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## Relationship between self-reported fish and shellfish consumption, carbon and nitrogen stable isotope values and total mercury concentrations in pregnant women (II) from Baja California Sur, Mexico



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### ABSTRACT

Seafood is a valuable source of nutrients important for fetal development. However, seafood consumption is the main route of exposure to monomethyl mercury (MeHg<sup>+</sup>) for humans. MeHg<sup>+</sup> is highly bioavailable and potentially adversely affects fetal neurodevelopment. MeHg<sup>+</sup> exposure from fish consumption varies significantly by age and trophic level of fish consumed as well as the frequency and amount of fish consumed. This study investigates total Hg concentrations ([THg]) in hair segments of pregnant Mexican women in relation to (1) self-reported frequency of fish and shellfish consumption, (2) maternal trophic level and marine diet contributions, determined using hair carbon (C) and nitrogen (N) stable isotopes, and (3) relates [THg] to various hair advisory thresholds. We also examined whether variation in C and N isotope values is explained by self-reported frequency of fish and shellfish consumption. A significant proportion of hair samples had [THg] higher than suggested agency thresholds and, for women within the range of the various advisory thresholds (1–20 μg g<sup>-1</sup>), the specific statistic used and threshold applied are important considerations for assessing and communicating risk. Individuals enriched in <sup>15</sup>N (δ<sup>15</sup>N values) had higher [THg] as did individuals that reported consuming fish and shellfish more frequently, suggesting that variation in [THg] can be explained by both consumer reported diet and diet as determined by C and N stable isotope assessment. However, at higher reported fish consumption levels the trophic level is maintained while [THg] is paradoxically lower. This suggests that THg exposure and assimilation are more complicated in higher fish frequency consumption categories. [THg] is more variable at the higher concentrations, possibly

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indicating some exposure to non-dietary Hg, heritable variations affecting Hg toxicodynamics, and BMI and tobacco exposure factors as outlined in our companion paper.

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## 1. Introduction

Finfish, mollusks, and crustaceans are seafood sources high in lean protein, omega-3 fatty acids, zinc, iron and, especially in the case of marine species, provide iodine, selenium, and vitamins A and D [1]. These nutrients, especially the omega-3 fatty acids, are vital to human fetal development with eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) contributing to development of the central nervous system [2,3]. Women consuming more fish during pregnancy have babies with higher IQs and fewer behavioral problems than mothers that ate little or no fish high in omega-3 fatty acids [4]. Additionally, fish consumption during pregnancy has been linked to a decrease in preterm delivery [5], increased likelihood of foveal stereoacuity in the child [6], and to development of the child's immune system [7,8].

Consumption of fish that accumulate monomethyl mercury ( $\text{MeHg}^+$ ) in their muscle is the primary pathway of exposure to mercury (Hg) in humans [9]. Inorganic forms of Hg enter ecosystems through natural sources such as volcanism, and anthropogenic sources such as mining, coal combustion, and cement production [10]. Hg is converted to the more toxic  $\text{MeHg}^+$  by bacteria [10] and in most systems biomagnifies with each trophic transfer.  $\text{MeHg}^+$  is highly bioavailable in humans [ $>95\%$  of ingested dose absorbed; Aberg et al. [11]], easily passes the placenta to the fetus, and crosses the blood brain barrier (BBB), potentially adversely affecting the developing nervous system [12–14]. Nearly 100% of the Hg that bioaccumulates in upper-trophic level fish skeletal muscle is  $\text{MeHg}^+$  [15].

Effects on brain function associated with prenatal  $\text{MeHg}^+$  exposure were found to be multi-focal and permanent [16] although these results may have been confounded by prenatal exposure to polychlorinated biphenyls [17]. The amount of Hg exposure to humans from fish consumption varies significantly by the age and trophic level of the fish consumed [18,19] and the frequency (Gaxiola-Robles et al. companion paper) and amount (mass) of the fish meals. Pouzaud et al. [20] found that a global increase in seafood consumption could lead to Hg exposure above some conservative consumption advisory limits for pregnant women, and examined the balance between Hg exposure risk and the beneficial effects of omega-3 long-chain polyunsaturated fatty acid intake. Pouzaud et al. [20] concluded pregnant women consuming a high proportion of some fatty fish, such as sardines and salmon, meet the omega-3 long-chain polyunsaturated fatty acid intake requirements without exceeding the advisory limits for Hg. In addition, Myers et al. [3] examined neurobehavioral effects in the Republic of Seychelles related to prenatal exposure to Hg resulting from a high fish diet and concluded that the beneficial influence of nutrients from fish may counter adverse effects of Hg on the developing nervous system.

Humans can be exposed to Hg through abiotic non-fish sources. Cigarette smoking and passive exposure, addressed in our companion paper (Gaxiola-Robles et al.), may be a substantial source of Hg not only to the smoker but also, through passive smoking, to nonsmokers [21], and has been shown to result in increased Hg concentrations in breast milk [22]. However, Gaxiola-Robles et al. (companion paper) did not find as strong a link between tobacco exposure and [THg] in hair in the population of women included in this study. Dental amalgam is a potentially significant source of exposure since it can contain up to 50% elemental Hg [23]. The use of Hg-containing beauty creams and other cosmetic products may also result in significant exposure to Hg [23]. Elemental Hg is used in some therapies, religions and other practices (e.g. Santería, Espiritismo) and can result in exposure with subsequent absorption and/or externally contaminated samples [e.g. hair; WHO [23]]. These are important confounders to consider in study designs and interpretation of fish consumption studies that determine [THg] in hair, blood, or both.

The feeding ecology/trophic level of individual mammals can be determined by naturally occurring variations in the ratio of heavy to light isotopes of carbon ( $^{13}\text{C}/^{12}\text{C}$ ,  $\delta^{13}\text{C}$ ) and nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ,  $\delta^{15}\text{N}$ ) and can be used to better understand contaminant exposure [24–27] including Hg bioaccumulation and biomagnification [28–30]. Enrichment of  $\delta^{15}\text{N}$  can be used to estimate trophic position because  $\delta^{15}\text{N}$  increases predictably with each trophic level transfer [31]. Changes in  $\delta^{13}\text{C}$  can provide information on the location of dietary resources [e.g. terrestrial vs. marine and pelagic vs. benthic; France [32], France and Peters [33], Newsome et al. [34]].

Understanding Hg pathways in human exposure is critical to assess risk and properly manage exposure, specifically in cohorts of concern, such as women of childbearing age. This is the 2nd of two papers examining [THg] in women in Baja California Sur, Mexico. We measured [THg] in the hair segments of pregnant women along with reported frequency of fish and shellfish consumption with the goal of evaluating whether [THg] varied with diet. Second, we measured  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  signatures in hair to determine longer-term dietary exposure to higher trophic level and marine derived protein sources, with the goal of evaluating whether [THg] varied in relation to the trophic level of the mother's diet and whether variation in C and N stable isotopes could be explained by reported frequency of consumption of fish and shellfish.

## 2. Materials and methods

### 2.1. Sampling

Samples of occipital scalp hair were collected from women in Baja California Sur, Mexico, following the established sample collection procedure [McDowell et al. [35],

see Gaxiola-Robles et al. companion paper]. The study site was chosen after Hg concentration in muscle samples from larger sharks (>200 cm LT) caught by local artisan fisheries in this area were found to exceed the permissible limit (>1 ppm wet weight) for human consumption set by numerous international agencies [36,37]. Informed consent and hair samples were collected the day of discharge from the hospital postpartum and in a follow-up interview, conducted 7–10 days after delivery, a survey was administered exploring food consumption 30 days prior to hair sample collection (between July and December 2011). No information was obtained about meal portion size, recipes, or preparation methods. Fish, shellfish, and dairy consumption frequency data were grouped into four categories: none consumed; consumed once a month; consumed once every two weeks; and consumed more than twice a week. 114 women contributed hair samples and 78 of these completed the survey. This research (project ID, CONACYT-SALUD 2010-C01-140272) was approved by the Baja California Sur Chapter of the National Mexican Academy for Bioethics.

This population consumes fish on a regular basis, generally sea bass, groupers, red and other snappers, sharks, rays, jacks, and dorados [38]. Beef (grass or corn-fed cattle) is consumed at most twice a week; corn-fed chicken is consumed more often than beef; generally, the population relies on eggs, corn, beans and rice for most meals [39]. Known consumption of corn or corn-fed cattle or chicken can affect the interpretation of C and N stable isotopes.

## 2.2. Sample preparation

Samples were analyzed for [THg] and stable isotopes of nitrogen (N) and carbon (C) values at the Wildlife Toxicology Laboratory (WTL), University of Alaska Fairbanks (UAF). Samples were provided with no indication of participant identification (de-identified). Samples were immersed in a 1% solution of Triton X-100<sup>®</sup> for 15–20 min to remove external contamination, then rinsed by an initial 10 min immersion in ultrapure water (NANOpure Model D4751, Barnstead International, Dubuque, Iowa), followed by a 5 min immersion and a further 3 sequential immersions. Cleaned samples were placed in labeled 4 oz polyethylene WhirlPak<sup>™</sup> bags and freeze dried for 48 h. Full length hair samples ( $n=97$ ) were subsampled into 3 sections (proximal, middle and distal segments) along the length of the hair, with the proximal sample representing the most recent hair growth, in order to assess temporal variability within an individual. Individual segments were analyzed for [THg], and the remaining hair was used for determination of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  stable isotope values. See Gaxiola-Robles et al. (companion paper) for additional details of the segmental analysis.

## 2.3. Mercury analysis

Total mercury concentration ( $\mu\text{g g}^{-1}$ ) was measured in hair segments using a DMA80 Direct Mercury Analyzer [Milestone Inc., Shelton, Connecticut; US EPA method 7473; Knott et al. [40], Castellini et al. [41], Rea et al. [30]; see Gaxiola-Robles et al. companion paper for additional

details of the segmental analysis]. Values from the three segments were used to establish the range and variability within each individual and for comparison with established thresholds, but for comparison with the diet surveys, only the value from the proximal (most recent) segment was used. Mean [THg] for each individual (across the three segments) was used in comparison with the carbon and nitrogen stable isotope values.

## 2.4. Stable isotope analysis

Hair samples ( $n=77$ ) were analyzed for stable isotopes of nitrogen (N) and carbon (C). The stable isotope sample was comprised of all the remaining hair after the segmental [THg] analysis was done. Approximately 0.5 mg of clean, dry hair was wrapped in ultrathin foil sheets (Elemental Microanalysis, Cambridge, UK) and analyzed at the Alaska Stable Isotopes Facility at the University of Alaska Fairbanks. An elemental analyzer–isotopic ratio mass spectrometer (Costech Elemental Analyzer [ESC 4010] and Finnigan MAT ConFlo III interface with a Delta+XP mass spectrometer) was used [28,30]. The ratio of stable isotopes is expressed in delta ( $\delta$ ) notation and calculated as:

$$\delta X = \left[ \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right] \times 1000$$

where  $X = {}^{15}\text{N}$  or  ${}^{13}\text{C}$  and  $R = {}^{15}\text{N}/{}^{14}\text{N}$  or  ${}^{13}\text{C}/{}^{12}\text{C}$  in the sample and standard.

## 2.5. Statistical Analysis

We generated mean total [THg] and 95% confidence interval for most individuals using 3 segments per individual to examine the percentage of women that had means and/or 95% confidence intervals significantly higher than various published health-related thresholds for women of child bearing age. The selected thresholds are  $1 \mu\text{g g}^{-1}$  [42],  $5 \mu\text{g g}^{-1}$  [43],  $10 \mu\text{g g}^{-1}$  [44–46],  $15.3 \mu\text{g g}^{-1}$  [47] and  $20 \mu\text{g g}^{-1}$  [46], as they represent a range of advisory levels that we are aware of. These advisory levels were generally developed to protect the most sensitive health outcomes of mercury exposure, the neurodevelopmental effects on the fetus of mothers who consume fish, but also young children.

We used general linear models (Proc GLM) to evaluate the relationship between the frequency of self-declared categorical consumption of fish and shellfish (never, once a month, every 2 weeks, or more than twice a week) as reported by the individual for the month prior to sampling, and [THg] in the proximal hair segment in pregnant Mexican women ( $n=78$ ) using 4 a priori candidate models. Only 78 women had both hair [THg] measured and completed diet recalls. Frequency of consumption of dairy products was also collected but not included in the model set as 72 individuals consumed dairy more than twice a week, 5 consumed dairy every 2 weeks, and 1 consumed dairy once a month resulting in a distribution not amenable to statistical evaluation. The proximal hair segment was chosen as it best correlates with the one month time frame of the diet data. Models in the candidate set included all

combinations of the variables (e.g.  $\text{Model}_{\text{fish}}$ ;  $\text{Model}_{\text{shellfish}}$ ;  $\text{Model}_{\text{fish} + \text{shellfish}}$ ;  $\text{Model}_{\text{fish} \times \text{shellfish}}$ ). [THg] was log transformed to improve normality.

We examined the relationship between [THg] and  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values using two separate simple linear regressions to test whether diet, as determined by  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ , affects mean [THg] (across segments; Proc REG;  $n = 73$ ). Seventy three women had hair [THg],  $\delta^{15}\text{N}$ , and  $\delta^{13}\text{C}$  values determined. We log-transformed the data to meet the assumption of homoscedasticity and examined for influential outliers. As we did not account for the negative sign associated with  $\delta^{13}\text{C}$ , a negative  $\beta$ -value indicates that [THg] decreased as  $\delta^{13}\text{C}$  is enriched (i.e. becomes less negative). Additionally, we ranked  $\delta^{13}\text{C}$  from 1 to 73 (from values of  $-18.52$  to  $-12.19$ ) and ran a regression on the ranks, reducing the influence of an outlying individual ( $\delta^{13}\text{C} = -12.19$ ).

Lastly, we used general linear models (GLM) to evaluate the relationship between the frequency of consumption of fish and shellfish as reported by the individual and  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  stable isotopes values ( $n = 61$ ), using 2 separate a priori candidate model sets, each with 3 models. Sixty one women had  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  measured and completed diet recalls. Models in the two candidate sets included all additive combinations of the variables (e.g.  $\delta^{15}\text{N}_{\text{fish}}$ ;  $\delta^{15}\text{N}_{\text{fish} + \text{shellfish}}$ ;  $\delta^{13}\text{C}_{\text{fish}}$ ;  $\delta^{13}\text{C}_{\text{fish} + \text{shellfish}}$ ). We added a constant (20) and square root-transformed  $\delta^{13}\text{C}$  to improve normality.

Values are reported as means  $\pm$  SE unless otherwise indicated. Analyses were conducted using SAS (SAS Institute, Cary, NC). We considered results significant at  $\alpha < 0.05$ . All statistical analyses were conducted with and without an individual with exceptionally high [THg] to ensure that this individual was not overly influential in our assessment. We used Akaike's information criterion adjusted for small sample size ( $\text{AIC}_c$ ) to select the best approximating models as it allowed us to evaluate a number of competing nested models without violating the rules of multiple comparisons and error rates [48]. We used Tukey's multiple comparison test to compare means.

**Table 1**

The number (%) of women with a mean and/or upper and lower 95% confidence limits of total mercury concentration ([THg]) significantly greater than various advisory thresholds or guidelines.

Agency/group	Threshold concentration ( $\mu\text{g g}^{-1}$ )	# (%) of participants with mean > threshold	# (%) of participants with upper 95% confidence interval > threshold	# (%) of participants with lower 95% confidence interval > threshold
US EPA <sup>a</sup>	1	64 (69%)	70 (75%)	49 (53%)
Alaska Statewide Hair Mercury Biomonitoring Program <sup>b</sup>	5	7 (8%)	9 (7%)	7 (8%)
Health Canada <sup>c</sup>	10	4 (4%)	6 (6%)	4 (4%)
Agency for Toxic Substances and Disease Registry <sup>d</sup>	15.3	3 (3%)	4 (4%)	2 (2%)
World Health Organization <sup>e</sup>	20	1 (1%)	3 (3%)	1 (1%)

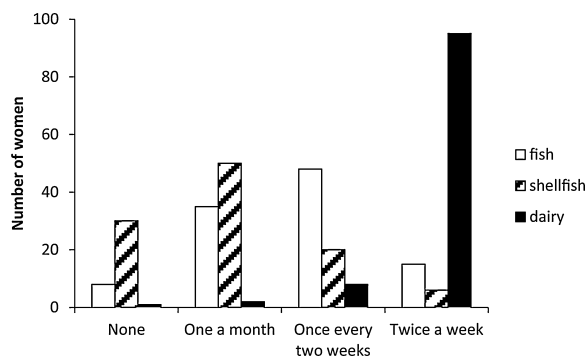
<sup>a</sup> U.S. EPA [42].

<sup>b</sup> Hamade [43].

<sup>c</sup> Feeley et al. [44]; NRC [45].

<sup>d</sup> Risher and DeWoskin [47].

<sup>e</sup> WHO [46].



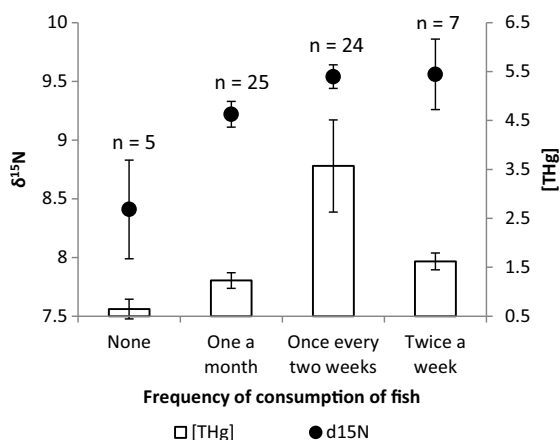
**Fig. 1.** Number of women ( $n = 78$ ) consuming fish, shellfish, and dairy in each consumption category (never, once per month, once every two weeks, or twice a week).

### 3. Results

#### 3.1. Total mercury concentrations

We measured [THg] in the proximal hair segments of 97 women. [THg] averaged  $3.26 \pm 0.97 \mu\text{g g}^{-1}$ , ranging from  $0.12$  to  $90.0 \mu\text{g g}^{-1}$  (750-fold range). When the individual with [THg] of  $90.0 \mu\text{g g}^{-1}$  was excluded as an outlier, [THg] averaged  $2.35 \pm 0.38 \mu\text{g g}^{-1}$  and ranged from  $0.12$  to  $24.20 \mu\text{g g}^{-1}$  (202-fold range). There was considerable variation in the number of women that had [THg] higher than specific thresholds for pregnant women or women of childbearing age, depending on which agency's threshold is considered (Table 1). However, including a measure of the variation in [THg] for an individual woman did not have a large effect on the number of women exceeding any given threshold (Table 1).

Frequency of self-reported consumption of fish, shellfish and dairy products are shown in Fig. 1. The best approximating a priori model describing [THg] in the proximal segment of hair of these pregnant women included the frequency of consumption of fish ( $\text{AIC}_c = -25.88$ ,



**Fig. 2.** Mean ( $\pm$ standard error) total mercury ([THg] concentration,  $\mu\text{g g}^{-1}$ ) and  $\delta^{15}\text{N}$  in relation to reported frequency of fish consumption.

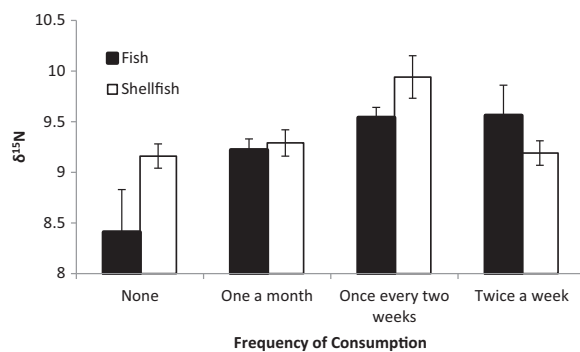
$w_i = 0.77$ ,  $K = 5$ ), and was 2.9  $\text{AIC}_c$  units from the next best model, which included an effect of shellfish consumption ( $\text{AIC}_c = -22.95$ ,  $w_i = 0.18$ ,  $K = 8$ ). [THg] varied significantly with fish consumption ( $F = 8.8$ ,  $p < 0.0001$ ; Fig. 2). Although the 2nd best model included an effect of shellfish consumption, the effect was not significant ( $F = 0.67$ ,  $p = 0.58$ ). These findings and results did not change significantly when the 90 ppm outlier was included.

### 3.2. Stable isotope associations

The  $\delta^{15}\text{N}$  values ranged from 7.43‰ to 10.70‰ (mean =  $9.35 \pm 0.08\%$ ) and  $\delta^{13}\text{C}$  ranged from  $-18.52\%$  to  $-12.19\%$  (mean =  $-16.62 \pm 0.09\%$ ). The [THg] increased with  $\delta^{15}\text{N}$  ( $F = 5.76$ ,  $p = 0.02$ ,  $R^2 = 0.08$ ), independent of the 90 ppm outlier, while [THg] decreased as  $\delta^{13}\text{C}$  became more enriched or less negative ( $F = 4.26$ ,  $p = 0.04$ ,  $R^2 = 0.06$ ), independent of the 90 ppm outlier. However, the relationship between  $\delta^{13}\text{C}$  and [THg] was not significant when  $\delta^{13}\text{C}$  was ranked ( $F = 0.7$ ,  $p = 0.41$ ) because the influence of an outlying individual is reduced. This individual had the lowest  $\delta^{15}\text{N}$  (7.43‰) as well as the most enriched  $\delta^{13}\text{C}$  ( $-12.19\%$ ) and the lowest mean [THg] ( $0.12 \mu\text{g/g}$ ), and reported consuming no fish or shellfish and dairy only once a month. The individual with the high [THg] ( $90 \mu\text{g g}^{-1}$ ) had values of  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  that fell near the mean (9.2‰,  $-16.58\%$ , respectively) and reported consuming fish once every two weeks, no shellfish, and dairy twice or more per week.

The best approximating a priori model describing variation in  $\delta^{15}\text{N}$  in the hair of these pregnant women in relation to reported diet included the frequency of consumption of fish and shellfish ( $\text{AIC}_c = -56.26$ ,  $w_i = 0.78$ , no. of parameters  $K = 8$ ), and was 2.56  $\text{AIC}_c$  units from the next best model, which did not include the effects of frequency of shellfish consumption ( $\text{AIC}_c = -53.70$ ,  $w_i = 0.22$ ,  $K = 5$ ).  $\delta^{15}\text{N}$  varied significantly with fish consumption ( $F = 5.6$ ,  $p < 0.01$ ) and shellfish consumption ( $F = 3.3$ ,  $p = 0.03$ ; Fig. 3).

The best approximating a priori model describing variation in  $\delta^{13}\text{C}$  in the hair in relation to reported diet included the frequency of consumption of fish ( $\text{AIC}_c = -182.91$ ,



**Fig. 3.** Mean ( $\pm$ standard error)  $\delta^{15}\text{N}$  in relation to reported frequency of consumption of fish and shellfish.

$w_i = 0.93$ ,  $K = 5$ ), and was 5.96  $\text{AIC}_c$  units from the next best model which included the effect of frequency of shellfish consumption ( $\text{AIC}_c = -176.94$ ,  $w_i = 0.05$ ,  $K = 5$ ).  $\delta^{13}\text{C}$  did not vary significantly with either fish or shellfish consumption ( $F < 1.95$ ,  $p > 0.13$ ).

## 4. Discussion

Total mercury concentrations in the hair of pregnant women living in Baja California Sur, Mexico were quite variable with a significant proportion higher than suggested agency thresholds or guidelines [43–47] and may be of concern that warrant follow up investigations. We evaluated both the total number of women whose mean [THg] was higher than suggested agency thresholds and the number of women whose upper 95% confidence were higher than the thresholds and found minor differences. We note large variability based on the advisory guidelines selected; for example, 1–53% exceeded various guidelines when using the lower 95% confidence limit for individuals and 1–69% when using mean [THg]. Most women with ‘high’ [THg] were considerably higher than the thresholds of 5 and  $10 \mu\text{g g}^{-1}$  and their [THg] were not variable enough between segments of hair (e.g. 95% confidence limit) to change the outcome. While it did not appear that there was a benefit to including variation around the mean when comparing [THg] to concentrations of concern for this ‘high’ group, it is apparent for women within the range of the various advisory thresholds ( $1\text{--}20 \mu\text{g g}^{-1}$ ) that the specific statistic and consumption threshold used are important considerations.

The variation in [THg] can be partially explained by reported consumption of finfish but not shellfish consumption. An increase in fish consumption from once a month to once every two weeks resulted in [THg] in hair increasing by more than  $2 \mu\text{g g}^{-1}$ , although women in the highest consumption category actually had lower [THg] (Fig. 2) while  $\delta^{15}\text{N}$  remained equivalent. The women in the study are consuming relatively low amounts of fish (Fig. 1); however, some are known to be predatory fish and both high in [THg] and of a high trophic position [4,36,38]. Finfish are, in general, of a higher trophic level than shellfish [49] and thus likely have higher [THg], so it is not surprising that there was no obvious link between shellfish consumption and [THg]. Greater variability at higher [THg] may indicate that

while diet (e.g. consumption of fish) explains most of an individual's [THg], some of the higher [THg] are attributable to non-dietary or non-fish dietary exposure [e.g. rice; Li et al. [50]] or to individual variation in genetic drivers; as well as BMI and tobacco exposure as indicated in our companion paper (Gaxiola-Robles et al. companion paper). One individual in particular illustrates this; the individual with 90.0 ppm THg had  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values near the mean values, as was the reported fish and shellfish consumption, suggesting that a gross measure of seafood diet was not the main driver of the relatively high [THg]. The authors recognize the benefits and limitations of dietary recall information and caution that detailed assessments are not warranted in many cases and that our findings will require more detailed follow up [51].

While consumption frequency provides evidence that [THg] is related to fish in the diet, interpretation is complicated by potential differences in meal size, age or size of the fish, and species consumed. Given these caveats,  $\delta^{15}\text{N}$  may be a better predictor of [THg] in hair, or may significantly supplement dietary information. In this study, the strength of conclusion varies by whether we are assessing [THg] in the proximal hair segment or mean [THg] across the hair sample. This is likely due to the fact that the time frame for the proximal hair segment better matches the diet recall survey while the mean hair [THg] time frame better matches the C and N stable isotope kinetics. The stable isotope sample was comprised of all the remaining hair after the segmental [THg] analysis was done.

Individuals that were relatively enriched in  $\delta^{15}\text{N}$  had significantly higher [THg], likely due to higher finfish consumption although  $\delta^{15}\text{N}$  values in this population did not have a wide range (7.43–10.7%). The relationship between  $\delta^{15}\text{N}$  and [THg] only explained 8% of the variability in [THg], thus we speculate this is likely due to the low protein consumption and multiple protein sources of this population and to additional abiotic Hg exposure. We will address this in future studies where we will include Hg, C and N data from actual food items related to observations in the hair of pregnant women. Women are consuming relatively little fish mass (Fig. 1), but as the fish consumed is generally of a high trophic level and associated high [THg], even at the consumption rates reported there could be link between fish consumption and [THg]. Future studies should collect data on meal size (mass), frequency, species of fish consumed (including fish size/age), and amount of consumption of other protein sources such as beef, chicken and eggs, as well as rice consumption [additional dietary source of Hg, Zhang et al. [52]] including measures of [THg] and C and N stable isotope values.

The variation in  $\delta^{13}\text{C}$  cannot be explained by reported diet and was not clearly related to [THg] possibly due to limitations of the study design (did not chemically characterize food items). In addition, this may be due to this population having a high use of maize, corn-based food additives (e.g. high fructose corn syrup), and marine protein sources [53]. Plants using the  $\text{C}_3$ -photosynthetic pathway (such as rice and beans) are depleted in  $^{13}\text{C}$  relative to  $\text{C}_4$ -photosynthetic plants [such as maize; Codron et al. [54]], allowing the determination of the relative contribution of  $\text{C}_3$  and  $\text{C}_4$  plants in the terrestrial diet. However,

$\delta^{13}\text{C}$  may help to identify consumers of marine resources if future studies were attempting to focus on that group and wanted to chemically exclude non-fish consumers. Including sulfur stable isotope analysis ( $\delta^{34}\text{S}$ ) would strengthen this ability even further [55] and is being considered for future studies.

Individuals that report consuming no fish are depleted in  $\delta^{15}\text{N}$  relative to higher fish consumers, but  $\delta^{15}\text{N}$  values are not significantly different in individuals that report consuming fish once per month. However, we can determine which individuals are consuming little to no marine derived protein using  $\delta^{15}\text{N}$ . The lack of a clearer differentiation may be due to the fact that we have information on frequency of fish consumption rather than mass consumed; mass of the marine based diet is important since changes in C and N isotope signatures are altered based on the proportion of the amount of C and N containing macromolecules that are ingested and assimilated into the consumer based on the total amount of those constituents (proportion marine derived C and N nutrients relative to total intake). This is illustrated by one individual who had the lowest  $\delta^{15}\text{N}$  (7.43‰), as well as the most enriched  $\delta^{13}\text{C}$  (−12.19‰), and the lowest mean [THg] (0.12  $\mu\text{g/g}$ ), and reported consuming no fish or shellfish and dairy only once a month. This individual is likely a vegetarian and additionally is consuming very little dairy, and her diet explains her low [THg] fairly well. This individual could be removed if one were attempting to study only fish consumers.

The variation in [THg] can, in part, be explained by both reported diet and diet as determined by C and N stable isotopes. Individuals that were enriched in  $\delta^{15}\text{N}$  had higher [THg] as did individuals that reported consuming fish and shellfish more frequently. However, the link between [THg], fish consumption, and  $\delta^{15}\text{N}$  gets more complicated with higher reported levels of fish consumption. While [THg] and  $\delta^{15}\text{N}$  (trophic level) increase with fish consumption at the lower reported levels of fish consumption, for the higher fish consumption levels, trophic level is maintained but [THg] is lower (Fig. 2). This apparent disconnect could be due to types of fish consumed or meal size (mass consumed vs. frequency). Given that trophic level ( $\delta^{15}\text{N}$ ) is maintained (although the values are more variable) at higher fish consumption levels, the decrease in [THg] may be due to types of fish consumed (e.g. [THg] varies with fish species, trophic level, and with age within species) than to a decreased or increased variability in actual mass of fish consumed. It seems unlikely that people reporting more frequent fish consumption would actually be consuming less fish, and  $\delta^{15}\text{N}$  values indicate that mean trophic level remains the same but we cannot account for the age of the fish consumed ([THg] are well known to increase with age of fish independent of trophic level). Lastly, the maintenance of trophic level with decreased [THg] could be due to a combination of more frequent fish consumption, but lower fish mass consumed from younger fish [36], and with increased consumption of beef or chicken protein (e.g., increases the proportion of non-marine N). This could result in maintenance of trophic position, relatively lower [THg], but relatively increased reported frequency of fish consumption.

## 5. Conclusions

There is a statistically significant relationship between increased [THg] and enriched  $\delta^{15}\text{N}$  (trophic position), and an increase in reported consumption of fish and increased [THg], suggesting that the increase in [THg] is due to fish consumption, at least at lower fish consumption frequencies and low to moderate [THg]. While we cannot completely tease apart the contribution of corn and corn-fed beef versus marine fish using C and N stable isotopes the significant relationship between  $\delta^{15}\text{N}$  values and reported consumption of fish supports the conclusion that fish consumption is an important pathway for Hg exposure in this population. Increased consumption of terrestrial fauna could result in an increase in trophic position but is unlikely to result in increased [THg]. We recommend that caution be used when consuming high trophic level fish during pregnancy based on our assessment of using various statistical measures (mean, lower and upper 95% CI) and a range of advisories based on [THg] in hair ( $1\text{--}20\ \mu\text{g g}^{-1}$ ).

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

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