1 **Title**: **Fetal membrane bacterial load is increased in histologically confirmed inflammatory**

2 **chorioamnionitis: A retrospective cohort study**

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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.

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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   dH2O 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$_2$, 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 (PERMAONVA 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)	*p. value* PTB+HCA PTB-HCA TB-HCA	*p. value* PTB+HCA PTB-HCA
Gestational age (mean (SD))	29.6 (2.9)	29.7 (4.0)	40.4 (0.6)	*0.001*	0.779
Birthweight (mean (SD))	1387.0 (504.4)	1736.7 (402.3)	3250.0 (495.6)	*0.001*	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
Mode of delivery 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 PPROM 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)	-	-	*<0.001*

| HCA Grade (mean (SD))	1.6 (0.5)	1.0 (0.0)	-	-	*0.036*
Clinical cases of chorioamnionitis	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=≤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 $\log_{10}$/mg vs 2.4 $\log_{10}$/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 $\log_{10}$/mg vs 2.4 $\log_{10}$/mg, p=0.002; Figure

243   1A). In chorion tissues, PTB+HCA bacterial loads were also greater compared with PTB-HCA

244   (3.3 $\log_{10}$/mg vs 2.3 $\log_{10}$/mg, p=0.042) and TB-HCA (3.3 $\log_{10}$/mg vs 2.3 $\log_{10}$/mg, p=0.031).

245   No difference was found between PTB-HCA and TB-HCA chorion (p=0.937, Figure 1B).
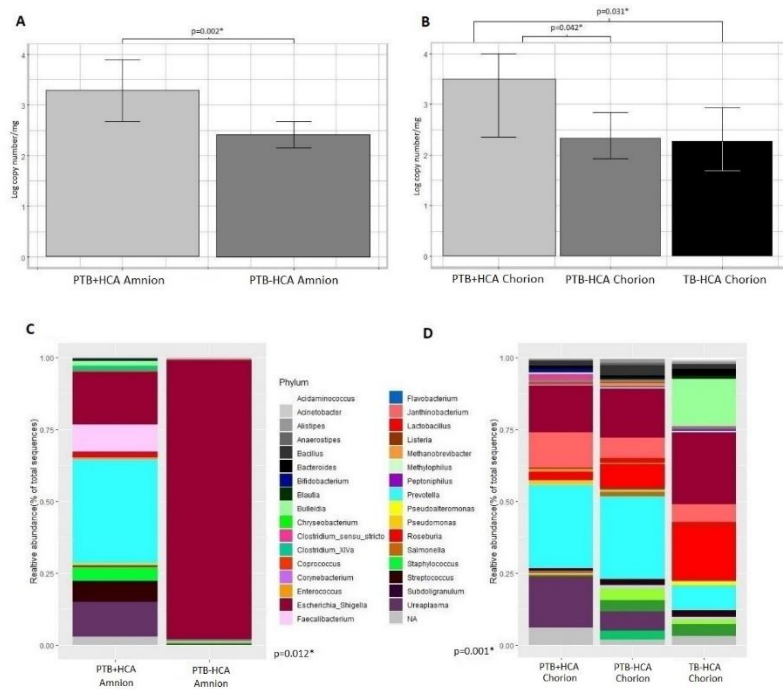
Figure 1: Quantitative PCR analysis of bacterial load (A+B) and NGS relative abundance (C+D). qPCR data displayed by log copy number/mg of sample from amnion (A) or chorion (B) with histological chorioamnionitis (PTB+HCA), preterm birth without chorioamnionitis (PTB-HCA) and term birth without HCA (TB-HCA). Significance was determined using Kruskal Wallis and Pairwise Wilcoxon Rank-Sum to a threshold of $p = \leq 0.05$. Relative abundance variation was further analysed between PTB+HCA, PTB-HCA and TB-HCA in amnion (C) and chorion (D) using GUniFrac PEMANOVA to a significance of $p = \leq 0.05$. Relative abundance was defined as the abundance of each individual genera relative to total percentage of bacterial genera.
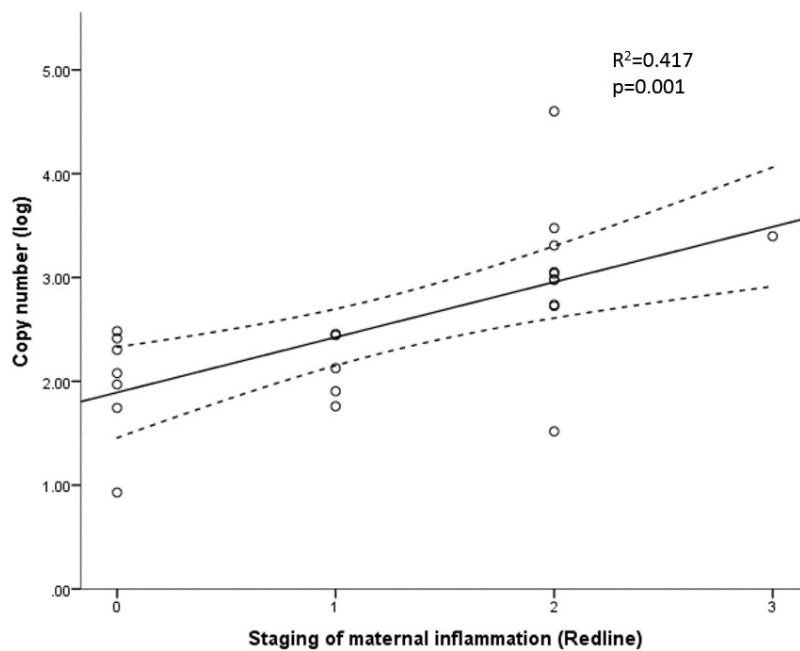
260 **Bacterial load positively correlates with histological staging in HCA**

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.

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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=≤0.05.

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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$_s$	*p. value*	Inflammatory marker	Spearman's R$_s$	*p. value*
TLR1	0.346	0.247	***TLR1***	***0.538***	***0.013***
TLR2	0.489	0.093	***TLR2***	***0.600***	***0.005***
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	***LY96***	***0.631***	***0.003***
IL8	***0.560***	***0.050***	IL8	0.655	0.002
IRAK2	0.489	0.093	***IRAK2***	***0.612***	***0.004***
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

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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=≤ 0.05, bold and italics).

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

The bacterial profile was significantly different between groups of PTB+HCA, PTB-HCA and 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). However, no specific genera were statistically significantly different when comparing between groups.

Alpha diversity does not differentiate between conditions

PTB+HCA samples had the higher overall bacterial diversity (0.7), with PTB-HCA (1.0) and TB-HCA lower (1.1), yet no difference between groups (p=0.220). When analysing by tissue type, although diversity was highest in both PTB+HCA amnion and chorion the differences across conditions were not statistically significant (Figure 3).
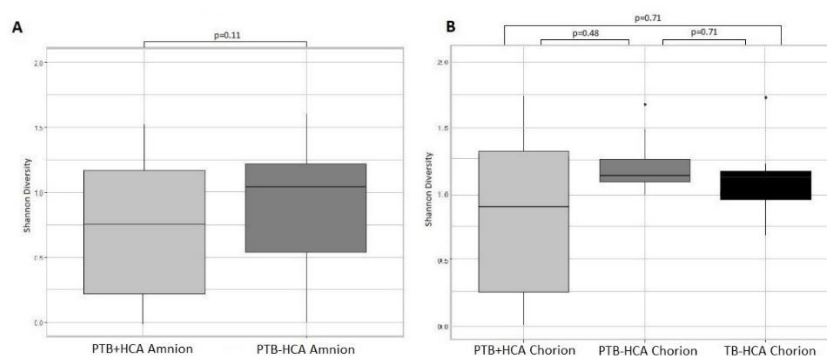
Figure 3: Alpha diversity analysis. Relative abundance sequencing data analysed by Shannon alpha diversity between amnion (A) and chorion membranes (B) from preterm birth samples with chorioamnionitis (PTB+HCA), preterm birth without chorioamnionitis (PTB-HCA) and term birth without chorioamnionitis (TB-HCA) to a threshold of p=≤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

Figure 4: Negative control comparison; Relative abundance (A) and qPCR bacterial load

(B). Analysis between kit negative controls (Negative Control) and non-HCA samples of

preterm (PTB-HCA) and term fetal membranes without chorioamnionitis (TB-HCA) were

compared. Relative abundance was analysed using GUniFrac PERMANOVA to a significance

of p=≤0.05. qPCR bacterial load (log copy number/mg) results displayed by comparison

between PTB-HCA, TB-HCA and Negative Control. Significance was determined using Kruskal

Wallis to a threshold of p=≤0.05.

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Discussion

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.

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 $\log_{10}$ 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   PPROM 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 PPROM 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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440   **Conflicts of Interests:** The Author(s) declare(s) that there is no conflict of interest.

441   **Contribution to authorship:** RH was responsible for qPCR planning, execution, data analysis

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

448   the study. All authors critically revised the manuscript and gave final approval for

449   publication.

450   **Details of ethical approval:** The samples were utilised for current research via a transfer

451   agreement, with prior approval from Newcastle and North Tyneside 1 Research Ethics

452   Committee (Ref:10/H0906/71).

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