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Impact of Pregnancy History and 17-hydroxyprogesterone caproate (17-OHPC) on Cervical Cytokines and Matrix Metalloproteinases

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Abstract

Objective—To evaluate the impact of pregnancy history and 17-hydroxyprogesterone caproate (17-OHPC) treatment on cervical fluid cytokines and matrix metalloproteinases (MMPs).

Study Design—Cervical fluid was obtained between 16 0/7 - 24 6/7 weeks from women with only prior term births (**controls, n=26**), women with one or more prior spontaneous preterm births (SPTBs) choosing to receive 17-OHPC (**17-OHPC, n=24**), or to not receive 17-OHPC (**refusers, n=12**). Cervical fluid collections were repeated two, four and eight, weeks after the 1st sample and concentrations of MMPs and cytokines were measured by multiplex immune assay.

Results—Among women whose earliest prior delivery occurred between 16–23 weeks, cervical fluid concentration of IL-6, IL-10 and TNF- α at baseline were significantly elevated when compared to cervical cytokines of women whose earliest delivery occurred between 32–36 weeks (relative risk ratio was 3.37 for IL-6 (95% CI 1.08–10.53, $p<0.05$), 2.81 for IL-10 (95% CI 1.39–5.70, $p<0.05$), and 6.34 for TNF- α (95% CI 2.19–18.68, $p<0.001$). Treatment with 17-OHPC had no significant impact on these cytokines.

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Conclusions—The cervical fluid of women with a history of an early prior SPTB is characterized by inflammation that is unaffected by 17-OHPC.

Keywords

17-hydroxyprogesterone caproate; cytokines; matrix metalloproteinases; cervicovaginal milieu inflammation

Introduction

Supplemental progesterone treatment is commonly utilized to reduce spontaneous preterm birth (SPTB) risk^{1,2}. One such progestin, intramuscular 17-hydroxyprogesterone caproate (17-OHPC) reduces the risk of SPTB in women with prior PTB by one-third¹. Despite widespread use of 17-OHPC for reducing PTB risk, the target of this treatment remains elusive. Identification of specific treatment-responsive pharmacodynamic marker or markers could provide insight into the biological pathways leading to preterm birth and could allow for development of more effective treatments.

The lack of a recognized target for 17-OHPC is not the only pharmacologic deficiency of this medication; the appropriate dose is also unclear. Current treatment utilizes a fixed dose of 250 mg weekly which is not based on any pharmacologic considerations. Ideally, dose-response studies are needed to define an optimal dose but these have not been performed for 17-OHPC. This omission hampers the assessment of efficacy for this medication which has been judged by the rates of SPTB in a treated versus a control group. The absence of dose-response studies to establish the optimal dose is clearly an issue for 17-OHPC. Plasma concentrations at steady state with the weekly 250 mg dose vary from 3–56 ng/ml and plasma concentrations impact efficacy³. If an intermediate pharmacodynamic endpoint for SPTB could be identified for this treatment, such a marker could be utilized to optimize a dosing regimen without the need for a large multicenter dose ranging study. Furthermore, by identifying a biomarker of SPTB risk in addition to history alone, further risk stratification would be possible. Identifying women at the highest risk utilizing both an individual's preterm birth history and a biomarker of risk would enable focused 17-OHPC treatments in an enriched high risk group.

This study focuses on the cervix for potential biomarkers of SPTB. A large body of literature has established that cervical length in the second trimester is an excellent predictor of SPTB risk^{4,5}. Cervical biomarkers during pregnancy that might serve as pharmacodynamic endpoints for 17-OHPC therapy are mostly limited to ultrasound imaging of cervical structure, digital examination and examination of the cervicovaginal fluid. Several inflammatory cytokines and MMPs have been associated with SPTB^{6–15}. The objective of this pilot study was to evaluate cervical cytokines and MMPs as potential targets of intramuscular 17-OHPC therapy and to determine if those potential targets are impacted by pregnancy history.

Materials and Methods

Study Design

This was a multicenter prospective observational cohort study with four participating centers of the NICHD-sponsored Obstetric–Fetal Pharmacology Research Units Network including University of Texas Medical Branch, Indiana University, University of Washington and the University of Pittsburgh. Approval was obtained from the IRB of each center. The primary aims of this study were to assess the relationship between pregnancy history and 17-OHPC treatment on the cervical fluid proteome, cytokines, MMPs and cervical length and density in 3 groups of women: control subjects (n=26) had a history of one or more term births and no preterm births. The 17-OHPC group (n=24) had a history of one or more prior SPTBs and chose to receive 17-OHPC intramuscular injections weekly starting between 16–20 weeks until 36 weeks gestation while refuser women (n=12) had a history of one or more SPTBs but declined to receive 17-OHPC. All subjects with a prior SPTB were enrolled after they and their providers had decided whether or not the subject would accept 17-OHPC treatment. They were not randomized to the two study groups; receipt or non-receipt of 17-OHPC was a clinical decision. We evaluated 4 MMPs including: MMP-1, MMP-2, MMP-8, MMP-9 and 8 cytokines including: IL-1 α , IL-1 β , IL-4, IL-6, IL-8, IL-10, IL-13 and TNF- α . We first compared baseline concentrations of these analytes and then compared concentrations during the 8 weeks study by group. In particular, we evaluated the impact of 17-OHPC treatment on trends in the MMP and cytokine concentrations over the 8 weeks of observation. The primary comparisons focus on the 17-OHPC group and the controls. Comparisons between the 2 groups of women with a prior SPTB i.e. 17-OHPC and refuser groups are also reported. This report focuses on the MMP and cytokine comparisons. Analyses of the proteomic, ultrasound, and pharmacogenomic analyses will be reported separately.

The study also planned to recruit a fourth group of asymptomatic women with a short cervix detected on routine ultrasound who agreed to receive vaginal progesterone. However, only 5 subjects were recruited in this arm, so this cohort of women was excluded from this report. Entry criteria for the 3 study groups are listed in the Appendix.

Study Procedures

All subjects fulfilling entry criteria and consenting to the study were scheduled to undergo 4 visits: the first within 1–2 weeks of enrollment and prior to any progestin therapy in the 17-OHPC group. This baseline visit was followed by 3 additional visits at 2 weeks, 4 weeks and 8 weeks after the baseline visit. These time point were selected both to detect early changes in measured parameters and also to allow adequate time for any treatment effects to be detected. At each of these visits a sterile speculum examination was performed without gel. Two Dacron-tipped swabs were placed in the external cervix for 1 minute for collection of cervical fluid. The first swab was designated for proteomic analysis; the second swab was designated for cytokine and MMP assay. Subsequently, a third swab was placed in the posterior fornix for 1 minute for future analyses of other potential biomarkers. A fourth swab was then placed in the posterior fornix for determination of bacterial vaginosis followed by a 5th swab which was placed in the cervix for determination of gonorrhea,

chlamydia and trichomonas using assay methods typically used at each institution. The swab for cytokines and MMP analysis was placed in a 2 ml cryovial containing 400 μ L of DPBS, swirled for 10 seconds, then the tip was cut and left in the vial, labeled and frozen at -80° C. The swabs for proteomic analysis and other future studies were placed into a 2 ml cryovial containing 1 ml DPBS and agitated for 10 seconds while in the DPBS then discarded. The cryovials were labeled and frozen at -80° C. The swabs for BV, trichomonas, chlamydia and gonorrhea were sent to the institutions local lab for analysis according to local standards. Women with bacterial vaginosis, Neisseria gonorrhea or chlamydia were excluded from the study. After all cervicovaginal fluids were obtained, an ultrasound probe was placed in the vagina with gel and a cervical length measurement was obtained and a digital scan of the cervix was stored. At each visit, a 7 ml venous blood sample was obtained, centrifuged and 1 ml aliquots of plasma frozen at -80° C until later analysis. The buffy coat was retained for DNA extraction and also frozen at -80° C.

Analytical Methods

MMP and Cytokine Analysis—For cytokine and MMP analysis, each specimen was thawed at room temperature and the swab along with the specimen diluent, was centrifuged in a spin-X centrifuge filter unit (Costar, Cambridge, MA) at 12,000 *rpm* for 20 min. Aliquots of the filtered solution were stored at -80° C until analysis. Protein levels of cytokines and MMPs in the cell-free supernatants were measured (pg/mL) in duplicate through multiplexing with the use of magnetic bead kits (EMD Millipore, Billerica, Massachusetts, USA) according to the manufacturer's instructions and analyzed on a MAGPIX (Luminex, Austin, Texas, USA) using the xPONENT® 4.2 software. Multiplexing allows for quantitative measurements of multiple analytes in a small volume of fluid (25 μ L) and is based on attachment of the cytokines and MMPs to magnetic beads and processing using LED excitation. The minimum detectable concentrations (pg/mL for all analytes) defined by the manufacturer were: IL-1 α = 9.4; IL-1 β = 0.8; IL-4 = 4.5; IL-6 = 0.9; IL-8 = 0.4; IL-10 = 1.1; IL-13 = 1.3; TNF- α = 0.7; MMP-1 = 3; MMP-2 = 200; MMP-9 = 2; MMP-8 = 137. For values below the detection limit, we imputed the mid-point between 0 and the lowest threshold.

Measurement of 17-OHPC concentration—The assay of 17-OHPC was performed by high performance liquid chromatography- mass spectrometry; our methodology for this assay has been reported. Assay limit of detection was 1 ng/ml¹⁶

The sample size calculation for each group was 25 subjects/group but the study was stopped before recruitment was completed as the funding cycle ended. SPSS version 23 was used for statistical analysis (IBM, 2014). Because the variables measured were skewed toward zero and did not conform to a normal distribution, generalized linear mixed models (GLMM) were used to analyze the estimated mean difference for each variable over time and between groups at each time point. P values <0.05 were considered significant and the estimated p value was adjusted to the least significant difference between Bonferroni and Sidak sequential adjustments. Statistical evaluation of trends in significant differences in inflammation markers over time and between the experimental and control groups were

examined through models that controlled for the demographic variables that significantly varied among the cohorts as shown in Table 1.

Mean and proportional differences corresponding to the demographic variables were estimated using analysis of variance (ANOVA) for the continuous variables that were normally distributed and Kruskal-Wallis tests for the non-continuous and continuous variables that were not normally distributed. A determination of normality for each demographic variable was made through the Shapiro-Wilk statistic. We present baseline data utilizing box and whisker plots to demonstrate the variability in the analyte concentrations. For the longitudinal data, we utilized mean data without adding a measure of variance in order to optimize the visual presentation of the data. Significant differences in both instances are based on the statistical analyses described above.

Results

Table 1 summarizes the recruited subjects by study group. There were no differences between groups in age, the proportion of African American and Caucasian subjects and BMI. There were anticipated differences between groups in the number of SPTBs based on inclusion criteria (e.g. control women had no prior preterm births). Notable differences were seen in pregnancy history among the 2 groups of women with a prior SPTB. The 17-OHPC women delivered earlier than the refuser women whether evaluated by the mean gestational age of the earliest SPTB 24.4 ± 5.6 vs 30.5 ± 4.3 or by the mean gestational age of all SPTBs, 24.6 ± 5.7 vs 30.9 ± 4.4 weeks, respectively ($p < 0.001$ for both comparisons (Table 1). The gestational age at baseline was significantly earlier in the 17-OHPC group (17.3 ± 1.3 weeks) compared to the control group (20.2 ± 2.2 weeks) or the refuser group (20.6 ± 2.6), $p < 0.001$. The cervical length in controls (39.5 ± 6.7 mm) was significantly longer than in the other 2 groups at baseline (36.4 ± 6.4 mm for the 17-OHPC group and 34.0 ± 5.4 mm for the refuser group) and at 8 weeks (39.7 ± 8.5 mm, 34.0 ± 8.7 mm, and 33.0 ± 8.4 mm for the controls, 17-OHPC and refusers, respectively). Despite these differences in cervical length and prior pregnancy outcome, the gestational age at delivery in the current pregnancy of women in 17-OHPC and refuser groups was similar and not significantly different than the gestational age at delivery of the controls.

MMPs and Cytokine Values at *Baseline* (Figures 1a, 1b and 2)

17-OHPC vs. Control Groups—At baseline, prior to any treatments, comparison of the control and 17-OHPC groups respectively (mean $\pm$ SD) demonstrated significant elevations of 5 cytokines in the 17-OHPC group (Figures 1a and 1b). TNF- α (38 ± 56 vs 136 ± 282 pg/ml), IL-6 (711 ± 1009 vs 1256 ± 1432 pg/ml), IL-8 (42830 ± 42284 vs 142226 ± 288667 pg/ml), IL-10 (42 ± 40 vs 84 ± 107 pg/ml) and IL-13 (24 ± 17 vs 35 ± 22 pg/ml). At baseline, none of the MMPs from the 17-OHPC group was significantly different than the control group but a trend toward significance was seen for MMP-1, $p < 0.08$ and MMP-9, $p < 0.07$ (Figure 2).

17-OHPC vs refuser groups—Baseline concentrations of the cytokines TNF- α (136 ± 282 vs 33 ± 47 pg/ml), IL-6 (1255 ± 1432 vs 392 ± 322 pg/ml), and IL-10 (84 ± 107 vs 39 ± 30 pg/ml), were significantly higher in the 17-OHPC group than in the refuser group while

concentrations of IL-8 were elevated but not significantly so ($p < 0.06$) in the 17-OHPC group when compared to the refuser group (Figures 1a and 1b). Among the MMPs, MMP-2 was significantly higher ($p < 0.005$) in the 17-OHPC group (5776 ± 5298 vs 1797 ± 1926 pg/ml) than the refuser group but there were no significant differences between any of the other MMPs (Figure 2).

Controls vs refuser groups—There were no significant differences at baseline between these 2 groups in any of the MMPs or cytokines evaluated.

Relationship between Gestational age at Earliest PTB and Cytokine Concentrations at Baseline (Figures 3a, 3b and 3c.)

To ascertain if gestational age at earliest delivery was predictive of cytokine concentration in those women with a prior SPTB, we evaluated the relationship between the earliest gestational age at delivery of all women with a SPTB (17-OHPC and refuser groups) and the plasma concentrations of the 5 cytokines that differed significantly at baseline from the controls (IL-6, IL-8, IL-10, IL-13 and TNF- α). Gestational age at earliest SPTB was significantly related to plasma concentrations of IL-6, IL-10 and TNF- α at baseline (Figures 3a, 3b and 3c) but not to the other 2 cytokines. Figures 3a, 3b and 3c depict the mean values for IL-6, IL-10 and TNF- α by 3 gestational age epochs. The relative concentration of cervical fluid cytokines differed significantly between those women whose earliest SPTB occurred at 16–23 week epoch as opposed to the other delivery epochs. Comparing cervical cytokine concentrations the women in the 16–23 epoch to those of women in the 32–36 week epoch (after adjusting for all the demographic variables included in Table 1), the relative cytokine concentration ratios were: 3.37 for IL-6 (95% CI 1.08–10.53, $p < 0.05$), 2.81 for IL-10 (95% CI 1.39–5.70, $p < .05$), and 6.34 for TNF- α (95% CI 2.19–18.68, $p < 0.001$). Similarly, the relative cytokine concentration ratio comparing women in the 16–23 weeks epoch to women in the 24–31 week epoch for TNF was 4.54 (CI 1.54 - 13.41, $p < 0.05$) and for IL-10 was 2.97 (CI 1.54–5.70, $p < 0.001$).

Gestational and Treatment Effects on MMPs and Cytokines (Figures 4a, 4b and 5)

The mean values at each of the 4-study time point for the 3 groups (controls, 17-OHPC, and refusers) are depicted in Figures 4a, 4b and 5. In the control group over the 8-week study period there were no significant gestational age-related changes from baseline in cytokines or MMPs. In the 17-OHPC group, TNF- α , IL-6 and IL-8 were elevated at baseline and remained significantly elevated compared with the control group over the entire 8-week period. These cytokines were frequently elevated compared to the refuser group but not at all time points. None of these 3 cytokines or any of the other measured cytokines was significantly impacted by 17-OHPC treatment (Figures 4a and 4b) however a decrease was seen in TNF- α , but the decrease was not significant either at the 4-week period ($p = 0.2$) or the 8-week period ($p = 0.2$). Among the MMPs evaluated, MMP-8 and MMP-9 did not differ over the 8-week study between the 3 groups but MMP-1 and MMP-2 were elevated in the 17-OHPC group throughout the first 4 weeks of study (Figure 5). Interestingly both MMP-1 and MMP-2 in the 17-OHPC group demonstrated a significant decrease in concentration between the 4 and 8-week time points ($p < 0.02$ and $p < 0.03$, respectively).

Figure 6 depicts trough 17-OHPC concentrations (mean $\pm$ SD) over the 8 weeks of the study. With weekly 250 mg intramuscular dosing, trough 17-OHPC plasma concentrations were 8.2 ± 4.5 ng/ml at week 2, 10.2 ± 4.5 ng/ml at week 4 and 11.5 ± 5.5 ng/ml at week 8.

Comment

The objective of this study was to compare cervical cytokines and MMPs in women with different risk profiles for preterm birth *prior to any treatment* and then to evaluate the impact of 17-OHPC on cervical cytokines and MMPs. Each subject served as her own control longitudinally during the study and we recruited parous women with no history of prior PTB to serve as controls for any potential gestational age effects. Each subject was studied longitudinally over 8 weeks to allow ample time for the treatment to exert its effect.

The most significant findings of this study were the elevated baseline cytokine profiles (IL-6, IL-8, IL-10, IL-13, and TNF- α) and MMP profiles (MMP-1 and MMP-2) observed in the cervical fluid of women with a history of prior preterm birth who accepted but had not yet received 17-OHPC treatment as opposed to women with a history of only term births. The baseline cervical fluid analysis was performed between 16–22 weeks; none of the women was at imminent risk of PTB nor did they have a short cervix or any other clinically apparent evidence that the parturitional process had started. These cervicovaginal profiles reflect the variations in the cervical fluid immunologic milieu of these subjects. It is not surprising that women with a prior preterm birth history differ from women with only term births since prior pregnancy history is well known to be a powerful predictor of preterm birth risk^{17–20}. The demonstration of significant differences in cytokine and MMP profile between the controls and those with an early prior SPTB birth is novel and provides a potential biological link for the impact of history on subsequent birth outcomes.

The other finding of clinical relevance was the baseline differences noted between those women with a prior SPTB who refused and those who accepted 17-OHPC treatment. The cytokine and MMP profiles of the women refusing 17-OHPC were more closely aligned with those of women with no prior PTB (controls). The pregnancy history of those refusing 17-OHPC differed from those accepting 17-OHPC (earliest PTB mean was 30.5 weeks for those who declined 17-OHPC vs 24.4 weeks for those accepting 17-OHPC). The mean gestational age of prior PTBs was 31.4 weeks for those who refused and 24.4 weeks for those who accepted the 17-OHPC. Thus, the choice to refuse 17-OHPC treatment is not altogether surprising given the differences in history in those women compared with the history of those who accepted treatment. The data presented here suggest that women with a prior preterm birth are a heterogeneous group relative to their cervical inflammatory milieu and PTB risk. What is unclear is how those differences in cervical inflammatory milieu impact SPTB risk. The cervical length in those who accepted 17-OHPC and those refusing 17-OHPC was similar throughout the study. Thus, one must conclude that the elevated cytokines or MMPs in the group accepting 17-OHPC were not major determinants of cervical length. Despite the fact that those who chose to receive 17-OHPC had very early preterm births and elevated cytokines and MMPs, the gestational age at delivery was similar to those women who refused 17-OHPC as well as those women without a prior SPTB.

There are two plausible explanations for the better than expected gestational age at delivery among the women who were at high risk for SPTB and who accepted 17-OHPC. First, one could suggest that the cytokine and MMP elevations and history had little to do with the gestational age at delivery and these women were destined to deliver at term. This explanation ignores the well-established relationship of prior history to recurrent SPTB and the predictive value of cytokines such as IL-6 in predicting risk for preterm birth^{12, 21–27}. The risk of recurrent preterm birth in women with a prior preterm birth is approximately 31%¹⁷. The risk is nearly 65% for those whose prior SPTB occurred prior to 28 weeks gestation¹⁷ and since the women who accepted 17-OHPC delivered at an average of 24 weeks gestation, their risk of SPTB would be expected to be in this range. Cervical IL-6 concentrations appear to predict the risk for PTB with a positive predictive value of 50%²⁴. In one study, an IL-6 value > 660 pg/ml at 30–31 weeks gestation had a positive predictive value for predicting PTB of 85%²⁶. The mean baseline value of IL-6 in the group accepting 17-OHPC in the current report was 1256 pg/ml. Thus, by both these predictors of risk, the group of women electing to receive 17-OHPC were at considerable risk for recurrent SPTB making the first possible explanation of our findings difficult to accept. The second possible explanation of our findings is that the 17-OHPC contributed to the good pregnancy outcome in women who received that treatment either by a delayed and unobserved effect on the biomarkers we studied or by affecting other progesterone responsive genes that modulate the parturitional process. It is conceivable that 17-OHPC affected some of the biomarkers we studied after the 8 weeks of observation and that with a longer period of surveillance, changes in the cytokines and MMPs would be observed. Plasma concentrations of 17-OHPC require approximately 80 days (11+ weeks) to achieve steady state²⁸ but concentrations in plasma in the current study were near steady state by 4 weeks. At 2 weeks, 17-OHPC trough plasma concentrations in our study averaged 8 ng/ml which our previous work suggests is sub-therapeutic³. It is intriguing that the MMP-1 and MMP-2 decreased significantly in our study between the 4 and 8 week measurements when plasma 17-OHPC concentrations would be near steady state. We did not attempt to measure 17-OHPC in the cervical fluid or cervical tissue as concentrations are likely to be too low to quantify. Such measurements might better reflect the potential impact of the medication on the biomarkers studied. Whether this reduction in both MMP-1 and MMP-2 can be attributed to 17-OHPC is unclear and requires confirmation.

In addition to the possibility that longer periods of observation might have demonstrated an effect of 17-OHPC on cytokines and MMPs, it is unclear if higher doses of 17-OHPC could have impacted the biomarkers investigated in this study. Relevant to this discussion are the data from Facchinetti et al who administered 682 mg 17-OHPC weekly to women after an episode of threatened PTB²⁹. Over a brief 21-day observation period, no changes were reported in cervical IL-6, IL-8 or TNF- α ; however, unlike our study, a significant reduction on IL-1 β was seen. These women in the study of Facchinetti et al demonstrated a lower rate of cervical shortening than the control women. Whether higher doses of 17-OHPC such as those used by Facchinetti et al would have affected any of the cytokines or MMPs in our 8-week study is unclear but we speculate that higher doses would lead to proportionally higher concentrations of drug in the cervix or cervical fluid.

If one assumes that cytokines and MMPs were not the targets of 17-OHPC in our study, 17-OHPC could have exerted a beneficial effect on pregnancy length by impacting other progesterone responsive genes. Progestins such as 17-OHPC affect many processes in the prematurity pathway that impact the cervix, myometrium and decidua. In the cervix, progesterone modulates the expression of genes involved in regulation of epithelial and endothelial permeability and metabolism of components of the extracellular matrix²³. Progesterone also inhibits the ripening process by suppressing the production of prostaglandins. In the myometrium progestins affect gap junctions and several membrane channels that affect uterine contractility.

Our study did not identify with certainty a pharmacodynamic target for 17-OHPC among those cervical cytokines and MMPs studied. Thus, these analytes could not be used as biomarkers by which to establish an optimal dosing regimen for 17-OHPC at this time. The finding that women with very early SPTBs exhibit an inflammatory cervical milieu suggests that enhanced risk profiling might be established utilizing history and a cervical biomarker such as IL-6 to better estimate risk and potentially target treatments. Such enhanced risk profiling could allow the enrollment of an enriched population of very high risk women for clinical trials evaluating therapies. The data from this study also demonstrate the potential for dilution of benefit in clinical studies that have evaluated or will evaluate 17-OHPC efficacy in the future. Inclusion of women such as those who refused 17-OHPC in this study would greatly dilute the effect size of any treatment since the risk of recurrence is small in such women.

The small sample size is a clear limitation of this study particularly our inability to recruit more subjects to the refuser group. This likely represents the widespread acceptance of 17-OHPC treatment for women with a prior preterm birth. Despite the small sample size, we were able to demonstrate a significant relationship between the cervical fluid milieu and gestational age at the earliest prior SPTB and to evaluate the impact of 17-OHPC treatment on multiple markers of inflammation and MMPs.

In conclusion, we have demonstrated that at a dose of 250 mg weekly, 17-OHPC does not significantly alter the cytokines studied but an effect on MMP-1 and MMP-2 cannot be ruled out. We have also identified heterogeneity of cytokine concentrations in the cervical fluid milieu among the women with a prior SPTB. Those women with a prior early preterm birth demonstrate an inflammatory milieu that is not seen in women with term deliveries or women with a prior SPTB that occurred at a later gestational age. This inflammatory signature suggests the possibility of additional risk stratification that would enable a more specific risk-targeted treatment approach.

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References

1. Meis PJ, Klebanoff M, Thom E, Dombrowski MP, Sibai B, Moawad AH, et al. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. Prevention of recurrent preterm delivery by 17 alpha-hydroxyprogesterone caproate. *N Engl J Med.* 2003 Jun 12; 348(24):2379–85. [PubMed: 12802023]
2. Romero R, Nicolaides KH, Conde-Agudelo A, O'Brien JM, Cetingoz E, Da Fonseca E, et al. Vaginal progesterone decreases preterm birth 34 weeks of gestation in women with a singleton pregnancy and a short cervix: An updated meta-analysis including data from the OPPTIMUM study. *Ultrasound Obstet Gynecol.* 2016 Sep; 48(3):308–17. [PubMed: 27444208]
3. Caritis SN, Venkataramanan R, Thom E, Harper M, Klebanoff MA, Sorokin Y, et al. Eunice Kennedy Shriver National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network and Obstetric-Fetal Pharmacology Research Units Network. Relationship between 17-alpha hydroxyprogesterone caproate concentration and spontaneous preterm birth. *Am J Obstet Gynecol.* 2014 Feb; 210(2):128e1–6. [PubMed: 24113254]
4. Iams JD, Goldenberg RL, Meis PJ, Mercer BM, Moawad A, Das A, et al. The length of the cervix and the risk of spontaneous premature delivery. National Institute of Child Health and Human Development Maternal Fetal Medicine Unit Network. *N Engl J Med.* 1996 Feb 29; 334(9):567–72. [PubMed: 8569824]
5. Owen J, Yost N, Berghella V, Thom E, Swain M, Dildy GA 3rd, et al. National Institute of Child Health and Human Development, Maternal-Fetal Medicine Units Network. Mid-trimester endovaginal sonography in women at high risk for spontaneous preterm birth. *JAMA.* 2001 Sep 19; 286(11):1340–8. [PubMed: 11560539]
6. Stygar D, Wang H, Vladic YS, Ekman G, Eriksson H, Sahlin L. Increased level of matrix metalloproteinases 2 and 9 in the ripening process of the human cervix. *Biol Reprod.* 2002 Sep; 67(3):889–94. [PubMed: 12193399]
7. Sennström MB, Brauner A, Byström B, Malmström A, Ekman G. Matrix metalloproteinase-8 correlates with the cervical ripening process in humans. *Acta Obstet Gynecol Scand.* 2003 Oct; 82(10):904–11. [PubMed: 12956839]
8. Wang W, Yen H, Chen CH, Jasani N, Soni R, Koscica K, et al. Prevention of inflammation-associated preterm birth by knockdown of the endothelin-1-matrix metalloproteinase-1 pathway. *Mol Med.* 2010 Nov-Dec; 16(11–12):505–12. [PubMed: 20809048]
9. Törnblom SA, Klimaviciute A, Byström B, Chromek M, Brauner A, Ekman-Ordeberg G. Non-infected preterm parturition is related to increased concentrations of IL-6, IL-8 and MCP-1 in human cervix. *Reprod Biol Endocrinol.* 2005 Aug 25; 3:39. [PubMed: 16122384]
10. Wei SQ, Fraser W, Luo ZC. Inflammatory cytokines and spontaneous preterm birth in asymptomatic women: a systematic review. *Obstet Gynecol.* 2010 Aug; 116(2 Pt 1):393–401. [PubMed: 20664401]
11. Holst RM, Mattsby-Baltzer I, Wennerholm UB, Hagberg H, Jacobsson B. Interleukin-6 and interleukin-8 in cervical fluid in a population of Swedish women in preterm labor: Relationship to microbial invasion of the amniotic fluid, intra-amniotic inflammation, and preterm delivery. *Acta Obstet Gynecol Scand.* 2005 Jun; 84(6):551–7. [PubMed: 15901266]
12. Lockwood CJ, Ghidini A, Wein R, Lapinski R, Casal D, Berkowitz RL. Increased interleukin-6 concentrations in cervical secretions are associated with preterm delivery. *Am J Obstet Gynecol.* 1994 Oct; 171(4):1097–102. [PubMed: 7943078]
13. Edwards RK, Ferguson RJ, Duff P. The interleukin-1 beta +3953 single nucleotide polymorphism: cervical protein concentration and preterm delivery risk. *Am J Reprod Immunol.* 2006 Apr; 55(4): 259–64. [PubMed: 16533337]
14. Woodworth A, Grenache DG, Gronowski AM. Cervicovaginal interleukin-6 as a predictor of preterm birth in African American women. *Clin Chim Acta.* 2011 May 12; 412(11–12):988–92. [PubMed: 21300046]
15. Dubicke A, Fransson E, Centini G, Andersson E, Byström B, Malmström A, et al. Pro-inflammatory and anti-inflammatory cytokines in human preterm and term cervical ripening. *J Reprod Immunol.* 2010 Mar; 84(2):176–85. [PubMed: 20096464]

16. Zhang S, Mada SR, Sharma S, et al. Simultaneous quantitation of 17 α -hydroxyprogesterone in human plasma using high-performance liquid chromatography-mass spectrometry(HPLC-MS/MS). *J Pharmaceutical BiomedAnal.* 2008; 48:1174–80.
17. Laughon SK, Albert PS, Leishear K, Mendola P. The NICHD Consecutive Pregnancies Study: Recurrent preterm delivery by subtype. *Am J Obstet Gynecol.* 2014 Feb; 210(2):131.e1–8. [PubMed: 24036403]
18. Spong CY, Meis PJ, Thom EA, Sibai B, Dombrowski MP, Moawad AH, et al. National Institute of Child Health and Human Development Maternal Fetal Medicine Units Network. Progesterone for prevention of recurrent preterm birth: impact of gestational age at previous delivery. *Am J Obstet Gynecol.* 2005 Sep; 193(3 Pt 2):1127–31. [PubMed: 16157124]
19. Esplin MS, O'Brien E, Fraser A, Kerber RA, Clark E, Simonsen SE, et al. Estimating recurrence of spontaneous preterm delivery. *Obstet Gynecol.* 2008 Sep; 112(3):516–23. [PubMed: 18757647]
20. Mercer BM, Goldenberg RL, Moawad AH, Meis PJ, Iams JD, Das AF, et al. The preterm prediction study: effect of gestational age and cause of preterm birth on subsequent obstetric outcome. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. *Am J Obstet Gynecol.* 1999 Nov; 181(5 Pt 1):1216–21. [PubMed: 10561648]
21. Brik M, Antonio P, Perales-Puchalt A, Diago V, Perales A. Cervical interleukin-6 as a predictive test for preterm delivery in symptomatic women: preliminary results. *Eur J Obstet Gynecol Reprod Biol.* 2011 Mar; 155(1):14–8. DOI: 10.1016/j.ejogrb.2010.11.001 [PubMed: 21130555]
22. Lange M, Chen FK, Wessel J, Buscher U, Dudenhausen JW. Elevation of interleukin-6 levels in cervical secretions as a predictor of preterm delivery. *Acta Obstet Gynecol Scand.* 2003 Apr; 82(4):326–9. [PubMed: 12716316]
23. Woodworth A, Grenache DG, Gronowski AM. Cervicovaginal interleukin-6 as a predictor of preterm birth in African American women. *Clin Chim Acta.* 2011 May 12; 412(11–12):988–92. [PubMed: 21300046]
24. Jung EY, Park JW, Ryu A, Lee SY, Cho SH, Park KH. Prediction of impending preterm delivery based on sonographic cervical length and different cytokine levels in cervicovaginal fluid in preterm labor. *J Obstet Gynaecol Res.* 2016 Feb; 42(2):158–65. [PubMed: 26556477]
25. Wei SQ, Fraser W, Luo ZC. Inflammatory cytokines and spontaneous preterm birth in asymptomatic women: a systematic review. *Obstet Gynecol.* 2010 Aug; 116(2 Pt 1):393–401. [PubMed: 20664401]
26. Torb A, Czajka R. Proinflammatory cytokines and other indications of inflammation in cervicovaginal secretions and preterm delivery. *Int J Gynaecol Obstet.* 2004 Nov; 87(2):125–30. [PubMed: 15491556]
27. Goepfert AR, Goldenberg RL, Andrews WW, Hauth JC, Mercer B, Iams J, et al. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. The Preterm Prediction Study: association between cervical interleukin 6 concentration and spontaneous preterm birth. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. *Am J Obstet Gynecol.* 2001 Feb; 184(3):483–8. Erratum in: *Am J Obstet Gynecol* 2008 Aug;199(2):e14–5. [PubMed: 11228507]
28. Caritis SN, Sharma S, Venkataramanan R, Hankins GD, Miodovnik M, Hebert MF, et al. Eunice Kennedy Shriver National Institute of Child Health and Human Development Obstetrical-Fetal Pharmacology Research Units Network. Pharmacology and placental transport of 17-hydroxyprogesterone caproate in singleton gestation. *Am J Obstet Gynecol.* 2012 Nov; 207(5): 398e1–8. [PubMed: 22967833]
29. Facchinetti F, Dante G, Venturini P, Paganelli S, Volpe A. 17 alpha-hydroxy-progesterone effects on cervical proinflammatory agents in women at risk for preterm delivery. *Am J Perinatol.* 2008 Sep; 25(8):503–6. [PubMed: 18756431]

Appendix

Inclusion Criteria

Controls (Parous Women, No Prior Preterm Birth, Normal Cervical Length)

1. One or more prior term births (>37 0/7 weeks)
2. Singleton gestation (16 0/7–23 6/7 weeks gestation)
3. No prior spontaneous birth at 16 0/7–36 6/7 weeks
4. Willing to provide informed consent
5. Age 18–50 years inclusive (*site-specific criterion*)
6. Cervical length > 25 mm at 16 0/7 – 23 6/7 week

17-OHPC and Refusers (Parous Women, Prior Preterm Birth, Normal Cervical Length)

1. Singleton gestation (16 0/7– 23 6/7 weeks gestation)
2. History of one or more prior spontaneous births at 16 0/7–34 0/7 weeks
3. Willing to provide informed consent
4. Age 18–50 years inclusive (*site-specific criterion*)
5. Cervical length greater than 25mm at 16 0/7 –23 6/7weeks

Exclusion criteria

- 1 Active labor or bleeding
- 2 Cervical dilation greater than or equal to 3 cm
- 3 On progestin therapy
- 5 Enrolled in another clinical trial (observational trials allowed)
- 6 Cerclage in place or anticipated
- 7 On chronic steroid or NSAID therapy
- 8 Major fetal malformation, lethal anomalies or anomalies that may lead to early delivery or increased risk of neonatal death (e.g., gastroschisis, spina bifida, or serious karyotypic abnormalities)
- 9 Amniotic membranes prolapsed beyond the external or protruding into the vagina
- 10 Pregnancy without a viable fetus
- 11 Congenital Mullerian abnormality of the uterus
- 12 Prenatal care or delivery planned elsewhere (unless the study visits can be made as scheduled and complete outcome information can be obtained)
- 13 Positive for bacterial vaginosis (BV) or sexually transmitted diseases (STDs) (Chlamydia, gonorrhea, trichomonas)
- 14 Known liver dysfunction or disease (active hepatitis, HIV)

And for those receiving 17-OHPC

1. Active deep vein thrombosis, pulmonary embolism, or history of these conditions
2. Active thromboembolic disease (for example, stroke and myocardial infarction), or a history of these conditions
3. Known or suspected breast cancer, other hormone-sensitive cancer, or history of these conditions
4. Cholestatic jaundice of pregnancy; liver tumors, benign or malignant, or active liver disease

Figure 1a

Figure 1b

Figure 1.

Figure 1a and 1b. Boxplot of pro-inflammatory (1a) and anti-inflammatory (1b) cytokines at baseline. In each box and whisker plot, the dark line in the box represents the mean log n value while the shaded area represents the interquartile range (i.e. between the 25th and 75th percentile). The whisker represents the maximum and minimum values. Significance levels between the 3 groups is indicated with the numerical value above the arrows. When differences were $p>0.1$, no values are indicated.

Figure 2.

Boxplot of matrix metalloproteinases at baseline. In each box and whisker plot, the dark line in the box represents the mean log n value while the shaded area represents the interquartile range (i.e. between the 25th and 75th percentile). The whisker represents the maximum and minimum values. Significance levels between the 3 groups is indicated with the numerical value above the arrows. When differences were $p > 0.1$, no values are indicated.

Figure 3a

Figure 3b

Figure 3c **Figure 3.**

Figure 3a, 3b and 3c. Relationship Between Gestational Age at Earliest SPTB and IL-6 (Fig 3a), IL-10 (Fig 3b) and TNF- α (Fig 3c) concentrations (pg/ml) at baseline. Concentration values at each gestational age epoch are mean $\pm$ SE. Significant differences between women with earliest SPTB at 16–23 weeks and the other groups indicated by asterisk.

Figure 4a

Figure 4b

Figure 4.

Changes in pro-inflammatory (4a) and anti-inflammatory (4b) cytokines over the 8-week study. Significant differences between the 17-OHPC group and controls group indicated by the $\square$ symbol. Significant differences between 17-OHPC group and refuser groups indicated by the $\blacklozenge$ symbol. Values are means.

Figure 5. Changes in Matrix metalloproteinases over the 8-week study. Significant differences between 17-OHPC and controls group indicated by the □ symbol. Significant differences between 17-OHPC and refuser groups indicated by the ◆ symbol. Values are means.

Figure 6.
Plasma concentrations (mean $\pm$ SD) of 17-OHPC during 8-week study.

Table 1

Demographic Characteristics of Study Population

	Controls	17-OHPC	Refusers	p value
	Term birth only	Prior SPTB Accepted 17-OHPC	Prior SPTB Refused 17-OHPC	
Cases	26	24	12	
Age (years)	29 ± 4.4	29 ± 5.9	31 ± 4.7	0.56
Race-AA	7	2	1	0.15
Race-CA	19	22	9	0.26
Race-Other	0	0	2 *	0.03
BMI	29 ± 6.6	28 ± 7.1	29 ± 5.9	0.80
Gravidity	2.8 ± 2.5	3.5 ± 1.5	5 ± 2.2 *	0.002
FT deliveries	1.6 ± 1	0.7 ± 0.9 *	1.5 ± 1.0	0.001
Prior SPTBs	0	1.0 ± 1.0	1.6 ± 1.0	<0.001
GA earliest SPTB (weeks)	NA	24.4 ± 5.6 *	30.5 ± 4.3	<0.001
Mean GA prior SPTBs (weeks)	NA	24.6 ± 5.7 *	30.9 ± 4.4	<0.001
GA- baseline CF obtained (weeks)	20.2 ± 2.2	17.1 ± 1.3 *	20.6 ± 2.6	0.001
GA -1 st inj (weeks)	na	17.4	na	
Cx length (mm) baseline	39.5 ± 6.7 *	36.4 ± 6.4	34.0 ± 5.4	0.04
Cx length (mm) at 8 weeks after baseline	39.7 ± 8.5 *	34.0 ± 8.7	33.0 ± 8.4	0.03
GA-delivery (weeks)	39 ± 1.3	37 ± 2.7	38 ± 1.5	0.19

Note: Mean ± SD is presented for continuous variables.

GA - Gestational age

AA - African American;

CA - Caucasian

FT - Full term

SPTB - Spontaneous Preterm Birth

GA - Gestational Age

Cx - Cervix

CF - Cervical Fluid

* Significantly different than other groups based Post-Hoc pairwise comparisons using Tukey HSD test.