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**Microbial mechanisms of methylmercury metabolism in humans.**

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## 1. Background and Purpose of study.

**1 A. Application Background.** This submission seeks approval of a new study that follows essentially the same protocol of two prior approved studies we have conducted, with a modification that includes an additional intervention of administration of a prebiotic to influence change in the gut microbiota. The prior studies were #RSRB00050613 entitled Methylmercury metabolism and elimination in humans and # RSRB00057934 entitled Determination of methylmercury metabolism and elimination status in humans.

**1 B. Purpose of the study.**

The overall objective of this study is to investigate the role of gut microbes in mediating how humans metabolize and excrete the environmental neurotoxicant methylmercury (MeHg). Our secondary objectives are: 1) to derive non-invasive tools to evaluate an individual's MeHg metabolizing potential, 2) to identify dietary supplement approaches to enhance an individual's capacity to metabolize and excrete toxic MeHg, 3) to discover symbiotic bacterial strains with probiotic potential. Successful completion of the study Aims will eventually lead to advanced methodologies to better assess the risks of Me Hg on human health and identify factors that mitigate this risk.

**1 C. Scientific Background.**

***MeHg impact on human health***. Exposure to MeHg through consumption of fish continues to pose a health risk for many populations globally. The developing nervous system is a preferred target for MeHg toxicity, making the fetus and young children most vulnerable. Conflicting results from large epidemiological studies has lead to uncertainty in advising the public on mercury risks associated with fish consumption [1-3]. At odds are the potential harmful effects of Me Hg and the beneficial effects of dietary nutrients and antioxidants in fish. A great deal of uncertainty for this conflict stems from the fact that MeHg metabolism and elimination rate is known to vary widely from individual to individual. This translates into the possibility that two individuals consuming the same amount of fish with the same frequency could, unknowingly, experience as much as 4-fold difference in MeHg in their bodies. Thus, there is a need for greater understanding of the mechanisms of MeHg metabolism and elimination, as well as for development of tools to assess these characteristics in people.

***MeHg toxicokinetics: the importance of elimination rate***. Orally ingested MeHg is absorbed very efficiently in the intestine with 95% of the dose typically being taken up and distributed to the bloodstream [4]. MeHg undergoes rapid transport and equilibration between blood and target organs [4]. Subsequent to an initial "spike" in concentration, mercury levels in blood resolve to a slow steady rate of decline that is dictated by the overall elimination rate of Me Hg from the body [4-6]. Elimination rates,

expressed as half time ($t_{1/2}$), range from <30 to >120 days, demonstrating the existence of "fast" and "slow" MeHg metabolizers [4-11]. The elimination rate of MeHg is a major determinant for accumulation of MeHg to the elevated steady state levels that occur in people who consume fish on a regular (e.g. weekly or daily) basis. The ability to determine the MeHg metabolism and elimination status of an individual is therefore a means of prospectively identifying "fast" versus "slow" metabolizers.

***Mechanisms of MeHg metabolism and elimination: the critical role of de-methylation***. Approximately 90% of the MeHg ingested and absorbed in the body is ultimately excreted via the feces [5, 7]. The current dogma holds that MeHg excretion occurs via the liver, where glutathione-MeHg conjugates are transported via bile to the small intestine [12]. However, a "vicious cycle" exists in that MeHg can be rapidly re-absorbed in the intestine and returned to circulation [13]. This cycle can be broken by MeHg de-methylation, which yields $Hg^{++}$, a poorly absorbed inorganic (iHg) form of mercury [14]. This mechanism is consistent with observations that Hg stemming from a MeHg dose is typically found as 90-100% iHg in the feces [15]. Rodent studies, using variations in diet and antibiotics, reinforce two principles regarding MeHg de-methylation: 1) it is a rate-limiting step in elimination of MeHg and, 2) it is supported by the microbiota of the gut [16] [17]. The mechanism by which human gut bacteria might de-methylate MeHg remains unclear. Furthermore, distinct species of bacteria with MeHg degrading activity within the human gut have yet to be characterized. Nonetheless, MeHg degrading bacteria in the environment, common to sediments and soils, have been well characterized. Such MeHg degrading strains carry a mercury (Mer) locus, which harbors genes encoding alkylmercury lyase (MerB) and mercury reductase (MerA) that encode enzymes capable of de-methylating MeHg and reducing Hg, respectively [18]. There is a possibility that related strains of microbes carry these genes in the human gut.

***A new method to measure MeHg metabolism in people***. We have recently overcome a significant barrier to implementing kinetic studies of MeHg in people by developing a simple, non-invasive protocol for sampling MeHg metabolism and excretion over time. We have published this in two papers, both stemming from prior IRB-approved human studies. In short, we accomplish this by assaying two samples of biological media: human hair and feces. Through application of current technology and instrumentation (i.e., laser ablation-ICP-mass spectrometry (LA-ICP-MS)) we have established a protocol to quantify MeHg elimination rate ($t_{1/2}$) and correlate it with demethylation (fecal iHg:MeHg ratio) in individuals who have previously eaten just three fish meals. We are now in a position to capitalize on this methodology to extend our investigations of the microbiota of the human gut to identify the mechanisms and the bacterial species responsible for MeHg metabolism and excretion.

**1D. Specific Aims**.

It should be noted that the human subject portion of this proposed study is part of consortium among the P.I. (M. Rand, U of R) and co-investigators at Montana State University (MSU), Drs. Seth Walk and Eric Boyd. This study will be supported by NIH ViCTER mechanism specifically designed to support such "Virtual Consortium" with three investigators. **As such, all aspects of human subject recruitment and bio-sample collection and sample coding will be performed in Aim 1.** Aims 2 and 3 will

involve studies in mice and with defined bacterial cultures and will be conducted with de-identified samples of feces or fecal DNA at Montana State University.

Our scientific premise is that symbiotic microbes in the human gut are required for the efficient biotransformation and excretion of toxic MeHg. There is substantial evidence, including our own, that the rate of MeHg elimination in the human body relies on gut microbiota. Importantly, we discovered that MeHg elimination kinetics in an individual can vary significantly over time and, furthermore, is significantly slowed when taking antibiotics [10, 11]. Our initial findings with microbiome characterization yielded logical candidate bacterial taxa that now require validation to test their role in MeHg elimination [19]. With this evidence, we **hypothesize that a susceptibility for reaching harmful levels of MeHg in the body is dictated by select members of the human gut microbiome that: a) carry out MeHg demethylation to enhance excretion, b) are variably present or variably abundant between and within individuals over time and c) are metabolically responsive to diet supplementation.** Our Specific Aims are:

**Aim 1. Establish gut microbiome samples that exhibit "fast" and "slow" MeHg kinetics in humans.**

**Aim 2. Validate the microbiome's role in MeHg kinetics using germ-free/gnotobiotic mouse modeling.**

**Aim 3. Identify and isolate microbial species responsible for MeHg demethylation in the human gut.**

## 2. STUDY DESIGN

### 2A. Overview

**Study site:** This study will involve human subjects recruited from the greater Rochester area and will be conducted in the URMC Department of Environmental Medicine. Hg analyses and DNA extraction of fecal samples will be performed in the U of R Environmental Health Science Center (EHSC) Elemental Analysis Facility. Fecal samples will also be evaluated for MeHg demethylation activity by assaying bacterial enzyme activity in Rochester. Hg analyses of hair samples by LA-ICP-MS will be done at the Trace Element Analysis lab at Dartmouth College, New Hampshire. Additional studies, exempt from IRB approval, will be done at Montana State University with de-identified samples of human feces and fecal DNA that are shipped to co-investigators Walk and Boyd at MSU.

**Protocol:** Subjects will follow a protocol of eating three fish meals within a two-week period to incur exposure to naturally occurring levels of MeHg in the fish. Fish meals will consist of 6-12 ounces of commercially available tuna steaks. This will be followed by an elimination period of 60 days where no fish or seafood is to be consumed. Single hairs collected at 60 days after the last of the three fish meals will be used to determine a MeHg elimination rate using LA-ICP-MS to perform longitudinal Hg elemental analysis as we have previously reported [10]. De-methylation of MeHg coming from the fish will be determined by direct analysis of iHg and MeHg in fecal samples collected

approximately four weeks following the final fish meal. Fecal samples will also be used for culture assays and for direct iHg/MeHg analysis to determine of MeHg demethylation rates *in vitro* and *in vivo*, respectively (Biotransformation, $B_{in\ vitro}$ and $B_{in\ vivo}$). Fecal DNA will be used for metagenomic analyses to profile type and abundance of all bacterial species present. Statistical correlations between elimination rate, demethylation rate and abundance of bacteria will be used to deduce strains of bacteria that contribute to demethylation and overall metabolism and elimination of MeHg. The above protocol will be performed in two separate "trials" (which are denoted as "Study Periods" to the participants in the consent form) within the course of approximately 10 months and separated by an intermediate "washout" period of 4 months (Figure 1). Subject will not be required to refrain from fish or seafood during this washout period. Prior to the second trial subjects will receive a commercially available over the counter prebiotic (Prebiotin™) to be orally self-administered on a regular schedule for the entire trial 2 period. This prebiotic is not the subject of this investigation in this study, and is simply selected for its known effects of shifting gut microbial composition to enrich for healthier strains of bacteria (e.g. Bifidobacteria). Fecal sampling prior to and subsequent to prebiotic will be used to document changes in the microbiota for each individual. The prediction is that prebiotic induced change in the gut microbiota will result in a change in MeHg metabolism kinetics in each individual.

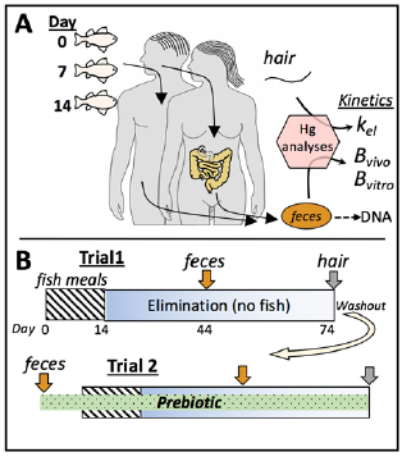


**Figure 1. Protocol Overview**. Elimination rate determination, $k_{el}$; Demethylation (Biotransformation) analysis , $B_{in\ vitro}$ and $B_{in\ vivo}$.

## 2B. Rationale for Study Design

The main rationale for this study design is based on our evidence that de-methylation of MeHg in the human gut is the driving force for faster elimination MeHg that comes with eating fish. We therefore predict that with the methods we now have available to measure MeHg kinetics, coupled that with high-resolution analyses of gut microbiome characteristics in individual people who have undergone a controlled exposure to MeHg, we can resolve a microbial basis for MeHg metabolism in humans. This is an ambitious undertaking, and hence, is designed as a consortium project to be executed by three investigators with respective expertise in 1) human MeHg kinetics, 2) mouse microbiome manipulations and 3) microbial metagenomics and culturing methods. By design, the human subject component of the study is to be carried out at URMC. The objective is to capture "slow" and "fast" MeHg metabolizing human microbiome samples, that can be handed off to colleagues at MSU for specialized analyses that do not involve the human subjects, simply their de-identified samples.

## 3. CHARACTERISTICS OF THE RESEARCH POPULATION

*(Many of the characteristics below are reiterated from our previously approved project: RSRB00050613 entitled Methylmercury metabolism and elimination in humans and RSRB00057934 entitled Determination of methylmercury metabolism and elimination*

*status in humans.)*

**3A. Subject Characteristics**

**Number of Subjects:** We aim to obtain data on 40 adults, 20 males and 20 females, for this study. We will therefore target consent of 46 participants (23 women, 23 men) to account for potential drop out. We expect the recruitment will be achieved locally in the Rochester area including the URMC community, but will not exclude interested people from outside this region. With the limited exclusion criteria below, we anticipate completing recruitment within the first several months of the study.

**Gender and Age of Subjects:** Study subjects will aim to be comprised of an equal number of men and women with no restriction on age ranging from 18 to 80.

**Racial and Ethnic Origin:** There will be no restrictions on racial or ethnic origin in subject recruitment. It is anticipated the racial and ethnic diversity of the study group will reflect the various ethnicities that comprise the general population of the greater Rochester metropolitan area.

**Dental amalgams**: Dental amalgams are a source of inorganic mercury. This study is not directed at inorganic mercury and prevailing literature indicates that inorganic mercury stemming from amalgams will not affect the biological and metabolic processes of MeHg under investigation here. Nonetheless, we will identify presence or absence of amalgams in subjects so that we can account for the contribution of this inorganic source of Hg to the Hg content measured in feces. This will be critical for evaluating the extent of MeHg demethylation that has occurred in each subject.

**Vulnerable Subjects:** Vulnerable subjects potentially include students and staff of the University of Rochester. Advertisements will be placed in areas where they may be seen by students, employees and the general public. For students and staff of the University of Rochester consent forms will clearly delineate that refusal to participate will have no consequence on job status, work place relationships, grades, or any other benefits to which the student/staff will otherwise be entitled.

**3B. Inclusion and Exclusion Criteria**

**Inclusion Criteria:**

- Subjects must be 18-80 years in age.
- Subjects must be in good general health based on self-reported health status, with the exception of self-reported conditions listed in the exclusion criteria.
- Subject must be willing to comply with study procedures.

**Exclusion Criteria:**

- Known allergy to fish.
- Pregnant or lactating women.
- Use of hair dyes or chemical treatments within the prior month and/or intent to use such treatments during the two trial periods. (Hair treatment during the washout period up to one month prior to the second trial is acceptable).
- Known gastrointestinal or renal disorders.
- Subjects with diminished mental capacity.
- Use of antibiotics within two months leading up to the study.

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**3C. Discussion of Subject Population**

The inclusion criteria are very broad and it is anticipated a large diversity in age and ethnicity will result. This is acceptable as age and ethnicity, although not primary study objectives in this pilot, are likely to produce variation in MeHg metabolism and elimination status, the determination of which is an objective of the study. Exclusion criteria are defined based on factors that are likely to result in either harm to the subject or to substantially alter the metabolic processes or analytical procedures of MeHg and thus obscure results. Of particular concern is exposure to the fetus or infant to MeHg, which is known to target the developing nervous system. This concern warrants exclusion of pregnant or breast-feeding women.

## 4. SUBJECT IDENTIFICATION, RECRUITMENT, CONSENT AND COMMUNICATION

### 4A. Method of Subject Identification and Recruitment

Subjects will be recruited through local advertising by posting RSRB-approved flyers around the University of Rochester Medical Center. Response via phone and email to these postings will be anticipated. The P.I. and Lab personnel will also inform friends and acquaintances about the study. Lab personnel will be prepared to tell interested persons of the existence of the study and provide interested persons with contact information for the P.I. for follow up. The Lab personnel will not inquire about health status or other personal information required of interested persons for consent. For phone inquiries the P.I. will perform an initial screening. Potential subjects will be asked if any of the following apply to them:

- Do you have any known allergy to fish?
- Are you pregnant or currently breastfeeding?
- Have you used hair dyes or treatments within the last month?
- Do you have any gastrointestinal or renal (kidney) disorders?
- Have you used antibiotics within the last month?

Subjects answering “no” to all of the above will be asked to follow up with the initial office visit.

**Process of Consent**

Subjects with Capacity: Only subjects with capacity will be recruited into the study. Subjects with capacity will be consented by the P.I. or study coordinator (TBD) listed on the protocol. The consent form will be reviewed with the subject directly, and the subject will also be offered the opportunity to review the consent form on their own. They will be encouraged to ask any questions and will be free to choose whether or not to participate.

Subject Comprehension: All subjects will be asked, in a private setting, if they understand all aspects of the consent form and expectations of participation in the protocol. They will be provided with contact information for the investigator and encouraged to ask for clarification at any time. Reading skills will be assessed by asking all participants, in private, if they would like any assistance in reading forms.

**Documentation of Consent.** All subjects will receive a signed copy of the consent form, and all consent forms will be kept on file in a locked office SMD Room 4-6818.

**Costs to Subjects.** There will be no costs incurred by subjects participating in the study.

**Payment for Participation.** Subjects will be reimbursed with "rewards cards" from Wegman's Supermarket. Renumeration will be prorated such that participants will receive $25 upon completion of trial 1 and an additional $50 ($75 total renumeration) upon completion of trial 2.

**Use of E-mail for Communication in Research**

E-mail communication will be used in recruitment and in scheduling visits and follow ups. E-mail will not be used in screening or consenting of subjects. E-mail will also be a means for subjects to contact Dr. Rand with any study concerns. When using e-mail to communicate with subjects in this study, the researcher cannot guarantee, but will use reasonable means to maintain security and confidentiality of e-mail information sent and received. Minimizing the risks for this is described below (9A, B).

## 5. METHODS AND STUDY PROCEDURES

### 5A. Overview:

The study will follow the protocol outlined below under "specific methods". A timeline of study activities can be seen in Figure 1 and Appendix 1. The study will engage human subjects in six activities to execute this protocol: 1) recruitment, 2) collection of body metrics (weight, height) and oral observation for presence/absence of amalgam fillings, 3) fish meal consumption 4) fecal sample collection and submission and 5) hair sample collection, 6) prebiotic self-administration (see below). A total of four visits will be mandatory, one at the beginning and one at the end of each trial. Fecal sample submission will be done with a shipping protocol, or scheduled pick up by lab staff, and will not require a visit. Steps 2-4 above are anticipated to occur over a period of approximately 74 days (see Figure 1 and Appendix 1). Step 5 will commence one week prior to the first fish meal in the second trial (Figure 1). Subjects will be asked to keep a food diary in a provided notebook and include notes on their general health, and particularly any illness or unusual diet habits, over the study period. The entire study, including pre-study preparations, subject enrollment, two trials of fish meal administration and hair and feces sampling, Hg measurements, microbiome analysis and statistical analysis of the data is anticipated to take place over a period of three years.

### 5B. Approach.

Our approach is summarized schematically in Figure 1. Subjects will consume three meals of tuna steaks, each meal separated by seven days (on days 0, 7 and 14"). This will be followed by an elimination period of 60 days where subjects will resume normal daily diets and activities. Hair will be sampled on or near the $60^{th}$ day after the last fish meal. Fecal samples will be taken at three time points: one each within 30 days following the last fish meal for each trial and one prior to, or upon the day of initiating the prebiotic administration in the second trial. Longitudinal Hg analysis of single hair strands will be performed with LA-ICP-MS. iHg and MeHg levels will be determined in fecal samples using tetra-ethylborate ($NaBEt_4$) derivatization and GC-ICP-MS methods.

Fecal DNA will be extracted and stored frozen. Hair Hg analyses will give Hg levels over time and will be used to determine a MeHg elimination rate ($t_{1/2}$). Fecal Hg analyses will be used to determine iHg/MeHg ratio as a proxy of MeHg de-methylation, and adjusted based on presence of dental amalgam. Fecal samples and isolated DNA will be shipped to MSU for germ-free mouse microbiome experiments and metagenomic sequencing and bacterial culturing and abundance analyses. Statistical analyses of correlations of elimination rate, MeHg demethylation and bacterial taxa abundance will be made.

**5C. Specific methods**.
*(Many of the specific methods below are reiterated from our previously approved project: RSRB00050613 entitled Methylmercury metabolism and elimination in humans and RSRB00057934 entitled Determination of methylmercury metabolism and elimination status in humans.)*

**Subject recruitment and subject information**. Potential subjects responding to flyer announcements of the study will be scheduled for a confidential visit in the office of the Principal Investigator (P.I.) who will serve as the study leader. The study methods and objectives and subject responsibilities, risks and benefits will be explained to prospective participants. Willing participants will sign a consent form provided by the P.I. at the time of the first visit. Alternatively, potential subjects will be given the opportunity to take home a written description of the study for further consideration before consenting. The study will target 40 participants, 20 women and 20 men. To achieve this subject recruitment will target 46 consenting participants (23 women, 23 men) to account for attrition due to drop out. When 46 participants have signed a consent form recruitment will cease. Once consent is obtained the subject will be assigned an alpha-numerical identifier that will be used to label all associated samples, data and record keeping throughout the study. This identifier number will be recorded on the consent form and kept in a locked and secure file in the P.I.'s office.

Information collected from the subjects at the initial study visit will be: age, sex, body weight, presence/absence of dental amalgams and medications currently taken. This will be collected without the need to access medical records. Subjects will be provided with a notebook carrying templates to fill in for a food diary. Alternatively, subjects who currently use a personal food tracking application (e.g. "My Fitness Pal" or "Healthwatch 360") can substitute a print out from this application of their food consumption record for period spanning the trial.

**Fish meal administration**. Just prior to each trial, individually weighed, wrapped and frozen portions of tuna steaks in 6-8 oz (170-227gm) portions will be prepared by and purchased from a local market (Wegman's). Portions will be kept frozen in a dedicated freezer in the P.I.'s lab until the time of distribution to the subjects. Frozen fish portions will keep over the time of distribution to enrolling participants (~ 2-3 months, https://stilltasty.com/fooditems/index/18559). Tuna portions will be prepared from one large fish fillet, so subjects will be receiving portions from the same fish within each Trial. A sample of the fish tissue will be set aside at the time of purchase to determine mercury content in the fish. Mercury measurements will be performed at the URMC EHSC Elemental Analysis Facility. Fish mercury values will be used to calculate predicted exposures to subjects based on individual body weights (see 9B, Protection

against risks, below). Where needed, instruction for adjustment of the portion size will be made to assure that MeHg exposure levels will not exceed federal guidelines for each subject. Each subject will be provided with the three frozen portions of tuna in a Thermosafe styrofoam shipping container affixed with prepaid return postage for later return (see below). Two polar-pack freezer blocks will be included to maintain the frozen steaks during transport home. For safety, subjects will be instructed on the steps necessary to keep the fish frozen until preparation for eating. Since cooking does not affect MeHg content in fish, fish meals can be prepared to the subject's own liking and eaten at home. Suggestions for preparation of the fish will be provided to the subjects. Fish meals will be eaten on Days 0, 7 and 14. Subjects will record the date and time of consumption of each meal. Subjects will be instructed not to eat any fish or seafood during the 60 days following the final tuna fish meal. Subjects will be asked to keep a record of all types of food and drink consumed at each meal during the 60-day elimination period using a provided template or their personal food tracking application

**Prebiotic administration.** Prebiotin™ (oligosaccharide enriched inulin), a commercially available prebiotic supplement from Jackson GI Medical (https://www.prebiotin.com/about-us/), will be distributed to the subjects for self-administration subsequent to the first trial and washout period and prior to the second trial (Figure 1). An intended use for Prebiotin™ is not the subject of investigation in this study. Prebiotic supplementation with this lawfully marketed over-the-counter dietary supplement is being used for its known properties of changing gut microbiome composition. Regarding FDA approval, use of Prebiotin™ in this study will be IND exempt in that all six (6) of the conditions under section 7.1 of Policy 605 of the Office of Human Subject Protection at the Univ. of Rochester are met. Briefly, with respect to Prebiotin™, this study: is not intended to support a new indication for use (7.1.1); is not intended to support a significant change in the advertising of Prebiotin™ (7.1.2); does not involve a change in route of administration, dosage level, or population at risk (7.1.3); will be conducted in compliance with the IRB review (7.1.4); will be conducted in compliance with requirements concerning the promotion and sale of Prebiotin™ (7.1.5); and, does not intend to request exception from informed consent requirements for emergency use (7.1.6). Individually packaged portions (4 grams) of the Prebiotin™ Prebiotic Fiber Stick Pac will be provided to subjects (https://prebiotin.net/collections/our-products/products/prebiotin-prebiotic-fiber-stick-pac-4g). Prebiotin™ is the prebiotic of choice in several published studies on gut microbiota control of obesity [20-22] and has been used in an NIH study on reducing inflammation in kidney patients [23]. For this study, we are targeting a dose of 8 grams/day. This dose amount is based on published effects of inulin on gut microbiota in the range of 4 to 16grams/day dosages [20, 24]. We have selected 8 grams/day based on the finding of Holscher et al [24], who demonstrated clear effects on shifting the microbiota composition and activity at the 7.5 gram/day dose level in healthy adults in a controlled double-blinded study. Prebiotin™ self-administration will be initiated one week prior to fish consumption in the second trial and extend through the entirety of the second trial (i.e. 11 weeks, Figure 1). Subjects will be given a 11-week supply of Prebiotin™ Stick Pacs and instructed on how to self-administer on either a once or twice daily dosing schedule. For the first week, subjects will be instructed to begin with a daily supplement of one Pac (4grams) a day, taken with meals. Starting the second week, and additional

Pac (2 Pacs total) will be consumed to achieve 8 grams/day. Both Pacs can be consumed at breakfast, or alternatively, divided between meals, e.g. breakfast and dinner. Prebiotin™ has a mild natural sweetness and will dissolve readily in any liquid. It can also be added to any food such as cereal, soups, salads, or sauces. If disruptive bloating or gas occurs with the 8gram dose, then the amount can be reduced to one pouch (4grams/day) until bloating and/or gas is manageable. While Prebiotin is safe to use in very high quantities, subjects will be advised on what to expect with prebiotic ingestion, including the anticipated digestive health benefits as well as possible feelings of bloating and excess flatulence that are harmless yet may produce some annoyance. Where bloating or gas is not manageable/tolerable and is disruptive to daily life, participants will be advised to contact study staff for further recommendations on reducing supplementation amounts to 2 grams/day or pursuing the option to withdraw from the study.

**Fecal sample collection, DNA isolation and shipping**. Feces will be used to capture the profile of the gut microbiota for each subject. Collection methods will therefore be aimed at capturing and preserving anaerobic species. At the initial visit, subjects will also be provided with instructions on the stool sampling protocol and provided with an easy to use commercially available collection container (Fisherbrand Commode Specimen Collection System) and an Omnigene-Gut collection tube for DNA subsampling (below). Stools will be sampled at three time points as indicated in Figure 1: approximately four weeks after the 3rd fish meal (for each trial) and at approximately one week prior to the 2nd Trial, preceding or contemporaneously with prebiotic supplementation (e.g. day 44 of the Trial 1 and day -7 and 44 of Trial 2). Collection of stools within 2-3 days centered on these target collection times will be acceptable. For all fecal samplings, the whole stool will be collected and frozen within 30 minutes to preserve microbe viability [25]. To stabilize a sample of the fecal DNA at the time of stool collection, a sub-sampling of feces with the Omnigene-Gut collection kit (DNA Genotek, Ottawa, ON, Canada) will be done by the subject according to the provided instructions. Subjects will be instructed to triple contain the stool collection with freezer-grade zip-lock bags provided. Subjects will ship the stool sample and the DNA stabilized sample to the P.I.'s lab using express shipping (FedEx) as soon as possible using the freezer packs and Thermosafe styrofoam packaging provided (see above). Alternatively, sample pick up by lab staff will be arranged. Fecal samples will be logged-in and coded for identity protection in the P.I.'s laboratory and kept frozen until analysis and DNA isolation. Fecal DNA will be isolated with the ZR fecal prep kit (Zymo Research) in the Rand lab and will be distributed to the Boyd lab at MSU. Samples of feces will also be distributed to the Walk lab at MSU. Human feces falls under the "exempt human/animal specimens" category for shipping. A portion of frozen stool for each subject will be prepared in appropriate triple-layer packaging and containment (http://www.fedex.com/us/packaging/guides/Clinical_fxcom.pdf) and marked appropriately with "exempt human specimens" for shipping to MSU.

**Hair sample collection and submission**. Hair samples will be collected during a visit of the subject to the P.I.'s laboratory on approximately day 74 (60 days after the last fish meal). Sampling within a five-day window of this target time will be acceptable. Hair will be sampled manually by direct withdrawal from the scalp. Sampling from the posterior region of the head will be done for all the subjects. Direct withdrawal of three

to five strands at once is found to elicit little discomfort and yield root material that can be used as a reference landmark for the start of the LA-ICP-MS analysis. A total of 20-25 hair strands will be collected for each subject and stored in a zip-lock bag. Hairs will then be mounted on double stick tape on a microscope slide and stored at room temperature until LA-ICP-MS analyses (see below). Hair samples will also be logged-in and coded for identity protection in the P.I.'s laboratory.

**Food and health diary**. Subjects will be requested to keep a food diary for the periods spanning each of the 75 days of Trial 1 and 2. The diary is to include a list of types of food eaten and drinks consumed at each meal. Careful accounting of amounts of food or caloric intake will not be requested. In addition, subjects will be asked to record incidents of illness (e.g. cold or flu) or stresses (e.g. work or personal) that are likely to contribute to unusual eating patterns, digestive distress or metabolic imbalance. A food diary will not be requested for the intervening 4-month washout period. However, participants, at the visit prior to Trial 2, will be asked to comment on fish/seafood eating frequency and if illness or undue stress was experienced during the washout period.

**Analytical procedures**.

**LA-ICP-MS of hair samples**. Hair analyses will be done on individual strands of hair using LA-ICP-MS at the Dartmouth College Trace Element Analysis (TEA) Lab at Dartmouth College (http://www.dartmouth.edu/~toxmetal/program-resources/trace-element-analysis/). These measurements will be done by the TEA lab staff on a fee for service basis. Laser ablation will be carried out with a UP213 laser (ESI, Bozeman, MT) equipped with a programmable X-Y stage for predetermining coordinates for ablations of a series of spots on the hair shaft. Parameters of laser power, gas flow rates, dwell time and acquisition time will be optimized with Dr. Jackson's assistance. Methods of Hg determination will follow previously described protocols [10, 11]. Briefly, spots of 50μm will be ablated at 333μm intervals along a 3.0cm length of the initial segment of hair closest to the scalp. Hair growth occurs at approximately 1.1 cm per month. Therefore, 3.3cm of the initial segment of hair will be analyzed to cover the 75 days previous in which MeHg administration and elimination have occurred. The $^{202}Hg$ isotope will be detected and normalized to the amount of hair sampled in the ablation event by detecting $^{34}S$ in parallel and determining the Hg/S ratio. Quantification of Hg will be done by comparing Hg/S ratios to a matrix match hair sample [11].

**Determination of MeHg elimination rate**. Elimination rate will be determined from the normalized relative Hg concentrations in an individual hair strand over time, which is determined from the length of hair that grew over the interval from the last fish meal to the 60 day collection point. Growth rate of each individual hair will be calibrated by measuring the distance between the "peaks" of Hg observed in the hair resulting from the first and last fish meal, a period representing 14 days. Elimination rate will be determined by linear regression analysis of Hg concentration over time in a semi-log plot and expressed as $t_{1/2}$ in units of days.

**Hg determinations in fecal samples**. iHg and MeHg analysis and quantitation will be done in the URMC EHSC Elemental Analysis Lab. Analyses will employ a standard protocol of acid digestion followed by tetra-ethylborate ($NaBEt_4$) derivatization and separation on GC-ICP-MS.

**Data & Specimen Banking for Future Research Use.** Samples (hair, feces)

will be stored in the P.I.'s laboratory until the analytical steps are completed. With subject consent, samples will be stored for future research purposes. Data emerging from the study will remain on the P.I.'s password protected personal computer.

**Genetic/Genomic Research Activities**

Genomic analysis will be carried out only on microbial samples from each subject. These studies are distinct from genomic testing of the subject's own DNA. Microbial genomic analyses will be done with a sequencing-based metagenomic method of identifying the presence and abundance of specific strains of bacteria. The relevance of the composition of the gut microbiota to MeHg toxicity will be explained to the subject. Recent focus on the gut microbiome has revealed several important roles for gut bacteria in health and disease. The subject will be made aware of the potential health benefits of management of gut microbiota composition and will have the opportunity to engage the P.I. in answering questions related to micriobiome research.

**Return of Individual Research Results**

A summary of the study results will be shared with the participants upon request in a one-page electronic document. Participants will also be alerted to any public forum where study results will be presented and they are welcome to attend.

## 6. CONCOMITANT AND DISALLOWED MEDICATIONS

Administration of antibiotic medication within two months prior to the study is an exclusion criterion. Antibiotic administration during the elimination period of the 75 day of protocol will not be disallowed, however, but must be reported to the P.I. No additional treatments or alteration of concomitant medication schedules will be necessary.

## 7. SUBJECT WITHDRAWALS

Subjects will be advised orally and in writing in the consent forms that they have the right to withdraw from the study at any time without prejudice. Subjects will be withdrawn from the research without their consent in instances of non-compliance or termination of funding for the study. Subjects withdrawn from the study will be replaced with a follow up recruitment.

## 8. SAFETY AND REPORTABLE EVENTS

At the first visit, subjects will be alerted to self-monitor for any adverse event and to contact study staff by phone or email with any concerns. To monitor subject adverse events, study staff will also contact subjects with a phone call or email within the week subsequent to the last fish meal in each Trial (e.g. between days 15-21 of the Trial). An additional call will be made on day "0" of Trial 2 to monitor effects of prebiotic self-administration. Study staff will not call subjects to monitor adverse events during the intermediate 4-month washout period. The most likely causes for an adverse event would be a reaction to consumption of tuna or the prebiotic administered in the second trial. Subjects will be advised to contact the P.I. by phone or email in the instance that an adverse symptom, sign, illness or experience is encountered. In the instance that this occurs before all tuna meals or prebiotic are consumed, the subject will be advised

to refrain from further fish or prebiotic consumption. Adverse events or problems are not expected but will be reported to the RSRB according to OHSP Policy 801 guidelines.

## 9. RISK/BENEFIT ASSESSMENT

### 9A. Potential Risks

The risk of MeHg toxicity from fish consumption remains a topic of considerable debate. MeHg poses the greatest risk for neural development in the fetus and young children, hence, the exclusion of pregnant and breast-feeding women and of children (17yrs and younger) in this study. The risk to adults (18yrs and older) is substantially less. Fish consumption implemented in this protocol will be carefully calculated so that blood MeHg levels incurred will not exceed that of the EPA reference dose (RfD) levels. The EPA reference dose includes a safety factor of 10. Thus, steps to avoid any potential risk due to MeHg have been averted in this study design.

There is a minimal risk of physical discomfort associated with hair collection and with examination for presence of dental amalgam surfaces. There is minimal risk for gastrointestinal discomfort with consumption of prebiotic.

There is a potential risk of infringement on privacy or confidentiality.

*Risks of e-mail use:*

Sending the subject's information by e-mail has a number of risks that the subject should consider. These include, but are not limited to, the following:

a) E-mail can be circulated, forwarded, stored electronically and on paper, and broadcast to unintended recipients.
b) E-mail senders can easily misaddress an e-mail.
c) Backup copies of e-mail may exist even after the sender or the recipient has deleted his or her copy.
d) Employers and on-line services have a right to inspect e-mail transmitted through their systems.
e) E-mail can be intercepted, altered, forwarded, or used without authorization or detection.
f) E-mail can be used to introduce viruses into computer systems.

### 9B. Protection Against Risks

Protection against MeHg toxicity will be implemented by careful calculations that consider: 1) the amounts of MeHg administration that will occur in the three fish meals, 2) known kinetic parameters of MeHg accumulation and elimination rates in the blood compartment and 3) each subject's individual body weight and 4) the EPA reference dose (RfD). These calculations are summarized below:

The EPA RfD is derived from a benchmark dose (BMD) analysis using a value of 5.8µg/L in blood, which includes a safety factor of 10 (http://www.epa.gov/iris/subst/0073.htm).

A study by Kershaw, et al. [4] concludes that 5.9% of a MeHg dose is deposited in the blood compartment after complete tissue distribution.

The blood compartment is ~7.1% of the body mass, equating to a range of 4.2 to 6.5 liters in 130 to 200 pound individuals, respectively.

Therefore, a blood level of 5.8µg/L equates to a single dose of:
5.8µg/L X (4.2-6.5L) X 1/0.059 = 413-639µg MeHg for individuals ranging from 130-200 pounds, respectively.

Fresh tuna steaks have an average mercury content of 0.39ppm* (µg/g wet weight) One 8oz (227gm) portion of tuna contains 88.5µg MeHg. Therefore, three 8oz portions of tuna will give a cumulative dose of 265.5µg MeHg, well below that needed to achieve blood levels equal to the EPA RfD.

*A recent in-house analysis of yellowfin tuna tissue obtained from the designated vendor (Wegman's) revealed a mercury content of 0.23-1.35ppm. For this highest level of mercury, three 8oz meals in 14 days would yield MeHg in blood exceeding to the RfD level for a 130-pound person. For this reason, the tuna used in this study will be sampled and analyzed for Hg content prior to distribution to the subjects. Subjects will be allocated tuna steak portion sizes according to their body weight and above calculations to ensure that EPA RfD levels are not exceeded.

Risks of physical discomfort of hair sampling will be managed by limiting sampling to 4-8 strands with each extraction. Subjects will be informed of what to expect with prebiotic administration, which could include feelings of bloating and excess flatulence, which have no adverse health effects beyond being an annoyance.

Privacy will be maintained as best possible by coding samples and storing documents under locked files in the P.I.'s office.

Risk of email breach will be minimized by utilizing teh U of R SecureMail Encryption process following the steps outlined here:
https://tech.rochester.edu/tutorials/sending-securemail-messages/

### 9C. Potential Benefits to Subjects

There will be no direct benefit to individual subjects in this study.

## 10. RESEARCH INFORMATION IN MEDICAL RECORDS

No information from this research will be included in the participants' medical records.

## 11. DATA ANALYSIS AND MONITORING

### Sample Size Determination

We will target 40 participants (20 women, 20 men)for the reason that some statistical power is expected while we also are aiming to keep the study size appropriate for the logistics of recruiting and retention of subjects for both trials. We aim to recruit and consent 46 participant (23 women, 23 men) in anticipation of some drop out. A

complete cohort of 20 women and 20 menwill afford the opportunity to assess sex-based differences. We have performed power analysis on the pairwise correlations between $k_{el}$ and MeHg demethylation rate and/or bacterial taxa abundance. With grouped sexes, 40 subjects will have at least 80% power to detect a linear relationship where 19% or more of the variance in elimination rate is explained by de-methylation status or particular bacterial species abundance.

**Planned Statistical Analysis**

Relationships of taxa abundance with kinetic parameters of MeHg elimination rate ($k_{el}$), biotransformation in vivo ($B_{vivo}$, %iHg in feces) and in vitro ($B_{vitro}$, %MeHg biotransformation in cultures) will be evaluated. A total of 117 numerical values for $k_{el}$ and $B_{vivo}$, $B_{vitro}$ will be available (37 from our prior study and 40 subjects X 2 trials in the present study). Evaluation of slopes and $R^2$ values for elimination rate calculations in the prior study demonstrates that a difference of two days in MeHg half-life between two subjects can be determined with significance. Pairwise t-tests will be used to assess significance among pre- vs. post-prebiotic elimination rates (Aim1). The correlation between the ($k_{el}$) and biotransformation ($B_{vivo}$ and $B_{vitro}$) will evaluated for the 40 X 2 = 80 new human subject determinations in Aim 1. Graphical and numerical diagnostics will be used to test the linearity of the relationships and the homogeneity of variance. If these conditions are met, linear regression will be used to estimate functions for predicting $k_{el}$ from $B_{vivo}$ or $B_{vitro}$. The R system for statistical computing will be used throughout [26].

**Data and Safety Monitoring.**

All records with information identifying a study subject will be kept in a secure, locked office or on a secure computer at the U of R site. Only the P.I. will have access to these records. All samples will be coded so as to remove any subject specific information. The laboratory staff will only have access to subject samples and various non-identifying analytical parameters. Samples shared with co-investigators at Montana State University (MSU) will be de-identified. In addition, the PI will consult with the MSU Co-Is to advise on practices of avoiding any possible disclosure of subject's personal information. In accordance with regulations, we may share a copy of the consent form and records that identify individual subjects with the U of R, MSU Department of Health and Human Services and National Institutes of Health.

**12. OVERSIGHT PLAN.** As PI, Dr. Rand will provide administrative and scientific oversight for all activities carried out by co-investigators Walk and Boyd at Montana State University. The PI will be responsible for conducting monthly meetings via Zoom or telephone to review study activities. Ongoing training of staff will be implemented to ensure protocol compliance. Data analysis will be reviewed on a monthly basis. In instances where there is a breach of privacy/confidentiality it will be promptly reported to the PI. Additional oversight and review of data analysis will occur at an annual meeting where PI, co-I's and students and staff members of the study will physically convene in either Rochester or Bozeman Montana.

**13. FOLLOW UP COMMUNICATIONS.** The Study PI will not initiate unsolicited contact with subjects but subjects may contact the Study PI to inquire about study status and

results subsequent to completing their study activities. It is anticipated subjects will contact the Study PI via email, at which point the Study PI will call the subject by phone to review an information sheet delineating the risks and conditions of communication via email, and proceed to obtain verbal consent for using email. The timeframe within which the Study PI will receive and respond to email inquiries will be through August 31st of 2023, when the project funding is anticipated to expire, and study results will be complete. Information about the study that is anticipated to be shared with subjects includes aggregate results and individual results specific to the subject only. Prior to sharing any data, the PI will confirm the subject data set matches the subject identity via a cross reference to the assigned alpha-numeric identifier assigned and recorded on the consent form as detailed in section 5C above. In addition, the Study PI, when requested by the subject, will inform the subject of published results and, where allowed by the publisher guidelines, share such document(s) via an electronic file (e.g. PDF file), or refer the subject to a publicly available site to access the publication.

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## Appendix 1

## Schedule of Activities.

| | | Trial 1 | | | | | | | Trial 2 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Activity | Up to day 0 | Day: 0 | 7 | 14 | 21* | 44# | 74$ | 4 months | (-7) | 0 | 7 | 14 | 21* | 44# | 74$ |
| confirm eligibility/enroll | X | | | | | | | | | | | | | | |
| informed consent | X | | | | | | | | | | | | | | |
| present protocol | X | | | | | | | | | | | | | | |
| provide fish meals | X | | | | | | | | | | | | | | |
| provide sample collection tools | X | | | | | | | | | | | | | | |
| consume fish meal at home | | X | X | X | | | | | | X | X | X | | | |
| washout period | | | | | | | | X | | | | | | | |
| fecal sample | | | | | | X | | | X | | | | | X | |
| hair sample | | | | | | | X | | | | | | | | X |
| consume prebiotic at home& | | | | | | | | | X | X | X | X | X | X | X |
| fill in food diary@ | | X | X | X | X | X | X | | X | X | X | X | X | X | X |
| adverse event monitoring phone call from study staff | | | | | X | | | | | X | | | X | | |
| ad hoc with phone calls from subjects | | X | X | X | X | X | X | X | X | X | X | X | X | X | X |

*Call will be made between day 15-21.
# Fecal sample can be collected within 2-3 day period centered on this day.
$Hair sample can be collected within 5-day period centered on this day.
&Prebiotic is to be consumed daily from days -7 to 74 of Trial 2.
@ Food diary is to be filled in every day during Trial 1 and 2, but not during washout.