Dear Editors:

Thank you for the opportunity to revise and resubmit our manuscript “Maternal Characteristics Associated with the Dietary Intake of Nitrates, Nitrites, and Nitrosamines in U.S. Women of Child-bearing Age” (MS 7934457943089536). Under the section “Comments and Responses,” we have addressed each of the peer reviewers’ comments and also have highlighted these changes in the text. With respect to the editorial comment that we “consider the implications of …findings for research in this area in a more general sense,” we have modified the conclusion of the abstract (Page 3) and the Discussion (pages 23-24). These changes are highlighted. We have also modified the title (added “a cross-sectional study”) and title page so that they conform to the journal style.

The following section provides the reviewers’ comments and our responses to each of these comments:

Comments and Responses

Reviewer: Barbara Thomson

Major Compulsory Revisions

1 The authors give a robust report of selected maternal characteristics in US women of childbearing age, from a large sample size of 5958 subjects. As a food
scientist however, the paper has significant weakness in the presentation of intake assessment of nitrates, nitrites and nitrosamines against which these maternal associations are assessed. The claimed associations are only as good as the underpinning data and whilst the authors cite methodology is published elsewhere, the reader is unable to evaluate the robustness of the dietary assessment. In my view Table F2A should be included in the body of the paper. Intake = concentration x consumption. Given the same average concentration values were used for each person, any observed differences reflect differences in amounts consumed or food choices. The intakes are based on 58 foods but the contribution of individual foods is not provided. This would be helpful in evaluating the reason for observed associations- as included in our paper

Thomson et al., (2007) Food Additives and Contaminants;24(2):113-121. Are the observed differences related to serving sizes or food choices?

Response:
To address the concern about amounts of foods consumed and food choices, Table 6 has been added that shows both food choices and quantity consumed by race/ethnicity and the relative contributions of each food category to the estimated daily intake of nitrates, nitrites, and nitrosamines. To be in compliance with the Journal’s requirement that tables in the paper be in portrait orientation, we needed to remove information regarding “Other” races. However, since that particular grouping is a conglomerate of races, we do not think that this information is necessary. These findings by food choice and consumption are discussed in the revised Results section under the heading Most frequently consumed food items, pages 15-17.

2 The discussion, in my view is weak on possible explanation/plausibility of the apparent associations. In part, this is because of the lack of detail provided on source foods.

Response:
As indicated in our response to Comment 1, Table 6 has been added and should support the discussion. Additional detail has been provided in Tables 5 and 6 as well as the results and discussion sections.

3 Page 11, nitrite intake, re dietary folate. The difference between >319> looks more significant than the difference between >685>. Consider rewording "Participants who consumed more than 319µg/day of dietary folate compared to participants with dietary folate consumption <319 µg/day were approximately twice as likely to also....."

Response:
The following text has been added on page 12..."With adjustment of other maternal characteristics, participants who consumed more than 319 µg/day of dietary folate compared to participants with dietary folate consumption <319 µg/day were approximately twice as likely to have dietary nitrite consumption at the highest quartile of intake across the second through fourth quartiles of dietary
folate intake.”

4 Total dietary nitrite intake. Any explanation for the difference in education status and nitrite intake compared with education difference and total nitrite intake?

Response:
As noted in the text, total dietary nitrite intake was the sum of dietary nitrite and 5% of dietary nitrate intake (page 7, Methods). In this population, estimated intake of nitrates tended to decrease with less education except for the least educated group for which there were the smallest proportions of women compared with other educational groups. In contrast, there appeared to be a strong trend between increased intake of nitrite with decreased educational attainment. Therefore, the crude odds ratios associated with total nitrite intake appeared muted compared with the odds ratios for nitrite intake. Maternal race/ethnicity was strongly associated with both nitrate/nitrite intake and educational attainment. When this variable was adjusted along with other maternal characteristics, educational attainment was no longer positively associated with total nitrite intake. In fact, compared with women in the highest education group, some of the lower education groups were less likely to have total nitrite intake at the highest quartile.

Minor essential revisions
1 Include in the introduction evidence of the role of alcohol in nitrosamine formation.

Response:
We have added the following statements to page 4, paragraph 2: In addition to dietary exposures, preformed nitrosamines have been found in beer and to a lesser extent, distilled spirits. Beer, presumably because of the malting process, contains volatile nitrosamines and has been implicated as a significant contributor to total dietary exposure of nitrosamines [26]. Ethanol has also been noted in several animal studies to increase internal exposure to nitrosamines by suppressing hepatic clearance of these compounds [27, 28].

2 Reduce the number of Tables by combining Tables 1&5, 2&6, 3&8 (landscape?) The first 5 columns of Tables 5-8 repeat the information in columns of Tables 1-4.

Response:
We combined the tables as recommended and the new Tables 1-4 reflect these changes. The Results section also refers to these revised tables.

3 Page 7, para 2 is more suitably placed in the results section - suggest Page 10, after para 1.

Response:
This paragraph has been moved to the Results section on page 10.
4 The number of decimal places in the intake assessments overstates the accuracy of these estimates. Suggest reducing to a maximum of 1 decimal place throughout the document.

Response:

We added the following sentences in the limitations on page 22… “The estimates are presented as two decimal places for nitrate, nitrites, and total nitrites and three decimal places for nitrosamines. The quartile breaks were established based on the actual distribution of participants and the number of decimal places reflects only the break between quartiles and should not be interpreted as an accurately measured value. The values are generated from estimates and, although useful for estimating consumption, may not be accurate representations of currently available food items.”

Reviewer: Mary H. Ward

This is an interesting article that describes maternal characteristics associated with consumption of N-nitroso compound (NOC) precursors (nitrate and nitrite) and nitrosamines. NOC are potent carcinogens and cause congenital malformations in animal studies and there have been few recent articles on consumption levels and factors associated with intake. The topic should be of interest to exposure scientists and epidemiologists.

Major compulsory revisions:

1. Background information about the database should be provided in order for the reader to be able to better interpret the intake patterns presented. The authors should describe the general approach to the development of the database perhaps including some supplementary tables with levels in foods (optional). Additional information about estimation of nitrosamine concentrations in the foods is needed especially since this was not provided in the database manuscript. After reviewing the database paper, it was not clear if individual nitrosamines concentrations from the published literature were summed or if one nitrosamine was used as an indicator of exposure. Most studies measured N-nitrosodimethylamine (NDMA) but some studies measured multiple nitrosamines. How were these data combined to come up with nitrosamine values for each food item?

Response:

General approach to database, p7. added …” Briefly, the database was developed by searching the literature for published articles and scientific reports presenting information about the nitrate, nitrite, and/or nitrosamine content in food items. The values were compiled and ranked with respect to time and country of origin. In general, information from 1980 or later and from countries with predominantly western diets were given highest priority and used to generate summary estimates of nitrate, nitrite, or nitrosamine content by food item.”

Nitrosamine determination…, p7. added…” Nitrosamine content from the
literature was frequently presented in terms of N-nitrosodimethylamine (NDMA), but was also presented as total nitrosamines, specifically identified as N-nitrosopyrrolidine (NPYR), N-nitrosopiperidine (NPIP), N-nitrosopropoline (NPOR), or as a combination of the aforementioned. For the purposes of estimating nitrosamine content, total nitrosamines were used either as reported or by adding the values from each of the specific types of nitrosamine—essentially summing the individual values from the reported literature.

2. Tables 1 through 4 present intake quartiles of nitrate, nitrite, total nitrite (5% of dietary nitrate intake plus nitrite intake), and nitrosamines by a priori factors of interest to adverse reproductive epidemiology studies including race/ethnicity, age at conception, education, income, pre-pregnancy BMI, folate intake and multivitamin use. These tables are not adjusted for caloric intake, which would be expected to be correlated with intake. Tables 5-8 show crude and adjusted odds ratios (the latter controlling for calories and other factors) and there is often a re-ranking of the factors associated with intake compared with the earlier tables. It is not clear how much of the confounding was due to caloric intake; however, BMI and caloric intake vary by racial and ethnic groups and it would not be surprising if nitrate or nitrite intake may be a function of racial/ethnic variation in these factors. Instead of Tables 1-4, the distribution of intakes by race/ethnicity in Table 9 could be presented standardized by total calorie intake. One approach would be to use the nutrient density method, which provides meaningful units of intake in which nitrate, nitrite, and nitrosamine intake are standardized by dividing by total calories (presented in units of mg of intake per 1000 calories).

Response:
We combined Tables 1-4 with Tables 5-8 to improve consistency. Additionally, a new Table 5 was added with unadjusted and energy-adjusted average daily intake of nitrates, nitrites, total nitrites, and nitrosamines. We also developed a new Table 6 that shows average intake of nitrate, nitrite, and nitrosamines per day for each food item and average serving sizes by race/ethnicity.

3. Additional information about the major food contributors to intake would help to further clarify the reasons for some of the different intake patterns that were observed across racial/ethnic groups in this study population. Animal and plant sources of nitrite should be separated because animal sources may be more important for formation of NOC because they also contain the necessary amine and amide precursors. Indeed, for some reproductive outcomes and specific cancers, animal and/or processed meat-derived nitrite shows a stronger association with risk than plant-derived nitrite. To that end, it would be particularly helpful if the authors separated meat and bean products and evaluated processed meat intakes separately from total meat or fresh meats when describing food group contributors to intake. Additional information about the top 3 to 5 individual food contributors to nitrate, nitrite, and nitrosamines intake by race/ethnicity would also be informative.

Response:
Table 6 lists all 58 food items and has superscript designation for top five food items by servings and by nitrate, nitrite, or nitrosamine intake.

Supplemental file 2A lists nitrate, nitrite, and total nitrite average daily intake by race/ethnicity and meat and bean products have been separated into distinct categories.

The following has been added to the results section (and is also shown in Table 6):

**Most frequently consumed food items**

Table 6 displays the average number of servings per day and the average daily intake of nitrates, nitrites, and nitrosamines by food item. The top five foods most frequently consumed and the top five foods with the most substantial contributions to nitrate, nitrite, and nitrosamine intake varied by race/ethnicity. The five most frequently consumed food items are described as follows.

Non-Hispanic white women most frequently consumed skim or lowfat milk, cereal, cheese, bread products, and orange juice. Non-Hispanic black women most frequently consumed cereal, bread products, whole milk, orange juice, and eggs. Hispanics reported tortillas, cereal, whole milk, orange juice, and fresh apples or pears as their most frequently ingested food. Asian/Pacific Islanders consumed rice or pasta, orange juice, cereal, skim milk, and fresh apples or pears most frequently.

The sources of nitrates, nitrites, and nitrosamines were explored by identifying the top five foods that contributed to the average daily intake by race/ethnicity. The foods that contribute the most to daily dietary nitrate, on average, for Non-Hispanic whites and blacks were spinach or collard greens, potatoes, broccoli, string beans, and orange juice. For Hispanics, the five foods contributing most to daily nitrate intake were spinach or collard greens, broccoli, potatoes, salsa, and orange juice. The five foods consumed contributing most to daily nitrate intake for Asian/Pacific Islanders were spinach or collard greens, broccoli, potatoes, cabbage (including cauliflower or Brussels sprouts), and rice or pasta.

The top five food items, starting with the largest, contributing to dietary nitrite intake for Non-Hispanic white women was beef (including pork, lamb or cabrito) as a main dish, beef (including pork, lamb, or cabrito) as a mixed dish, chicken or turkey, rice or pasta, and hot dogs. For non-Hispanic black women the top five contributors to dietary nitrite intake were beef (including pork, lamb or cabrito) as a main dish, chicken or turkey, beef (including pork, lamb or cabrito) a mixed dish, hot dogs, and bacon. Daily intake of dietary nitrites from beef (including pork, lamb or cabrito) as a main dish, refried beans, beef (including pork, lamb or cabrito) as a mixed dish, chicken or turkey, and rice or pasta were the top five contributors for Hispanics. The top five foods contributing to nitrite intake among Asian Pacific Islanders were beef (including pork, lamb or cabrito) as a main dish, rice or pasta, beef (including pork, lamb or cabrito) as a mixed dish, chicken or turkey, and fish.

The five foods consumed contributing most to daily dietary nitrosamine intake for
non-Hispanic whites were cereal, skim or low fat milk, beef (including pork, lamb or cabrito) as a main dish, beef (including pork, lamb or cabrito) as a mixed dish, and cheese. For non-Hispanic blacks, the top five foods were cereal, beef (including pork, lamb or cabrito) as a main dish, skim or low fat milk, bacon, and beef (including pork, lamb or cabrito) as a mixed dish. The top five foods contributing to daily nitrosamine intake for Hispanics were cereal, beef (including pork, lamb or cabrito) as main dish, skim or low fat milk, beef (including pork, lamb or cabrito) as a mixed dish, and whole milk. For Asian/Pacific Islanders, the top five foods with respect to daily nitrosamine intake were cereal, skim or low fat milk, beef (including pork, lamb or cabrito) as a main or mixed dish, and fish.

4. Dietary nitrate has shown qualitatively different associations with risk in epidemiologic studies than drinking water nitrate or dietary nitrite presumably because vegetables are the major source of intake. Some, but not all, vegetables also contain high levels of vitamin C and other antioxidants that inhibit the in vivo formation of NOC. This should be discussed especially in conjunction with the rationale for summing nitrite derived from dietary nitrate and nitrite derived from processed meats and other foods.

Response:

We added the following sentence to the discussion on page 22…” For total nitrite (dietary nitrite + 5% dietary nitrate), it is important to note that vegetables are the main contributor for dietary nitrate, many of which are also rich in vitamin C—a vitamin known to inhibit the formation of nitrosamines under normal gastric conditions.”

5. According to the database paper, there were fairly high nitrate and nitrosamine levels in the “beef, pork, and lamb”, liver, and fish line items. These high values may have been due to the equal weights that were assigned for the individual food values that comprised these line items. Assigning equal weights to each type of these foods included in the database may overestimate intake for certain line items. For example, consumption varies greatly across types of fish – fresh fish is eaten much more commonly than smoked and pickled fish by the U.S. population. Weighting intake equally across types of fish overestimates nitrite and nitrosamine intake from fish since values are high in smoked and pickled fish but low in fresh fish. Please comment on the approach you used versus using weights based on sex- and age-specific USDA consumption data. How might your weighting approach have affected the dietary patterns you observed?

Response:

We reviewed the values from the literature that were used to develop nitrite and nitrosamine estimates for the “beef, pork, and lamb”, liver, and fish. Because more samples and estimates were available for fresh versus smoked items especially fish, smoked and cured items were weighted less than fresh items in the generation of the final estimates. However, it is likely that estimates of nitrite and nitrosamine intake would be more accurate had the types of fish and meat (smoked or cured versus fresh) been on the food frequency questionnaire. In this study, all participants were women and over 85% were between 20 and 39 years
of age. Therefore, the USDA consumption data on sex and age might not be that helpful in distinguishing relative differences between groups. Consumption data by race/ethnicity would have been useful in distinguishing differences however and in improving estimates.

We have added the following sentences in the Discussion on page 23 regarding limitations on estimates for meat and fish in this study: “Broad designations of food groups such as “beef, pork, and lamb” and “fish” decreased accuracy of estimated nitrite and nitrosamine intake in two ways. First, estimates of nitrite and nitrosamine content in these food items were based on an average of these foods from data published in the literature including values for fresh, smoked, and pickled items. Second, the intake of fresh or cured items could not be distinguished among participants. These limitations most likely led to an overestimation of nitrite and nitrosamine intake among women who reported consumption of these food items.”

Discretionary Revisions

1. It was not clear why logistic regression was chosen for the analysis presented in Tables 5-8 instead of linear regression. Consider using linear regression models that include all factors of interest as independent variables in relation to continuous mg/day intakes of nitrate, nitrite, and nitrosamines as this would evaluate factors associated with intake across the complete range of intakes in the study population (compared with logistic models that compare high versus low intake quartiles). It would be useful to present parameter estimates for all variables that are significantly associated with one or more intakes of nitrate, nitrite, and nitrosamine so the reader can compare the magnitude of the parameter estimates for the various intakes (versus only presenting significant variables for each type of intake).

Response:

We appreciate that linear regression would have been another appropriate approach in assessing factors related to intake of nitrates, nitrites, and nitrosamines. To aid in interpretation of the risk estimates provided, crude associations have been included for all variables considered, although only those variables that were significant and included in the final model have adjusted odds ratios and 95% confidence intervals.

2. The authors could comment on the fact that the highest dietary nitrate intake quartile captures the healthiest members of the population, who are high consumers of vegetables and who also tend to have higher income and folate intake.

Response:

We added the following comments on page 18…”Increased consumption of dietary nitrites and nitrosamines is generally considered unhealthy, and foods high in nitrite and nitrosamine content (processed meat, alcohol, dairy products) should be consumed in moderation. However, vegetables are the largest
contributor to dietary nitrate and, in contrast to nitrites and nitrosamines, increased intake of vegetables is widely accepted as a healthy behavior associated with higher income, especially given the higher cost of fresh fruits and vegetables compared to less expensive processed foods. Increased consumption of vegetables at the highest quartile of intake would also increase folate consumption and other phyto-nutrients, reflecting a diet associated with the healthiest members of the population.”

We would like to thank the reviewers for their comments and suggestions; in our opinion, incorporation of these suggestions has substantially improved the manuscript.

Sincerely,

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Associate Dean for Research
Texas A&M University Health Science Center
School of Rural Public Health