Author’s response to reviews

Title: Para-occupational exposure to pesticides, PON1 polymorphisms and hypothyroxinemia during the first half of pregnancy in women living in a Mexican floricultural area.

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Author’s response to reviews:

Response to Reviewers

Reviewer reports:

Reviewer #1: ENHE-D-18-00411

Para-occupational exposure to pesticides, PON1 polymorphisms and hypothyroxinemia during the first half of pregnancy in women living in a Mexican floricultural area.

The study is based on 381 pregnant women (< GW 17) living in a floricultural area in Mexico where pesticides, inclusive organophosphates (OPs), are routinely used. Associations between hypothyroxinemia and PON1 genotype and para-occupational pesticide exposure were investigated. The MS is mostly well written, but the aims and hypothesis of the study are less clear. I have some concerns mainly related to the exposure classification and the statistical analyses as listed below:
Pesticide exposure classification

The women were classified as para-occupationally exposed if their partner was occupationally exposed to pesticides but since they all live in a floricultural area and probably have a higher "background exposure level" it is not oblivious that a classification based on the partners "take-home exposure" is meaningful. Biomonitoring data would have strengthened the study but since such data are not available more information on the exposure situation are warranted to evaluate the risk of exposure misclassification:

Reply: First of all, thank you very much for your suggestions.

We agree with the reviewer; however, due to budgetary reasons, we were not able to determine pesticide metabolite concentrations. We are searching for other financing sources to solve this limitation. In the discussion, we recognize that this is one of the limitations of the study (please, see Discussion section, page 16, second paragraph). Nevertheless, many pesticides, such as organophosphates are non-persistent compounds and the information provided by exposure biomarkers, although relevant, also has limitations because it shows exposure during the previous 48 hours, and not long term exposure. We assumed that women who have partners whose jobs expose them to pesticides, would be more exposed than women whose partners do a different kind of work, because previously we have found that workers frequently take the pesticide spraying equipment home, they store pesticides in their house and they bring work shoes and clothes into the house as well. It is important to mention that most of the time, the women are in charge of washing the clothes, which is frequently done by hand, since these are low-income women who don’t have access to automatic washers, which increases dermal exposure (Blanco-Muñoz and Lacasaña. J Agromedicine. 2011; 16:117-26).

On the other hand, recently we have measured the six dialkylphosphate (DAP) metabolites of organophosphate pesticides in a subsample of 65 pregnant women (please see Materials and Methods section, Pesticide exposure subsection, page 8, last paragraph). Although we found no significant differences, para-occupationally exposed women had a higher proportion of DAP metabolites above the limit of detection and a median of total DAP higher than non para-occupationally exposed women (1.16 µmol/g creatinine vs 0.83 µmol/g creatinine, p =0.56 by Mann-Whitney test). We have add a paragraph showing this result (please see the last paragraph of the Results section, first paragraph, page 11-12)

Additionally, we evaluated the association between total DAP exposure and hypothyroxinemia in this subsample of women (n=65) and, after adjusting for maternal age, gestational age and urinary creatinine concentration, no association was statistically significant (OR= 1.00, CI 95% 0.99, 1.004) This is consistent with the results observed when we use para-occupational exposure as a proxy for pesticide exposure, where no association was found between para-occupational exposure and hypothyroxinemia. (Please see the last paragraph of the Results section, page 13)

a) residential exposure - did all the women live in close proximity to pesticide floricultural areas?
Reply: All women reside in the same areas, close to flower crops and are environmentally exposed to pesticides. However, we think that it is not risky to assume that para-occupationally exposed women will be more exposed to pesticides than the women who are only environmentally exposed.

b) partner’s exposure - work function, which pesticides, were OPs used by all the partners?

Reply: Unfortunately we do not have specific information about the pesticides used by the partners. However, previous findings from our research group in the study area showed that flower growers regularly applied OPs: omethoate, oxydemeton, metamidophos, chlorpyrifos, and malathion. (Lacasaña M. et al. Toxicology and Applied Pharmacology 2010; 243: 19–26)

c) was the exposure different between summer and winter?

Reply: Previously we also found that the pesticide use was more frequently reported during the rainy season (from June to December) compared with the dry season (from January to May). (Schilmann et al. Occup Environ Med. 2010; 67: 323-329). More than 90% of the floricultural workers had at least one DAP metabolite present in the urine during the rainy season against 69% of workers during the dry season. Furthermore, total dialkylphosphates (DAP) levels were four times higher during the rainy than during the dry season (2.00 vs.0.48 μmol/g creatinine; p=0.001). (Lacasaña M. et al. Toxicology and Applied Pharmacology 2010; 243: 19–26).

In the current study we adjusted by the “season” of blood collection in the analyses. However, although the frequency of hypothyroxinemia was lower in the winter period than in the summer (please, see table 2), the inclusion of the “season” variable in the models did not substantially change the results of the previous analyses, compared to the ones that were carried out without including it.

d) did the families report residential use of pesticides/OPs?

Reply: Yes, 19.9 % of women reported residential use of pesticides, but this exposure was not associated with hypothyroxinemia (OR=0.88, CI95% 0.48, 1.58) and did not confound the associations observed between para-occupational exposure or PON1 polymorphisms and hypothyroxinemia.

Statistics and results

A high fraction, 54%, of the women in this study had hypothyroxinemia. Iodine deficiency (even mild) is a risk factor for hypothyroxinemia and accordingly "consumption of iodine supplements from the 3 months before pregnancy to the date of the interview" was included as a binary covariate (Yes or No). However, it is not explained how iodine supplements for a shorter period was handled, i.e., if a woman took supplement from 1 month before pregnancy - was she then categorized as No? Please clarify.
Reply: The consumption of iodine supplements in any of the months (from the 3 months before pregnancy to the date of the interview) was categorized as “Yes”. It is worth to mentioning that in a subsample of 103 women we measured the urinary iodine concentration. The median urinary iodine concentration was 246 µg/L. Women who reported iodine supplements intake, had higher median urinary concentrations (285 µg/L) than those who did not (242 µg/L), p=0.09, (Hernandez-Mariano et al., Environ Res. 2017;156:597-604). According to the criteria of the World Health Organization (WHO) for pregnant women, iodine deficiency was considered as a median urinary concentration <150 µg/L (WHO 2014). So, our population doesn't seem to have iodine deficiency.

Compared to winter sampling, a higher percentage of those who gave samples in summer had hypothyroxinemia (Table 2). Did use of pesticide deviate between summer and winter and how?

Reply: please see response to c) question.

If season is related to pesticide exposure level, "season" would be an obvious potential confounder, but this variable was apparently not included in the data analysis, why?

Reply: We agreed with the reviewer. We included the season of blood collection in the data analyses (please see Material and Methods section, Description of covariates subsection, page 10).

Furthermore, there are studies that have shown a circannual variation in thyroid hormone concentrations. These changes are more evident in regions where variations in light and temperature during the year are pronounced. In Mexico, these variations are less evident and the difference between spring, summer, fall and winter is not as marked as in other latitudes. For this reason, we operationalized the “season” variable in a dichotomous manner, according to changes in summer time and winter time. We considered “winter” to be the period between October and March of the following year, and “summer” to be the period between April and September. However, although the frequency of hypothyroxinemia was lower in the winter period than in the summer (table 2), the inclusion of the “season” variable in the models did not substantially change the results of the previous analyses, compared to the ones that were carried out without including it: para-occupational exposure was not associated with hypothyroxinemia. The PON155LM polymorphism was independently associated with this variable and no significant interactions were observed between para-occupational exposure and genetic polymorphisms (please see models in Supplementary material). Therefore, in the manuscript we consider pertinent to present the model without including season.

At page 11 the authors states that "None of the three studied polymorphisms was found to be in Hardy Weinberg equilibrium;"

How did the authors take this result into consideration in the further data-analysis?
The authors further state that: "no linkage disequilibrium was observed among the three polymorphisms".

Other studies report significant linkage disequilibrium between PON1 192 and PON1 55. Therefore, some data illustrating the inter-individual distribution of the polymorphism would be relevant to illustrate the lack of linkage disequilibrium.

Reply: Our working group has published several works on the subject. The frequencies reported in this study are in accordance with the frequencies of previous work in these kind of population. For example, on effect on non-recurrent spontaneous abortion in Mexican women [Muñoz-Blanco J, Gene 689 (2019) 69–75] and in miscarriage in Mexican women exposed to pesticides. [J. Blanco-Muñoz et al; Science of the Total Environment 449 (2013) 302–308] and others manuscripts.

The fact that they are not in balance of HW, may be due to the fact that the inhabitants are in closed villages, with a small number of inhabitants and in which it is common to find that marriages occur among the inhabitants of the same community, which would limit genetic diversity. Therefore, these values were considered for subsequent analyzes. We have add a paragraph in the Discussion section (page 15, second paragraph)

To evaluate the precision and fidelity of the test, we reviewed the results at two different time point’s moments by the same molecular biologist and also evaluated the results through intraobserver and between inter-observer.

In Table 3, the associations between para-occupational pesticide exposure and hypothyroxinemia were adjusted for maternal PON1 genotype and associations between PON1 genotype and hypothyroxinemia were adjusted for para-occupational exposure (according to the Table footnote). Please explain the rationale for these adjustments and whether the ORs were affected by these adjustments.

Reply: Although the ORs were not substantially affected by these adjustment, but we wanted to include PON1 genotype or para-occupational pesticide exposure in the adjusted models because these variables were the main independent factors evaluated.

The crude results (unadjusted ORs) should be included in Table 3 and 4 to provide information on the robustness of the associations.

Reply: According to reviewer suggestion we included the unadjusted ORs in Table 3 and 4 in the revised manuscript.

Discussion
Some discussion of the high percentage of women with hypothyroxinemia in relation to the general population would be relevant. Is this fraction (54%) higher than in other areas in Mexico or representative for Mexico? What is known about the iodine intake in Mexico or the specific region compared to the levels recommended for pregnant women by the World Health Organization (250 mcg daily).

Reply: We agree with the reviewer.

During pregnancy, the physiological increase in the hormone thyroxine-binding globulin (TBG) and the decrease in albumin, affect the concentration of free T4 and complicate the interpretation of the laboratory results. According to a recent review, which included studies that measured the free T4 concentration by immunooassay, the prevalence of isolated hypothyroxinemia varied between 1.3% and 24% during pregnancy and the authors attribute this wide variation to the ethnic differences and iodine bioavailability of the studied populations, as well as to cut-off points selection (Dosiou and Medici, European Journal of Endocrinology 2017;176: R21-R38). Therefore, the American Thyroid Association (Alexander et al. Thyroid. 2017;27:315-389) has proposed that reference ranges for free T4 during pregnancy should be established for each specific population and trimester of pregnancy, in samples of women without thyroid disease, without thyroid antibodies and with adequate iodine consumption. According to these criteria, in Mexico, we only have found a study conducted in Yucatan (Quinn et al, Clin Chem Lab Med. 2014;52:1305-1311), which established a reference range for the first trimester from 0.75 to 1.7 ng/dL, not far from the range proposed by our laboratory (0.76 to 2.24 ng/dL).

Recently, a study conducted in Mexico City, including pregnant women without history of thyroid disease, found that the prevalence of clinical and subclinical hypothyroidism was 12.8% and 21.1%, respectively; the prevalence of isolated hypothyroxinemia was 12.8% (Cruz-Cruz et al. Ginecol Obstet Mex. 2014;82:717-724). These results suggest that the prevalence of thyroid disorders during pregnancy in Mexico, could be greater than that reported by the literature.

Given that the prevalence of iodine deficiency is low in our study population, immunological factors and exposure to other endocrine disruptors as synthetic fertilizers that include nitrates in their composition, could explain the high frequency of isolated hypothyroxinemia we find, a hypothesis that should be evaluated in future studies.

We have add a paragraph in the Discussion section (please page 17, last paragraph).

According to the last section (before the conclusion), the authors adjusted for both season and iodine supplementation but conclude that these variables did not confound the associations. Does this statement mean that the crude associations were changed less than 10% for each of these variables? Please explain.

Reply: Yes, the crude associations were changed less than 10% for each of these variables.

Reviewer #2: The study shows that paraoccupational exposure to pesticides does not appear to be significantly correlated with hypoparathyroxinemia. However, the authors observed that certain
PON1 polymorphisms are correlated to hypothyroxinemia. The topic (role of the maternal thyroid function in early pregnancy) is highly relevant, but the study is essentially observational.

Comments:

- There are two different investigations in this study: one attempting to correlate paraoccupational exposure to pesticides (in a highly exposed region) to thyroid toxicity, the other assessing the possible interaction between PON1 polymorphisms and thyroid function. The link is that PON1 is involved in the metabolism of organophosphates (OP). But it should be stressed that PON1 has many other functions and the effects of the genetic polymorphisms of PON1 may be correlated to cardiovascular outcomes and possibly to differences in pesticide metabolism.

Reply: First of all, thank you for your comment and suggestions.

We agree with the reviewer. The introduction specifies that PON1 is involved in the detoxification of organophosphate pesticides and also in the metabolism of lipids preventing the peroxidation of HDL and LDL (please see Introduction section, page 4, second paragraph). Because we found no significant interaction between para-occupational exposure to pesticides and PON1 polymorphisms we hypothesize that the observed independent association between PON155MM genotype and hypothyroxinemia could be related with the role of PON1 in the oxidative stress. (please see Discussion section, page 14, second and third paragraph)

- I am puzzled by the frequency of hypothyroxinemia in pregnant women in this study. More women have low T4 than normal levels. How does this compare to other studies?

Reply: During pregnancy, the physiological increase in the hormone thyroxine-binding globulin (TBG) and the decrease in albumin, affect the concentration of free T4 and complicate the interpretation of the laboratory results. According to a recent review, which included studies that measured the free T4 concentration by immunoassay, the prevalence of isolated hypothyroxinemia varies between 1.3% and 24% during pregnancy and the authors attribute this wide variation to the ethnic differences and iodine bioavailability of the studied populations, as well as to cut-off points selection (Dosiou and Medici, European Journal of Endocrinology 2017;176: R21- R38). Therefore, the American Thyroid Association (Alexander et al. Thyroid. 2017;27:315-389) has proposed that reference ranges for free T4 during pregnancy should be established for each specific population and trimester of pregnancy, in samples of women without thyroid disease, without thyroid antibodies and with adequate iodine consumption. According to these criteria, in Mexico, we only have found a study conducted in Yucatan (Quinn et al, Clin Chem Lab Med. 2014;52:1305-1311), which established a reference range for the first trimester from 0.75 to 1.7 ng/dL, not far from the range proposed by our laboratory (0.76 to 2.24 ng/dL).

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and 21.1%, respectively; the prevalence of isolated hypothyroxinemia was 12.8% (Cruz-Cruz et al. Ginecol Obstet Mex. 2014;82:717-724). These results suggest that the prevalence of thyroid disorders during pregnancy in Mexico, could be greater than that reported by the literature.

Given that the prevalence of iodine deficiency is low in our study population, immunological factors and exposure to other endocrine disruptors as synthetic fertilizers that include nitrates in their composition, could explain the high frequency of isolated hypothyroxinemia we find, a hypothesis that should be evaluated in future studies.

We have add a paragraph in the Discussion section (please page 17, last paragraph).

- The evidence for a role of OPs in this study is not sufficiently supported. The authors did not assay OPs through biomonitoring for budgetary reasons. They do not have evidence for the actual pesticides that are used by the workers. Maybe they do not all use OPs (?) and this could make a difference in statistical analysis.

Reply: We agree with the reviewer; however, due to budgetary reasons, we were not able to determine pesticide metabolite concentrations. We are searching for other financing sources to solve this limitation. In the discussion, we recognize that this is one of the limitations of the study (please, see Discussion section, page 16, second paragraph). Nevertheless, many pesticides, such as organophosphates are non-persistent compounds and the information provided by exposure biomarkers, although relevant, also has limitations because it shows exposure during the previous 48 hours, and not long term exposure. We assumed that women who have partners whose jobs expose them to pesticides, would be more exposed than women whose partners do a different kind of work, because previously we have found that workers frequently take the pesticide spraying equipment home, they store pesticides in their house and they bring work shoes and clothes into the house as well. It is important to mention that most of the time, the women are in charge of washing the clothes, which is frequently done by hand, since these are low-income women who don’t have access to automatic washers, which increases dermal exposure (Blanco-Muñoz and Lacasaña. J Agromedicine. 2011; 16:117-26).

On the other hand, recently we have measured the six dialkylphosphate (DAP) metabolites of organophosphate pesticides in a subsample of 65 pregnant women (please see Materials and Methods section, Pesticide exposure subsection, page 8, last paragraph). Although we found no significant differences, para-occupationally exposed women had a higher proportion of DAP metabolites above the limit of detection and a median of total DAP higher than non para-occupationally exposed women (1.16 µmol/g creatinine vs 0.83 µmol/g creatinine, p =0.56 by Mann-Whitney test). We have add a paragraph showing this result (please see the last paragraph of the Results section, first paragraph, page 11-12).

Additionally, we evaluated the association between total DAP exposure and hypothyroxinemia in this subsample of women (n=65) and, after adjusting for maternal age, gestational age and urinary creatinine concentration, not association was statistically significant (OR= 1.00, CI 95% 0.99, 1.004) This is consistent with the results observed when we use para-occupational exposure
as a proxy for pesticide exposure, where no association was found between para-occupational exposure and hypothyroxinemia. (Please see the last paragraph of the Results section, page 13).

- Because of the number of studied individuals, the number of actual hypothyroidism is low. The clinical characterization of the individuals with high TSH is lacking.

Reply: We agree with the reviewer. However this is an epidemiological study and we have not information about the clinical characterization of the individuals with high TSH. The ten women with 4 mIU/L were referred to their physician in order to confirm the diagnosis.

- The observed correlation between PON1 polymorphisms and hypothyroxinemia is interesting although it is quite descriptive at this stage. There could be some biases as mentioned by the authors since the hypothyroxinemia group differs from the control group in many respects.

Reply: We believe that the observed association between PON1 polymorphisms and hypothyroxinemia is interesting, but recognize that additional epidemiological and basic studies are required to confirm or refute these results.

On the other hand, although the control group differ from hypothyroxinemia group in many variables, in the multivariate analyses we controlled for the main potential confounders (please see Material and Methods section, Description of covariates subsection, page 10).

This study addresses some important questions. There are some interesting observations but none has been explored sufficiently. Assessing OP levels at least in a subgroup of individuals would improve the study as well as a better characterization of the type of pesticide used if this information is available.

Reply: we agree with the reviewer and additional studies on this topic are needed.

On the other hand, as we previously said, recently we have measured the six dialkylphosphate (DAP) metabolites of organophosphate pesticides in a subsample of 65 pregnant women. (please see the answer above)

Reviewer #3: In this report by Torres Sanchez et al., the authors evaluate the association between para-occupational exposure (defined as living with a partner who is involved in pesticide application) during pregnancy and hypothyroxinemia; and whether this association is modified by PON1 polymorphisms. The authors do not find an association between exposure and hypothyroxinemia but do observe significant associations between PON1 polymorphisms and hypothyroxinemia. It is difficult to know how to interpret the significance of this finding independent of pesticide exposure.
Reply: First of all, thank you for your comments and suggestions.

PON1 is a multifunctional enzyme involved in the detoxification of organophosphate pesticides and also in the metabolism of lipids preventing the peroxidation of HDL and LDL (please see Introduction section, page 4, second paragraph). Because we found no significant interaction between para-occupational exposure to pesticides and PON1 polymorphisms we hypothesize that the observed independent association between PON155MM genotype and hypothyroxinemia could be related with the role of PON1 in the oxidative stress. (please see Discussion, page 14, second and third paragraph).

Major Concerns:

1. Exposure classification is based on self-reported occupation of partners of pregnant women and was dichotomized as "para exposed" vs. not. There is a lot of potential for exposure misclassification, particularly among pregnant women who live in an area where the primary industry is floriculture. This authors discuss this limitation but in my opinion, underestimate its importance as a possibility for the lack of observed association between exposure and thyroid function, which was the primary goal of this study. This is a major limitation.

Reply: We agree with the reviewer; however, due to budgetary reasons, we were not able to determine pesticide metabolite concentrations. We are searching for other financing sources to solve this limitation. In the discussion, we recognize that this is one of the limitations of the study (please, see Discussion section, page 16, second paragraph). Nevertheless, many pesticides, such as organophosphates are non-persistent compounds and the information provided by exposure biomarkers, although relevant, also has limitations because it shows exposure during the previous 48 hours, and not long term exposure. We assumed that women who have partners whose jobs expose them to pesticides, would be more exposed than women whose partners do a different kind of work, because previously we have found that workers frequently take the pesticide spraying equipment home, they store pesticides in their house and they bring work shoes and clothes into the house as well. It is important to mention that most of the time, the women are in charge of washing the clothes, which is frequently done by hand, since these are low-income women who don’t have access to automatic washers, which increases dermal exposure (Blanco-Muñoz and Lacasaña. J Agromedicine. 2011; 16:117-26).

On the other hand, recently we have measured the six dialkylphosphate (DAP) metabolites of organophosphate pesticides in a subsample of 65 pregnant women (please see Materials and Methods section, Pesticide exposure subsection, page 8, last paragraph). Although we found no significant differences, para-occupationally exposed women had a higher proportion of DAP metabolites above the limit of detection and a median of total DAP higher than non para-occupationally exposed women (1.16 µmol/g creatinine vs 0.83 µmol/g creatinine, p =0.56 by Mann-Whitney test). We have add a paragraph showing this result (please see the last paragraph of the Results section, first paragraph, page 11-12)

Additionally, we evaluated the association between total DAP exposure and hypothyroxinemia in this subsample of women (n=65) and, after adjusting for maternal age, gestational age and
urinary creatinine concentration, not association was statistically significant (OR= 1.00, CI 95% 0.99, 1.004) This is consistent with the results observed when we use para-occupational exposure as a proxy for pesticide exposure, where no association was found between para-occupational exposure and hypothyroxinemia. (Please see the last paragraph of the Results section, page 13.

2. I am not sure how to interpret the association between PON1 polymorphisms and hypothyroxinemia independent of exposure. The authors do a lot of speculation about why this is important but if the primary role of this gene is to detoxify OP exposure, it is not clear why genotype is important if not to modify the effect of exposure, which it does not appear to do in this population.

Reply: PON1 is a multifunctional enzyme involved in the detoxification of organophosphate pesticides and also in the metabolism of lipids preventing the peroxidation of HDL and LDL; PON1 polymorphisms have been linked with cardiovascular disease and with some adverse reproductive effects related with oxidative stress (preeclampsia, pregnancy loss). Because we found no significant interaction between para-occupational exposure to pesticides and PON1 polymorphisms we hypothesize that the observed independent association between PON155MM genotype and hypothyroxinemia could be related with the role of PON1 in the oxidative stress. (please see Discussion, page 14, second and third paragraph).

We think that the observed association between PON1 polymorphisms and hypothyroxinemia is novel, but recognize that additional epidemiological and basic studies are required to confirm or refute these results.

3. It is not clear why the authors did not consider thyroid hormone parameters as continuous measures. This would give them more statistical power to observe associations with exposure (and interactions with PON1 polymorphisms).

Reply: We wanted to evaluate the effect on the maternal thyroid hormone concentrations that have been found to be related with poor infant neurodevelopment.

We run the analysis using thyroid hormone parameters as continuous measures. Lineal regression was used to evaluate the associations between para-occupational exposure and PON1 polymorphisms with log-transformed free T4. After adjusting for potential confounders we did not found significant association with para-occupational exposure. PON1 55MM and PON1 -108TT genotypes were negatively associated with free T4 (please see model in the Supplementary material). These results were consistent to those found when we used free T4 categorized as hypothyroxinemia (Yes or No). In order to evaluate whether maternal genotypes modify the association between para-occupational exposure and log-transformed free T4, We evaluate this association stratifying by maternal genotype. No effect modification was found.

Therefore, in the manuscript we consider pertinent to present the model using hypothyroxinemia as dependent variable.
Minor Concerns:

1. There were ~100 women who were eligible and agreed to participate but were not included in this report due to missing information. While there were no observed demographic differences between those who were and were not included, the authors could employ techniques (e.g., inverse probability weighting) to evaluate the potential bias that this selection might introduce.

Reply: We apologize. We have no experience with the inverse probability weighting technique.

Women with or without complete information were compared by selected characteristics (socio-demographic, reproductive, tobacco and alcohol consumption and gestational age at baseline. In the case of categorical variables we used the $\chi^2$ test, while Student’s t test or non-parametric tests (Mann-Whitney test) were used for continuous variables. No significant differences were observed between these women and the remaining participants with respect to these variables. We therefore believe that selection bias is unlikely.

2. Lines Page 7, 14-18. It is not clear to me what intra-assay and inter-assay variation refers to. Were control samples analyzed in each batch? It is not clear to me how these ranges were calculated.

Reply: In each test, controls were used which are included in the commercial kit and depending on the results of these controls, the variability within the assay (intra-assay variation) and between the other tests performed (interassay variation) is calculated.

3. It is somewhat surprising that over half the study participants were classified as hypothyroxic. While it is common condition, a rate of 54% seems rather high. A relatively small proportion of participants took iodine supplements yet iodine supplementation status was not associated with hypothyroxinemia (Table 2). Are these results consistent with previous reports in this population?

Reply: We are also was quite surprise with the incidence of hypothyroxinemia taking into account the apparently iodine sufficiency in this population (Hernández-Mariano et al. 2016).

During pregnancy, the physiological increase in the hormone thyroxine-binding globulin (TBG) and the decrease in albumin, affect the concentration of free T4 and complicate the interpretation of the laboratory results. According to a recent review, which included studies that measured the free T4 concentration by immunoassay, the prevalence of isolated hypothyroxinemia varied between 1.3% and 24% during pregnancy and the authors attribute this wide variation to the ethnic differences and iodine bioavailability of the studied populations, as well as to cut-off points selection (Dosio and Medici, European Journal of Endocrinology 2017;176: R21-R38). Therefore, the American Thyroid Association (Alexander et al. Thyroid. 2017;27:315-389) has proposed that reference ranges for free T4 during pregnancy should be established for each specific population and trimester of pregnancy, in samples of women without thyroid disease, without thyroid antibodies and with adequate iodine consumption. According to these criteria, in
Mexico, we only have found a study conducted in Yucatan (Quinn et al, Clin Chem Lab Med. 2014;52:1305-1311), which established a reference range for the first trimester from 0.75 to 1.7 ng/dL, not far from the range proposed by our laboratory (0.76 to 2.24 ng/dL).

Recently, a study conducted in Mexico City, including pregnant women without history of thyroid disease, found that the prevalence of clinical and subclinical hypothyroidism was 12.8 % and 21.1%, respectively; the prevalence of isolated hypothyroxinemia was 12.8 % (Cruz-Cruz et al. Ginecol Obstet Mex. 2014;82:717-724). These results suggest that the prevalence of thyroid disorders during pregnancy in Mexico, could be greater than that reported by the literature.

Given that the prevalence of iodine deficiency is low in our study population, immunological factors and exposure to other endocrine disruptors as synthetic fertilizers that include nitrites in their composition, could explain the high frequency of isolated hypothyroxinemia we find, a hypothesis that should be evaluated in future studies.

We have add a paragraph in the Discussion section (please page 17, last paragraph).

4. What are the implications of the fact that none of the three polymorphisms studied were in Hardy Weinberg equilibrium (Page 11, line 39). Is this unexpected?

Reply: Our working group has published several works on the subject. The frequencies reported in this study are in accordance with the frequencies of previous work in these kind of population. For example, on effect on non-recurrent spontaneous abortion in Mexican women [Muñoz-Blanco J, Gene 689 (2019) 69–75] and in miscarriage in Mexican women exposed to pesticides. [J. Blanco-Muñoz et al; Science of the Total Environment 449 (2013) 302–308] and others manuscripts.

The fact that they are not in balance of HW, may be due to the fact that the inhabitants are in closed villages, with a small number of inhabitants and in which it is common to find that marriages occur among the inhabitants of the same community, which would limit genetic diversity. Therefore, these values were considered for subsequent analyzes. We have add a paragraph in Discussion section (page 15, second paragraph).

To evaluate the precision and fidelity of the test, we reviewed the results at two different time point’s moments by the same molecular biologist and also evaluated the results through intraobserver and between inter-observer.

5. Page 11, Line 50-52: I would not reword this. Given the 95% CI, I would not consider the observed results to be an increase in hypothyroxinemia.

Reply: We have modified the sentence according to reviewer suggestion.
6. Page 16, Line 37: It is difficult to understand how the authors conclude that there could be a potential interaction between PON1-108CT and OP para exposure when they did not observe such an interaction. If the authors think that this is a real possibility, they should discuss why they did not observe it. If the authors believe that their study was underpowered to detect this interaction, they should provide a power calculation to demonstrate how many women they would potentially need to observe this association.

Reply: We have suppressed this conclusion.