Author's response to reviews

Title: Second trimester amniotic fluid cytokine concentrations, Ureaplasma spp. colonisation status and sexual activity as predictors of preterm birth in Chinese and Australian women

Authors:

Matthew S Payne (matthew.payne@uwa.edu.au)
Zhenhua Feng (fengzhenhua1222@163.com)
Shaofu Li (shaofu.li@uwa.edu.au)
Dorota A Doherty (dorota.doherty@uwa.edu.au)
Biyun Xu (biyunxu@163.com)
Jie Li (jie1967@126.com)
Lenan Liu (victorialiu1203@sina.com)
Jeffrey A Keelan (jeff.keelan@uwa.edu.au)
Yi-Hua Zhou (zgr03summer@126.com)
Jan E Dickinson (jan.dickinson@uwa.edu.au)
Yali Hu (yalihu@nju.edu.cn)
John P Newnham (john.newnham@uwa.edu.au)

Version: 3 Date: 12 September 2014

Author's response to reviews: see over
11th September 2014

To the Editor, BMC Pregnancy and Childbirth,

Attached you will find a revised version of the manuscript entitled ‘Second trimester amniotic fluid cytokine concentrations, *Ureaplasm*a sp. colonisation status and sexual activity as predictors of preterm birth in Chinese and Australian women’, which we have modified based on the comments made by the reviewers.

We have addressed all reviewers’ comments with corresponding changes made directly into the manuscript where appropriate (highlighted in yellow). In addition, we are providing a figure that is not for inclusion in the manuscript but will clarify one reviewer’s query.

We trust that we have addressed all the comments and questions and are grateful for the reviews which we believe have improved the manuscript.

We would like to thank you for considering our submission.

Kind regards,

John P Newnham AM
Winthrop Professor of Obstetrics (Maternal Fetal Medicine),
Head, School of Women’s and Infants’ Health,
The University of Western Australia.
Honorary Director of Obstetrics, Drum Tower Hospital, Nanjing University
Reviewer 1

Comments:

1) **The methods portion of the abstract lacks any mention of the lifestyle factors being measured.**

The text: “Lifestyle factors, including history of smoking and sexual activity during pregnancy, were obtained through completion of questionnaires upon recruitment to the study.” Has been inserted into the abstract at lines 38-40.

2) **In the introduction, mention of the potential role of sexually transmitted infections, bacterial vaginosis and organisms, other than Ureaplasma, in the progression of infection-associated preterm birth would improve this section and the rationale of the study.**

The text beginning at line 72 has been changed to: “In terms of infection, numerous organisms have been associated with PTB. These range from common sexually transmitted disease-associated organisms such as *Chlamydia trachomatis* [6] to the large number of anaerobic genera associated with bacterial vaginosis [7]. However, intrauterine tissues from preterm pregnancies are most frequently colonised with *Ureaplasma* spp. and there is strong evidence from both animal models [8, 9] and human pregnancies [10, 11] for a causal relationship between this organism and PTB.”

3) **Was screening for other organisms considered?**

Screening for other organisms was not considered in this study, as based on previous data from studies that looked at bacterial colonisation of the amniotic fluid and preterm birth, *Ureaplasma* sp. were the most commonly reported organism. This is not to say that other organisms are not relevant, however, in this study, our focus was on *Ureaplasma* sp. based on previous findings.

4) **Previous studies or supporting evidence for the rationale of studying the role of sexual activity during pregnancy and its association with preterm birth is missing from the introduction. Please add this.**

The text beginning at line 64 has been changed to: “The reasons underpinning the different rates of PTB in China and Australia are not understood. We previously speculated that lifestyle differences, particularly attitudes towards smoking and sexual activity during pregnancy, may be involved. A subsequent study by Zhang et al. [5] reported a significant association between sexual activity and PTB in Chinese women. However, evidence to support a similar association in western populations is conflicting [6].”

In addition, we have added an extended discussion on this topic. This begins at line 409: “This result is similar to previous studies which either found no association between sexual activity during pregnancy and recurrent PTB [45] or actually described a reduced risk of PTB following intercourse during late pregnancy [6]. These data are all in contrast to the data presented by Zhang et al. [5] however, which suggested an almost two-fold increased risk of PTB in Chinese women who had sexual intercourse during pregnancy. It is important to note, however, that this study did not document the amount of intercourse had; it instead documented whether intercourse occurred at all during pregnancy and attempted to ascertain during what trimesters this occurred but did not
receive a high compliance rate with this question (32.1% of subjects). To our knowledge, our study is the first of its kind to document sexual activity during pregnancy alongside levels of amniotic fluid inflammatory cytokines as potential indicators for risk of inflammation-driven PTB.”

5) **Hypothesis should be written in the present tense (line 114).**

We respectfully disagree with the reviewer in regards to this. The article is written in past tense and is written based on a hypothesis that was formulated several years ago. As such, we feel that past tense is the best way to present this.

6) **The examination of lifestyle factors in this study is omitted from the aims described in the introduction (lines 118-121).**

The text beginning at line 110 has been changed to: “The aims of this study were, therefore, to describe the levels of inflammatory cytokines IL-1β, IL-6, IL-10, TNF-α and the chemokine MCP-1 and colonisation rates of *Ureaplasma parvum* and *Ureaplasma urealyticum* in 2nd trimester amniotic fluid samples from Chinese and Australian women; and to evaluate the association between these variables, lifestyle factors including smoking and sexual activity during pregnancy, and PTB.”

7) **The final two sentences of the introduction may be better suited to inclusion in the discussion (lines 121-126). Consider deleting or moving.**

The text “Adjustment factors were required to account for the fact that all laboratory measurements had be done in the country of origin of the sample as it is illegal to import or remove human samples to or from China” has been moved to the statistical analyses section of the methods, beginning at line 231.

We respectfully disagree that the text at the end of the introduction “If individual, or a combination, of inflammatory and infectious markers were to be strong predictors of early PTB, amniocentesis early in the 2nd trimester in cases suspected to be at high risk could become a useful clinical tool” needs to be removed or moved as it provides clinical justification for the aims of the study.

8) **Methodological data for the sexual activity and lifestyle questionnaire is lacking.**

The following text has been added from line 144: “The questionnaire inquired about smoking practices as yes or no, and then sought to quantify the number of cigarettes smoked each day in those who declared themselves to be smokers. Sexual activity during pregnancy was assessed by inquiring about the number of episodes of sexual intercourse during the pregnancy. Responses to this question were categorised as: 1) On most days/daily; 2) 2-3 times each week; 3) About once each week; 4) Less than once each week; and 5) Not at all. Information regarding any vaginal bleeding in the two weeks preceding amniocentesis was also recorded.”

9) **Previous use or validation of the questionnaire for its use in two different cultural and language settings is necessary when comparing behavioral data between varied populations. This is particularly an issue, given the much lower response rate to the questionnaire in the Chinese population, which may greatly skew the results reported. Self-reporting of smoking is also known to be highly inaccurate in many settings. Were any objective measures used to assess the validity of this data?**
Although we were unable to validate our questionnaire for use in two different cultural/language settings, we sought lengthy consultation with the obstetricians involved in the study as to the appropriateness of this document for the target population. In addition, the document was written in English and professionally translated into Chinese to ensure that the data obtained from all questions was valid.

Regarding self-reporting of smoking, we did not measure cotinine levels to confirm smoking practices and we also had no method of validating the sexual history. The low rate of smoking within the Chinese population in our study was consistent with our previous population level data obtained during our previous study (Newnham et al. 2011).

In regards to the lower response rate to the questionnaire in the Chinese population, this only related to the question about sexual activity during pregnancy. Unfortunately, inquiries about lifestyle practices in China are not straightforward and account for the infrequent data in the literature. For example, similar results exist in the Chinese study by Zhang et al. (2012), who also received a very low compliance rate regarding an optional sexual activity during pregnancy question in their study.

10) Please clarify the statistical comparisons made between the populations for data other than the cytokine analysis (lines 240-242) eg. gestational age, smoking and sexual intercourse data.

The text at line 236 has been changed to “Univariable comparisons of cytokines/chemokines and lifestyle and pregnancy features/outcomes were based on Mann-Whitney or Kruskal-Wallis tests and Chi-square or Fisher exact tests for continuous and categorical data as required.”

11) Racial/ethnic background of both populations is not described other than Australian and Chinese. Given the rationale behind the study and discussion of the involvement of racial factors in determining immune and inflammatory responses, the inclusion (or discussion) of ethnic background would enhance the paper.

Data on ethnicity of women in the study has been inserted into table 1.

12) The discussion does not include mention of the poor response rate from the Chinese population and the implications for the reliability of this data.

Please see answer to question 9.

13) Description of the clinical management, method of dating pregnancies, choice to perform amniocentesis in the different populations that may confound the data collected should be clarified in the methods or commented on in the discussion. These factors, for example, could influence the incidence of reported threatened abortion prior to amniocentesis if different courses of management are prescribed in the different settings. Please clarify and discuss.

The following text has been inserted into the methods at line 120: “In the Australian cohort, indications to conduct this procedure were primarily to exclude aneuploidy based on high risk findings at either first trimester screening (ultrasound measurement of fetal nuchal translucency and
maternal biochemistry) or second trimester maternal serum screening (maternal biochemistry) with no apparent fetal structural anomalies. In the Chinese cohort, the indications for amniocentesis included advanced maternal age, nuchal translucency screening and maternal request. In all pregnancies, gestational age was determined by ultrasound imaging and biometry in the first or early second trimester. Participants were required to possess language fluency sufficient to understand the implications of participation. Cases subsequently shown to be complicated by aneuploidy were withdrawn and the amniotic fluid not analysed further.”

14) Selection of the population by those high-risk pregnancies indicating the need for genetic amniocentesis also warrants comment. Please discuss if this could affect the outcomes measured in this study?

There were differences in the indications for amniocentesis in the two populations, manifested as different median ages at the time of the procedure (China 29 years, Australia 35 years). This different selection process, however, would be unlikely to influence the rate of amniotic fluid microbial colonisation or cytokine level.”

15) Given that infection-associated preterm birth generally occurs earlier in gestation, could the absence of preterm birth at <28 weeks in the Chinese population explain the low rate of infection in the population studied? Please provide more discussion on the low incidence of Ureaplasma infection.

Although infection-associated PTB is especially associated with deliveries <28 wks GA, it is still a major factor in deliveries <32 wks GA, which constituted 1% of Chinese births. However, we are not sure what the reviewer is trying to indicate by this comment as *Ureaplasma* sp. DNA was found on two occasions in the Chinese population. In both cases, it was associated with preterm birth (29 and 34 weeks GA) and significantly elevated cytokine levels for every cytokine measured relative to mean levels (Table 3). We have inserted text at line 348 which adds further discussion to this finding: “The two Chinese cases where *Ureaplasma* sp. DNA was detected were associated with significantly elevated levels of all cytokines measured. A similar result was reported by Jacobssen et al. [37], who described an association between TNF-α levels and presence of *Ureaplasma* sp. DNA in amniotic fluid from women with PTL and PPROM. However, in contrast this study found no association between levels of IL1-β, IL-6 and IL-10 and detection of *Ureaplasma* sp. DNA.”

As for the request to provide more discussion regarding the low incidence of *Ureaplasma* sp. infection, we feel this is suitably dealt with in the existing text at lines 358-388. This includes the newly inserted text regarding the reviewer’s question below (18), which provides evidence of *Ureaplasma* sp. in cervical swabs from Chinese pregnant women, demonstrating a potential reservoir for amniotic fluid infection.

16) Data relating to the frequency of sexual activity during pregnancy is duplicated from Table 1 in Figure 2. Please consider deleting the graph or the data from the table to avoid unnecessary repetition.

We have removed these data from the table as per the reviewer’s request.

17) Differences in parity and presence of previous preterm birth in the Australian population (with much lower incidence of nulliparity) are not mentioned in the results or discussion.
Other factors that should be included in the demographic data or at least discussed are the presence of other sexually transmitted infections, short cervix and use of antibiotics during pregnancy, particularly given the incidence of PROM in the populations.

The rate of nulliparity was different in the two populations but this difference would be unlikely to influence the results as nulliparity is not strongly related to risk of preterm birth.

Although it would be of interest in retrospect to discuss the presence of other sexually transmitted infections, short cervix and use of antibiotics during pregnancy, we are unable to generate this data. However, we feel that this in no way detracts from major conclusions drawn from our data.

18) Given that the identification of Ureaplasma infection was a primary aim of this study, why was the presence of Ureaplasma in vaginal/cervical swabs (at amniocentesis or delivery) or amniotic fluid at the time of birth not determined in this study?

Although the collection of vaginal/cervical swabs at the time of amniocentesis or delivery for detection of *Ureaplasma* sp. would have been interesting, the primary focus of this study was to determine whether detection of *Ureaplasma* sp. in the amniotic fluid could be used as a biomarker of preterm birth when combined with amniotic fluid inflammatory markers and/or lifestyle factors. Data already exists demonstrating that most amniotic fluid infections originate in the vagina and we were not attempting to add to this.

19) Some data on the presence of vaginal/cervical *Ureaplasma* infection is available in some Chinese populations. This should at least be mentioned alongside the similar Australian data (line 369).

The text beginning at line 373 has been changed to: “This is comparable to that reported in a range of vaginal prevalence studies conducted on different geographical cohorts of pregnant women [17].

The rate of colonisation in cervical swabs, however, is somewhat lower than that reported previously in a Chinese population. Kong et al. [38] collected 400 cervical swabs from sex workers (200), women attending a sexually-transmitted infection (STI) clinic (100) and pregnant women (100), and detected *Ureaplasma* sp. in 185/400 (46.3%) swabs. Interestingly, *Ureaplasma* sp. was detected significantly more frequently in swabs from pregnant women (58%) compared to sex workers or women attending the STI clinic (42.3%). These studies at the very least provide evidence that *Ureaplasma* sp. have the potential to invade the amniotic cavity in both Australian and Chinese women, based upon the theory of vaginal ascension [7]. Our results instead suggest that if *Ureaplasma* sp. do ascend into the amniotic cavity during pregnancy in these women, then they do so primarily at a time point after 20 weeks’ gestation. Taking this into account, particularly within the Australian setting, amniocentesis prior to 20 weeks gestation for detection of *Ureaplasma* sp. as an early predictive marker of PTB is unlikely to be a useful investigational tool. Unfortunately, it is rarely clinically possible to obtain amniotic fluid samples opportunistically after 20 weeks gestation as is the case with genetic amniocentesis in the early second trimester [39].”
Reviewer 2

Comments:

1) *Were any of the Australian subjects of Chinese ethnicity? If so, please report the number/percentage and comment on whether they should be included or not. As the authors are aware, ethnicity is a different consideration than culture, and perhaps if the Discussion is focused on ethnicity, then Chinese Australians should be removed from the study.*

We have inserted ethnicity data on women in the study in table 1. Unfortunately, amongst Australian women, we only obtained information as to whether they were Caucasian, Asian or Aboriginal. ‘Asian’ may include women of South-East Asian descent, in addition to women of Japanese, Chinese or Taiwanese descent. We also had a high number of women that chose not to answer this question. Although we agree that ethnicity is a different consideration than culture, our previous study (Newnham et al. 2011) observed that migration of Chinese women from mainland China to westernised populations was associated with a subsequent increase in risk of PTB, irrespective of ethnicity. As such, in this study, we don’t feel that Asian-Australians should be removed from the Australian data.

2) *Would it be of interest to plot the amniotic fluid cytokine concentrations as a function of Gestational Age at Delivery and then determine whether a significant relationship exists as opposed to the way the data are displayed in Figure 1? As Term vs. PTB, there is an 8:1 ratio difference in the number of subjects in each group.*

As per the reviewer’s suggestion, we plotted the cytokine concentrations as a function of gestational age at delivery (see figure below). As we did not see any relationship between the two we don’t think it’s necessary to include this as a replacement for our existing figure 1 (term vs PTB). In addition, this study was powered to look at an endpoint of either term or preterm birth as opposed to stratification by gestational age at delivery. As a result, doing this would greatly reduce the statistical validity of the data.

3) *Many preterm birth cytokine studies, and many studies examining allostatic load, determine C-reactive protein concentrations. Why was this factor not determined?*

In the current study we did not measure C-Reactive Protein (CRP) concentrations as previous studies by Mazor et al. (1993), Lee et al. (2007) and Park et al. (2014) have demonstrated that CRP, unlike IL-6 for example, is not a particularly sensitive marker of intraamniotic inflammation.

4) *The Figure 1 legend should include . . . ‘increased’ IL-10 . . .*

This correction has been made and appears at line 472.

5) *In Table 1 was there a statistically significant difference in the mean age of the Chinese and Australian mothers (29 vs. 35)? If so, should this be commented on?*

There were differences in the indications for amniocentesis in the two populations, manifested as different median ages at the time of the procedure (China 29 years, Australia 35 years). This different selection process, however, would be unlikely to influence the rate of amniotic fluid microbial colonisation or cytokine level.
The graphs show the relationships between gestational age and cytokine levels for different cytokines:

- **IL-1b**
  - Equation: $y = 0.0005x + 0.004$
  - $R^2 = 0.004$

- **IL-6**
  - Equation: $y = -2.866x + 12.265$
  - $R^2 = 0.0152$

- **MCP-1**
  - Equation: $y = 25.532x$
  - $R^2 = 0.642$

- **IL-10**
  - Equation: $y = 0.0902x$
  - $R^2 = 0.613$

- **TNFα**
  - Equation: $y = 0.1449x$
  - $R^2 = 0.0319$

Each graph plots cytokine level against gestational age, showing the trend for each cytokine.