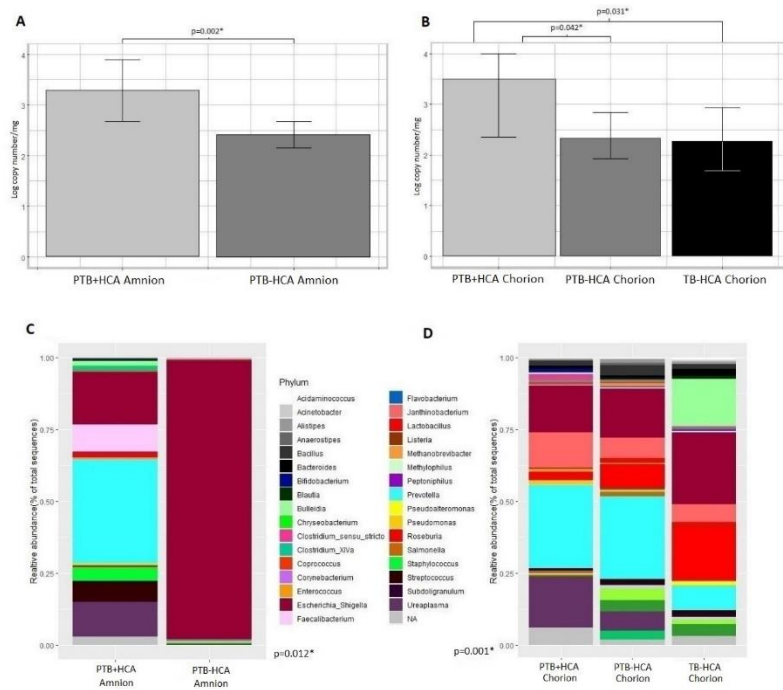
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230 **Table 1: Sample characteristic data.** Assessed between conditions of histological  
231 chorioamnionitis (PTB+HCA), plus preterm (PTB-HCA) and term birth without  
232 chorioamnionitis (TB-HCA). Comparison between all three groups was performed using  
233 Kruskal-Wallis and Pearson's chi-squared. Characteristics monitored in PTB+HCA and PTB-  
234 HCA only using Wilcoxon Rank-Sum and Fisher's exact test. Significance threshold for  
235 comparisons was  $p \leq 0.05$  (bold and italics). Results are displayed as n (%) or mean (SD).  
236 Data unavailable for term subjects (-).

237

### 238 **Bacterial load is increased with HCA**

239 Fetal membranes from participants with PTB+HCA displayed a greater mean bacterial load  
240 than those with PTB-HCA (3.4  $\log_{10}/\text{mg}$  vs 2.4  $\log_{10}/\text{mg}$ ,  $p < 0.001$ ). When investigating  
241 individual membranes; significantly greater bacterial load was evident in PTB+HCA amnion  
242 tissues compared to PTB-HCA amnion tissues (3.3  $\log_{10}/\text{mg}$  vs 2.4  $\log_{10}/\text{mg}$ ,  $p = 0.002$ ; Figure  
243 1A). In chorion tissues, PTB+HCA bacterial loads were also greater compared with PTB-HCA  
244 (3.3  $\log_{10}/\text{mg}$  vs 2.3  $\log_{10}/\text{mg}$ ,  $p = 0.042$ ) and TB-HCA (3.3  $\log_{10}/\text{mg}$  vs 2.3  $\log_{10}/\text{mg}$ ,  $p = 0.031$ ).  
245 No difference was found between PTB-HCA and TB-HCA chorion ( $p = 0.937$ , Figure 1B).



246

247 **Figure 1: Quantitative PCR analysis of bacterial load (A+B) and NGS relative abundance**248 **(C+D).** qPCR data displayed by log copy number/mg of sample from amnion (A) or chorion

249 (B) with histological chorioamnionitis (PTB+HCA), preterm birth without chorioamnionitis

250 (PTB-HCA) and term birth without HCA (TB-HCA). Significance was determined using Kruskal

251 Wallis and Pairwise Wilcoxon Rank-Sum to a threshold of  $p \leq 0.05$ . Relative abundance

252 variation was further analysed between PTB+HCA, PTB-HCA and TB-HCA in amnion (C) and

253 chorion (D) using GUniFrac PEMANOVA to a significance of  $p \leq 0.05$ . Relative abundance

254 was defined as the abundance of each individual genera relative to total percentage of

255 bacterial genera.

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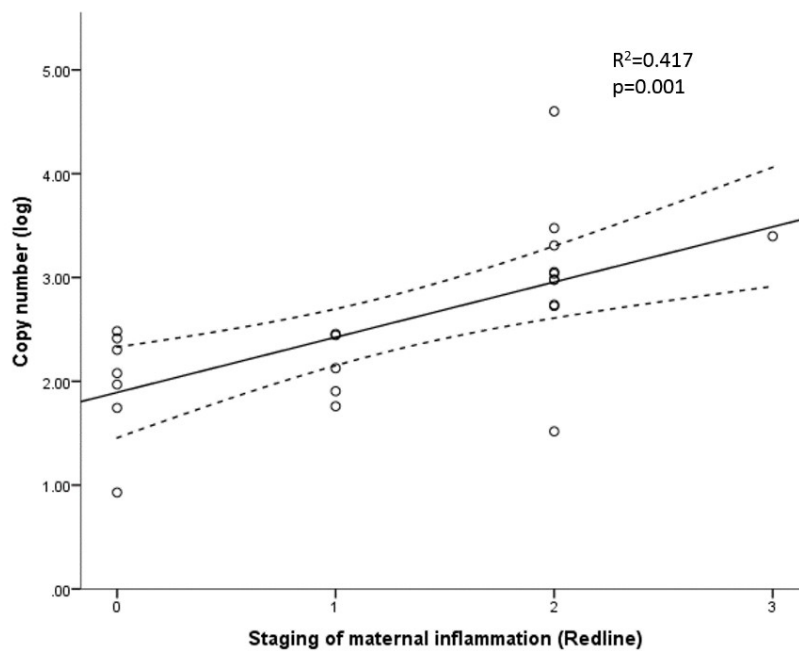
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260 **Bacterial load positively correlates with histological staging in HCA**

261 There was a significantly positive correlation between bacterial load and histological staging  
 262 of membrane inflammation ( $p=0.001$ ; Figure 2), with higher bacterial load related to higher  
 263 stage of HCA.

264



265

266 **Figure 2: Linear regression analysis.** Analysis between bacterial load (log copy number) and  
 267 histological staging of membrane inflammation using linear regression to a threshold of  
 268  $p \leq 0.05$ .

269

270 **Bacterial load is positively correlated with inflammatory gene expression**

271 Bacterial loads in amnion and chorion were significantly correlated to the expression of  
 272 some inflammatory markers (Table 2). In the chorion, bacterial load was positively

273 correlated with IL8 ( $p=0.002$ ), LY96 ( $p=0.003$ ), IRAK2 ( $p=0.004$ ), TLR2 ( $p=0.005$ ) and TLR1  
 274 ( $p=0.013$ ). In the amnion, only IL8 was significantly correlated with bacterial load ( $p=0.050$ ).

Amnion			Chorion		
Inflammatory marker	Spearman's $R_s$	<i>p. value</i>	Inflammatory marker	Spearman's $R_s$	<i>p. value</i>
TLR1	0.346	0.247	<b><i>TLR1</i></b>	<b><i>0.538</i></b>	<b><i>0.013</i></b>
TLR2	0.489	0.093	<b><i>TLR2</i></b>	<b><i>0.600</i></b>	<b><i>0.005</i></b>
TLR4	0.363	0.224	TLR4	0.147	0.524
TLR6	-0.093	0.764	TLR6	0.261	0.252
SARM1	-0.302	0.315	SARM1	0.117	0.613
MyD88	0.346	0.247	MyD88	0.061	0.793
LY96	0.357	0.232	<b><i>LY96</i></b>	<b><i>0.631</i></b>	<b><i>0.003</i></b>
<b><i>IL8</i></b>	<b><i>0.560</i></b>	<b><i>0.050</i></b>	<b><i>IL8</i></b>	<b><i>0.655</i></b>	<b><i>0.002</i></b>
IRAK2	0.489	0.093	<b><i>IRAK2</i></b>	<b><i>0.612</i></b>	<b><i>0.004</i></b>
HMGB1	0.050	0.878	HMGB1	0.284	0.211
SIGIRR	0.368	0.216	SIGIRR	0.139	0.549
TIRAP	0.088	0.778	TIRAP	0.234	0.306

275

276 **Table 2: Correlation of bacterial load against inflammatory gene fold change.** Significant  
 277 differences displayed individually by amnion or chorion were determined using Spearman's  
 278 Rank Bonferroni ( $p \leq 0.05$ , bold and italics).



279 **There is varied range of bacterial genera present irrespective of histological phenotype**

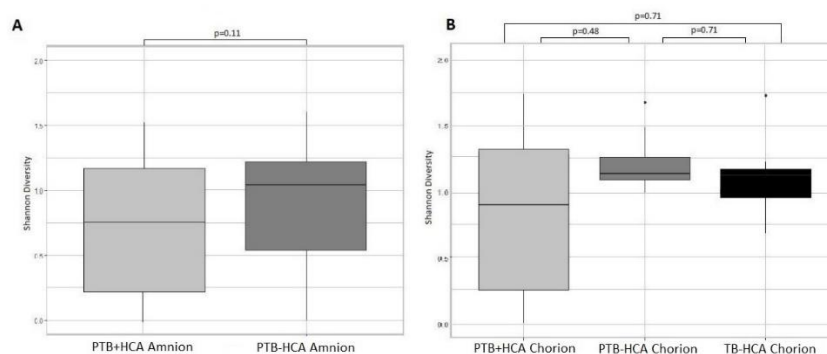
280 The bacterial profile was significantly different between groups of PTB+HCA, PTB-HCA and  
 281 TB-HCA in both chorion ( $R^2=0.2$ ,  $p=0.010$ ), and amnion ( $R^2=0.2$ ,  $p=0.012$ ; Figure 1C and 1D).  
 282 However, no specific genera were statistically significantly different when comparing  
 283 between groups.

284

285 **Alpha diversity does not differentiate between conditions**

286 PTB+HCA samples had the higher overall bacterial diversity (0.7), with PTB-HCA (1.0) and TB-  
 287 HCA lower (1.1), yet no difference between groups ( $p=0.220$ ). When analysing by tissue  
 288 type, although diversity was highest in both PTB+HCA amnion and chorion the differences  
 289 across conditions were not statistically significant (Figure 3).

290



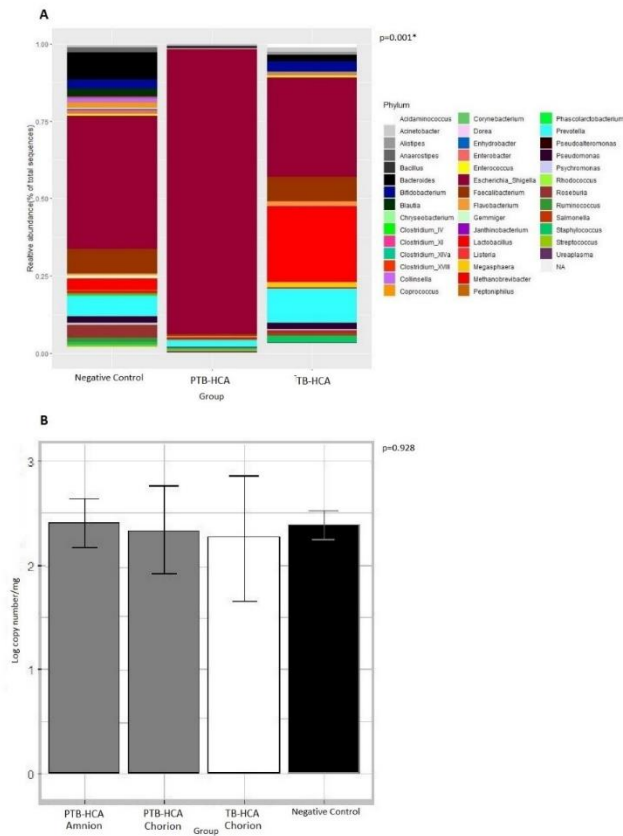
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292 **Figure 3: Alpha diversity analysis.** Relative abundance sequencing data analysed by  
 293 Shannon alpha diversity between amnion (A) and chorion membranes (B) from preterm  
 294 birth samples with chorioamnionitis (PTB+HCA), preterm birth without chorioamnionitis  
 295 (PTB-HCA) and term birth without chorioamnionitis (TB-HCA) to a threshold of  $p \leq 0.05$ .

296 **Non-HCA samples and negative controls differ in bacterial profiles and specific genera, but**  
297 **not bacterial load**

298 Sequencing and qPCR results from preterm and term patients without HCA were compared  
299 to negative controls to investigate genuine microbiota detection from non-HCA fetal  
300 membranes. The overall bacterial profiles were significantly different between non-HCA  
301 samples and negative controls ( $r^2=0.2$ ,  $p<0.001$ ; Figure 4A). Further significance was  
302 detected between specific genera. *Dorea* was detected in negative controls (average read  
303 number=163.1), but not detected in non-HCA samples ( $p=0.001$ , FDR=0.027). The mean  
304 abundance from *Pseudomonas* was significantly greater in negative controls (91.7)  
305 compared to PTB-HCA (4.8) and TB-HCA samples (2.8;  $p=0.002$ , FDR=0.030). *Escherichia* was  
306 significantly reduced in TB-HCA (45.5), compared to similar levels from PTB-HCA (2295.2)  
307 and negative controls (2237.2;  $p<0.001$ , FDR= $<0.001$ ). There was no variation in  
308 *Lactobacillus* ( $p=0.050$ , FDR=0.303), *Ureaplasma* ( $p=0.073$ , FDR=0.308) or *Prevotella*  
309 ( $p=0.608$ , FDR=0.730).

310 No significant difference was detected when comparing bacterial loads of non-HCA samples  
311 to negative controls (2.4  $\log_{10}$ ,  $p=0.9277$ ; Figure 4B). For clarification, no bacterial loads  
312 were detected from NTCs for all qPCR experiments.



313

314 **Figure 4: Negative control comparison; Relative abundance (A) and qPCR bacterial load**  
 315 **(B).** Analysis between kit negative controls (Negative Control) and non-HCA samples of  
 316 preterm (PTB-HCA) and term fetal membranes without chorioamnionitis (TB-HCA) were  
 317 compared. Relative abundance was analysed using GUniFrac PERMANOVA to a significance  
 318 of  $p \leq 0.05$ . qPCR bacterial load (log copy number/mg) results displayed by comparison  
 319 between PTB-HCA, TB-HCA and Negative Control. Significance was determined using Kruskal  
 320 Wallis to a threshold of  $p \leq 0.05$ .

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## 325 Discussion

### 326 Main findings

327 Findings indicate that a greater bacterial load is associated with HCA and a greater bacterial  
328 load is positively correlated with greater histological staging and inflammatory markers. This  
329 supports the suggestion that bacteria act as inflammatory agents in a dose dependent  
330 manner in HCA.

### 331 Interpretation

332 **The key finding of this study is that inflammation in the fetal membranes is associated**  
333 **with presence of bacterial infection and increased bacterial load.** Previous research  
334 supports the theory that bacterial presence is linked to HCA [19,20], with 97% of HCA cases  
335 presenting with bacterial colonisation [40], leading to microbial associated inflammation of  
336 the amnion [40]. Bacterial loads of up to 5.2 log<sub>10</sub> copies/μl have also been detected in fetal  
337 membranes with HCA [16], consistent with our findings. In contrast, Romero *et al* (2014)[41]  
338 detected bacteria in 11% of amniotic fluid samples with PTB and intra-amniotic  
339 inflammation, compared to 26% with a sterile inflammatory response [41]. Studies have  
340 linked HCA to bacterial loads of specific genera, including *Prevotella* [17] and *Ureaplasma*  
341 [40]. The expansion of *Ureaplasma* in HCA was supported here yet did not reach  
342 significance. Although inflammation has not been attributed to specific organisms here,  
343 investigation of the species-specific bacterial load may play a role in this multifactorial  
344 inflammatory condition. As the likely passage of bacteria is ascending, lower bacterial load  
345 would be expected in the chorion. Although consistent bacterial load was present across  
346 membranes with HCA here, the inflammatory response may differ across membranes  
347 impacting clinical relevance and requiring further investigation.

348 **Findings show that bacterial load is positively correlated with HCA severity in a dose**  
349 **dependent manner.** This observation is supported across multiple methodologies and tissue  
350 types [19,42,43]. Research on chorioamniotic membranes has suggested that as HCA  
351 severity increased, so did bacterial load [19]. Bacteria were detected in 87% of membranes  
352 with stage three HCA, compared to 33%, 40% and 60% with stage zero, one and two HCA,  
353 respectively [19]. In amniotic fluid, bacterial load was  $10^6$  copies/ml with stage three HCA,  
354 compared to  $10^3$  copies/ml in stages zero, one and two [42]. However, the link between  
355 bacterial load and inflammation in HCA has been questioned, with the suggestion that any  
356 increase in bacterial load or inflammation is due to active labour rather than specific to HCA  
357 [23]. In this study all patients recruited were in spontaneous active labour, limiting variation  
358 and controlling for vaginal contamination, and the relationship between histological grading  
359 and bacterial load remained consistent. Although the focus here was on preterm patients,  
360 studies addressing HCA at term are required.

361 **Data suggests that bacterial load correlates to inflammation via activation of the TLR**  
362 **signalling pathway.** We have previously reported an increase in gene expression of TLR1  
363 and TLR2 in HCA in the same samples, with a correlation between the increase in TLR gene  
364 expression and HCA stage in both amnion and chorion [21]. Correlation between HCA  
365 bacterial load with TLR1/2 suggests that the number of gram-negative bacteria in the fetal  
366 membranes may be important in the development of HCA, as the TLR1/2 heterodimer  
367 recognises lipopeptides from gram negative bacteria. Although a trend was present, we  
368 were unable to identify significant differences in specific genera (including gram-negative  
369 bacteria) between groups. IL8 was the only inflammatory marker that correlated with  
370 bacterial load in both membranes. The IL8 ligand has been detected in greater  
371 concentrations from HCA patients compared to without HCA, as supported by Kacerovsky *et*

372 *al* (2009) [44]. IL8 levels have previously been used to predict HCA staging in amniotic fluid,  
373 with high specificity [45]. Alternatively, danger signals including HMGB1 also activate the  
374 TLR/MyD88 dependent pathway [46], known as the sterile inflammatory response theory  
375 [41]. However, our work suggests that bacterial load is the key driver to inflammation in the  
376 fetal membranes studied here.

377 **Findings show that non-HCA samples and negative controls differ in few specific bacterial**  
378 **genera but display no difference in bacterial load.** Previous studies have also detected  
379 genera originating mainly from negative controls, including *Dorea* and *Pseudomonas* when  
380 establishing bacterial profiles of placental samples [18,47]. These genera are suggested to  
381 be contaminants in low biomass research [18,47], thus findings indicating clinical relevance  
382 of these bacteria are to be carefully analysed and ensure that correct methodology and  
383 negative controls have been included to avoid misinterpretation.

#### 384 **Research and clinical implications**

385 Conflicting literature highlights the difficulty of reaching a conclusion on the fetal membrane  
386 microbiome in HCA [2,16,23,46]. Although a linear relationship between bacterial load and  
387 inflammation was detected here, the threshold overall bacterial load required to activate  
388 the inflammatory response warrants further study. Investigating selected inflammatory  
389 markers as potential biomarkers for HCA, including TLR signalling mediators, may be  
390 important, including a focus on LY96 (MD2) which links cell surface TLR to bacterial LPS.  
391 PPRM was the most prevalent cause of PTB, occurring in 75% of HCA and 50% of PTB  
392 patients, thus it may be of interest to investigate the variation in HCA between PPRM and  
393 sPTB. Additional research may also aim to understand the origin of bacteria using multiple  
394 body site analysis

## 395 **Strengths and limitations**

396 The absence of a known healthy fetal membrane microbiome complicates the ability to  
397 determine a microbiome linked to HCA. Thus, fetal membranes without chorioamnionitis  
398 from preterm and term labour are required for within study comparisons, as incorporated  
399 into this study. The histological threshold for HCA was set at stage two inflammatory  
400 response. However, only one stage three sample was available from the HCA subgroup,  
401 limiting conclusions at this level. Excluding stage one subchorionitis ensures specificity to  
402 HCA rather than subclinical chorioamnionitis, and is an established reproducible diagnostic  
403 criterion for HCA. Other studies may have included stage one, leading to different  
404 conclusions as to the role of infection and inflammation in HCA.

405 The fetal membrane is a low biomass sample [45,47], which increases the risk of  
406 contamination [48]. To minimise this, negative controls were included and compared to  
407 samples and all samples displayed progressive labour, limiting variation. A 24-patient  
408 sample set from one tertiary unit was utilised increasing consistency of sample handling. A  
409 larger sample set would have strengthened findings to cover heterogeneity of maternal and  
410 fetal response, though the low incidence of early preterm birth and HCA is a recognised  
411 challenge in this field of research. For a subset of patients only amnion or chorion were  
412 available, which could bias results and is a known limitation of human tissue collection.  
413 Bacterial origin cannot be determined as only fetal membrane samples were analysed. The  
414 inclusion of vaginal, oral, skin and blood samples would allow greater understanding of the  
415 source of bacteria and allow further investigation into the link between reproductive,  
416 placental and fetal membrane health [49].

## 417 **Conclusions**

418 The data indicates that inflammation of the fetal membranes is associated with infection  
419 and increased bacterial load in a dose dependent relationship, rather than specific bacterial  
420 profiles. Bacterial load is positively correlated to HCA severity and activation of the TLR  
421 signalling pathway. Further research investigating the bacterial threshold level required to  
422 generate an inflammatory response leading to HCA requires attention.

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442 and interpretation, plus analysis and interpretation of the NGS data, also for drafting the  
443 manuscript. GW designed the study, was involved in approval, tissue collection and  
444 providing data and analysis for the correlation section. GT and CO were involved in the  
445 design, monitoring and support of the study. CO was also involved in qPCR planning. SPC  
446 and SCR were responsible for the initial concept and approval for the study. AN was  
447 responsible for planning and design of the study, executing the NGS method and support of  
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