THE ROLE OF GUT MICROBIOTA IN FETAL METHYLMERCURY EXPOSURE: INSIGHTS FROM A PILOT STUDY

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ABSTRACT

PURPOSE:The mechanisms by which gut microbiota contribute to methylmercury metabolism remain unclear. Among a cohort of pregnant mothers, theobjectives of our pilot study were to determine 1) associations between gut microbiota and mercury concentrations in biomarkers (stool, hair and cord blood) and 2) the contributionsof gut microbial mercury methylation/demethylation to stool methylmercury.

METHODS: Pregnant women (36-39 weeks gestation, n=17) donated hair and stool specimens, and cord blood was collected for a subset (n=7). The diversity of gut microbiota was determined using16S rRNA gene profiling (n=17). For 6 stool samples with highest/lowestmethylmercury concentrations, metagenomic whole genome shotgun sequencing was employed to search forthe mercury methylation gene (*hgcA*), and two *mer* operon genes involved in methylmercury detoxification (*merA* and *merB*).

RESULTS: Seventeen bacterial genera were significantly correlated (increasing or decreasing) with stool methylmercury, stool inorganic mercury,or hair total mercury; however, aside from one genus, there was no overlap between biomarkers. There were no definitive matches for *hgcA* or *merB*, while *merA* was detected at low concentrations in all six samples.

MAJOR CONCLUSIONS:Proportional differences in stool methylmercury were not likely attributedto gut microbiota through methylation/demethylation. Gut microbiotapotentially altered methylmercury metabolism using indirect pathways.

KEYWORDS: prenatal, mercury, gut, microbiota,microbiome, metabolism

78 1. INTRODUCTION[1]

79 Microbes modulate the toxicity of mercury (Hg) through methylation of less toxic
80 inorganic Hg(II) (IHg)and demethylation (i.e., detoxification) of methylmercury (MeHg)
81 (Barkay et al 2003; Gilmour et al. 2013; Parks et al. 2013; Smith et al. 2015). The distal
82 gastrointestinal tract is one of the most densely populated ecosystems, with greater than 10^{11}-10^{12}
83 organisms per mL of luminal content. Thus the gut ispotentially an important reservoir for Hg
84 cycling and MeHg metabolism.

85 Several animal and human studies confirmed gut microbiotamodulate the enterohepatic
86 cycling of MeHg. Co-consumption of high-fiber foods, including wheat and fruit, were
87 associated with lower absorption of MeHg into tissues (Passos et al. 2007; Rowland et al. 1984,
88 1986), presumably due to higher elimination of MeHg.Treatment of animals with antibiotics
89 reduced decomposition of MeHg in the large intestine compared to controls (Seko et al. 1981),
90 and increased the half-time of MeHg elimination (Rowland et al. 1984), implicating gut
91 microbes in MeHg metabolism. Microbial MeHg detoxification involvesMerA, the mercuric
92 reductase, andMerB, the organomercurial lyase(Barkay et al., 2003). Both genes encoding these
93 proteinswere recovered from human and non-human primate feces, verifyingdecomposition of
94 Hg species by gut microbiota potentially occurred (Liebert et al. 1997).Tanzanian pregnant
95 women who consumed probiotics daily (i.e., yogurt) had significantly lower blood Hg
96 concentrations compared to controls, matched by age, nutritional status and fish intake (Bisanz et
97 al. 2014). Methylation of IHg by gut microbes is also possible;to date, one commensal
98 methanogen (*Methanomassiliicoccus luminyensis*)isolated from human feces (Dridi et al. 2012)
99 contained the gene cluster (*hgcA* and *hgcB*) required for IHg methylation (Parks et al.
100 2013).However, the capacity of intestinal bacteria to methylate Hg was observed by some

[1] Abbreviations: BMI, body mass index; CH_2Cl_2, dichloromethane; CMMR, Alkek Center for Metagenomics and Microbiome Research; CVAFS, cold vapor atomic fluorescence spectrometry; DDI, double-distilled; USEPA, U.S. Environmental Protection Agency; GC, gas chromatography; Hg, mercury; Hg(0), elemental mercury; IHg, inorganic mercury(II); IOM, Institute of Medicine of the National Academies; KEGG, Kyoto Encyclopedia of Genes and Genomes; MeHg, methylmercury; NIH, U.S. National Institutes of Health; NRC, National Research Council; ORNL, Oak Ridge National Laboratory; OTU, Operational Taxonomic Units; PCoA, Principal Coordinates Analysis; QIIME, Quantitative Insights Into Microbial Ecology; THg, total mercury; WGS, whole genome shotgun sequencing

researchers (Rowland et al. 1975), but not others (Zhou et al. 2011). The mechanisms by which gut microbes contribute to MeHg cycling in the human body remain unclear.

This pilot study involves trimester 3 pregnant women because the fetus is the most vulnerable population to the deleterious effects due to MeHg exposure [National Research Council (NRC) 2000], and trimester 3 coincides with the period when brain growth is most rapid. Pregnancy is also associated with changes in gut microbiota composition (Koren et al. 2012; Santacruz et al. 2010), which may alter MeHg metabolism, and hence fetal MeHg exposure. The following hypotheses were investigated:1) gut microbiota alter the speciation and toxicity of Hg through Hg(II) methylation and/or MeHg demethylation, and 2) gut microbiota affect MeHg bioavailability to the developing fetus by altering Hg speciation.

2. MATERIALS AND METHODS

Recruitment. Between November-December 2013, pregnant women from the Greenville Health System (Greenville, South Carolina,USA)were invited to participate, who were 36-39 weeks pregnant, at least 18 years of age, and in good general health. Nineteen mothers provided informed consent, including17 mothers who donated both hair and stool samples (89%). Cord blood samples were obtained for a subset (n=7, 37%). Trimester 1 (8-12 weeks gestation) body mass index (BMI) (kg/m^2), weight change during pregnancy, ethnicity, and antibiotic treatment were determined from the medical record (Table 1).Adherence to Institute of Medicine (IOM) of the National Academies Guidelines for healthy weight gain during pregnancy (IOM 2009)was determined from trimester 1 BMI and weight change at recruitment. Protocols were approved by the Institutional Review Boards at Greenville Health System and the University of South Carolina.

Biomarker collection and preservation. A hair sample wascollected from the occipital region, which was tied with a string and stored in a plastic bag at room temperature. Mothers were given a stool collection kit including sterile collection containers (Thermo Fisher02-544-208) and detailed instructions adapted from the Human Microbiome Projectprotocols [Human Microbiome Project (HMP) 2010]. Participants shipped stool samples overnightto the University of South Carolina. Upon receipt, stool samples were aliquoted using sterile microspatulas (Corning 3012)into 2 mL sterile cryovials (Thermo Fisher50-476-502), and then a separate sample was aliquoted using acid-washed utensils intopre-weighed acid-washed 50 mL

polypropylene vials. Vials were stored frozen (-80°C) until analysis.Whole blood wascollected from the umbilical cord into vials (Becton Dickinson, K2EDTA, Royal Blue) and frozenat -20°C and then at -80°C.

Hg analyses for hair. Hair samples corresponding to trimester 3 were analyzed, based on the monthly growth rate of hair (Loussouarn et al. 2005), assuming a 10-day lag between MeHg intake and absorption into the hair shaft (Cernichiari et al. 1995). Hair samples were washed in 1% (v/v)2-mercaptoethanol, shaken for 1 hour, then triple rinsed withdouble-distilled (DDI)-H_20, and air-dried overnight in a Class II Biosafety Hood (Baker Company, Sanford, ME, USA). Hair total Hg(THg) (~10 mg) was analyzed with a portable Hg vapor analyzer (Lumex, Model RA-915+/PYRO-915+, St. Petersburg, Russia) using thermal decomposition and atomic absorption spectrophotometry[U.S. Environmental Protection Agency (USEPA) Method 7473](USEPA 2007). There was insufficient hair for MeHg analysis.

Hg analyses for stools. For THg, stool specimens (~0.6 g) were digested in 10 mL of freshly prepared 7:3 nitric:sulfuric acid, samples were gently refluxed for 3 hours in a water bath (85°C), then oxidized with 0.2 N bromine monochloride. Excess oxidant was neutralized with hydroxylamine hydrochloride, and Hg was further reduced with stannous chloride, converting IHg to Hg(0). Quantification was by gold amalgamation followed bycold vapor atomic fluorescence spectrometry (CVAFS) (Brooks Rand Model III, Seattle, WA, USA)(EPA Method 1631) (USEPA 2002). For MeHg, stool samples (~0.6 g) were leached in 1.5 ml 1 M coppersulfate, 7.5 mL 25% nitric acid, and 10 mL dichloromethane (CH_2Cl_2) and MeHg was back-extracted into DDI-H_2O(Liang et al. 2004).MeHg extracts were analyzed following EPA Method 1630 (USEPA 2001)using gas chromatography (GC)-CVAFS (Brooks Rand Model III).Stool THg and MeHg concentrations were reported in dry weight, which was determined after drying a subsampleat 105°C overnight.

Hg analyses for cord blood. For THg,~0.7 g of blood were analyzed using EPA Method 7473 (22) (as described above).For blood MeHg, the same steps for stool MeHg were followed, except leaching procedures differed:~0.6 g of blood were dried in a 70°C oven overnight, then leached in2 mL 25% potassium hydroxide:methanol (w/v) for 3 hours in a 75°C oven, then 10 mL CH_2Cl_2 and 2 mL hydrochloric acid were added (Liang et al. 2000). MeHg was quantified by EPA Method 1630 (EPA 2001) using GC-CVAFS (as described above).

Hg quality assurance/quality control(QA/QC). QA/QC parameters are summarized in Table S1. Matrix-specific detection levels were based on the average mass of sample analyzed (hair: 0.01 g, stool: 0.7 g, and blood: 0.6 g) and the region of the calibration curve where there was a significant change in sensitivity, includinghair (THg: 9.5 ng/g), stool (THg: 0.033 ng/g, MeHg: 0.0017 ng/g), and blood (THg: 0.14µg/l, MeHg: 0.0017 µg/l).Two values were below the detection level, and half the detection level was imputed.

Gut microbiota and 16S rRNA gene profiling. Frozen stool samples (-80^{o}C) were shipped overnight to the Alkek Center for Metagenomics and Microbiome Research (CMMR) at Baylor College of Medicine. Microbial genomic DNA was extracted using the PowerSoil DNA Isolation Kit according to the manufacturer's instructions (MoBio Laboratories, CA, USA), and the concentration and purity of the extracted DNA were evaluated through gel electrophoresis and PicoGreen assays (Invitrogen, NY, USA).

For all stool samples (n=17), the 16S rDNA V4 region was amplified by polymerase chain reaction (PCR) using bacteria/archaeal primers 515Fand 806R (see Table S2). Sequencing was performed on the MiSeq platform (Illumina, CA, USA) using the 2 ×250 bp paired-end protocol,which yielded pair-end reads that almost completelyoverlapped. The primers contained adapters for MiSeq sequencing and dual-index barcodes so that the PCR products werepooled and sequenced directly (Caporaso et al. 2012), targeting at least 15,000 reads per sample. The 16S rRNA gene pipeline data incorporates phylogenetic and alignment-based approaches to maximize data resolution. The read pairs were demultiplexed based on unique molecular barcodes, and merged using USEARCH v7.0.1001 (Edgar 2010). The CMMR pipeline for 16S analysis leverages the QIIME (Quantitative Insights Into Microbial Ecology) software package (Caporaso et al. 2010), as well ascustom analytic packages.16S rRNA gene sequences were classified into Operational Taxonomic Units (OTUs) at a similarity cutoff value of 97% using the UPARSE pipeline in QIIME and the SILVA Database (Quast et al. 2013), and abundances were recovered by mapping the demultiplexed reads to the UPARSE OTUs database. An OTU table was constructedfor taxonomic summaries and calculate alpha- and beta-diversity(Lozupone and Knight 2005).

Whole genome shotgun (WGS) sequencing. For 6 (of 17) samples, metagenomic libraries were subjected to WGS (Illumina), including 3 samples with high (≥ median) stool MeHg (# 134, 146, and 157) and 3 with low (< median) stool MeHg (# 101, 118, and 163). To search Hg

methylation genes, nucleotide sequence reads were converted into amino acid sequences by six-frame translation. Although both HgcA and HgcB are considered essential for microbial Hg methylation (Parks et al. 2013; Smith et al. 2015), most organisms possessing HgcA also contain HgcB.Sequences of 77 HgcA homologs from bacteria and archaea with complete genome sequences (http://www.esd.ornl.gov/programs/rsfa/data.shtml) were obtained from GenBank. A 30-amino-acid-stretch encompassing the highly conserved 'cap helix' region of HgcA, containing the conserved motif TxG[IV]N[VI]WCA[AGS][GA][KE] (Parks et al 2013; Smith et al. 2015) was used to search for the presence of HgcAin the metagenomic libraries. For MerA and MerB, a reference database was created using the found sequences in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (Kanehisa et al. 2014). Metagenomic libraries were searched using USearch's usearch_local method with target coverage set to 0.8 (80% coverage) and ID threshold of 0.5 (50%).

Statistics. Alpha- and beta-diversity were used to examine associations between gut microbiota and dichotomized (high: ≥median, low: <median) Hg concentrations in biomarkers (stool MeHg, stool IHg, and hair THg). Alpha-diversity (within sample diversity) was measured with the Chao1 index (richness) (Chao 1984), the Shannon diversity index, and the observed number of sequences per sample. Beta-diversity (between sample diversity), defined as a measure of the evolutionary distance between gut microbiotas, was evaluated with the Unifrac distance metric(Lozupone and Knight, 2005).

Bivariate associations between Hg biomarkers (continuous, not dichotomized) and phylum- and genus-level gut microbiota abundances were assessedusing Spearman's and Pearson's correlation. Spearman's correlation was calculated with the untransformed variables. For Pearson's correlation, a $\log_{10}$-transformation was applied to right-skewed variables for gut microbiota abundancesafter increasing all values by 0.001% due to the number of 0's; 0.001%was considered a minimum detection level. For one genus (unclassified 00r39*Peptostreptococcaceae*), 0.001% was disconnected from the remaining observations, and therefore this value was not imputed for 0.Bivariate associationswere also determined between categorical data from the medical record and Hg biomarkers (hair, stool and cord blood), and phylum- and genus-level gut microbiota abundances using Wilcoxon rank-sum test or Student's 2-tailed t-test. Multiple test comparisons were applied using the Benjamini-Hochberg False Discovery Rate (FDR) procedure(Yekutieli and Benjamini, 1999), using a q-value of

0.20.Statistical analyses were completed using the R-platform or Stata (Version 9.2, College Station, TX, USA).

Accession numbers.This study is registered in the U.S. National Institutes of Health (NIH) Database for Genotypes and Phenotypes (dbGaP) under accession number phs000970.v1.p1.

3. RESULTS

Demographics. Demographic data and body measurements are included in Table 1.Trimester 1 BMI averaged 29 kg/m^2 (range 16-40 kg/m^2), including 76% of pregnant mothers classified as overweight or obese (i.e., BMI $\geq$ 25 kg/m^2). More than half the mothers exceeded IOM guidelines for healthy weight gain during pregnancy (IOM 2009). Gestational weight gain was similar for obese and non-obese mothers(t-test, p=0.80).

Biomarker Hg concentrations. Hair and cord blood THg and/or MeHg indicated low dietary intake of MeHg through fish consumption for most mothers. Hair THg concentrationsaveraged 57 ng/g (Table 2), which was 5-10 times lower compared to cohorts of U.S. pregnant women (average: 290-550 ng/g) (Oken et al. 2005; Xue et al. 2007). Cord blood THg averaged 2.9 times lower compared to blood Hg for U.S. adults(0.99 µg/L, n=10,673) (Nielsen et al. 2014), while the geometric mean for blood Hg was 1.9 times lower than Rhode Island mothers (this study: 0.28 µg/L; Rhode Island: 0.52 µg/L, n=538, from King et al. 2012).

Biomarker Hg correlations. Maternal hair THg and cord blood MeHg (or THg) are established biomarkers for fetal MeHg exposure (Cernichiari et al 1995; NRC 2000). Hair THg and cord blood MeHg concentrations were highly correlated when $\log_{10}$-transformed(Pearson's rho=0.91, p<0.01, n=7) (Figure 1).Stool MeHg was more positively correlated with cord blood MeHg (Pearson's rho=0.58, p=0.18, n=7) than hair THg (Pearson's rho=0.16, p=0.54, n=17) (when $\log_{10}$-transformed); however, both were non-significant(Figure 1).Results did not differ when the sample size was limited to mothers with cord blood data (Pearson's rho=0.24, p=0.61, n=7). These data suggested stool MeHg did not contribute significantly to fetal MeHg exposure.

Medical record data and Hgbiomarkers.There were no significant associations between Hg biomarkers [hair THg, stool MeHg, and stool IHg (IHg=THg-MeHg)], and categorical data from the medical record, including 1) trimester 1 weight status (obese/non-obese), 2) weight gain during pregnancy (excessive weight gain versus below or within IOM guidelines), and 3)

antibiotic treatment (yes versus no or uncertain) (Wilcoxon rank-sum test, p = 0.15-0.92, n=17; t-test, p = 0.17-0.90, n=17).The latter results were surprising because antibiotic treatment altered the enterohepatic cycling of Hg in mice (Rowland et al. 1984; Seko et al. 1981). For both animal studies, MeHg-chloride was administered near the same time as antibiotics(seven days after antibiotics started, Rowland et al., 1984; two days before antibiotics started, Seko et al., 1981). In the present study, antibiotic treatment occurred within the previous three months and possibly did not coincide with dietary MeHg intake.

16S rRNA gene profiling of gut microbes. The total number of mapped reads for 17 stool samples was 356,456, averaging 20,968 reads per sample; analyses were performed on an equal number of reads per sample (15,928).16S rRNA reads were assigned to 7 phyla, including the following 5 phyla representing 98 ± 0.94 % of gut microbes. On average (± 1 SD), *Firmicutes* comprised 56% ± 17%, followed by *Bacteroidetes* (17% ± 17%), *Actinobacteria* (17% ± 19%), *Verrucomicrobia* (3.9% ± 7.2%), and *Proteobacteria* (3.1% ± 6.9%).The 7 phyla were represented by 81 genera (averaging 52 ± 7.2 genera per mother), including 21 genera with abundances >1% (Supplemental Material, Figure S1). The most abundant genera were*Bifidobacterium*(15% ± 18%), *Bacteroides*(11% ± 13%), *Alistipes* (4.1 ± 5.3), *Subdoligranulum* (5.7% ± 4.6%), *Blautia*(5.5% ± 4.3) unclassified *Lachnospiraceae* spp. (4.8% ± 3.9%), and *Akkermansia* spp. (3.9%± 7.2%). The latter was the sole representative of the *Verrucomicrobia* phylum, and therefore results/discussion for *Verrucomicrobia* were omitted.

Indices for microbial richness (i.e., alpha-diversity)did not differ significantly for gut microbiotas with high (≥ median) and low (< median) concentrations of hair THg and stool IHg (p=0.44-0.85) (Figure S2). Microbial richness estimated by the Chao1 index was significantly higher for mothers with high stool MeHg concentrations ($p<0.05$), while there were no significant differences using other indices (p=0.29-0.50). Chao1 is more sensitive to rare species, whereas the Shannon index estimates the uniformity of sequences (Chao 1984), suggesting differences between high/low stool MeHg are due to the presence/absence of rare gut microbial species.Beta-diversity patterns were visualized using Principal Coordinates Analysis (PCoA) (Figure S3). For all three outcomes (stool MeHg, stool IHg, and hair THg)beta-diversity did not differbetween high/low groups using unweighted and weighted Unifrac distance metrics (p=0.11-0.64).

285 Medical record data and gut microbiota. Like Hg biomarkers, there were no significant
286 associations between gut microbiota phyla and categorical data from the medical record,
287 including 1) trimester 1 weight status (obese/non-obese), 2) weight gain during pregnancy
288 (excessive weight gain versus below or within IOM guidelines), and 3) antibiotic treatment (yes
289 versus no or uncertain) (Wilcoxon rank-sum test, p=0.07-0.92). Among gut microbiota
290 genera,abundance of *Blautia*(phylum: Firmicutes)was significantly higher for mothers who
291 gained excessed weight (Wilcoxon rank-sum, $p<0.05$), and unclassified members of the
292 genus*Ruminococcaceae*(phylum: Firmicutes)were significantly higher for mothers prescribed
293 antibiotics (Wilcoxon rank-sum, $p<0.05$); however all correlations were not significant at a FDR
294 of 20%.The abundance of *Akkermansia* spp. was higher (but not significantly) for mothers that
295 gained excessive weight compared to normal weight (Wilcoxon rank-sum, p=0.09). This trend
296 differed from Santacruz et al. (2010), who reported significantlylower abundance of
297 *A.muciniphilia* amongSpanish pregnant women that gained excessive weight (n=16)compared to
298 normal weight (n=34).
299 Bacterial diversity and Hg biomarkers. For continuous Hg biomarkers (stool MeHg,
300 stool IHg, and hair THg), significant associations were observed between stool MeHg and
301 *Proteobacteria*using Pearson's correlation (rho = -0.61, $p<0.01$), but not Spearman's correlation
302 (rho = -0.45, respectively, p=0.07). However, thisinverse association was driven by a single
303 observation; when this observation was removed Pearson's correlation was no longer significant
304 (p=0.13). Seventeen genera (of 81) were significantly correlated (Spearman's and/or Pearson's)
305 with stool MeHg, stool IHg or hair THg ($p<0.05$), including three genera that were significantly
306 correlated at a FDR of 20% (Figure 2, Table S3).Two genera were positively associated with
307 stool MeHg (both were unclassified members of the *Ruminococcaceae* genus), and one genus
308 was inversely associated with stool IHg (*Moryella*). There was no overlap between taxonomic
309 groups significantly correlated with stool MeHg, stool IHg or hair THg, aside from one genus
310 (*Subdoligranulum*) (Figure 2, Table S3).
311 Metagenomic WGS results. Among 6 stool samples, including 3 with low MeHg and 3
312 with high MeHg, there were no definitive matches for HgcA. Although some of the matches
313 overall showed a high level of sequence identity to known HgcA sequences, significant sequence
314 variability was observed near the strictly conserved cysteine in the 'cap-helix' region, which was
315 expected to be the least variable region (Parks et al. 2013; Smith et al. 2015). For MerA, low

316 abundant hits (<0.001%)were observed across all six samples with good identity scores, while
317 MerB produced no hits.
318
319 4. DISCUSSION
320 Stool MeHg and dietary MeHg intake. Results suggested stool MeHg concentrations did
321 not likely reflect biotransformation by gut microbiota through Hg(II) methylation or
322 demethylation, thus rejecting hypothesis 1. To date, one commensal methanogen (*M*.
323 *luminyensis*) isolated from human fecescontained the gene cluster required for IHg methylation
324 (Dridi et al. 2012; Parks et al. 2013). In this study, the proportional abundance of this genus was
325 less than 0.001%, suggesting: 1) the abundance of *M*. *luminyensis*was too low to be detected, or
326 2) dietary MeHg intake contributed to stool MeHg. For 6 samples, including 3 with the
327 highest/lowest stool MeHg content, both *hgcA*and *merB*genes were not present. Microbial
328 methylation/demethylation did not likely contribute to the net stool MeHg concentrations,
329 supporting the second assumption. Alternatively, MeHg was demethylated abiotically, for
330 example, via phagocytosis (Suda et al. 1992). Gut microbiota possibly demethylated MeHg
331 using a different metabolic pathway (i.e., oxidative demethylation), which was reported in
332 anaerobic freshwater sediment (Barkay et al. 2003; Oremland et al. 1991); however no specific
333 genes have been identified with this process.
334 Ishihara (2000) published the only other study to date (to the best of our knowledge)
335 including IHg and MeHg concentrations for stool specimens from 4 Japanese men, who ingested
336 fish regularly (unlike most mothers in this study). Stool MeHg averaged 7.0 ng MeHg/g wet
337 weight and stool %MeHg (of THg) averaged 17%, while hair THg averaged 4800 ng/g (Ishihara
338 2000), which were 72, 22, and 84 times higher than corresponding values for this study (Table
339 2). The magnitude difference for stool MeHg was likely higher (i.e., 200-300, not 72), because
340 Ishihara (2000) recorded stool Hg concentrations in wet weight (median wet:dry ratio for this
341 study = 3.50).
342 The distal gut is considered the primary site where MeHg is demethylated to IHg, which
343 is less likely to be absorbed through the intestinal wall and thus excreted, reducing human MeHg
344 body burden (Clarkson and Magos 2006). Results from this study and Ishihara (2000) indicated
345 not all MeHg was demethylated before excretion. Combined with the analysis of

hair/blood/stool (Figure 1), stoolMeHg did not correlate significantly with fetal MeHg exposure, i.e., MeHg was not bioavailable.

Hg biomarkers and gut microbial taxa. Seventeen genera were significantly associated with Hg biomarkers (stool MeHg, stool IHg, and hair THg), and aside from one genus (*Subdoligranulum*), there was no overlap between biomarkers (Figure 2). A majority of genera were not significantly associated with Hg biomarkers at a FDR of 20%; however these modest associationssuggestedother pathways were possibly more important for MeHg metabolism and exposure. For some genera their functions are well known, which are discussed below.

Akkermansia spp. were positively correlated with stool MeHg (when $\log_{10}$-transformed) (Pearson's rho=0.50, $p<0.05$). To date, no species in this phylum (*Verrucomicrobia*) have been identified, which containthe gene cluster required to methylate IHg (i.e., *hgcA* and *hgcB*) (Parks et al. 2013). The intestinal tract is covered by a mucus layer, which insulates gut microbes from host tissues and protects the epithelium from pathogenic microorganisms, as well as toxins and acids(Derrien et al. 2004). *A. muciniphila*is the dominant human bacterium that resides within the mucus layer and degrades mucin, providing usable energy to non-mucolytic bacteria (Derrien et al. 2004).Enrichment of *A. muciniphila* is associated with stronger gut barrier function (Png et al. 2010), and is considered a biomarker for a healthy intestine (Belzer and de Vos 2012).

We hypothesize that gut microbiota contributed indirectly to MeHg metabolism through improved gut barrier function, whichprevented re-absorption of MeHg through the intestinal epithelial layer and concentrated MeHg in the stool.Using an in vitro model, Vázquez et al. (2013) reported the mucus layer represented a barrier to transport of MeHg and inorganic Hg(II), trapping 70% and 40%, respectively, supporting an association between intestinal permeability and Hg absorption into systemic circulation.Higher absorption of other metals (cadmium and lead) was reported for germfree mice compared to controls, which was attributed to differences in the gut barrier function and expression of host-genes such as metallothioneins (Breton et al. 2013). Therefore, gut microbiotamay reduce MeHg bioavailability and fetal exposure through indirect pathways (i.e., intestinal permeability), as well asdirect pathways (i.e., methylation/demethylation).

Other genera were significantly associated with stool MeHg (positive: *Subdoligranulum*, negative: *Streptococcus* spp.). *Streptococcus* spp. is one of the most dominant microbes in breast milk, and some *Streptococcus* spp. are associated with greater pathogen resistance among

preterm neonates (Martín et al. 2004). Both genera were previously reported in maternal stool and breast milk, and the authors suggestedthe maternal gut was a source of microbiota to breast milk (Jost et al. 2014). From this study, both positive and inverse associations indicatedietary MeHg intake may impact maternal stool gut microbiota, and possibly breast milk microbiota composition, which should be further investigated.

5. CONCLUSIONS

Methylation of IHg and reductive detoxification of MeHg mediated by the *mer* operon are two known pathways that microbes use to alter Hg cycling (Barkay et al. 2003; Gilmour et al. 2013; Liebert et al. 1997; Parks et al. 2013; Smith et al. 2015). It is thought that gut microbes demethylate MeHg and reduce bioavailability to cross the intestine into systemic circulation (Clarkson and Magos 2006). Although this was a small data set, results from this pilot study suggested gut microbes possiblyreduced MeHg bioavailability and exposure byindirect pathways. This conclusion is supported in part by an absence of *hgcA* and *merB* in 6 stool samples with highest/lowest stool MeHg concentrations, and in part by associations between Hg biomarkers and gut microbial taxa with known functions(e.g., *Akkermansia*spp.).Future research should consider both direct and indirect pathways by which gut microbiota affect MeHg metabolism and exposure. Direct pathways include microbial methylation/demethylation and indirect pathways include changes in the gut barrier function.

Findings from this analysis are limited. A food frequency questionnaire was not administered, and dietary MeHg intake was inferred from Hg biomarkers. In addition, diet profoundly affects the microbiome (Wu et al. 2011), and observed associations between Hg biomarkers and gut microbiota taxa may be confounded by diet. Dietary MeHg intake was low for most mothers, and therefore results were not necessarily applicable to populations ingesting more fish.Most mothers were overweight or obese, which possibly affected MeHg metabolism (Rothenberg et al. 2015). However, aside from one genus (*Blautia*), there were no significant associations between obesity and gut microbiota, and there were no genera significant at the FDR of 20%. Stool specimens do not accurately reflect microbial activity in proximal regions of the gastrointestinal tract (Eckburg et al. 2005), where IHg methylation and/or demethylation possibly occurred, and may not represent the mucosa-associated bacteria (Png et al. 2010). This is a cross-sectional study, which precludes us from making any inferences about direction or

causality. Lastly, this study was limited to a few weeks in late-stage pregnancy and may only be suggestive of windows of susceptibility to toxic insult from MeHg for certain endpoints, while missing susceptibility windows for other endpoints.

In spite of these limitations, profiles of gut microbes differed dramatically between Hg biomarkers, and their roles in the transformation of MeHg and/or reducing MeHg exposureshould be elucidated.

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8. FIGURE LEGENDS

Figure 1. Bivariate scatterplots relating total mercury (THg) or methylmercury (MeHg) concentrations between a) hair-cord blood, b) stool-cord blood, and c) stool-hair, including Pearson's correlation (rho).

Figure 2. Venn diagram including gut microbial genera that were significantly correlated with stool methylmercury (MeHg), hair total mercury (THg), or stool inorganic mercury(II) (IHg) using Spearman's and/or Pearson's rho (n=17) ($p<0.05$).(*) indicates significance at a False Discovery Rate of 20%, arrows represent the direction of correlation, and bolded numbers indicate relative abundance was >1% (see Table S3).

(Graphical Abstract also provided)

Table

TABLES

Table 1. Demographic data for 17 participants.

	Average ± 1 SD (range)
Gestation (weeks)	37 ± 0.94 (36 - 39)
Trimester 1 BMI (kg/m^2)	29 ± 6.7 (16 - 40)
Weight gain (kg)	14 ± 7.8 (1.4 - 33)
	n (%)
Ethnicity	
White	7 (41)
Black	7 (41)
Hispanic	3 (18)
Weight class	
Underweight	1 (5.9)
Normal	3 (18)
Overweight	5 (29)
Obese	8 (47)
IOM Guidelines	
Below	2 (12)
Within	6 (35)
Above	9 (53)
Antibiotic treatment previous 3 months	
No	10 (59)
Yes	6 (35)
Uncertain	1 (5.9)

Institute of Medicine (IOM), trimester 1 body mass index (BMI)

Table 2. Summary statisticsfor total mercury, methylmercury and percent methylmercury (of total mercury) in biomarkers, including maternal hair, maternal stool, and cord blood.

Parameter	**Hair (ng/g) (n=17)**		**Stool (ng/g) (n=17)**		**Cord Blood (μg/L) (n=7)**	
	Average (range)	**Median**	**Average (range)**	**Median**	**Average (range)**	**Median**
THg	57 (BDL-230)	27	150 (2.1-810)	30	0.34 (BDL-0.67)	0.35
MeHg	NA	NA	0.097 (0.0025-0.39)	0.060	0.23 (0.061-0.73)	0.18
%MeHg (of THg)	NA	NA	0.78 (0.0058-5.8)	0.12	69 (25-124)	50

Below detection level (BDL), methylmercury (MeHg), not applicable (NA), total mercury (THg)

Figure

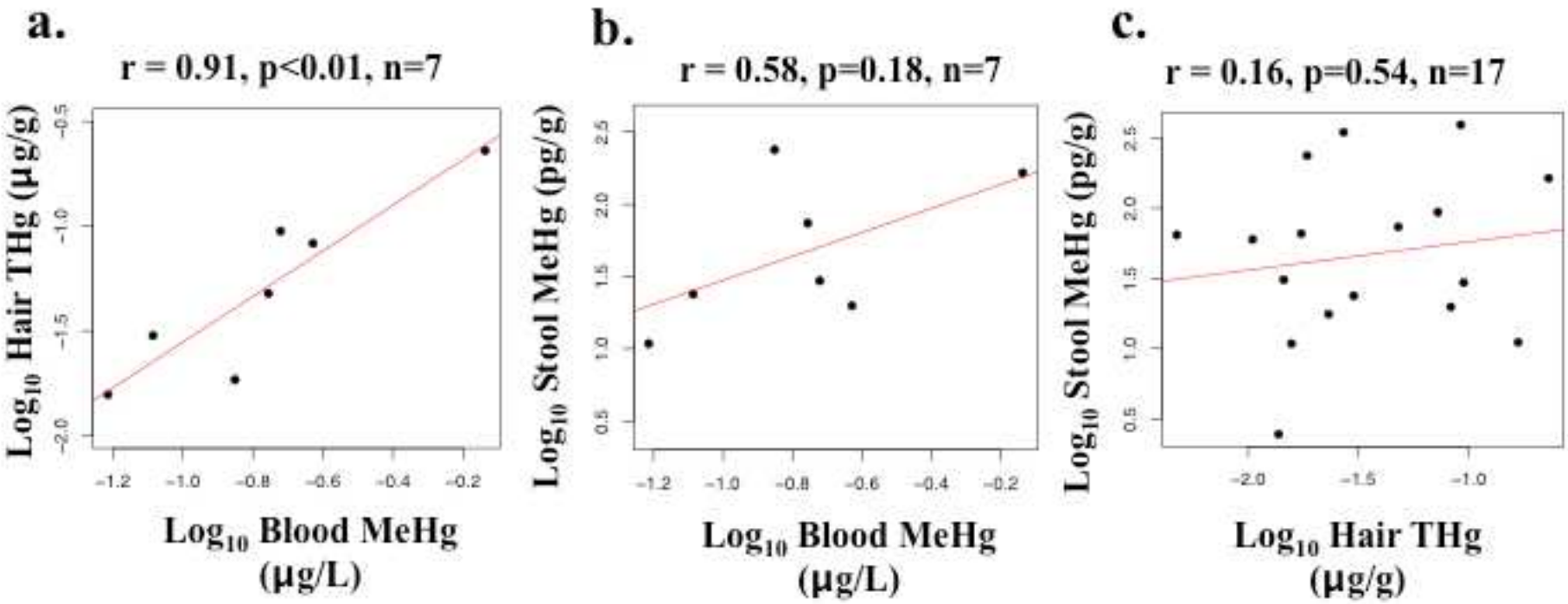

Figure 1.

Figure

Genera

1. ***Akkermansia* spp.**
2. ***Clostridiales*, Uncl. 0065e**
3. Erysipelotrichaceae, Uncl. 62248
4. ***Faecalibacterium***
5. *Lachnospiraceae*, Uncl. B9304
6. ***Lachnospiraceae*, Uncl. 00bd9**
7. *Moryella**
8. ***Peptostreptococcaceae*, Uncl. 00r39**
9. *Ruminococcaceae*, Uncl. 00jsh
10. *Ruminococcaceae*, Uncl. 76946
11. *Ruminococcaceae*, Uncl. 02cp1*
12. *Ruminococcaceae*, Uncl. 02fdk
13. *Ruminococcaceae*, Uncl. B1957*
14. *Ruminococcaceae*, Uncl. 009c6
15. ***Streptococcus***
16. ***Subdoligranulum***
17. Turicibacter

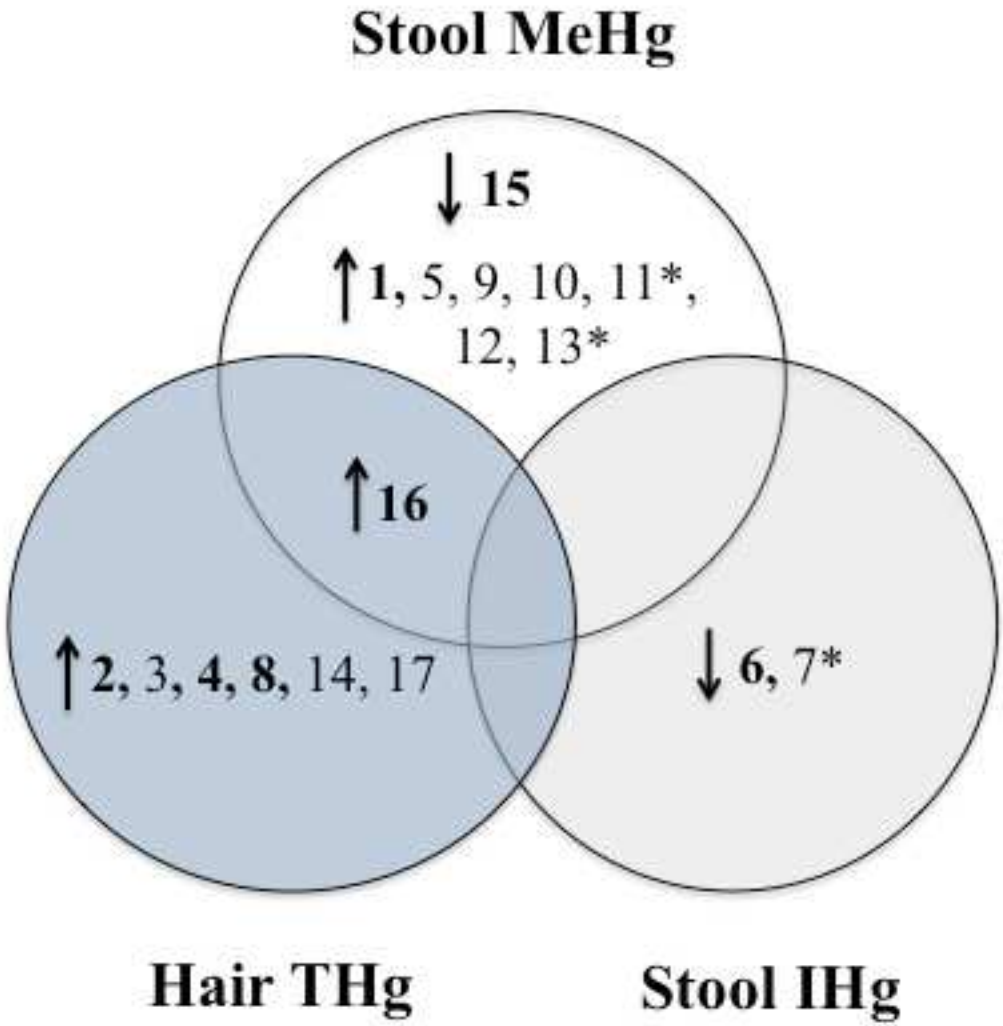

Figure 2