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

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Abstract

Introduction

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

Methods

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

Results

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

Discussion

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

Short title: Fetal membrane bacterial load is increased in HCA

Highlights:

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

Keywords: Histological chorioamnionitis; Placenta; Fetal membrane; Microbiome; Inflammation; Bacterial load.

Introduction

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

The origin of bacteria within the healthy fetal membrane microbiome is widely debated [6]. Conflicting studies have suggested that the placenta and fetal membranes are: (i) sterile [7,8,9], with any detection of bacteria linked to the mode of delivery [10]; (ii) typically sterile, with any bacteria detected arising due to co-existent maternal conditions, such as periodontal disease [10,11], vaginal infection [12], or gestational diabetes [13]; (iii) universally colonised with low abundant, non-pathogenic bacteria [14]. Although the existence of a unique microbiome in healthy membranes remains debated [6,14], the healthy bacterial profile (composition and proportion of bacteria) is suggested to consist mainly of *Escherichia spp.* [14,15]. Alternatively, HCA membranes from preterm and term labour have presented with *Ureaplasma spp.* in 59% and 60% of cases respectively [2], suggesting any involvement is independent of gestation. Whilst other studies link HCA and inflammation with increased bacterial load (measurable quantity of bacteria) [16], with a positive correlation between the load of *Prevotella spp.* and HCA severity [17]. Alternatively, lower bacterial diversity has been implicated in preterm HCA membranes compared to controls [15], with monomicrobial characteristics in 83% of HCA cases [2]. In contrast, studies using shotgun and 16S rRNA gene sequencing have reported no distinct bacterial profiles in HCA membranes [6].

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

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

Aims and objectives

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

Methods

Tissue selection and preparation

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

Fetal membrane samples were collected from 24 patients. Following histological diagnosis of HCA, patients were prospectively assigned to spontaneous preterm birth with histological chorioamnionitis (PTB+HCA, n=12), spontaneous preterm birth without HCA (PTB-HCA, n=6) and spontaneous term birth without HCA (TB-HCA, n=6). Amnion and chorion were available for a subset of patients (PTB+HCA=8, PTB-HCA=5, TB-HCA=0). In the remainder, amnion (PTB+HCA=1, PTB-HCA=0, TB-HCA=0), or chorion only were processed (PTB+HCA=3, PTB-HCA=1, TB-HCA=6). Samples were processed in triplicate and prepared with nine DNA extraction kit negative controls. Negative controls were processed identical to samples, with dH2O replacing tissue samples.

HCA was defined by standardised criteria, at maternal stage 2 and above [27]. Subchorionitis was defined as inflammatory stage one [27]. Labour was defined as the presence of regular spontaneous uterine contractions with progressive cervical dilation leading to delivery. Term was defined as a gestational age of >37 weeks, term patients were excluded if presenting with histologically indicated chorioamnionitis. Preterm samples were 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.

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

Expression of inflammatory markers

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

Microbiota analysis

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

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

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

Statistical analyses

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

For the analysis of bacterial load, copy numbers of 16S rRNA gene/mg of tissue were calculated and $\log_{10}$ transformed. Comparison between conditions were conducted using Kruskal Wallis followed by Pairwise Wilcoxon Rank-Sum and visualised with ggplot2 [35]. The correlation of bacterial load to histological staging or inflammatory marker fold change was performed using linear regression [36] and Spearman's Rho Bonferroni, respectively [35].

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

Results

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

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

229

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).

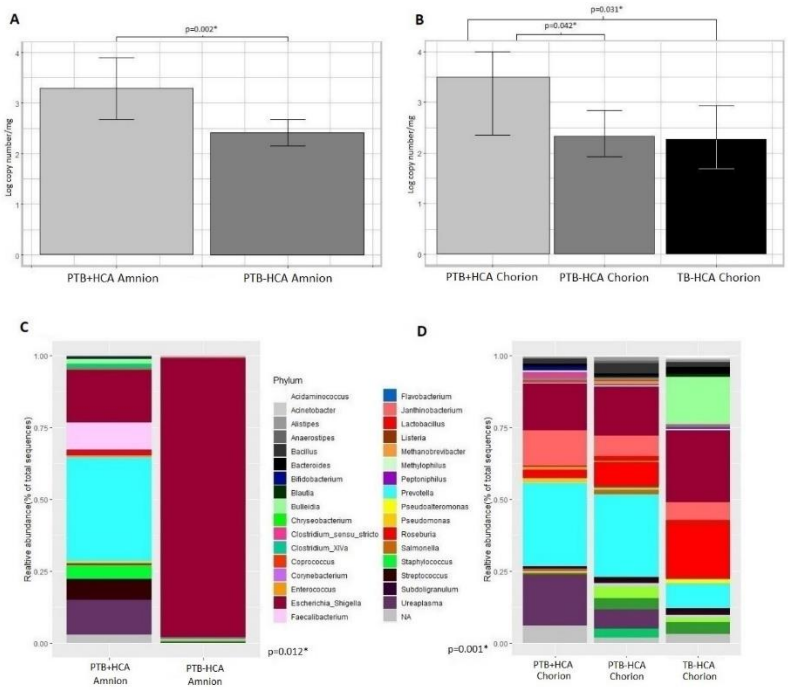

Figure 1: Quantitative PCR analysis of bacterial load (A+B) and NGS relative abundance (C+D). qPCR data displayed by log copy number/mg of sample from amnion (A) or chorion (B) with histological chorioamnionitis (PTB+HCA), preterm birth without chorioamnionitis (PTB-HCA) and term birth without HCA (TB-HCA). Significance was determined using Kruskal Wallis and Pairwise Wilcoxon Rank-Sum to a threshold of p=≤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=≤0.05. Relative abundance was defined as the abundance of each individual genera relative to total percentage of bacterial genera.

Bacterial load positively correlates with histological staging in HCA

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

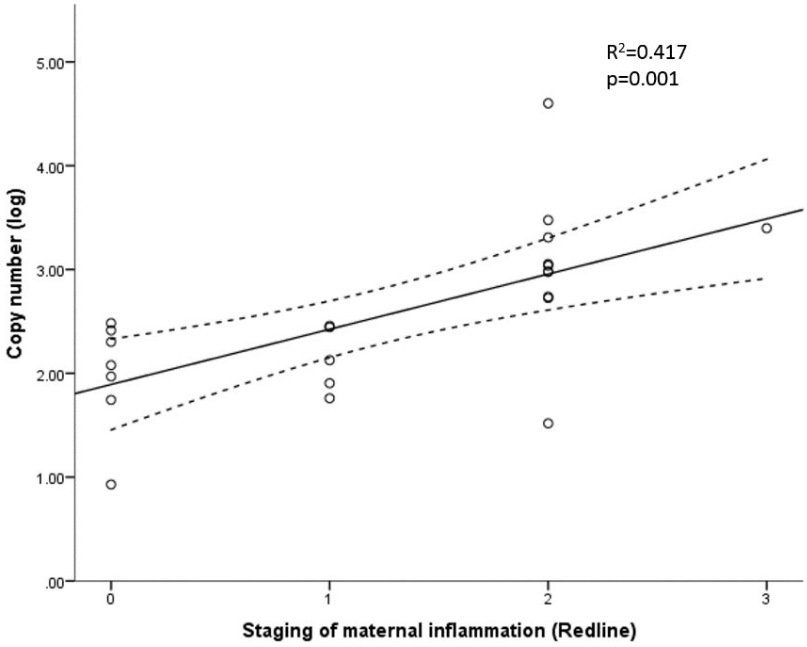

Figure 2: Linear regression analysis. Analysis between bacterial load (log copy number) and histological staging of membrane inflammation using linear regression to a threshold of p=≤0.05.

Bacterial load is positively correlated with inflammatory gene expression

Bacterial loads in amnion and chorion were significantly correlated to the expression of 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

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=≤ 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).

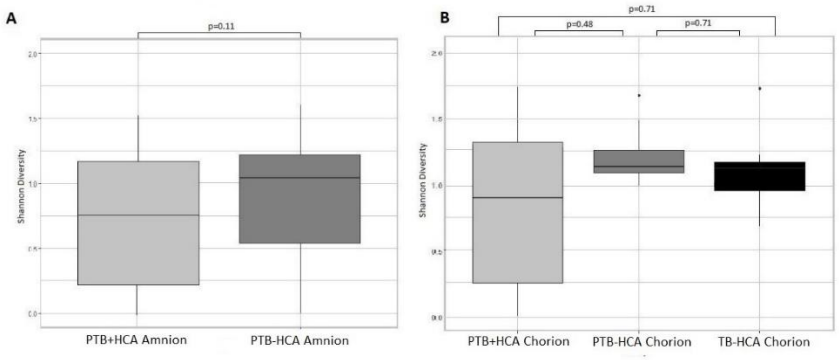

Figure 3: Alpha diversity analysis. Relative abundance sequencing data analysed by Shannon alpha diversity between amnion (A) and chorion membranes (B) from preterm birth samples with chorioamnionitis (PTB+HCA), preterm birth without chorioamnionitis (PTB-HCA) and term birth without chorioamnionitis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

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

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

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

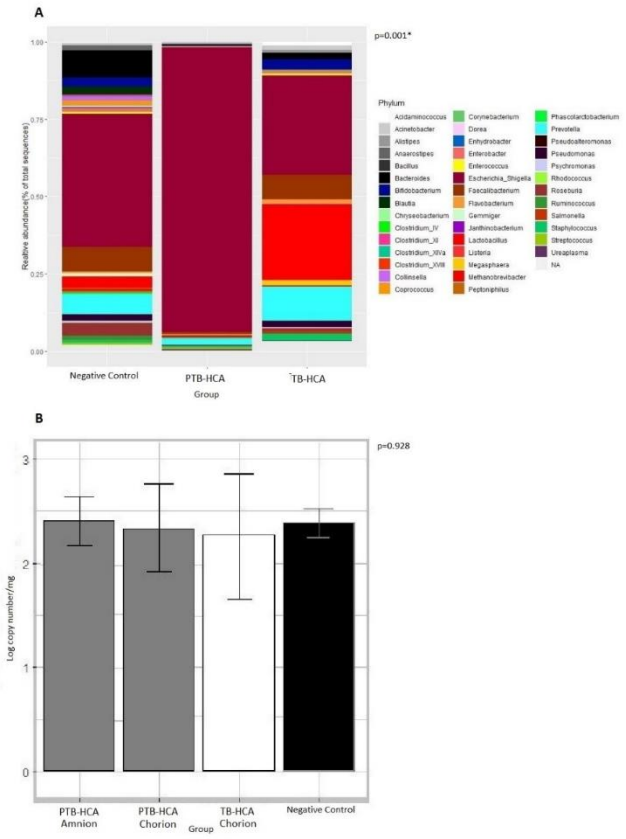

Figure 4: Negative control comparison; Relative abundance (A) and qPCR bacterial load (B). Analysis between kit negative controls (Negative Control) and non-HCA samples of preterm (PTB-HCA) and term fetal membranes without chorioamnionitis (TB-HCA) were compared. Relative abundance was analysed using 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.

Discussion

Main findings

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

Interpretation

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

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

Data suggests that bacterial load correlates to inflammation via activation of the TLR signalling pathway. We have previously reported an increase in gene expression of TLR1 and TLR2 in HCA in the same samples, with a correlation between the increase in TLR gene expression and HCA stage in both amnion and chorion [21]. Correlation between HCA bacterial load with TLR1/2 suggests that the number of gram-negative bacteria in the fetal membranes may be important in the development of HCA, as the TLR1/2 heterodimer recognises lipopeptides from gram negative bacteria. Although a trend was present , we were unable to identify significant differences in specific genera (including gram-negative bacteria) between groups. IL8 was the only inflammatory marker that correlated with bacterial load in both membranes. The IL8 ligand has been detected in greater 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

Strengths and limitations

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

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

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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Contribution to authorship: RH was responsible for qPCR planning, execution, data analysis and interpretation, plus analysis and interpretation of the NGS data, also for drafting the manuscript. GW designed the study, was involved in approval, tissue collection and providing data and analysis for the correlation section. GT and CO were involved in the design, monitoring and support of the study. CO was also involved in qPCR planning. SPC and SCR were responsible for the initial concept and approval for the study. AN was responsible for planning and design of the study, executing the NGS method and support of the study. All authors critically revised the manuscript and gave final approval for publication.

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