

Microbiologic and Inflammatory Markers of Periodontitis are Different to those of Preterm Birth

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Abstract

Background: Comparing microbiologic and inflammatory markers of periodontitis with those of preterm birth (PTB).

Methods: Secondary analysis of a prior prospective case-control study done at the Geneva University Hospitals. Cases were women delivering at 22-34 6/7 weeks (early PTB, n=30) and controls were women delivering at ≥ 37 weeks (term delivery, n=87). We collected dental plaque during labour to quantify RNA levels from *Aggregatibacter actinomycetemcomitans* (Aa), *Tannerella forsythia* (Tf), *Porphyromonas gingivalis* (Pg) and *Treponema denticola* (Td). We also collected cord blood for cultures and cytokine quantification (IL-1Ra, IL-6, IL-8, IL-10, IL-17, TNF- α , MCP-1 and RANTES). Periodontal status was evaluated at the immediate postpartum using the American Consensus definition. Data was analysed by univariate and multivariate logistic regression.

Results: Levels of Pg and Td were significantly higher in dental plaque of women with severe and moderate periodontitis. Microbiologic composition of dental plaque was not different between preterm and term delivery groups. There were no differences in cord blood cultures between groups. Cord TNF- $\alpha \geq 8$ pg/ml and IL-10 ≥ 1.3 pg/ml were associated with a higher risk of periodontitis (OR 2.78, 95% CI: 1.09-7.13, P=0.033; OR 2.78, 95% CI: 1.09-7.08, P=0.032 respectively), but differences were not significant at multivariate analysis. Cord MCP-1 ≥ 350 pg/ml was associated with a higher risk of PTB at both univariate (OR 40.25, 95% CI: 6.79-238.48, P<0.001) and multivariate analysis (OR 53.71, 95% CI: 7.02-410.95, P<0.001).

Conclusion: In labour, microbiologic markers and inflammatory responses associated with periodontitis and PTB are different. The mechanisms linking periodontitis and PTB therefore still need to be elucidated.

Keywords: Periodontitis; Premature birth; Oral microbiology; Cord blood; Cytokines; Inflammation

Introduction

Periodontal disease (PD) is a group of disorders that affect the tissues surrounding and supporting the teeth (periodontium), and is generally caused by Gram-negative anaerobic bacteria colonising the dental plaque. Two forms exist: gingivitis (inflammation of the gums) and periodontitis (inflammation and destruction of the supporting connective tissue and alveolar bone) [1].

Preterm birth (PTB), defined as delivery occurring before 37 gestational weeks, is the leading cause of neonatal mortality and morbidity and also generates important medical costs [2]. In high-income countries, the prevalence of PTB increased over the last 30 years reaching a plateau in the last decade, and currently accounts for 4% to 12% of all life births [3,4].

Infection is the main cause of PTB [5]. Although the mechanism is not fully understood, evidence suggests that infection activates a local inflammatory response, resulting in production of cytokines and prostaglandins by cells of the fetal membranes and decidua, which in turn trigger uterine contractions, membrane rupture and cervical ripening [6]. Whereas the link between PTB and loco-regional infections such as chorioamnionitis, bacterial vaginosis and urinary infections is well established [7], its association with anatomically more "distant" sites of infection such as oral infections, is becoming more accepted [8].

PD affects approximately 74% of pregnant women and 9% of them have periodontitis [9]. Multiple studies controlling for the effect of

demographic, socioeconomic and other pregnancy-related risk factors, have revealed a significant association between periodontitis and PTB [8,10-13]. This association was strongest in the more severe periodontitis [14]. Both microbiologic [15,16] and inflammatory markers [17,18] of periodontitis have been reported to be associated with PTB. Different hypothetical mechanisms could explain this link [19]: (i) haematogenous dissemination of inflammatory mediators from the periodontium into the systemic circulation; (ii) haematogenous dissemination of bacterial toxins (such as LPS) from the periodontium to the feto-placental unit; and (iii) bacteremia.

In 2012, we published the results of a case-control study, which showed that the presence of severe periodontitis using the American consensus definition significantly increased the risk of early PTB (OR

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2.38, 95% CI: 1.36-4.14, $P=0.002$) [20]. In this secondary analysis, we aim to compare the type of bacteria (in dental plaque and cord blood) and cytokines/chemokines (in cord blood) in women with severe periodontitis with those in women with early PTB. The objective is to determine if the microbiological and/or inflammatory pathways in periodontitis are the same as those involved in early PTB. A shared molecular pathway would explain the aetiology of the association between periodontitis and increased risk of PTB that was observed in our previous study.

Methods

Study design

This is a planned secondary analysis of women included in a prior prospective case-control (1:4) study performed between 09 November, 2007 and 18 March, 2010 at the maternity unit of the Geneva University Hospitals, Switzerland [20]. Briefly, the study evaluated the association of periodontitis in cases (women delivering spontaneously between 22 and 34, 6/7 weeks of gestation) compared to controls (women delivering as term [≥ 37 weeks]). The study was approved by the Ethics Committee of Geneva (study number 07-008, approved 28 March 2007). All participants signed an informed consent form. Exclusion criteria were: women aged <18 years, still birth/miscarriage, multiple gestation, cervical cerclage, hydramnios (amniotic fluid index >95 th percentile for gestational age or greatest pocket >8 cm), placenta praevia or abruptio placentae, antibiotic treatment 1 week prior to delivery, any other maternal or foetal pathology requiring preterm delivery. Controls were included after delivery of a case. We collected maternal and neonatal information using a standardized questionnaire, and dental and umbilical cord blood samples during delivery and the immediate post-partum.

Maternal questionnaire

All women enrolled in the study completed a standardised questionnaire, details of which have been published elsewhere [20]. Briefly, the information collected was: age; marital status; socioeconomic status (using a continuous score ranging from 2 to 12 based on the years of formal education of the women and professional activity of the partner); tobacco consumption (previous and current); health status prior to pregnancy; obstetric history including history of previous preterm delivery; infections during pregnancy and their treatment, dental visits and treatment (other than tooth cleaning) in the previous year and during pregnancy; antibiotic treatment during pregnancy; pregnancy complications. Additional data about delivery and the newborn was collected from medical records. A strict protocol was followed to minimize bias, as interviewers were not blinded to the mother's status (early PTB or term delivery).

Oral examination

All women enrolled in the study had a full-mouth oral and periodontal examination within 24-72 hours after delivery, details of which have been published elsewhere [20]. Briefly, clinical parameters recorded on all present teeth (excluding 3rd molars) included: (i) dental plaque index measured on four sites/teeth (three buccal sites, one lingual site), according to O'Leary [21]; (ii) probing pocket depth measured on six sites/teeth (three buccal sites, three lingual sites) using a graduated periodontal probe (Williams' probe, diameter 0.3 mm); (iii) clinical attachment level measured on six sites/teeth (three buccal sites, three lingual sites) with the cemento-enamel junction serving as reference point; (iv) bleeding on probing to the bottom of the pocket measured on four sites/teeth (two buccal sites, two lingual sites), according to Ainamo

and Bay [22]. The number of absent teeth was also recorded. All oral examinations were performed by the same person (SR), who had over 20 years of professional experience, was calibrated at the beginning of the study, and was blinded to the women's obstetric status.

The presence of periodontitis was defined using the USA consensus definitions [23]. These include (i) a case definition for moderate periodontitis ("two or more interproximal sites with attachment loss ≥ 4 mm, not on the same tooth, or two or more interproximal sites with probing depths ≥ 5 mm, not on the same tooth"); and (ii) a case definition for severe periodontitis ("two or more interproximal sites with attachment loss ≥ 6 mm, not on the same tooth, and one or more interproximal sites with probing depth ≥ 5 mm"). For this study, we considered both moderate and severe periodontitis.

Dental and cord sampling

Obstetricians and midwives were trained to perform dental and cord sampling. It was not possible to collect samples from all participants due to high workload in labour units (especially true for cases).

Dental samples: During labour, oral samples of subgingival plaque were collected. If women had to receive antibiotics during labour, oral samples were collected prior to administration. Briefly, after careful removal of supragingival plaque with a curette (without penetrating the pocket), the sampling site was air-dried. Using sterile tweezers, a paper point was inserted into the periodontal pockets of teeth number 16, 26, 36, 46 according to the International Standards Organisation Designation System [24] for 10 seconds to obtain subgingival plaque. Each paper point was then inserted into a test tube containing stabilising buffer and stocked at -80°C until all the tubes were analysed using the IAI PadoTest 4-5[®] (Institute for Applied Immunology AG, Solothurn, Switzerland). This was a RNA hybridisation test that used specific oligonucleotide probes directed against the small subunit ribosomal RNA (SSU rRNA) of *Aa*, *Tf*, *Pg* and *Td*, four anaerobic bacteria that had been associated with PD [25]. By dosing RNA rather than DNA, a reliable estimate of the viable bacteria was obtained, as RNA degraded rapidly (unlike DNA). Presence and concentrations were measured.

Cord samples: Immediately after delivery, 2 ml of venous umbilical cord blood were collected in an anaerobic blood culture bottle (BACTEC[®]) and incubated during 5 days at 38°C in the BACTEC FX[®] incubator (Becton-Dickinson, Sparks, MD, USA), and processed as recommended by CLSI guidelines. An additional 2 ml of cord blood were immediately centrifuged and plasma supernatant stored at -20°C .

Main pro-inflammatory cytokines (tumor necrosis factor [TNF]- α ; interleukin [IL]-6; IL-17), chemokines (IL-8; monocyte chemoattractant protein [MCP]-1; regulated on activation normal T-cell expressed and secreted [RANTES]) as well as two anti-inflammatory cytokines (IL-10; IL-1Ra) were selected to evaluate the cord blood inflammatory status. These cytokines were selected based on existing literature linking them to periodontitis and PTB, for practical reasons (quantifying different cytokines using a single multiplex panel), and also for biological reasons. For example, IL-1Ra was chosen over IL-1 β as the latter is hardly ever detected in circulation, but because IL-1Ra is produced by hepatocytes stimulated by IL-6 and IL-1 β , IL-1Ra may reflect the production of IL-1 β and can be used as its surrogate marker [17].

Cytokines were quantified using the commercial kit Fluorokine MAP Multiplex Human Cytokine Panel (R&D Systems, Minneapolis, MN, USA) according to the supplier's instructions. Briefly, analyte-specific antibodies are conjugated onto beads with defined spectral property. Beads, samples, standards and controls were pipetted into a filter-bottom 96-well plate and incubated at room temperature for 3

hour while gently shaking. After three washes, the biotinylated detector antibody cocktail was added to the wells and incubated for 1 hour at room temperature. After several washings, streptavidin conjugated with R-phycoerythrin (SA-PE) was added to the wells and incubated for 30 min. The data (mean fluorescence intensity) was then read by the Bioplex 200 array reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Sample concentrations were calculated using standard curves. All measurements were performed in the same run assay to avoid inter-assay variability. Intra-assay variation coefficients were below 8%.

Statistical analysis

Categorical variables were described as number and relative frequencies; continuous variables were presented by their median and the interquartile range due to small numbers and therefore due to skewed distributions. Categorical variables were compared between the groups (periodontitis [severe and moderate] vs no-periodontitis; early PTB vs term delivery) using Fisher's exact test. Continuous variables were compared between the groups using non-parametric Mann-Whitney test.

We assessed if the microbiologic and cytokine variables were independently associated with the probability of periodontitis or early PTB. We first identified the variables associated with the probability of periodontitis and early PTB separately by using univariable logistic regression models. Then we performed a backward stepwise procedure to select the independent variables using a p-value <0.20 at univariable analysis; all variables significantly associated with the outcome were kept in the multivariable model after adjustment on main confounders previously identified in our first study (smoking, vaginal bleeding) [20]. Because we did not respect loglinearity when we used cytokine values in their continuous format, we created dichotomous variables using cut-offs with clinical significance and then we assessed whether concentrations above thresholds increased the odds of periodontitis (then PTB) compared to concentrations under the threshold. Logistic regression provided maximum likelihood estimates of the odds ratios (OR) and their confidence intervals (95% CI). Goodness-of-fit was verified using the Hosmer-Lemeshow test. Amount of variation in the risk of periodontitis or early PTB explained by the model was explored by the Cox and Snell R2 and the Nagelkerke R2 test. The discriminant capacity of the model was evaluated by the area under the curve (AUC) assorted with its 95% CI.

To further assess the combined effect of periodontitis with PTB, we created four subgroups: early PTB with periodontitis; early PTB without periodontitis; term delivery with periodontitis; and term delivery without periodontitis. Microbiologic and cytokine variables between the four groups were compared using Kruskal-Wallis non-parametric test for continuous variables and Fischer exact test for dichotomous variables.

All statistical analyses were performed using the Stata statistical package, version IC 14.0 (Stata Corp, College Station, TX, USA). P-values <0.05 were considered significant.

Results

A total of 429 women were included in the original study (84 early PTB; 345 term delivery). In this secondary study we included 117 women (30 early PTB; 87 term delivery) for whom dental bacterial samples were collected. Amongst the 30 women with PTB, 11 cord blood culture bottles (36.7%) and 13 cord cytokine samples (43.3%) were collected. Amongst the 87 women with term delivery, 73 cord blood culture bottles (83.9%) and 71 cord cytokine samples (81.6%) were collected (Figure 1).

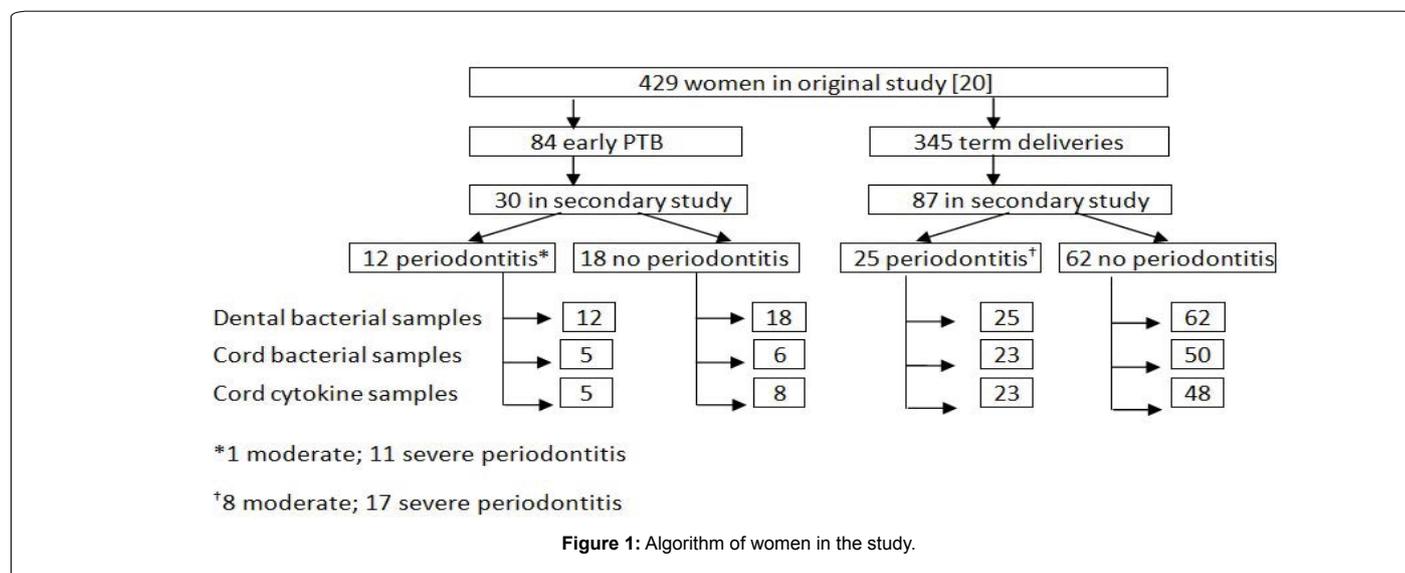
Patient characteristics

Sociodemographic and obstetrical characteristics of women depending on dental status and gestational age at delivery are shown in Table 1.

Mean maternal age was 32.2 years (\pm 5.6, median 32.7), 40 (34.2%) were of Swiss nationality and 40 (34.2%) were Caucasian, with no differences between groups (PTB vs term, $P=0.944$; periodontitis vs no periodontitis, $P=0.233$). There were significantly more women living with their partners among women delivering at term than in the group delivering preterm. During pregnancy, significantly more women with PTB were hospitalized for other reasons than preterm labour compared to women delivering at term. There was no difference between women with and without periodontitis regarding the various variables tested (Table 1).

Dental bacterial results

Levels of *Pg*, *Td* and total bacterial load were significantly higher in women with periodontitis (moderate and severe) compared to



Variables	Periodontitis (N=37)	No periodontitis (N=80)	p	PTB (N=30)	Term (N=87)	p
Sociodemographic characteristics						
Family situation, n (%)			0.205			0.001
Live with partner	33 (89.2)	77 (96.3)		24 (80.0)	86 (98.8)	
Alone, living with family, or widow	4 (10.8)	3 (3.7)		6 (20.0)	1 (1.2)	
Median socioeconomic status (interquartile range)	4 (3-7)	4 (3-6)	0.365	4.5 (3-9)	4 (3-6)	0.06
Health insurance status, n (%)			0.765			0.088
Standard	32 (86.5)	70 (88.6)		29 (96.7)	73 (84.9)	
Private	5 (13.5)	9 (11.4)		1 (3.3)	13 (15.1)	
Professional activity, n (%)			0.209			0.633
Student or at home	10 (27.0)	13 (16.3)		5 (16.7)	18 (20.7)	
Independent or employed	27 (73.0)	67 (83.7)		25 (83.3)	69 (79.3)	
Obstetrical history						
Chronic blood hypertension, n (%)			0.999			0.256
No	37 (100.0)	79 (98.8)		29 (96.7)	87 (100.0)	
Yes	0 (0)	1 (1.2)		1 (3.3)	0 (0)	
Primiparity, n (%)			0.764			0.498
Yes	26 (70.3)	54 (67.5)		22 (73.3)	58 (66.7)	
No	11 (29.7)	26 (32.5)		8 (26.7)	29 (33.3)	
Extrauterine pregnancy/early miscarriage, n (%)			0.727			0.082
None	17 (46.0)	34 (42.5)		9 (30.0)	42 (48.3)	
>=1	20 (54.0)	46 (57.5)		21 (70.0)	45 (51.7)	
Prior PTB			0.681			0.99
No	35 (94.6)	77 (96.3)		29 (96.7)	83 (95.4)	
Yes	2 (5.4)	3 (3.7)		1 (3.3)	4 (4.6)	
Smoking during pregnancy, n (%)			0.395			0.235
No	27 (73.0)	64 (80.0)		21 (70.0)	70 (80.5)	
Yes	10 (27.0)	16 (20.0)		9 (30.0)	17 (19.5)	
Hospitalisation during pregnancy, n (%)			0.235			<0.001
No	30 (81.1)	72 (90.0)		19 (63.3)	83 (95.4)	
Yes	7 (18.9)	8 (10.0)		11 (36.7)	4 (4.6)	
PTB			0.253			
No	25 (67.6)	62 (77.5)		---	---	---
Yes	12 (32.4)	18 (22.5)		---	---	---
Periodontitis (moderate and severe)						0.253
No				18 (60.0)	62 (71.3)	
Yes				12 (40.0)	25 (28.7)	

Table 1: Sociodemographic and obstetrical characteristics based on dental status and gestational age at delivery.

those without (Table 2). Similar results were obtained in women with only severe periodontitis (data not shown). There were no significant differences in median levels of *Aa*, *Tf*, *Pg*, *Td* and total bacterial load between women delivering preterm and those delivering at term (Table 2). The proportion of samples in which bacteria *Pg* was present was significantly higher amongst women with periodontitis ($P < 0.001$). No such differences were seen between the proportions of other bacteria.

Cord Results

We found no association between the presence of bacteria in cord blood and dental status (4% of women with periodontitis vs 11% of women without periodontitis had positive cord blood cultures; $P = 0.416$). Similarly, there was no link between the presence of bacteria in cord blood and risk of PTB (0% of women delivering preterm vs 10% of term birth had positive cord blood cultures; $P = 0.587$).

Results of umbilical cord cytokines are shown in Table 3. Women with periodontitis expressed significantly higher cord levels of IL-10 than women without periodontitis and there was a trend for higher TNF- α in women with periodontitis compared to those without periodontitis. Results were similar when only women with severe periodontitis were selected (data not shown). Women with early PTB expressed significantly lower levels of IL-1ra but available upon request and significantly higher levels MCP-1 with respect to women delivering term. IL-17 was undetectable in all samples.

Multivariate models assessing the cytokines associated with periodontitis and PTB

At univariable, the likelihood of periodontitis was increased if levels of TNF- α were above the cut-off 8 pg/ml, or if levels of IL-10 were above the cut-off 1.3 pg/ml. In multivariable, the association between

Bacteria	Periodontitis (N=37)	N (%)	No periodontitis (N=80)	N (%)	p	PTB (N=30)	N (%)	Term (N=87)	N (%)	p
Levels (x10 ⁶)										
<i>Aa</i>	0 (0-0)	1 (3)	0 (0-0)	0 (0)	0.815	0 (0-0)	1 (3)	0 (0)	0 (0)	0.786
<i>Tf</i>	0 (0-0.224)	17 (46)	0 (0-0.224)	33 (41)	0.616	0.014 (0-0.246)	15 (50)	0 (0-0.224)	35 (40)	0.377
<i>Pg</i>	0 (0-0.130)	16 (43)	0 (0-0)	9 (11)	0.004 [†]	0 (0-0)	7 (23)	0 (0-0)	18 (21)	0.736
<i>Td</i>	0.033 (0-0.204)	22 (59)	0 (0-0.017)	26 (33)	0.0009 [†]	0 (0-0.129)	14 (47)	0 (0-0.033)	34 (39)	0.283
Total load (x10 ⁶)	33.67 (28.92-40.98)	37 (100)	31.38 (22.22-37.74)	80 (100)	0.024	30.89 (26.03-46.68)	30 (100)	32.63 (24.29-38.01)	87 (100)	0.583

Data presented as median (inter-quartile range).
[†]Number (percentage) of samples with bacteria present
[†]P-values <0.05 were considered significant.

Table 2: Dental bacterial levels and frequencies based on dental status and gestational age at delivery.

	Periodontitis (N=28)	No periodontitis (N=56)	p	Early PTB [†] (N=13)	Term (N=71)	p
IL-1ra	4457.5 (2796.5-7367)	4169 (2173-6646.5)	0.563	1708 (958.0-6359.0)	4383 (2894.0-7320.0)	0.037 [†]
IL-6	2.96 (1.5-8.99)	3.03 (1.58-6.13)	0.826	2.51 (1.50-4.14)	3.04 (1.50-7.00)	0.689
IL-8	66.5 (18.5-193)	38 (18-102)	0.265	26.0 (18.0-81.0)	42.0 (19.0-148.0)	0.354
IL-10	1.40 (1.05-2.19)	1 (1-1.6)	0.011 [†]	1.09 (1.00-1.35)	1.12 (1.00-1.79)	0.683
MCP-1	187.5 (133-279.5)	191.5 (147-262)	0.951	388.0 (175.0-466.0)	182.0 (132.0-246.0)	0.007 [†]
TNF-α	8.50 (7.19-9.53)	7.75 (6.76-8.64)	0.053 [†]	8.16 (7.09-10.41)	7.92 (6.82-8.85)	0.432
Rantes	86590.5 (43136-118993.5)	72226.5 (51795-113022.5)	0.663	53561.0 (39808.0-127081.0)	75580.0 (52632.0-114806.0)	0.469

Data presented as median (quartile 1- quartile 3).
[†]P-values <0.05 were considered significant.
[†]PTB: preterm birth

Table 3: Cord cytokines (pg/ml) based on dental status and gestational age at delivery.

periodontitis and higher levels of TNF-α or IL-10 was not significant. The likelihood of early PTB was independently associated with levels of MCP-1 above the cut-off 350 pg/ml (Table 4).

Association between PTB and periodontitis

We then assessed the combined effect of periodontitis with PTB by creating four subgroups (early PTB with periodontitis; early PTB without periodontitis; term delivery with periodontitis; and term delivery without periodontitis). At the microbiological level, *Pg* and *Td* levels were significantly higher in the two subgroups with periodontitis (with and without PTB) (Table 5). At the inflammatory level, we found that the frequencies of patients with MCP-1 ≥ 350 pg/ml were significantly higher in the two groups with PTB (with and without periodontitis) compared to the two groups delivering at term (with and without periodontitis) (Figure 2).

Discussion

The pathophysiology of PTB remains poorly understood, although infection is thought to be the main trigger [6]. Literature has provided inconsistent data on the association between periodontitis and PTB [8,10,14,26]. Periodontitis and PTB are associated with infectious aetiology and elicit an activation of the inflammatory cascade. In this study, we evaluated microbiologic status of the dental plaque and umbilical cord as well as the inflammatory response in the umbilical cord in women included in a prior case-control study evaluating the

association for periodontitis and early preterm birth (<34 weeks). To our knowledge, this is the first study reporting data about dental and cord blood microbiology, dental status and delivery outcome.

As expected, total bacterial load and levels of *Td* and *Pg* in dental plaque were significantly higher in patients with periodontitis. However, surprisingly, none of the four evaluated bacterial species were increased in women delivering preterm. Previous studies have already described the association between *Td*, *Pg* and *Tf* and periodontitis [27], but have also failed to find a link between oral levels of these bacteria and PTB [28]. Therefore rather than specific oral bacterial species and their load, severity (aggressive versus chronic) and extension (localised versus generalized) of periodontal disease might be the key factors determining the type of immune response generated and the risk/kind of bacteremia, consequently increasing the risk of PTB. For example, *Aa* is found at higher levels in patients with aggressive generalized forms of periodontitis [29]. Similarly, patients with localized periodontitis had low IgG titers to *Tf* unlike patients with generalized periodontitis, although both were colonized by *Tf*, suggesting different immune responses depending on disease extension [30]. Significantly low IgG titers to *Pg* have also been observed in women with PTB (although they had higher levels of periodontal bacteria in dental plaque than women undergoing term delivery), suggesting a weaker immune response to *Pg* increased the risk of PTB, perhaps by dissemination of the infection to extra-oral sites [31].

Independent predictors for periodontitis						
Cytokine categories (pg/ml)*	Univariate (N=84)			Multivariate [‡] (N=84)		
	OR	95% CI	p-value	OR	95% CI	p-value
TNF-α						
<8	1	-		1		
≥ 8	2.78	1.09-7.13	0.033 [†]	2.37	0.89-6.31	0.085
IL-10						
<1.3	1	-		1		
≥ 1.3	2.78	1.09-7.08	0.032 [†]	2.48	0.94-6.60	0.068
MCP-1						
<350	1	-		1		
≥ 350	2.83	0.69-11.50	0.147	2.91	0.67-12.60	0.154
Independent predictors for early PTB						
Cytokine categories (pg/ml)*	Univariate (N=84)			Multivariate [§] (N=84)		
	OR	95% CI	p-value	OR	95% CI	p-value
TNF-α						
<8	1	-		1		
≥ 8	1.34	0.41-4.40	0.626	1.46	0.32-6.69	0.625
IL-10						
<1.3	1	-		1		
≥ 1.3	0.51	0.14-1.82	0.3	0.33	0.06-1.92	0.218
MCP-1						
<350	1	-		1		
≥ 350	40.25	6.79-238.48	<0.001 [†]	53.71	7.02-410.95	<0.001 [†]
Periodontitis[¶]						
No	1	-		1		
Yes	1.3	0.38-4.43	0.67	0.76	0.13-4.46	0.763

*using the median as cut off values.
[†]P-values <0.05 were considered significant.
[¶] Periodontitis is included as a variable in the multivariate analysis of the predictors of early PTB. This is done to confirm that the significant increase in MCP-1 observed in women with early PTB is an independent factor to periodontitis.
[‡]AUC=69.9% (58.2-81.6); pseudo R²=9.3%; Hosmer-Lemeshow test p=0.872.
[§]AUC=79.4% (64.5-94.2); pseudo R²=32.1%; Hosmer-Lemeshow test p=0.797.

Table 4: Independent predictors for periodontitis and early PTB (logistic regression model).

Our study failed to find an association between presence of bacteria in cord blood of women with periodontitis and of women delivering preterm. Nevertheless, this does not mean that oral bacteria are not implicated in the process leading to preterm birth as several studies have confirmed the dissemination of periodontal pathogens to extraoral sites in pregnancy [32,33]. Aagaard et al. have also reported a very close similarity between placental and oral microbiomes [34]. A reason that may explain why we were unable to detect bacteria in cord blood is the timing of our samples. Dental bacteria may have colonised the foeto-placental unit at early stages of pregnancy, altered the placenta microbiota and activated throughout pregnancy the inflammatory cascade leading to preterm birth. Unfortunately, we did not perform microbiological exams of the placenta and foetal membranes and cannot confirm our hypothesis. Clearly, a sample of cord blood obtained immediately after delivery provides a limited view of the pathophysiological mechanisms of bacterial invasion that may have occurred throughout pregnancy.

In terms of inflammatory markers, women with periodontitis expressed significantly higher levels of IL-10 and a trend for higher levels of TNF-α. This increase in pro-inflammatory TNF-α during periodontitis has been well documented [35], and it is known to play a substantial role in periodontitis mediated bone loss [36]. The

concomitant increase in anti-inflammatory IL-10 in the periodontitis group is however more surprising, although it has been previously observed [37-39]. One hypothesis explaining the observed concomitant pro- and anti-inflammatory response is that aggressive periodontitis would stimulate a strong increase in TNF-α, which in turn could activate a counteracting anti-inflammatory IL-10 response to avoid over-activation of inflammation. Another hypothesis would be that during chronic generalised periodontitis, IL-10 would increase resulting in an “anti-inflammatory” state in the mouth, facilitating the dissemination of dental bacteria to extra-oral sites, which then would activate a pro-inflammatory response in the foetal-placental unit and therefore trigger PTB. For example, Havemose et al. showed a concomitant increase in TNF-α and IL-10 in patients with generalised aggressive periodontitis, but IL-10 alone was not increased in patients with localised aggressive periodontitis [38].

High concentrations of MCP-1 (≥ 350 pg/ml) in umbilical cord blood significantly increased the likelihood of PTB. This finding was also described by Matoba et al. who quantified twenty-seven types of cytokines in cord blood at different gestational ages and found that MCP-1 was the one that was most strongly associated with PTB [40]. Significantly higher levels of MCP-1 have also been measured in cervico-vaginal fluid [41] and amniotic fluid of women undergoing PTB [42].

	PTB with periodontitis (N=12)		PTB without periodontitis (N=18)		Term with periodontitis (N=25)		Term without periodontitis (N=62)		<i>p</i> [†]
Bacteria									
Levels (x10 ⁶)		N(%)*		N(%)*		N(%)*		N(%)*	
<i>Aa</i>	0 (0-0)	1 (8)	0 (0-0)	0 (0)	0 (0-0)	0 (0)	0 (0-0)	0 (0)	0.974
<i>Tf</i>	0.048 (0-0.493)	7 (58)	0 (0-0.224)	8 (44)	0 (0-0.157)	10 (40)	0 (0-0.224)	25 (40)	0.752
<i>Pg</i>	0.028 (0-0.448)	6 (50)	0 (0-0)	1 (6)	0 (0-0.130)	10 (40)	0 (0-0)	8 (13)	0.033 [†]
<i>Td</i>	0.140 (0-0.392)	8 (67)	0 (0-0.017)	6 (33)	0.025 (0-0.173)	14 (56)	0 (0-0.017)	20 (32)	0.007 [†]
Total load (x10 ⁶)	36.00 (29.67-47.12)	12 (100)	28.53 (23.96-46.68)	18 (100)	33.67 (28.84-40.87)	25 (100)	32.05 (21.89-37.59)	62 (100)	0.147
	PTB with periodontitis (N=5)		PTB without periodontitis (N=8)		Term with periodontitis (N=23)		Term without periodontitis (N=48)		<i>p</i> [†]
Cord cytokines (pg/ml)									
		N(%)*		N(%)*		N(%)*		N(%)*	
IL-1ra	1708 (645-3074)	12 (100)	2267 (1006-6800)	18 (100)	5388 (2963-7392)	25 (100)	4340 (2846-6622)	62 (100)	0.146
IL-6	1.5 (1.5-2.51)	12 (100)	3.19 (1.69-13.82)	18 (100)	3.52 (1.5-9.3)	25 (100)	3.03 (1.54-6.13)	62 (100)	0.681
IL-8	18 (13-20)	12 (100)	39 (24-108.5)	18 (100)	117 (25-195)	25 (100)	37.5 (16.5-102)	62 (100)	0.182
IL-10	1.18 (1.09-1.35)	12 (100)	1 (1-1.56)	18 (100)	1.61 (1-2.2)	25 (100)	1 (1-1.61)	62 (100)	0.104
MCP-1	388 (168-466)	12 (100)	349 (185-567.5)	18 (100)	171 (129-234)	25 (100)	183 (140.5-249)	62 (100)	0.061
TNF-α	8.16 (7.28-9.82)	12 (100)	8.12 (5.98-10.5)	18 (100)	8.55 (6.82-9.52)	25 (100)	7.75 (6.81-8.44)	62 (100)	0.233
Rantes	41639 (30218-121358)	12 (100)	56344 (46013-161785)	18 (100)	86661 (53131-116629)	25 (100)	73765 (52002-108354)	62 (100)	0.700
TNF-α ≥ 8	5 (55.6)	12 (100)	5 (45.5)	18 (100)	16 (66.7)	25 (100)	20 (36.4)	62 (100)	0.084
IL-10 ≥ 1.3	4 (44.4)	12 (100)	3 (27.3)	18 (100)	16 (66.7)	25 (100)	24 (43.6)	62 (100)	0.132
MCP-1 ≥ 350	4 (44.4)	12 (100)	5 (45.5)	18 (100)	2 (8.3)	25 (100)	1 (1.8)	62 (100)	<0.001

Continuous data are presented by median and interquartile range, binary variables are presented by frequency and relative proportion.
[†] Kruskal-Wallis nonparametric test was used to compare continuous variables and Fisher exact test was used to compare binary variables. P-values <0.05 were considered significant.

* Number (percentage) of samples with bacteria present
 PTB: preterm birth
 Periodontitis: severe and moderate

Table 5: Dental bacterial and cord results.

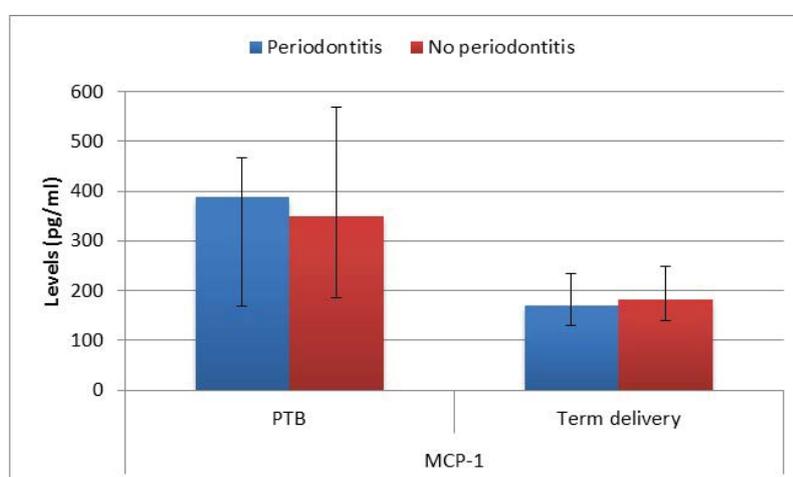


Figure 2: Levels of MCP-1 based on delivery and periodontal status.

MCP-1 plays an essential role in the initiation and progression of the inflammatory response, as it is one of the main chemokines regulating migration and infiltration of monocytes/macrophages. This explains why MCP-1 is involved in a variety of inflammatory diseases, such as cardiovascular disease, multiple sclerosis and rheumatoid arthritis [43]. During labour, expression of MCP-1 and other chemokines in chorionic decidua increases, allowing infiltration of macrophages and other leucocytes into the uterus [44]. Therefore, although the exact role of MCP-1 in PTB is unknown, it may mediate leucocyte trafficking in a similar way as during term delivery and could therefore be partially responsible for PTB. However, this increase doesn't seem to be related to periodontitis.

Low levels of IL-1Ra were associated with PTB in the univariable analysis, but this association was no longer sustained in the multivariable model probably due to the small sample size. Other studies have shown that IL-1Ra is decreased in gingival fluid and in cervico-vaginal fluid but not in serum of women with pre-term labor [45]. The physiological role of IL-1Ra is to antagonize IL-1 inflammatory effects, but it is produced as an acute phase protein [46]. High serum levels could reflect an active inflammatory process rather than a protective role, explaining the discrepancies between the studies and the weakness of the association.

Unlike other published studies, we did not find significantly increased levels of IL-6, IL-8 and TNF- α in women delivering preterm [47,48]. There is a vast controversy in the literature regarding cytokine profiles and PTB. This is probably due to variations in sample size and sampling sites, variations in the methods used for quantifying the cytokines and their detection limits, the complexity of cytokine systems and their redundant nature, and the effect of other confounding variables such as ethnicity, age and other obstetrical complications that also increase risk of PTB [49,50].

In the study we published in 2012, we found a significant association between periodontitis and early PTB [20]. However, this secondary study shows that the oral bacteria and cord cytokines involved in periodontitis and PTB are different. This suggests periodontitis is not directly the cause triggering PTB, but it simply a marker of an inflammatory state, and therefore a risk factor for PTB. Our results are in agreement with other studies published [11].

Our study has several strengths. We have evaluated simultaneously presence/absence of periodontitis using an accepted consensus definition, done bacteriological assessment in the mouth, and quantified bacteria and cytokines in cord blood in women undergoing early PTB versus term delivery. This allows us to compare whether differences observed in oral and cord samples in women with early PTB were also present in women with periodontitis. Hasegawa et al. have been the only group to also compare the association between periodontal status, serum cytokine levels and delivery outcomes in 88 women [48]. They found that women with threatened preterm labour had worsened periodontal status, significantly higher levels of *Tf* in oral samples, and significantly higher levels of serum IL-8 and IL-1 β versus women without it. Their study however failed to dose MCP-1 (only four cytokines were quantified: IL-6, IL-8, IL-1 β , TNF- α) and no microbiological analysis on cord blood was carried out. Finally, we used multiplex technology for the analysis of cytokines allowing to measure multiple proteins simultaneously, and thus to reduce inter-assay variability.

The main limitation in our study is the reduced sample size, which decreased the statistical power but also the generalizability of our findings. Also, since blood cultures in women with PTB were negative, we were unable to characterize bacteria type in umbilical blood

cultures, thus making it impossible to determine if PTB in patients with periodontitis was due to bacteremia of oral bacteria.

Conclusion

Although a significant association between severe periodontitis and early PTB was found in our primary analysis of this study (data previously published), this planned secondary analysis shows that periodontitis and PTB have different periodontopathogens and cord cytokines at delivery. PTB is a highly complex process, driven by a MCP-1 inflammatory response. Periodontitis is probably simply a marker of an inflammatory state, and thus a risk factor of PTB, but not the cause triggering it. The molecular pathways linking periodontitis and early PTB still need to be clarified.

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Declaration of Interest

Authors declare no conflict of interest related to this study. The contributions of individual authors to this paper is as follows: planning research (BMT, OI and PB); executing research (BMT, PB); analysing data (AGA); interpreting data (MLA, BMT, AGA, PRL); writing (MLA, BMT, AGA, PRL, JS, PB).

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