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Mercury, polychlorinated biphenyls, and immune response indicators in the National Health and Nutrition Examination Survey (NHANES)

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Carolyn M. Gallagher

We, the dissertation committee for the above candidate for the

Doctor of Philosophy degree, hereby recommend

acceptance of this dissertation.

Jaymie R. Meliker, Ph.D. – Dissertation Advisor
Associate Professor, Department of Preventive Medicine, Graduate Program in Public Health

Dylan M. Smith, Ph.D. - Chairperson of Defense
Associate Professor, Department of Preventive Medicine

Marc G. Golightly, Ph.D.- Dissertation Committee Member
Professor, Department of Pathology

Anne E. McElroy, Ph.D. – Dissertation Committee Member
Professor, School of Marine and Atmospheric Sciences

This dissertation is accepted by the Graduate School

Charles Taber
Interim Dean of the Graduate School

Abstract of the Dissertation

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Autoimmune diseases are among the leading causes of death among young and middle-aged women, and prevalence is increasing; however, autoimmune etiology is poorly understood. The need to investigate relationships between environmental factors and human autoimmune responses has been prioritized by the World Health Organization and the National Institutes of Health (NIH), yet epidemiologic research is sparse. Mercury (Hg) and polychlorinated biphenyls (PCBs) are ubiquitous and persistent environmental contaminants with early evidence of immunotoxic associations. Immunotoxic responses fall on a continuum, with the normal range of immune responses at the center, immunostimulation and autoimmunity at one end, and immunosuppression and infection at the other. Common immune response indicators include antibodies against “self”, that is, the body’s own normal cells, and antibodies against pathogens such as wild-type or vaccine-type viral antigens. Serum vaccine antibody concentration is an increasingly recognized parameter of children’s immune system response to environmental

contaminants in population studies. The NIH and the Centers for Disease Control and Prevention have prioritized the need to evaluate differential immune responses to vaccination; and the U.S. Department of Health and Human Services (DHHS) identified the need to evaluate the relationships between environmental exposures and immunologic outcomes in susceptible subpopulations. Further, sex, nutritional deficiencies and related metabolic cofactors may represent important immune susceptibility cofactors.

The objective of this dissertation research is to evaluate the relationships between total blood Hg and serum PCBs with human immune response indicators across the continuum of immunotoxicology among susceptible subpopulations.

My dissertation research uses cross-sectional data obtained from U.S. probability samples of the National Health and Nutrition Examination Survey to address gaps in epidemiologic research of the relationships between PCBs and Hg and immune response indicators in each of four separate studies A, B, C and D. Study A evaluates the relationships between total blood Hg, serum PCBs, and antinuclear antibody (ANA) positivity, an indicator of systemic autoimmune response, stratified by sex. Study B evaluates the relationship betweenHgand indicators of organ-specific immune response, specifically, thyroid autoantibody positivity, as well as a risk factor for hypothyroidism, stratified by sex and iodine status in order to evaluate these recognized autoimmune thyroid disease susceptibility factors. Studies C and D evaluate the relationships between Hg exposure and serum concentrations of measles antibodies, and between Hg exposure and serum concentrations of rubella antibodies, in nutritionally-susceptible subpopulations of children.

Key findings are: (A) There were no associations between Hg and ANA, or between nondioxin-like PCBs and ANA, among males or females; however, among females, dioxin-like PCBs were significantly and positively related to ANA positivity. (B) Hg was positively associated with the hypothyroidism risk factor among females with lower iodine and iodine deficient females, and in these same subsamples, positive associations were also observed between Hg and thyroid peroxidase autoantibodies. In contrast to females, who showed an overall pattern of elevated odds ratios for thyroid measures that included autoantibodies, most consistently among females with lower and deficient iodine levels, an overall inverse pattern was evident among males with higher and excessive iodine levels. (C) Positive associations were observed between Hg and serum measles antibodies in boys with lower folate and B-12, and higher homocysteine levels, but inverse associations were observed in all other children. (D) Positive associations were observed between Hg and serum rubella antibodies in the same nutritionally susceptible subset as in study C, with inverse associations among the remaining subset; however, findings were observed in both boys and girls, combined.

In this thesis, I present novel findings of positive associations between (A) dioxin-like PCBS and ANA positivity in females; (B) Hg exposure and a risk factor for hypothyroidism in women with lower or deficient iodine levels; and (C) Hg exposure and elevated serum measles IgG antibodies in boys with lower folate and vitamin B-12 and higher homocysteine levels, as well as elevated rubella IgG antibodies in boys and girls with this same nutritional susceptibility. Moreover, these are the first studies to show these relationships in a general population with lower immunotoxicant exposure levels to these ubiquitous contaminants as compared with studies in populations with known higher exposures due to industrial pollution and/or contaminated dietary sources. Given the divergent findings by sex and nutritional cofactors, an

overarching recommendation is for environmental epidemiology to identify susceptible subpopulations and to conduct epidemiological research stratified by these vulnerability subsets. Taken together, these findings shine the spotlight on the need for prospective studies that take an integrative approach to evaluate these relationships across the immunotoxicological continuum, and have the potential to guide public health decision-making for the protection of the most vulnerable.

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List of Abbreviations

ANA antinuclear antibody

β regression coefficient

BMI body mass index

CI confidence interval

EPA eicosapentaenoic acid

Hg mercury

IFN-α interferon alpha

IFN- interferon gamma

IgG immunoglobulin G

IU international unit

MMA methylmalonic acid

NHANES National Health and Nutrition Examination Survey

PCB polychlorinated biphenyls

RNA ribonucleic acid

SLE systemic lupus erythematosus

SSPE subacute sclerosing panencephalitis

TgAb thyroglobulin autoantibody

TPOAb thyroid peroxidase autoantibody

µg/L micrograms per liter

µmol/L micromoles per liter

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Introduction to Dissertation Research

Public Health Need

Autoimmune diseases are among the leading causes of death among young and middle-aged women (Walsh and Rau, 2000; NIH, 2005); however, current International Classification of Disease and Related Health Problems- (ICD-9-) based methodology to establish leading causes of death underestimates this fact (Walsh and Rau, 2000). Trend studies show increasing incidence from 1960-1996 for Type I diabetes worldwide (Onkamo et al., 1999), increasing prevalence from 1965-1995 for multiple sclerosis, myasthenia gravis, primary billiary cirrhosis, and scleroderma in the U.S. (Jacobson et al., 1997), and an almost 3-fold increase in the incidence of systemic lupus erythematosus between 1950 and 1992 across two population cohorts from Rochester, Minnesota (Uramoto et al., 1999). Yet, autoimmune etiology is poorly understood (WHO, 2006). The leading working hypothesis, according to a National Institute of Environmental Health Sciences expert panel workshop, states that "autoimmunity results from a susceptible genetic background and the impact of specific environmental factors" (Selmi et al., 2012). This is an area of critical public health need, which has seen few investigations into environmental factors; a need recognized both nationally (Miller, 2011) and worldwide (WHO, 2006).

Immunotoxicants interact with the immune system to induce adverse effects of immunosuppression, as well as immunostimulation and autoimmunity (Klaassen, 2008). Whereas antibodies against "self", that is, the body's own normal cells, are indicators of autoimmune responses, antibodies to pathogens are an indicator of the body's response to non-self antigens such as wild-type or vaccine-type viral antigens. Microbial pathogens,

environmental toxi cants and host susceptibility are cofactors that may interact to contribute to disease risk, and therefore, it has recently been proposed that environmental epidemiological research integrate toxicological and infectious disease models to evaluate potential interactions (Feingold et al., 2010). The National Institutes of Health and the Centers for Disease Control and Prevention recently identified the evaluation of differential immune responses to vaccination as a research priority (NIH, CDC, 2011). Serum vaccine antibody concentration is an increasingly recognized parameter of immune system response to environmental contaminants in population studies (Grandjean et al., 2012; Jusko et al., 2010, 2011) and the U.S. Department of Health and Human Services identified the need to evaluate relationships between environmental exposures and immunologic outcomes in susceptible subpopulations (US DHHS, 2011a).

Exposures of Concern for Human Health

Mercury and PCBs are contaminants found in the U.S. food supply, particularly fish (ATSDR, 1999; 2000). The Environmental Protection Agency recently prioritized regulation of mercury emissions from power plants (US EPA, 2012a) and issued national guidance on disposal of PCB-containing fluorescent lights (US EPA, 2012b) to protect Americans from potential health impacts of current exposure sources. Although PCBs have not been manufactured in the U.S. since 1977, U.S. residents may still be exposed to PCBs by eating contaminated food, particularly fish, and breathing contaminated air (ATSDR, 2000). Healthy People 2020 targets mercury and PCBs for reduced human exposure, as well as other environmental chemicals with evidence of immune toxicity, such as arsenic, lead, cadmium, halogenated aromatic hydrocarbons, perchlorate and pesticides (Klaassen, 2008; US DHHS, 2011b). Here I focus on mercury and PCBs, where early evidence exists to suggest associations with immunomodulation

and/or autoimmune-mediated diseases, and sufficient data on exposures and outcomes of interest are available from the National Health and Nutrition Examination Survey (NHANES), a nationally-representative sample of the noninstitutionalized U.S. population (CDC, 2011a). This dissertation represents some of the first epidemiologic studies of the relationships between these contaminants and immune response indicators.

The continuum of immunotoxicology

Immunotoxicology is a continuum, with the normal range of immune responses at the center, morbidity and mortality secondary to immunostimulation and autoimmunity at one end, and immunosuppression and infection at the other (Figure 1; Klaassen, 2008). Autoimmune diseases may be systemic, e.g., systemic lupus erythematosus (SLE), and characterized by non-organ specific antibodies, such as antinuclear antibodies, which attack normal proteins within the nucleus of cells, or organ-specific, as in autoimmune thyroiditis with thyroid-specific antibodies (Golightly and Golightly, 2002). Elevated thyroglobulin autoantibodies (TgAb) and thyroid peroxidase autoantibodies (TPOAb) have been observed in patients with other autoimmune diseases, for example, elevated TgAb in patients with primary biliary cirrhosis and autoimmune hepatitis, and elevated TPOAb in patients with primary biliary cirrhosis (Nakamura et al., 2008). Further, there may be overlap between infectious agents and autoimmune disease; for example, infections with the rubella virus may be comorbid with autoimmune thyroiditis (Jaume, 2011), some autoimmune diseases may be triggered by an infectious agent (NIH, 2006-2007; Munz et al., 2009), and evidence of an association between chronic arthritis after rubella vaccination, as well as between thrombocytopenic purpura after measles vaccination, is recognized by the Advisory Committee on Immunization Practices (Miller, 2011). Autoimmune diseases have been

associated with elevated levels of viral antibody titers; for example, associations have been observed between the measles, mumps, and Epstein-Barr virus-IgG antibody levels with multiple sclerosis (Kinnuen et al., 1990); serum antibodies to measles and rubella viruses with multiple sclerosis and rheumatoid arthritis (Shirodaria et al., 1987); measles-specific IgG antibody concentrations and juvenile idiopathic arthritis (Heijstek et al., 2012); and serum antibodies to the Measles-Mumps-Rubella-II vaccine with increased myelin basic protein antibodies in children with autism (Singh et al., 2002), a neuro-developmental disorder of poorly understood etiology. Autism has more recently been characterized by brain-specific autoantibodies (Cabanlit et al., 2007; Wills et al., 2009; Goines et al., 2011) and elevated serum IgG4 antibodies (Croonenbergs et al., 2002; Enstrom et al., 2009); the latter may indicate an underlying autoimmune disorder or immunosuppression with consequent chronic viral infections (Croonenberghs et al., 2002). Further, Munz et al. (2009) reviewed bi-directional aspects of the immunological continuum, for example, how pathogens might trigger autoimmune disease on the one hand, yet on the other, how autoimmunity could trigger reactivation of pathogens. Xenobiotics, including environmental contaminants, can interact with immune function to exaggerate immune responses, both immunostimulation and immunosuppression, and so, induce autoimmune and infectious diseases, respectively (Klaassen, 2008).

Mercury, immunosuppression, and immunostimulation

Mercury is present in the environment in several forms. Methyl mercury is an organomercurial metal that humans are exposed to by eating contaminated fish (ATSDR, 1999). Although low amounts of Hg occur naturally in the environment, large releases of elemental Hg from industrial sources, particularly coal-fired power plants, are widespread and persistent

(ATSDR 1999). Deposition to marine environments leads to conversion to methylmercury by aquatic microorganisms, concentration of Hg in the muscle tissue of fish (USGS, 2009), and bioaccumulation in the marine food chain (ATSDR, 1999; USGS, 2009). Ethylmercury is an organomercurial metal found in small amounts in multi-dose vials of medicines and vaccines, such as some influenza vaccines, and is more rapidly eliminated from the body (CDC, 2011b), as is phenylmercury, an organomercurial which has been used as a fungicide in paint and as a preservative in contraceptive and cosmetic products (IARC, 1993). Mercury released from dental amalgam fillings or broken fluorescent light bulbs are potential sources of exposure to metallic, or elemental, Hg.

The specific form of Hg may differentially influence the immunologic effects over time by acting on B cells that make antibodies against antigens, and T cells, important for cell-mediated immunity. In addition to cytotoxic T cells that kill pathogens, T cells include both T helper cells that assist B cells to mature into plasma cells for humoral immunity and other T cells that can inhibit B cell development (Chin and Alonazi, 2012). Pusey et al. (1990) showed that repeated low dose injections of inorganic Hg in rats induced polyclonal activation, whereby multiple antibodies are produced when different antigens are recognized, with short lived increases in thyroglobulin autoantibodies and more sustained increases in total immunoglobulin G (lgG) antibodies and cellular autoantibodies to single-stranded DNA (ssDNA) and to double-stranded DNA (dsDNA). Both methyl mercury and ethylmercury have immunosuppressive effects, with subsequent immunostimulation and autoimmune effects in mice (Havarinasab and Hultman, 2005). For example, in genetically susceptible mice, subcutaneous injections of 540 µg of methylmercury/kg body weight per day caused a 47% reduction of B-cells and a 9% reduction

of T-cells, and a similar dose of thimerosal (ethylmercury) caused a 65% reduction of T- and B-cells; these reductions were observed five days post-exposure (Havarinasab et al., 2005). After 9 days of methylmercury exposure, and after 30 days of ethymercury exposure, splenic T- and B-cells showed an increase (Haggqvist et al., 2005; Havarinasab et al., 2005). Moreover, ethylmercury induced strong autoimmunity, e.g., antinucleolar antibody (ANoA) positivity, in this second phase, which may be due in part to the metabolism of ethylmercury to inorganic Hg (Havarinasab et al., 2005). Recently, Zhang et al. (2011) showed that exposure to 50μM inorganic Hg in drinking water from gestational day 8 to post-natal day 21 induced T-cell dependent polyclonal B-cell production of brain reactive IgG antibodies against nuclear proteins in male and female mice with known susceptibility to Hg-induced nephritis. In a mouse model absent susceptible genes, Abedi-Valugerdi (2009) showed that long term exposure to inorganic Hg induced B-cell activation and increased anti-nucleolar autoantibodies with anti-fibrinollarin specificity, and thus, raised the question of whether environmental factors may be more important than genetic susceptibility for induction of autoimmunity.

Another type of T cell releases cytokines, cell-signaling proteins such as interferon gamma (IFN-γ) that can induce inflammation, e.g. in patients with autoimmune thyroiditis (Bossowski et al., 2011), or drive viral clearance, e.g., in measles virus-infected brain tissue (Stubblefield et al., 2011). Human cellular studies showed that inorganic Hg and methyl mercury increased pro-inflammatory cytokine release, whereas ethyl mercury decreased the release of the cytokine IFN-γ (Gardner et al., 2010a). Methylmercury was also observed to inhibit IFN-γ in vitro (de Vos et al., 2007). IFN- γ activates phagocytic cells to confer protection against viruses (Steward-Tharp et al., 2010). Pellisso and colleagues (2008) showed that in vitro exposure of bottlenose dolphin

leukocytes to 5 and 10 ppm inorganic Hg significantly decreased phagocytosis by 20% and 40%, respectively, and interpreted findings as evidence that Hg exposures reduce host resistance to disease. Whereas B-cell secretion of IFN- γ has been linked to the inflammatory cascade of autoimmunity (Vaughan et al., 2011), an emerging insight is that IFN- γ deficiency exacerbates autoimmunity mediated by IL-17 producing T-cells (Steward-Tharp et al., 2010). Further, Nyland et al. (2011) found that, among fish consumers in Amazonian Brazil, total blood Hg was associated with increased antiviral IFN-γ, with this exception: IFN-γ decreased with methylmercury exposure in the subpopulation with elevated ANA. While there may be differences from exposure to different forms of Hg, both inorganic and organic forms appear to exert immunological effects that span the immunotoxicological continuum, and subpopulation susceptibility may be an important effect modifier.

Schiraldi and Monestier (2009) suggest that Hg's sulf-hydryl-binding properties, i.e., high affinity for thiol molecules such as the T-cell intracellular tripeptide glutathione, may underlie the immunological effects of Hg. Glutathione is a prime source of intracellular thiols and is required for induction of IFN-□, and therefore, inhibition of IFN-□ production by Hg in susceptible rats may be attributable to Hg-glutathione interactions (Schiraldi and Monestier, 2009). Zhu et al. (2000) also suggest that Hg's toxicity is attributable to its sulf-hydryl-binding capacity, and thus, acts as an enzyme inhibitor; emerging evidence from human studies lends support to this broader mechanism. Gundacker et al. (2007) reported increased hair Hg concentrations, a measure of longer term methylmercury exposure, in male and female subjects with deletion polymorphisms in genes that express glutathione-S-transferase, an enzyme that catalyzes the binding of glutathione with Hg for transport out of the cell, and consequently,

detoxification. In a more recent study, although specific polymorphisms were inconsistent with the former study, Goodrich et al. (2011) found that polymorphisms in a gene that expresses glutathione synthetase, an enzyme that catalyzes glutathione synthesis, influenced hair Hg levels following exposure to methylmercury from fish consumption. Mechanistic studies have also shown that methyl- (Smith and Smith, 1990) and ethyl- (Waly et al., 2004) Hg inhibit the enzyme methionine synthase, which converts homocysteine into methionine, a key methyl donor for biological functions driven by the vitamin B-12- and folate-dependent methylation cycle (Waly et al., 2004), and that Hg inhibits the coupling of iodine-containing thyroid proteins by the enzyme thyroperoxidase (Kawada et al., 1980; Nishida et al., 1986). Thus, Hg's enzyme binding capacity may relate to nutritional as well as genetic susceptibility in sensitive subpopulations.

Different human biomarkers of Hg indicate different forms of Hg exposures. Total blood Hg is primarily a biomarker of organic Hg exposure, and although it also may reflect exposure to inorganic Hg, total blood Hg is an established biomarker of exposure to methyl mercury over the past 1.5 to 2 months (EFSA, 2004). Mahaffey et al. (2004) showed a significant association between total self-reported fish intake over the past 30 days and total blood Hg in U.S. women of childbearing age (adjusted multiple correlation coefficient=.54; $p<0.0001$). Ursinyova et al. (2012) reported positive correlations between the number of maternal amalgam fillings and total Hg in maternal blood and cord blood ($r=0.460$; $p<0.001$ and $r=0.460$; $p<0.001$) and between the number of maternal amalgam fillings and methylmercury in maternal ($r=0.476$; $p=0.005$) and cord ($r=0.475$; $p=0.006$) blood in a sample of 75 mother-child pairs from Eastern Slovakia. Urine Hg, rather than blood Hg, is a recognized biomarker of human exposure to inorganic Hg that is eliminated over a period of several weeks to months (ATSDR, 1999).

There are few epidemiologic studies that examine associations between Hg exposure and autoantibodies that target the body's own cells or organs, or antibodies to infectious agents such as viruses; however, emerging evidence suggests associations between Hg exposure and immune response indicators. Specifically, high ANA titers (≥1:80 relative to <1:80) showed a significant positive association with incremental log-transformed changes in blood Hg in a homogeneous, riverine population (OR=2.8; 95% CI=1.1, 7.5) (Nyland et al., 2011). Moreover, Stern and Korn (2001) used simulation to show that large-scale epidemiologic research often fails to detect a dose-response relationship between Hg and neurodevelopmental endpoints if it only exists in sensitive subpopulations that comprise less than 5-10% of the total population (Stern and Korn, 2001). Subgroups with nutritional deficiencies may represent subpopulations susceptible to metal uptake (Gochfeld, 1997; Gallagher et al., 2011b). For example, iron deficiency was associated with 0.044 µg/g creatinine greater urine cadmium (95% CI=0.020, 0.069) and 0.162 µg/L greater blood cadmium (95% CI=0.132, 0.193) in U.S. women (Gallagher et al., 2011b). Further, the combined methylation cycle/transsulfuration pathway involving folate, vitamin B12, and homocysteine is emerging as a biologically plausible modifier of the association between environmental toxicants and immune-related disorders (Ji and Khuraran Hershey, 2012).

Homocysteine may represent a susceptibility cofactor through several biological mechanisms; for example, as a consequence of vitamin B12 and folate deficiency (Schroecksnadel , 2003), as an indicator of altered amino acid metabolism (Dufault et al., 2012), and as either a cause or consequence of immune-inflammatory activation characteristic of autoimmunity (Lazerini et al. (2007). Further, experimental research found that homocysteine inhibited metallothionein (Barbato et al., 2007), a protein that reduces Hg's bioavailability (Aschner, 1997; ATSDR, 1999)

and cytotoxicity (Rising et al., 1995; Vitarella et al., 1996; Yao et al., 1999), suggesting the potential for a Hg-homocsyteine interaction. My prior research showed an inverse relationship between Hg and homocysteine in boys with lower folate and B-12 levels, but not in other children; however, this subset showed higher homocysteine levels relative to boys and girls with higher B vitamin levels (Gallagher and Meliker, 2011). Therefore, studies that evaluate Hg dose-response in susceptible subpopulations may refine scientific knowledge regarding Hg toxicity.

In summary, mechanistic evidence indicates that Hg has both immunosuppressive and immunostimulatory effects. Epidemiologic research provides evidence to suggest that Hg is associated with systemic autoimmunity and decreased antiviral interferon in high exposure populations. Overall, however, epidemiologic studies to assess the potential role of Hg within the immunotoxicological continuum, and that stratify by susceptible subpopulations, are sparse.

Polychlorinated biphenyls, immunosuppression, and immunostimulation

Less is known about PCBs' effects on the immune system. Cord blood levels of Hg and PCBs were inversely and weakly correlated with naïve helper T-cells, and plasma IgG levels were higher in newborns from a subsistence fishing subgroup compared to a reference subgroup (Belles-Isles et al., 2002). Human studies have also shown an inverse relationship between prenatal PCB exposure and children's immune response to vaccines (Heilman et al., 2006), as well as increased odds for respiratory infection among infants with the highest non-dioxin-like PCB prenatal exposure and lowest dioxin-like PCB prenatal exposure (Glynn et al, 2008). Experimental studies have shown that PCBs reduce immune system function by binding to the aryl hydrocarbon receptor (AhR) (Silkworth and Grabstein, 1982; Kerkvliet, 1995; ATSDR, 2000; US EPA, 2012c). Mechanistic evidence also supports the biological plausibility of aryl

hydrocarbon receptor (AhR)-mediated xenobiotic-induced autoimmunity (Stockinger et al., 2011). The AhR is part of a transcription factor family that senses environmentally induced changes such as cytokines (immune mediator proteins), hormones and chemicals with consequent gene expression that can shift immune balance towards either immune suppression by regulatory T cells (Tregs) or autoimmunity by Th17 cells, a class of T cells that express the AhR (Stevens and Bradfield, 2008). Experimental research showed that dioxin enhanced Treg activity and suppressed autoimmunity in mouse models of experimental autoimmune encephalitis (Quintana et al., 2008), autoimmune uveoretinitis (Zhang et al., 2010), and Type 1 diabetes (Kerkvliet et al., 2009); however, the immune effects of dioxin-like and non-dioxin-like PCBs' binding with the AhR are uncertain, and epidemiologic studies to evaluate associations between PCBs and autoimmune function are sparse. AhR-mediated proinflammatory response is another mechanism of action for dioxin-like PCBs (Hennig et al., 2002; Kim et al., 2012), and for nondioxin-like PCBs, as suggested by a recent study of children with asthma (Tsuji et al., 2012). Further, a National Institute of Environmental Health Sciences expert panel workshop recently concluded that incomplete knowledge regarding the role of environmental contaminants in promoting Th17-mediated autoimmunity by ligation to AhR represents an important gap in scientific understanding (Selmi et al., 2012). A cross-sectional NHANES study reported a positive association between PCBs and rheumatoid type arthritis, an autoimmune disease, in women (Lee et al., 2007), and a study of highly PCB-exposed Taiwanese women showed increased risk for systemic lupus erythematosus mortality (Tsai et al., 2007). Cebecauer et al. (2009) observed a significant positive association between ANA prevalence and higher serum PCB concentrations among adults from East Slovakian districts that included one area with known heavy PCB pollution and two others with background PCB pollution. The relationship

between serum PCB concentrations and ANA prevalence has not been evaluated in a U.S. probability sample. Further, although ANA prevalence is higher among women compared to men (Satoh et al., 2012), associations between PCBs and ANA have not been investigated separately for males and females. In addition, associations between ANA and PCBs by congener type, i.e., dioxin-like versus nondioxin-like, have not been evaluated. This represents an important gap in public health knowledge because, per the Toxic Equivalency Factor (TEF) model for calculating PCB risk, the immunotoxicity of dioxin-like PCBs, but not nondioxin-like PCBs, is mediated by additive congener activation of the aryl hydrocarbon receptor (AhR) (Giesy and Kannan, 1998). Moreover, nondioxin-like PCBs may antagonize the immunotoxic effects of dioxin-like PCBs via non-AhR mediated mechanisms (Henry and DeVito, 2003; Tyrphonas and Feely, 2001). Therefore, studies to evaluate associations between ANA prevalence and dioxin-like and nondioxin-like PCBs, separately, and for males and females, separately, may contribute to scientific knowledge regarding toxicity of this persistent pollutant.

Data Source

My dissertation research utilizes publicly available data from The National Health and Nutrition Examination Survey (NHANES) to evaluate associations between antinuclear autoantibodies and Hg and PCBs, between thyroid autoantibodies and Hg, and between serum measles and rubella virus antibody concentratins and Hg. These measures were selected in consideration of their relevance to the proposed research and are described in greater detail in the pertinent chapters. Choice of exposure and outcome measures are limited by requirements for their dual availability in NHANES sample subsets of adequate sample size with consistent analyte measurement methods across survey years. NHANES uses a complex, multistage,

probability sampling design to select participants representative of the civilian, non-institutionalized US population. Each sample person is assigned a sample weight that reflects adjustments for complex survey design (including oversampling), survey nonresponses, and post-stratification, to ensure that calculated estimates are representative of the non-institutionalized US population (CDC, 2011c). NHANES is an unparalleled resource for environmental epidemiologic investigations because its data files contain the exposure and outcome variables of interest, as well as measures of potential confounders, effect modifiers and related covariates. NHANES data is intended for use in epidemiologic research to identify possible risk factors for disease and related outcomes (CDC, 2011a).

Overview of Dissertation Research

My dissertation research aims to address gaps in epidemiologic research on the relationships between environmental contaminants and immune response indicators in each of four studies A, B, C and D, presented in Chapters 1, 2, 3, and 4, respectively. **The primary aims of Study A are to evaluate the relationships between total blood Hg, seruumPCBs, and antinuclear antibody (ANA) positivity**, and is the first epidemiologic study to do so using a large and diverse US sample and stratifying by males and females in order to evaluate susceptibility by sex. Thus, my dissertation research extends knowledge of the relationship between Hg and PCBs and ANA positivity beyond high exposure populations. Further, this is the first study to evaluate PCB congener type-specific associations with ANA positivity**. I hypothesize that Hg and PCBs will be positively associated with ANA positivity.**

The primary aim of Study B is to evaluate the relationship between Hg and thyroid autoantibody positivity, and is the first to do so in US-representative sample stratified by sex

and iodine status in order to evaluate these recognized autoimmune thyroid disease susceptibility factors (Baskin, 2002; WHO, 2007). **I hypothesize that Hg will be positively associated with indicators of thyroid autoimmunity in females, and in additional stratification by iodine status, particularly for females with either iodine deficiency or iodine excess as defined by the World Health Organization (2007).**

The primary aims of Studies C and D are to evaluate the relationships between Hg exposure and serum concentrations of measles antibodies, and between Hg exposure and serum concentrations of rubella antibodies, in nutritionally-susceptible subpopulations of children; this study is unique in its assessment of dose-response for the outcome of IgG antibodies, measures of T cell-dependent functional immunity (Jusko et al., 2010; Dietert and Holsapple, 2007) and persistence of viral-specific B-cell immunity (Haralambieva et al., 2011) in sensitive subpopulations. **I hypothesize that Hg will be positively associated with serum measles and rubella antibody concentrations in a nutritionally susceptible subpopulation of children.**

Figure 2 presents an overview study diagram, Figure 3 presents epidemiologic knowledge and gaps, and Figure 4 presents study hypotheses. Together, these study aims address the immunotoxicology continuum, by addressing outcome indicators of both autoimmune and infectious response, and their relationships with chemical exposures of prime public health concern. The rationale for the proposed exposure-outcome analyses are supported by experimental studies that support biologically plausible mechanisms of action, epidemiologic studies that suggest relationships in human populations, as well as the availability of rigorously measured biomarkers of exposure and outcome in a dataset intended for hypothesis testing

(CDC, 2011a). Moreover, the proposed approaches are unique by virtue of utilizing larger, diverse samples with heterogeneous exposures, and susceptible subsets.

Statistical Analysis

Statistical analysis is conducted using SAS (Cary, N.C.) version 9.3 and incorporates primary sampling units, strata, and appropriate sample and subsample weights in accordance with complex survey design recommendations (CDC, 2011c). Logistic regression procedures are conducted to evaluate dichotomous outcomes and linear regression procedures are conducted to evaluate continuous outcomes. Odds ratios and 95% confidence intervals are reported as measures of effect for logistic regression and beta coefficients and 95% confidence intervals are reported as measures of effect for linear regression. Unweighted models are used to assess robustness of the direction and magnitude of the effect estimate. Bivariate linear regression and residual plots are visually inspected in order to determine whether linearity assumptions are met for linear regression, with re-examination using log-transformations. Results of multiple linear regression are presented in both tabular and plotted graph format; the latter using residualized exposure variables, as in previous work (Gallagher et al., 2011a). Sample weights were plotted against exposure measures to evaluate outliers per NHANES recommended methodology (CDC, 2011d). For both linear and logistic regression models, multicollinearity is assessed using tolerance statistics (Allison, 2006). Exposure variables are entered in the models as both continuous and categorical variables. A benefit of using continuous exposure variables is that incremental effects may be estimated in the absence of scientific knowledge regarding specific exposure levels associated with the outcomes of interest; however, a disadvantage is lack of information regarding dose-response. Therefore, categorical exposure variables were also used

e.g., quartiles or quintiles, with p-values reported for trend tests to evaluate dose-response relationships. In consideration of the reduced power of models that use categorical variables, continuous measures offer the additional benefit of preserving power, as well as the means to detect possible threshold effects by evaluating departures from linearity. Both multivariable-adjusted and unadjusted models are evaluated. Multivariable models are built based upon initial consideration of candidate covariate's relationships with the exposure and outcomes per the scientific literature, and based upon final consideration of model estimated effects using backward elimination methods. The rationale for stratified analyses, e.g., sex, nutrient status, is based upon prior evidence in the literature and statistical tests for interactions. Model performance is compared using the Akaike Information Criterion. Only models demonstrating adequate fit per the convergence criterion are presented. Alpha significance levels are set a priori at 5 percent.

Public Health Significance

As many as 23.5 million Americans have an autoimmune disease according to the National Institutes of Health (NIH, 2006-2007). The prevalence is increasing (US DHHS, 2005) and women are disproportionately affected (Walsh and Rau, 2000); however, the causes remain largely unknown (NIH, 2006-2007). Mercury and PCBs are environmental contaminants of concern because of widespread human exposure and mechanistic evidence of their association with immune response (Havarinasab and Hultman, 2005; Havarinasab et al., 2005; deVos et al., 2007; Gardner et al., 2010a, 2010b; Silkworth and Grabstein, 1982; Kerkvliet, 1995; US EPA, 2012c), yet epidemiologic studies of their association with immunity are sparse, particularly in general populations with background environmental, rather than higher exposures from known

industrial or occupational sources. In recognition of the threats that these ubiquitous contaminants pose to human health, the US EPA prioritizes Hg and PCBsfor environmental regulation, and Healthy People 2020 has targeted a 30 percent reduction in the level of blood Hg in U.S. children and women of childbearing age, as well as a 30 percent reduction in the level of serum PCBs in the U.S. population ages 12 years and older (US DHHS, 2011b). By investigating results separately for women, as well as looking into susceptible subpopulations such as children with nutritional deficiencies, any positive findings will shine the spotlight on the need for prospective studies of these relationships within the immunotoxicological continuum, and guide public health decision-making for the protection of the most vulnerable.

Figure 1.

Continuum of Immunotoxicology

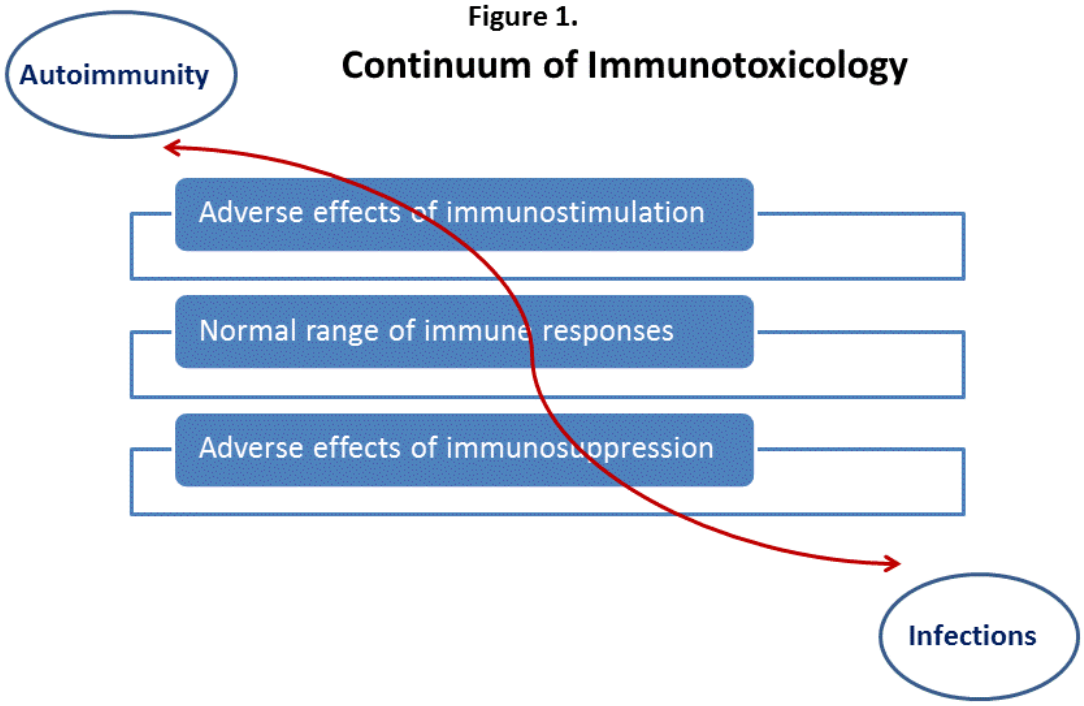

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Figure 2.

Overview Study Diagram:

Mercury, PCBs, and immune response indicators in NHANES

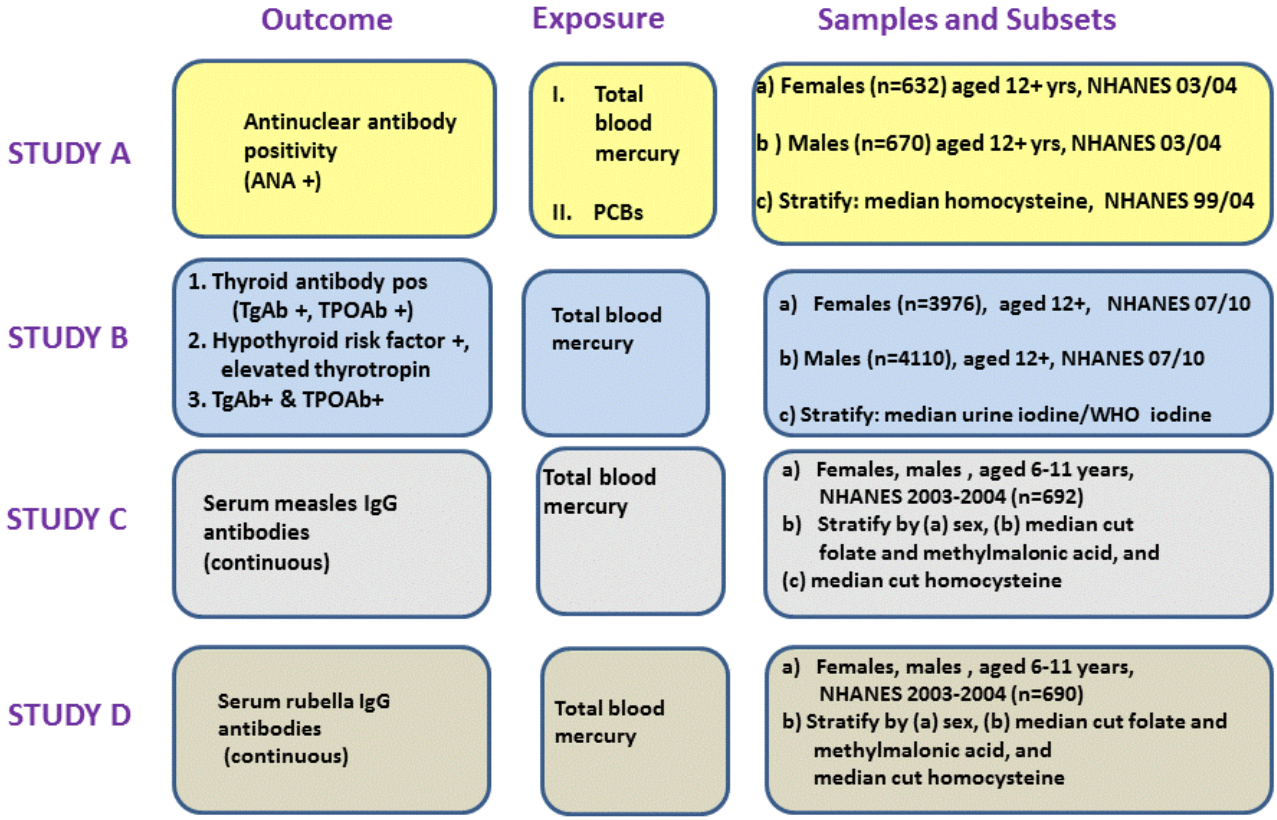


Figure 3

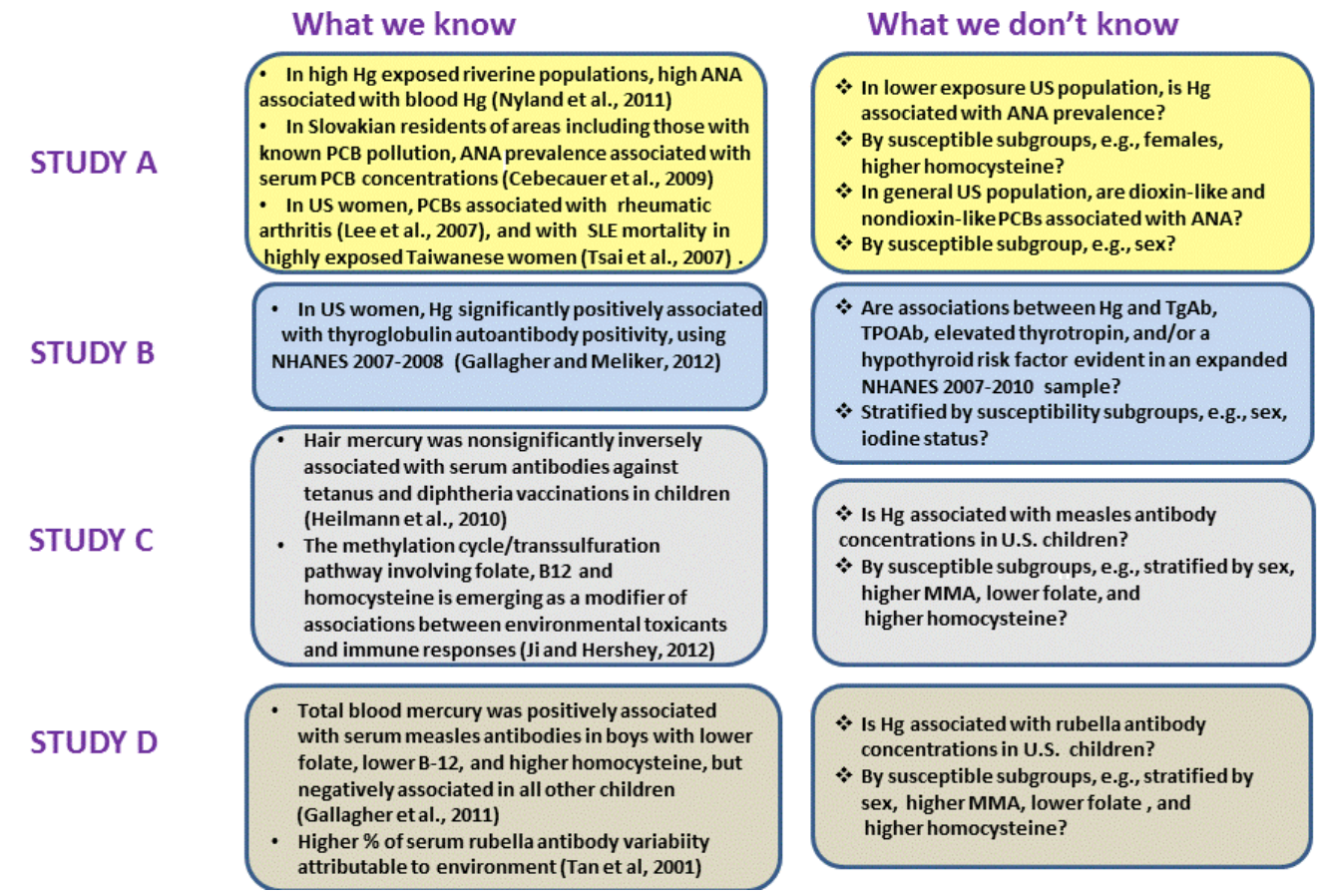
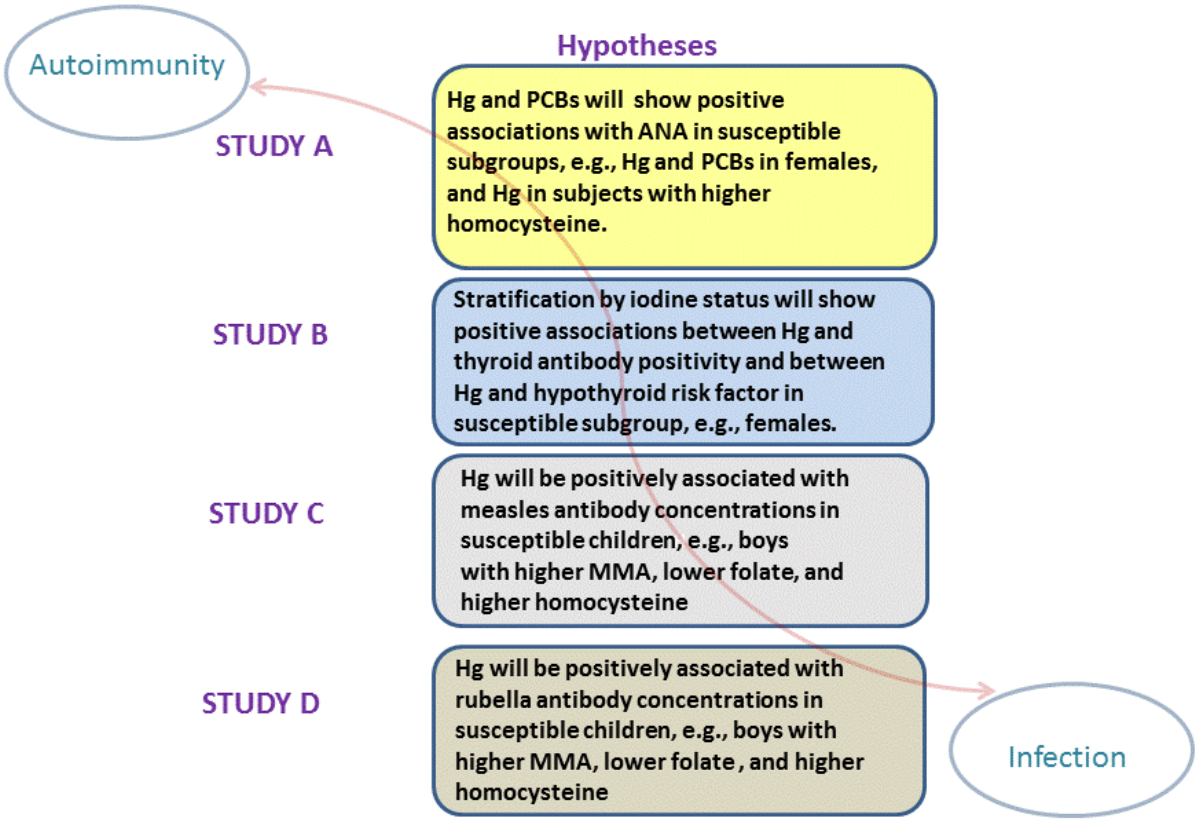

Hg, PCBs and immune response indicators: Epidemiologic Knowledge & Gaps
What we know
What we don't know
STUDY A
• In high Hg exposed riverine populations, high ANA associated with blood Hg (Nyland et al., 2011)
• In Slovakian residents of areas including those with known PCB pollution, ANA prevalence associated with serum PCB concentrations (Cebecauer et al., 2009)
• In US women, PCBs associated with rheumatic arthritis (Lee et al., 2007), and with SLE mortality in highly exposed Taiwanese women (Tsai et al., 2007) .
❖ In lower exposure US population, is Hg associated with ANA prevalence?
❖ By susceptible subgroups, e.g., females, higher homocysteine?
❖ In general US population, are dioxin-like and nondioxin-like PCBs associated with ANA?
❖ By susceptible subgroup, e.g., sex?
STUDY B
• In US women, Hg significantly positively associated with thyroglobulin autoantibody positivity, using NHANES 2007-2008 (Gallagher and Meliker, 2012)
❖ Are associations between Hg and TgAb, TPOAb, elevated thyrotropin, and/or a hypothyroid risk factor evident in an expanded NHANES 2007-2010 sample?
❖ Stratified by susceptibility subgroups, e.g., sex, iodine status?
STUDY C
• Hair mercury was nonsignificantly inversely associated with serum antibodies against tetanus and diphtheria vaccinations in children (Heilmann et al., 2010)
• The methylation cycle/transsulfuration pathway involving folate, B12 and homocysteine is emerging as a modifier of associations between environmental toxicants and immune responses (Ji and Hershey, 2012)
❖ Is Hg associated with measles antibody concentrations in U.S. children?
❖ By susceptible subgroups, e.g., stratified by sex, higher MMA, lower folate, and higher homocysteine?
STUDY D
• Total blood mercury was positively associated with serum measles antibodies in boys with lower folate, lower B-12, and higher homocysteine, but negatively associated in all other children (Gallagher et al., 2011)
• Higher % of serum rubella antibody variabiity attributable to environment (Tan et al, 2001)
❖ Is Hg associated with rubella antibody concentrations in U.S. children?
❖ By susceptible subgroups, e.g., stratified by sex, higher MMA, lower folate , and higher homocysteine?

Figure 4

Hg, PCBs and immune response indicators: Study Hypotheses



Chapter 1

Polychlorinated biphenyls, mercury, and antinuclear antibody positivity, NHANES 2003-2004

Abstract

Serum antinuclear antibody positivity (ANA) has been associated with elevated serum PCBs among residents in PCB-polluted areas; however, associations in general populations have not been reported by congener type or with adjustment for Hg. Cross-sectional data on serum PCBs, total blood Hg, ANA, and potential confounders age, race, body mass index, menopausal status, and dietary eicosapentaenoic acid (EPA) were obtained from the 2003-2004 National Health and Nutrition Examination Survey (NHANES) for males and females aged 12-85. PCB congeners were summed separately for dioxin-like and nondioxin-like PCBs; the former were weighted for toxic equivalent factors. Total PCBs by congener type and Hg were analyzed as both continuous log-transformed variables and as categorical quintiles. Logistic regression models were stratified by sex. There were no associations between nondioxin-like PCBs or Hg and ANA among males or females. Among females (n=114 affected and 518 unaffected), adjusting for potential confounders, the prevalence odds for ANA positivity were significantly elevated per incremental increase in log-transformed dioxin-like PCBs (Odds Ratio {OR}=1.66; 95% Confidence Interval {CI}=1.24, 2.23); the highest dioxin-like PCB quintile (>0.00425 - 0.04339 ng/g) was significantly associated with 4.04 (95% CI=2.43, 6.70) greater prevalence odds for ANA positivity relative to the lowest quintile ($p_{trend}<0.001$). We present novel findings of an association between low-level dioxin-like PCBs and ANA among women. No associations were observed between Hg and ANA at Hg levels common to the US population.

Introduction

Autoimmune diseases are common, of increasing prevalence, and are characterized by antibodies that attack the body's own normal cells (USDHHS, 2005). Antinuclear antibodies (ANA) are antibodies directed against proteins within the cell nucleus, and are indicators of systemic autoimmunity in humans (Golightly and Golightly, 2002), as well as higher mortality risk (Chou et al., 2011). Polychlorinated biphenyls are persistent environmental contaminants. Higher serum dioxin-like chemical concentrations including PCBs have been associated with increased mortality risk in the U.S. population (Lin et al., 2012). Although PCBs have not been manufactured and used in the U.S. since 1977, U.S. residents may still be exposed to PCBs by eating contaminated food, particularly fish (ATSDR, 2000). Contaminated air is another important exposure source (ATSDR, 2000); for example, the United States Environmental Protection Agency issued guidelines for the safe removal and disposal of PCB-containing fluorescent light ballasts because leaks may expose school-children, teachers, and other school personnel to unsafe levels of PCBs in the air they breathe (US EPA, 2012b). Immunosuppression and immunostimulation are recognized biological effects of PCB exposure (Kramer et al., 2012; Strauss and Heiger-Bernays, 2012). Experimental studies have shown that PCBs reduce immune system function by binding to the aryl hydrocarbon receptor (AhR) (Silkworth and Grabstein, 1982; Kerkvliet, 1995; ATSDR, 2000; US EPA, 2008; Stockinger et al., 2011). AhR-mediated proinflammatory response is another mechanism of action for dioxin-like PCBs (Hennig et al., 2002; Kim et al., 2012), and for nondioxin-like PCBs, as suggested by a recent study of children with asthma (Tsuji et al., 2012). Human studies have shown an inverse relationship between prenatal PCB exposure and children's immune response to vaccines (Heilman et al., 2006), as

well as increased odds for respiratory infection among infants with the highest non-dioxin-like PCB prenatal exposure and lowest dioxin-like PCB prenatal exposure (Glynn et al, 2008). A cross-sectional NHANES study reported a positive association between PCBs and rheumatoid type arthritis, an autoimmune disease, in women (Lee et al., 2007), and Tsai et al. (2007) reported higher death rates caused by systemic lupus erythematosus among females exposed to PCBs by consuming contaminated rice; both of these autoimmune diseases are characterized by detectable antinuclear antibodies (Chellingworth et al., 1984; Golightly and Golightly, 2002). Cebecauer et al. (2009) observed a significant positive association between ANA prevalence and higher serum PCB concentrations among adults from East Slovakian districts that included one area with known heavy PCB pollution and two others with background PCB pollution.

The relationship between serum PCB concentrations and ANA prevalence has not been evaluated in a U.S. probability sample. Further, although ANA prevalence is higher among women compared to men (Satoh et al., 2012), associations between PCBs and ANA have not been investigated separately for males and females. Nor have associations between ANA and PCBs by congener type, i.e., dioxin-like versus nondioxin-like, been evaluated. This represents an important gap in public health knowledge because, per the Toxic Equivalency Factor (TEF) model for calculating PCB risk, the immunotoxicity of dioxin-like PCBs, but not nondioxin-like PCBs, is mediated by additive congener activation of the aryl hydrocarbon receptor (AhR) (Giesy and Kannan, 1998). An additional consideration is that nondioxin-like PCBs may antagonize the immunotoxic effects of dioxin-like PCBs via non-AhR mediated mechanisms (Henry and DeVito, 2003; Tyrphonas and Feely, 2001). Therefore, the primary aim of this study

is to evaluate associations between ANA prevalence and dioxin-like and nondioxin-like PCBs, separately, and for males and females, separately, in the general U.S. population.

Total blood Hg, a biomarker of methylmercury from dietary fish consumption, may potentially confound the relationship between PCBs and ANA, or be associated with ANA independent of PCBs. Among fish consumers in Amazonian Brazil, high ANA titers (≥1:80, relative to <1:80) were positively associated with an incremental increase in log-transformed blood Hg (OR=2.8; 95% CI=1.1, 7.5) (Nyland et al., 2011). Therefore, the primary analysis of the relationship between ANA prevalence and dioxin-like and nondioxin-like PCBs considers total blood Hg as a potential confounder, and a secondary aim is to evaluate associations between total blood Hg and ANA prevalence.

Methods

Study design

Cross-sectional, secondary data on serum autoantibodies to human cellular antigens , serum polychlorinated biphenyl (PCB) concentrations, total blood Hg, 24-hour estimated dietary intake of EPA, body mass index, menopausal status and demographic data were obtained from the 2003-2004 National Health and Nutrition Examination Survey (NHANES), a stratified multistage probability sample of the civilian non-institutionalized population of the U.S.

Analytic sample

A one third subsample of NHANES 2003-2004 participants aged 12 years and older in the subsample with serum measures of dioxin-like and nondioxin-like PCBs had stored sera used for testing of antinuclear antibody positivity (ANA). Data on these measures were not available in

other years of data with the exception of the 1999-2002 NHANES datasets; however, serum PCB data from these years were limited by considerably higher limits of detection, so were not included in the current study. The analytic sample excluded pregnant or lactating women in order to minimize potentially related hormonal influences and was limited to survey participants with data on the variables described in the preceding section on study design. The analytic sample domain was stratified by sex yielding 632 females and 670 males.

Outcome measure

Testing of IgG autoantibodies to human cellular antigens was performed by the HEp-2 cell immunofluorescence assay (CDC, 2012a). Antinuclear antibody (ANA) positivity was defined as intensities of 3 and 4 (Satoh et al., 2012).

Exposure measures

PCBs were measured in serum by high-resolution gas chromatography/isotope dilution high-resolution mass spectrometry (CDC, 2008). Values for serum PCB concentrations below the limit of detection were calculated by dividing the detection limit by the square roof of 2 (CDC, 2008).This study used values reported on a lipid-adjusted basis. Serum lipid-adjusted PCB levels are recognized biomarkers of body burden and/or exposure, with half-lives greater than one week (ATSDR, 2000). Exposure source and amount influence serum half-life. For example, for large exposures to PCBs in river waters, the estimated half-life is 8-9 months (ATSDR, 2000).

Dioxin-like PCBs included the following PCB congeners, with corresponding (toxic equivalency factor {Van den Berg et al., 2006}) and [limit of detection {CDC, 2009}]: PCB 81 (0.0003) [13.1 pg/g]; PCB 105 (0.00003) [0.4 ng/g]; PCB 118 (0.00003) [0.6 ng/g); PCB 126

(0.10) [13.9 pg/g]; PCB 156 (0.00003) [0.4 ng/g]; PCB 157 (0.00003) [0.4 ng/g]; PCB 167 (0.00003) [0.4 ng/g]; PCB 169 (0.03) [15.9 pg/g]; PCB 189 (0.00003) [0.4 ng/g]. After applying the toxic equivalency factors, toxic equivalents (TEQs) of dioxin-like PCBs were summed and log-transformed to address skewness in order to create a continuous measure of total dioxin-like PCBs. Nontransformed total dioxin-like serum PCB TEQs were grouped into quintiles based upon the analytic sample frequency distribution in order to create a categorical variable for evaluating dose-response.

Nondioxin-like PCBs included the following PCB congeners, with corresponding [limits of detection {CDC, 2009}]: PCB 28 [1.7 ng/g]; PCB 44 [0.4 ng/g]; PCB 49 [0.4 ng/g]; PCB 52 [0.8 ng/g]; PCB 87 [0.4 ng/g]; PCB 99 [0.6 ng/g]; PCB 101 [0.6 ng/g]; PCB 110 [0.8 ng/g]; PCB 128 [0.4 ng/g]; PCB 138 [0.4 ng/g]; PCB 146 [0.4 ng/g]; PCB 149 [0.4 ng/g]; PCB 151 [0.4 ng/g]; PCB 153 [1.1 ng/g]; PCB 170 [0.4 ng/g]; PCB 172 [0.4 ng/g]; PCB 177 [0.4 ng/g]; PCB 178 [0.4 ng/g]; PCB 180 [0.4 ng/g]; PCB 183 [0.4 ng/g]; PCB 187 [0.4 ng/g]; PCB 187 [0.4 ng/g]; PCB 194 [0.4 ng/g]; PCB 195 [0.7 ng/g]; PCB 196&203 [0.4 ng/g]; PCB 199 [0.4 ng/g]; PCB 206 [0.7 ng/g]; PCB 209 [0.7 ng/g]. Nondioxin-like PCBs were summed and log-transformed to address skewness in order to create a continuous measure of total nondioxin-like PCBs. Nontransformed total nondioxin-like serum PCB concentrations were grouped into quintiles based upon the analytic sample frequency distribution in order to create a categorical variable for evaluating dose-response.

Covariates

Satoh et al. (2012) observed that, in addition to female sex, other factors associated with ANA prevalence in a U.S. representative sample were older age, African American race and normal

weight. Therefore, these factors were considered as potential confounders in statistical models. Body mass index (BMI) was calculated as weight (kilograms) divided by height (meters squared). For adults, standard BMI cut points were used to define underweight (BMI <18.5), normal weight (referent; 18.5 ≥BMI< 25), overweight (BMI 25 ≥BMI <30, and obese (BMI≥30). For adolescents, corresponding sex-specific BMI-for-age weight status categories were used (CDC, 2000). Age was measured in years as a continuous covariate. Race/ethnicity was categorized into three groups: nonHispanic white (referent), nonHispanic black and other. Eicosapentaenoic acid (EPA), an n-3 polyunsaturated fatty acid derived from fish oil, is another potential confounder, as a recent review interpreted scientific findings to suggest that induction of Tregs by EPA may inhibit autoimmune response (Iwami et al., 2011). Dietary intake of EPA was estimated by trained NHANES interviewers based upon 24-hour recall of respondents, and was limited to participants with reliable recall, also as determined by trained NHANES interviewers (CDC, 2007). A categorical variable was developed for EPA using the following groupings: none (referent), above sample median (≥0.009 gm), and below sample median. Whole blood Hg concentrations were measured using inductively coupled plasma mass spectrometry (CDC, 2006b), with a lower detection limit of 0.2 µg/L. A continuous measure of total blood Hg was log-transformed to address skewness; Hg was also grouped into quintiles based on the frequency distribution to address dose-response. Menopausal status was also included as a covariate in order to control for the potentially confounding effect of related hormonal status (Deng and Liu, 2009; Lahita et al., 1979; Lahita et al., 1981; Lahita et al., 1982; Weidler et al., 2004); a woman was considered menopausal if she was not an adolescent, not pregnant or lactating, and reported not having menstruated over the past 12 months.

Statistical Analysis

Statistical analysis was conducted using SAS (Cary, N.C.) version 9.3. Primary sampling units, strata, and dioxin subsample weights were incorporated in accordance with complex survey design recommendations for hypothesis testing and the generation of U.S. population-representative statistics (CDC, 2011c). In the primary analysis, logistic regression analysis was used to evaluate the relationships between dioxin-like and non-dioxin-like PCBs and ANA positivity, stratified by sex. In the secondary analysis, relationships between total blood Hg and ANA positivity were similarly evaluated. Odds ratios and 95% confidence intervals were reported as measures of estimated effects, both unadjusted and adjusted for covariates. Simple and multiple logistic regression were used to evaluate associations between the binary outcome measure of ANA positivity and dioxin-like and non-dioxin-like PCBs and Hg, as both continuous log-transformed measures and categorical quintiles, with p-values reported for trend tests to evaluate dose-response relationships. A sensitivity analysis was conducted using unweighted measures of association by omitting sampling weights from the model but incorporated sample strata and primary sampling units to account for the complex, multistage, probability sampling design (CDC, 2011c). Multicollinearity was assessed using tolerance statistics, with a value of <0.40 considered indicative of collinearity (Allison 2006). Additional sensitivity analysis was conducted to assess a model that omitted a covariate with a borderline tolerance statistic. Adequacy of model fit was evaluated using the convergence criterion; alpha significance levels were set a priori at 5 percent.

Results

There were no statistically significant associations between PCBs or Hg and ANA among males; results are presented in Tables for females, only. Analyte concentrations did not differ between ANA positive and ANA negative females. Menopausal status was the only significantly different covariate between ANA positive and ANA negative females, with a greater proportion of ANA negative females menopausal (39%) relative to ANA positive females (29%) (Table 1).

There were no statistically significant associations between nondioxin-like PCBs and ANA in females; results are presented in Table 2 for dioxin-like PCBs, only. Multicollinearity diagnostics for the fully-adjusted Model 4 indicated tolerance < 0.40 for age (0.23), log-transformed dioxin-like PCBs (0.30) and log-transformed nondioxin-like PCBs (0.20). Model 5 omitted collinear variables age and nondioxin-like PCBs, and showed that, per unit increase in log-transformed total dioxin-like PCBs, the odds for ANA positivity in women were significantly increased by 66%, adjusted for total blood Hg, race, body mass index, menopausal status and dietary EPA (OR=1.66; 95% CI=1.24, 2.23) (Table 2). In the unadjusted Model 1, the odds for ANA positivity per unit increase in log-transformed total dioxin-like PCBs was 1.17 (95% CI=0.91, 1.52), but increased by 32% to 1.54 (95% CI=0.88, 2.70) when log-transformed total nondioxin-like PCBs were added in Model 2. Nondioxin-like PCBs showed a nonsignificant inverse relationship with ANA in the model with both dioxin-like and nondioxin-like PCBs (Table 3).

Relative to the lowest dioxin-like PCB quintile, the highest dioxin-like PCB quintile showed significantly increased odds for ANA positivity in women in Model 2 that adjusted for nondioxin-like PCBs (OR=3.83; 95% CI=1.12, 13.06); in Model 3 that additionally adjusted for total blood Hg (OR=4.30; 95% CI=1.32, 14.04); in Model 4 that additionally adjusted for age, race, body mass index, menopausal status, and dietary EPA (OR=5.36; 95% CI=1.55, 18.54);

and in Model 5 that omitted collinear covariates of age and non-dioxin-like PCBs (OR=4.04; 95% CI=2.43, 6.70). Corresponding p-values for trend for these adjusted models were 0.047, 0.021, 0.021, and <0.001, respectively (Table 2).

In the secondary analysis, log-transformed total blood Hg was nonsignificantly inversely associated with ANA in women in unadjusted (OR=0.74; 95% CI=0.42, 1.30), fully adjusted (Model 4: OR=0.69; 95% CI=0.42, 1.12), as well as in the analysis that omitted collinear variables (Model 5: OR=0.63; 95% CI=0.40, 1.01) (Supplementary Table). There was no evidence of a statistically significant trend in the models using Hg quintiles; however, the odds ratios comparing Hg quintiles to the lowest quintile decreased as the Hg quintiles (Q) increased. For example, in Model 5: Q2: OR=2.15; 95% CI=1.04, 4.46, Q3: OR=2.04; 95% CI=0.71, 5.88, Q4: OR=1.50; 95% CI=0.90, 2.52; Q5: OR=0.95, 95% CI=0.64, 1.39) (Supplementary Table).

Discussion

We report the novel finding of a positive association between dioxin-like PCBs and ANA in U.S. females, but not males, consistent with the greater prevalence of ANA positivity among women (Satoh et al., 2012). Previous research of the relationship between non-TEF-weighted total serum PCB concentrations of dioxin-like and non-dioxin-like PCBs, combined, i.e., PCBs 28, 52, 101, 105, 114, 118, 123, 138, 153, 156, 157, 167, 170, 180, and 189) and ANA in East Slovakian adults from regions with both known and background PCB pollution reported an approximate twofold greater prevalence of ANA positivity among males and females, combined, with PCB levels in the 4^{th} (1,517-2,734 ng/g lipid) and 5^{th} (2,740-32,273) highest quintiles relative to the lowest PCB quintile (149-679 ng/g lipid) (Cebecauer et al., 2009). Comparable lipid-adjusted serum PCB exposure levels in the U.S. are substantially lower. In the current

NHANES study, we conducted a supplemental frequency distribution analysis, without TEF weighting, in males and females, combined, limited to these same PCBs, with the exception of PCBs 114 and 123 which were not included in the CDC's National Exposure Report, and the addition of PCB 158, which is co-reported with PCB 138 (CDC, 2010). Corresponding U.S. upper quintile limits for the 1st, 4^{th} and 5^{th} quintiles were 31.01 ng/g, 176.0 ng/g, and 1624.3 ng/g, respectively. Therefore, we show for the first time a positive association between ANA positivity and lower background environmental exposures to dioxin-like PCBs in U.S. women.d

Evidence of a suppressive confounding effect (MacKinon et al., 2000) on the estimate for dioxin-like PCBs was observed for nondioxin-like PCBs; this is consistent with scientific knowledge regarding the apparent antagonistic effects of some nondioxin-like congeners on the immunotoxic effects of dioxin-like congeners, particularly through non-AhR mediated biological mechanisms (Henry and DeVito, 2003; Tryphonas and Feely, 2001), although statistical influences also merit consideration. Specifically, we considered the statistical influences of multicollinearity among independent variables as a possible explanation for the observed elevated risk from dioxin-like PCB exposure. Using the 0.40 cutoff tolerance statistic suggested by Allison (2006), multicollinearity diagnostics indicated multicollinearity between log-transformed dioxin-like PCBs (Tolerance=0.30), log-transformed nondioxin-like PCBs (Tolerance=0.20), and age in years (Tolerance=0.23), with approximately twofold inflation of standard errors. Among females, log-transformed dioxin-like PCBs were significantly correlated with log-transformed nondioxin-like PCBs (r=0.81), limiting our ability to adequately tease out dioxin-like effects independent of non-dioxin-like effects. However, it is notable that Glynn et al. (2008) also reported strong correlations among dioxin-like and nondioxin-like PCBs ($r \geq 0.93$)

in infants, as well as differential effects on immune response, although in an opposite direction with increased risk for respiratory infections associated with nondioxin-like PCB exposures, and protective association with dioxin-like PCB exposures.

In the current study, age in years was also significantly correlated with dioxin-like PCBs (r=0.68) and nondioxin-like PCBs (r=0.80). This finding is consistent with work from others (Cebecauer and et al., 2009; Lee et al., 2007; Lin et al., 2012) suggesting multicollinearity and difficulty statistically adjusting for age. Nonetheless, age is a possible confounder for many outcomes hypothesized to be associated with PCBs; therefore most investigators choose to adjust for age.

In light of possible multicollinearity for highly correlated covariates age, nondioxin-like PCBs, and dioxin-like PCBs , we chose to present results both with and without adjustment for these covariates. Model 5, with dioxin-like PCBs and without correlated nondioxin-like PCBs and age variables, generated results similar to the fully adjusted Model 4, with slightly attenuated estimates and more stable confidence intervals. This model included the menopause covariate in order to control for the statistically indicated and biologically plausible confounding effects of estrogen-related influences (Deng and Liu, 2009; Lahita et al., 1979; Lahita et al., 1981; Lahita et al., 1982; Weidler et al., 2004).

Results presented use data weighted in accordance with complex survey design so that estimates would be representative of the U.S. population (CDC, 2011c). In sensitivity analyses, we also examined robustness of results without sampling weights; in Model 5, the estimate for associations between the continuous measure of dioxin-like PCBs and ANA was attenuated (OR per unit increase in log-transformed total dioxin-like PCBs=1.34 (95% CI=1.12, 1.61). The odds

ratio comparing the highest to the lowest PCB quintile was similarly attenuated (OR=2.04; 95% CI=1.29, 3.22; $P_{trend}<0.001$) (results not shown in tables). The attenuation of the relationship between dioxin-like PCBs and ANA in the unweighted model suggests the potential for underestimating associations in the absence of complex survey weighting and reveals the influence of several heavily weighted data points.

Menopause showed statistically significant, albeit weaker, correlations with log-transformed dioxin-like PCBs (r=0.49), log-transformed nondioxin-like PCBs (r=0.61), and age (r=0.76). Sensitivity analysis was also conducted using a weighted model that, in addition to omitting age and nondioxin-like PCBS, omitted the menopause covariate, which showed a borderline tolerance statistic of 0.42. Observed associations were attenuated for continuous log-transformed dioxin-like PCBs (OR=1.31; 95% CI=1.01, 1.71). Relative to the lowest dioxin-like PCB quintile, the highest dioxin-like PCB quintile's association with ANA was also attenuated (OR=2.23; 95% CI=1.37, 3.64; $P_{trend}=0.015$).

Inclusion of highly correlated variables into the same regression model has been shown to cause unstable results that may result in incorrect conclusions (Allison, 2006; Steinmaus et al., 2009); therefore, consideration of multicollinearity in epidemiologic studies of PCBs is critical. Weighted Model 5 balanced controlling for confounding by menopause with minimizing multicollinearity, and generated stable and elevated odds ratios for the relationship between dioxin-like PCBs and ANA positivity. Further research is merited to confirm dioxin-like PCB effects on human immune responses.

The aryl hydrocarbon receptor (AhR) is part of a transcription factor family that senses environmentally induced changes such as cytokines (immune mediator proteins), hormones and

chemicals with consequent gene expression that can shift immune balance either towards immune suppression or autoimmunity (Stevens and Bradfield, 2008). Our findings of a positive association between dioxin-like PCBs and ANA positivity in females, together with other human studies that report associations between PCB exposures and cellular autoimmunity (Cebecauer et al., 2009) and autoimmune disease (Lee et al., 2007; Tsai et al., 2007) lend support to mechanistic evidence of dioxin-like PCB proinflammatory effects via the Aryl hydrocarbon receptor (Hennig et al., 2002; Kim et al., 2012). Mechanistic research indicates that the direct association of the AhR with estrogen or androgen receptors mediates the modulation of estrogen/androgen signaling by dioxins (Ohtake et al., 2008; 2011), and that the dioxin-like PCB congener 126 exerts estrogenic effects (Mortenson and Arukwe, 2008). Further, the AhR also mediates estrogen signaling through the ubiquitin system, which senses environmental stressors such as dioxin (Ohtake et al., 2011), and may play a role in preventing systemic autoimmunity (Tavares et al., 2010; Kool et al., 2011) with deregulation of this system linked to autoimmune diseases (Fierabracci, 2012). Additional mechanistic research is warranted to elucidate how PCBs might differentially affect immune response in males and females via the aryl hydrocarbon receptor.

Inclusion of total blood Hg in the models did not appreciably alter the effect estimates for dioxin-like PCBs; therefore, there was a lack of evidence for confounding. Moreover, total blood Hg was not associated with ANA in the secondary analysis. This is in contrast to findings of Nyland et al. (2011) in a higher methylmercury-exposed riverine population. The authors noted that, although the range of values of blood Hg levels in their population included the range of values reported in the U.S. population, the geometric mean in the riverine population (42.5

µg/L) was significantly higher compared to the U.S. population (1.02 µg/L) (Mahaffey et al., 2004; Nyland et al., 2011). The upper quintile of Hg in our study population begins at 1.8 µg/L. Therefore, we interpret the current null findings as indicative of the absence of a Hg-ANA association at lower levels of Hg exposure characteristic of the U.S. population.

A major strength of the current study is the use of a U.S. probability sample to generate prevalence odds ratios that are generalizable to the U.S. population of women, thus enhancing external validity. Moreover, restricting analysis to the NHANES dataset with the most sensitive serum PCB detection enhances internal validity. An important limitation is that, as in any cross-sectional epidemiologic study, causality cannot be determined in the absence of an established temporal association. Further, this study has limited power given reliance on only 2003-2004 data; moreover, attenuation in unweighted analyses and in analyses without age or menopause adjustment suggests the possibility of chance findings cannot be ruled out. Residual confounding also may have influenced our findings, although potential confounders were identified from the scientific literature and models statistically adjusted for their possible influences. We were not able to include serum measure of EPA; however, our variable for estimated 24 hour dietary EPA intake was significantly, albeit weakly, correlated with serum EPA in a small subsample of 194 NHANES 2003-2004 participants with serum EPA measures within the dioxin subsample (r=0.30). Given recent findings that ANA positivity was associated with a higher mortality risk among children and adolescents (Chou et al., 2011) and their potential for exposure in school environments (US EPA, 2012b), further research is warranted to address potential health risks among this vulnerable subpopulation. Future studies of PCBs also warrant special consideration of multicollinearity.

In conclusion, using a U.S. probability sample, our findings suggest a positive association specific to dioxin-like PCBs and ANA positivity in women, but do not lend support to previous findings of associations between ANA positivity and Hg at levels of exposure common in the U.S. Results do, however, lend support to U.S. public health objectives for the reduction of human exposure to PCBs (US DHHS, 2011b). Prospective studies using larger sample sizes are called for to validate our findings, and to further investigate PCBs and autoimmune diseases.

Table 1. Sample weighted mean (standard error) values, frequency distributions (#) and weighted proportions (%) by antinuclear antibody (ANA) positivity[a] status, U.S. females aged 12-85 years, NHANES 2003-2004.

	ANA positive (n=114)	ANA negative (n=518)
Mean (SE) total serum dioxin-like PCBs[b] (ng/g) Min/max	$3.61x10^{-3}$ ($0.4x10^{-3}$) $0.44x10^{-3}$/$43.39x10^{-3}$	3.58x10-3 ($0.2x10^{-3}$) $0.44x10^{-3}$/$30.73x10^{-3}$
Mean (SE) total serum nondioxin-like PCBs (ng/g)[c] Min/max	143.24 (15.84) 14.53/1411.68	150.87 (7.24) 11.94/1032.36
Mean (SE) total blood mercury (µg/l) Min/max	1.30 (0.13) 0.10/8.9	1.47 (0.09) 0.10/24.20
Mean (SE) age (years) Min/max	39 (2) 12/85	39 (1) 12/85
# (%) with: No reported EPA intake[d] EPA intake below the sample median EPA intake above the sample median	 31 (28) 41 (33) 42 (39)	 153 (30) 181 (33) 184 (37)
# (%): NonHispanic white NonHispanic black Hispanic and other	 51 (72) 31 (12) 32 (16)	 247 (73) 122 (12) 149 (15)
# (%): Normal weight Underweight Overweight Obese	 55 (51) 2 (2) 26 (21) 31 (27)	 215 (40) 7 (3) 128 (26) 168 (31)
# (%) Menopausal	32[e] (29)	181 (39)

a. ANA positivity defined as intensity ≥ 3 (Miller et al., 2012).
b. Sum of lipid-adjusted serum dioxin-like PCBs weighted per World Health Organization toxic equivalent factors (WHO 2005).
c. Sum of lipid-adjusted serum nondioxin-like PCBs
d. 24 hour intake of dietary eicosapentaenoic fatty acid (EPA) (gm) estimated by trained NHANES interviewers
e. Statistically significant difference at α=0.05 level

Table 2. Odds ratios (95% CI) for the relationships between antinuclear antibody (ANA) positivity[a] (n=114 affected + 518 unaffected) and total dioxin-like PCBs[b], U.S. females aged 12-85 years, NHANES 2003-2004.

	Model 1: Unadjusted	Model 2: Adjusted for NonDioxin-Like PCBs	Model 3: Additionally adjusted for Hg	Model 4: Additionally adjusted for age, race, body mass index, menopausal status, and estimated 24-hour dietary intake of EPA[c]	Model 5: Omit covariates with tolerance<0.40 (age and non-dioxin-like PCBs)
Per unit increase log-transformed total dioxin-like PCBs	1.17 (0.91, 1.52)	1.54 (0.88, 2.70)	1.61 (0.92, 2.83)	1.82 (0.93, 3.54)	1.66 (1.24, 2.23)
Dioxin-like PCB quintiles (ng/g):					
Q1: ≤0.00117	Referent	Referent	Referent	Referent	Referent
Q2: 0.00117 <PCB≤0.00165	0.88 (0.38, 2.05)	1.02 (0.42, 2.48)	1.06 (0.44, 2.58)	1.06 (0.43, 2.63)	0.99 (0.41, 2.36)
Q3: 0.00165<PCB≤0.00245	1.78 (0.97, 3.26)	2.59 (1.08, 6.18)	2.67 (1.10, 6.46)	2.62 (0.90, 7.63)	2.19 (1.03, 4.68)
Q4: 0.00245<PCB≤0.00425	1.15 (0.68, 1.97)	1.97 (0.82, 4.76)	2.18 (0.90, 5.26)	2.18 (0.88, 5.41)	1.79 (1.01, 3.17)
Q5: >0.00425	1.72 (1.00, 2.94)	3.83 (1.12, 13.06)	4.30 (1.32, 14.04)	5.36 (1.55, 18.54)	4.04 (2.43, 6.70)
	P_{trend}=0.117	P_{trend}=0.047	P_{trend}=0.021	P_{trend}=0.021	P_{trend}<0.001

a. ANA positivity defined as intensity ≥ 3 (Miller et al., 2012).

b. Dioxin-like PCBs weighted per World Health Organization toxic equivalent factors (WHO 2005).

c. 24 hour intake of dietary eicosapentaenoic fatty acid (gm) estimated by trained NHANES interviewers (none, below and above sample median)

Table 3. Odds ratios (95% CI) for the relationships between antinuclear antibody (ANA) positivity[a] (n=114 affected + 518 unaffected) and total dioxin-like PCBs[b], total nondioxin-like PCBs and total blood mercury (Hg), U.S. females aged 12-85 years, NHANES 2003-2004.

	Model 1[c]: Unadjusted	Model 2[d]: Dioxin-like PCBs and NonDioxin-Like PCBs	Model 3[d]: Dioxin-like PCBs, NonDioxin-Like PCBs, and Hg	Model 4[d]: Additionally adjusted for age, race, BMI, menopause, est. 24-hr EPA[e]	Model 5[d]: Omit covariates with tolerance <0.40[f]
Per unit increase log-transformed total dioxin-like PCBs	1.17 (0.91, 1.52)	1.54 (0.88, 2.70)	1.61 (0.92, 2.83)	1.82 (0.93, 3.54)	1.66 (1.24, 2.23)
Dioxin-like PCB quintiles (ng/g):					
Q1: ≤0.00117	Referent	Referent	Referent	Referent	Referent
Q2: 0.00117 <PCB≤0.00165	0.88 (0.38, 2.05)	1.02 (0.42, 2.48)	1.06 (0.44, 2.58)	1.06 (0.43, 2.63)	0.99 (0.41, 2.36)
Q3: 0.00165<PCB≤0.00245	1.78 (0.97, 3.26)	2.59 (1.08, 6.18)	2.67 (1.10, 6.46)	2.62 (0.90, 7.63)	2.19 (1.03, 4.68)
Q4: 0.00245<PCB≤0.00425	1.15 (0.68, 1.97)	1.97 (0.82, 4.76)	2.18 (0.90, 5.26)	2.18 (0.88, 5.41)	1.79 (1.01, 3.17)
Q5: >0.00425	1.72 (1.00, 2.94)	3.83 (1.12, 13.06)	4.30 (1.32, 14.04)	5.36 (1.55, 18.54)	4.04 (2.43, 6.70)
	P_{trend}=0.117	P_{trend}=0.047	P_{trend}=0.021	P_{trend}=0.021	P_{trend}<0.001
Per unit increase log-transformed total nondioxin-like PCBs	1.02 (0.82, 1.26)	0.73 (0.44, 1.20)	0.74 (0.45, 1.23)	0.62 (0.26, 1.48)	1.39 (0.96, 2.00)
NonDioxin-like PCB quintiles (ng/g):					
Q1: ≤40.46	Referent	Referent	Referent	Referent	Referent
Q2: 40.46 <PCB≤66.31	1.16 (0.48, 2.82)	0.99 (0.41, 2.39)	1.04 (0.41, 2.60)	0.89 (0.35, 2.26)	1.23 (0.50, 3.04)
Q3: 66.31<PCB≤124.36	1.23 (0.42, 3.64)	0.87 (0.25, 3.03)	0.95 (0.26, 3.41)	0.66 (0.15, 2.95)	1.54 (0.43, 5.51)
Q4: 124.36<PCB≤237.68	1.31 (0.52, 3.32)	0.69 (0.24, 1.99)	0.77 (0.27, 2.21)	0.59 (0.14, 2.43)	2.29 (0.76, 6.88)
Q5: >237.68	0.86 (0.33, 2.30)	0.29 (0.06, 1.33)	0.30 (0.06, 1.44)	0.19 (0.03, 1.43)	1.65 (0.53, 5.11)
	P_{trend}=0.892	P_{trend}=0.163	P_{trend}=0.199	P_{trend}=0.211	P_{trend}=0.165
Per unit increase log-transformed Hg	0.74 (0.42, 1.30)	---	0.69 (0.39, 1.21)	0.69 (0.42, 1.12)	0.63 (0.40, 1.01)
Hg quintiles (µg/l):					
Q1: ≤0.3	Referent	---	Referent	Referent	Referent
Q2: 0.3 <Hg≤0.6	1.92 (1.05, 3.53)		2.01 (1.02, 3.96)	2.12 (1.01, 4.47)	2.15 (1.04, 4.46)
Q3: 0.6<Hg≤1.0	1.90 (0.80, 4.54)		1.92 (0.75, 4.92)	1.99 (0.66, 5.99)	2.04 (0.71, 5.88)
Q4: 1.0<Hg≤1.8	1.49 (0.96, 2.31)		1.46 (0.91, 2.33)	1.46 (0.84, 2.54)	1.50 (0.90, 2.52)
Q5: >1.8	1.07 (0.61, 1.87)		1.01 (0.58, 1.73)	1.05 (0.70, 1.58)	0.95 (0.64, 1.39)
	P_{trend}=0.565		P_{trend}=0.384	P_{trend}=0.411	P_{trend}=0.243

a. ANA positivity defined as intensity ≥ 3 (Miller et al., 2012).

b. Dioxin-like PCBs weighted per World Health Organization toxic equivalent factors (WHO 2005).

c. Model 1 presents unadjusted odds ratios and 95% CIs for each analyte as generated by separate logistic regression models that exclude the other analytes
d. Models 2 – 5 present adjusted odd ratios and 95% CIs for each analyte as generated by separate logistic regression models that adjust for other analytes using their log-transformed continuous values.
e. 24 hour intake of dietary eicosapentaenoic fatty acid (gm) estimated by trained NHANES interviewers (none, below and above sample median)
f. In Model 5, variables omitted due to multicollinearity: for dioxin-like PCBs, age and nondioxin-like PCBs were omitted. In Model 5 for nondioxin-like PCBS, age and dioxin-like PCBs were omitted. In Model 5 for mercury, age and non-dioxin-like PCBs were omitted.

Chapter 2

Total blood mercury and thyroid autoantibodies in U.S. females and males aged 12 years and older,

NHANES 2007-2010

Abstract

Mercury (Hg) accumulates in the thyroid gland, and prior research suggests a positive association between higher Hg exposures and thyroglobulin autoantibody positivity in U.S. women. Iodine deficiency and excess are risk factors for autoimmune thyroid disease; however, the relationships between Hg and thyroid autoantibodies have not been examined in models that stratify by iodine status. Further, although autoimmune thyroid disease shows a clear sex-bias, findings of sexual dimorphism have not been reported regarding associations between Hg and thyroid autoimmunity.

Data on total blood Hg, urine iodine, thyroglobulin autoantibody positivity (TgAb), thyroid peroxidase positivity (TPOAb), and thyrotropin were obtained from the 2007-2010 National Health and Nutrition Examination Survey for females and males aged 12 years and older. Multiple logistic regression was used to evaluate associations between Hg and TgAb, TPOAb, elevated thyrotropin, a risk factor for hypothyroidism that included each of these measures, and dual thyroid autoantibody positivity. Models were stratified by sex and median-cut urine iodine levels, and by World Health Organization iodine status categories.

Mercury was positively associated with the hypothyroidism risk factor among females with lower iodine and iodine deficiency, who also showed positive associations between Hg and

thyroid peroxidase autoantibodies, but not thyroglobulin autoantibodies. Hg was positively associated with dual thyroid autoantibody positivity in females with iodine excess. An overall inverse pattern for the relationship between Hg and thyroid autoantibody-containing measures was evident among males with higher and excessive iodine levels. Among iodine deficient subjects, Hg and elevated thyrotropin showed a nonsignificant positive relationship in females and a significant positive association in males.

Study findings provide novel evidence to suggest an interaction between sex and Hg-associated thyroid autoimmune response indicators in humans. Higher Hg exposures are a potential risk factor for thyroid autoimmune response indicators in females with either deficient or excessive iodine levels. Longitudinal studies using larger sample sizes are merited.

Note: This chapter represents a substantially updated version of a study results previously published:

Gallagher, CM, Meliker JR. Mercury and thyroid autoantibodies in U.S. women, NHANES 2007-2008. Env Int 2012;40:39-43.

Introduction

Environmental toxicants may trigger autoimmune thyroid disease in susceptible individuals (Brent, 2010) and there is emerging evidence that the heavy metal Hg, a ubiquitous environmental contaminant, may play a role. Mercury accumulates in the human thyroid gland, as shown in studies of occupationally exposed workers (Falnoga et al., 2000; Nylander and Weiner, 1991) and industrially exposed residents (Falnoga et al., 2000). Higher levels of blood and hair Hg, indicators of organic Hg exposure (ATSDR, 1999), have been associated with detectable antinuclear autoantibodies (ANA) and antinucleolar autoantibodies (ANoA), respectively, biomarkers of cellular autoimmunity, in non-occupationally-exposed human subjects (Silva et al., 2004; Nyland et al., 2011). In addition, removal of inorganic Hg-containing dental amalgams resulted in significantly decreased levels of the thyroid autoantibodies thyroglobulin antibody (TgAb) and thyroid peroxidase antibody (TPOAb) (Sterzl et al., 2006) suggesting a positive association between Hg and these antibodies. Pusey et al. (1990) showed that repeated low dose injections of inorganic Hg in rats induced polyclonal activation with increases in thyroglobulin autoantibodies.

Thyroglobulin autoantibodies (TgAb) and thyroid peroxidase autoantibodies (TPOAb) are recognized markers of thyroid autoimmunity (McLachlan and Rapoport, 2004; Baskin, 2002). Thyroglobulin antibody is an antibody against the thyroid protein thyroglobulin, and thyroid peroxidase antibody is directed against thyroid peroxidase, a thyroid enzyme. Elevated levels of these thyroid autoantibodies have been observed in patients with autoimmune thyroiditis (Baskin, 2002), and in patients with other autoimmune disorders: TgAb in patients with systemic lupus erythematosus (Lu et al. 2006; Parente et al. 2009; Porkodi et al., 2004) and both TgAb

and TPOAb in patients with rheumatoid arthritis (Atzeni et al., 2008; Porkodi et al., 2004), pernicious anemia (Chan et al., 2009), fibromyalgia (Bazzichi et al., 2007; Pamuk and Cakir, 2007) and diabetes (Prazny et al., 2005). Women are at increased risk for autoimmune disease (Dunaif, 2010) and mortality (Walsh and Rau, 2000, and therefore, represent a susceptible subpopulation.

We recently reported findings of a positive association between total blood Hg and thyroglobulin autoantibodies in U.S. women (Gallagher and Meliker, 2012). Using data on 2,047 women aged 20 years and older from the NHANES 2007-2008 dataset, we observed that, relative to women with the lowest blood Hg levels (≤0.40 µg/L), women with Hg >1.81 µg/L (upper quintile) showed 2.24 (95% CI=1.22, 4.12) greater odds for thyroglobulin autoantibody positivity (p_{trend}=.032), but not TPOAb positivity) (Gallagher and Meliker, 2012). This original analysis used dietary sample weights as this sample was restricted to survey participants with data on NHANES-estimated 24 hour intake of the beneficial fatty acid eicosapentanoic acid, a potential confounder (Mahaffey et al., 2008; Makino et al., 2001). Although iodine status, both deficiency and excess, plays a role in autoimmune thyroid disease (Baskin, 2002; WHO, 2007; Brent, 2010), sample size was not sufficient in our previous study to stratify by this potential susceptibility cofactor (Gallagher and Meliker, 2012). Therefore, to increase power for iodine-stratified analysis, the first aim of the current study is to examine the relationships between total blood Hg and TgAb and TPOAb in US females using the expanded sample for NHANES 2007-2010 that also includes younger ages 12-19 years, as well as survey participants without dietary data..

Studies have also shown that thyroid autoantibodies TgAb and TPOAb are prognostic indicators for long-term risk of hypothyroidism (Hutfless et al., 2011; Li et al., 2008; Vanderpump et al., 1995; Walsh et al., 2010), a disorder of thyroid hormone deficiency more prevalent in women and most commonly caused by autoimmune thyroiditis (NIH, 2011). The measurement of thyroid stimulating hormone, or thyrotropin, is the most valuable test to diagnose hypothyroidism and subclinical hypothyroidism, or mildly elevated thyrotropin (Baskin, 2002). Walsh et al. (2010) showed that, among women with baseline thyrotropin levels above 4.0 μIU/ml coincident with TgAb or TPO positivity, the prevalence of hypothyroidism after 13 years was 85.7%. Although we previously reported an elevated odds ratio for the association between total blood Hg ≥1.81 μg/L and thyrotropin >4μIU/mL coincident with thyroid autoantibody positivity (OR=1.35; 95% CI=0.58, 3.09), findings were not statistically significant. Therefore, the second aim of the current study is to evaluate the associations between total blood Hg and elevated thyrotropin coincident with thyroid antibody positivity, in the expanded 2007-2010 sample of US women, with stratification by iodine status. Consistent with our previous study, associations between Hg and elevated thyrotropin will be similarly re-examined.

Further, Pedersen et al. (2003) observed higher thyroid antibody concentrations in the presence of simultaneous thyroglobulin and thyroid peroxidase autoantibody positivity, and interpreted this as an indication that thyroid antibody positivity represents general immune system activation, consistent with Pusey and colleagues' observation of polyclonal activation of thyroglobulin autoantibodies in rats exposed to inorganic Hg (1990). Thus, the third aim of this study is to evaluate the association between total blood Hg, an indicator of exposure to both

organic and inorganic Hg, and the additional outcome of coincident thyroglobulin autoantibody and thyroid peroxidase autoantibody positivity, in the overall sample and stratified by iodine status.

Sexual dimorphism in the endocrine-immune response, particularly autoimmunity, is unequivocal (Da Silva, 1995, 1999; Gaillard and Spinedi, 1998; McCombe et al., 2009; Lee and Chiang, 2012, Pennell et al., 2012, Quintero et al., 2012). Although mechanistic evidence supports Hg's endocrine disruptive effects (Zhu et al, 2000) and epidemiological studies link high Hg exposures with cellular autoimmune response (Silva et al., 2004; Nyland et al., 2011), there is no clear evidence of an interaction between sex and Hg-induced autoimmunity in humans (Pollard, 2012).

Autoimmune thyroid disease shows a clear sex-bias (Gaillard and Spinedi, 1998). The female: male prevalence ratio for Grave's disease, or hyperthyroidism, is 3.5:1 and, for Hashimoto's thyroiditis, or hypothyroidism, is 5.2:1 (McCombe et al., 2009). We previously examined cross-sectional associations between total blood Hg and indicators of thyroid autoimmune response in females (Gallagher and Meliker, 2012), but not in males; therefore, the fourth aim of the current study is to evaluate these same relationships in males.

Methods

Study design

Cross-sectional data on total blood Hg , serum concentrations of thyroglobulin antibody (TgAb), thyroid peroxidase antibody (TPOAb), and thyrotropin, and urine iodine concentrations were obtained from the 2007-2010 National Health and Nutrition Examination Survey

(NHANES) files (CDC, 2012b,c). This study is an expanded version of the study we previously conducted using NHANES 2007-2008 data (Gallagher and Meliker, 2012).

Analytic sample

For females, the analytic sample subpopulation was restricted to females aged 12 years and older without missing values for Hg, TgAb and TPOAb, thyrotropin and urinary iodine, as iodine excess and deficiency have been linked with autoimmune thyroid disorders (Powell et al., 1999; WHO, 2006). In consideration of pregnancy (Baskin, 2002) and estrogen (Klecha et al., 2008) as potential influences with regard to thyroid function, pregnant or lactating women were excluded from the analytic sample domain. For males, the analytic sample subpopulation was restricted to males aged 12 years and older without missing values for Hg, TgAb and TPOAb, thyrotropin and urinary iodine. A difference from the original study is that this expanded study did not restrict subjects to those with dietary measures of eicosapentaenoic acid.

Outcome measures

Dichotomized variables were created for positive laboratory results for thyroglobulin antibody (>4.0 IU/mL) and for thyroid peroxidase antibody (>9 IU/mL) using CDC definitions for normal laboratory values derived from the NHANES sample, per National Academy of Clinical Biochemists criteria for establishing a normal reference range for thyroid antibody testing (CDC, 2009). In addition, a dichotomous variable was created for coincident thyroglobulin antibody and thyroid peroxidase antibody positivity. A dichotomous variable was also created for thyrotropin >4.0 μIU/mL, as well as for hypothyroid risk, defined as thyrotropin > 4.0 μIU/mL with either thyroglobulin antibody >4.0 IU/mL or thyroid peroxidase antibody >9

IU/mL, per longitudinal study findings of Walsh et al. (2010). Data on other thyroid autoantibodies were not available from NHANES.

Exposure measures

Whole blood Hg concentrations were measured using inductively coupled plasma mass spectrometry (CDC, 2012b,c). Total blood Hg is primarily a biomarker of organic Hg exposure, and though it may also reflect exposure to inorganic Hg, total blood Hg is an established biomarker of exposure to methylmercury Hg over the past 1.5 to 2 months (EFSA, 2004). Mahaffey et al. (2004) showed a significant association between total self-reported fish intake over the past 30 days and total blood Hgin U.S. women of childbearing age (adjusted multiple correlation coefficient=.54; $p<0.0001$). More recently, however, Ursinyova et al. (2012) showed that total blood Hg and methylmercury in maternal and cord blood also correlate with the number of dental amalgams, with correlation coefficients ≥0.46, among residents of Eastern Slovakia where ambient air exposure to Hg levels is typical for the European Union. A continuous measure of total blood Hg was log-transformed to address skewness. In the overall female sample, Hg was also grouped into the following quintiles (Q) based on the frequency distribution to address dose-response: Q1: Hg ≤ 0.37 µg/L, Q2: 0.37<Hg≤0.62 µg/L, Q3: 0.62<Hg≤1.00 µg/L, Q4: 1.05<Hg≤1.92 µg/L, Q5: Hg>1.92 µg/L. In the iodine-stratified subsamples, Hg was grouped into the following tertiles (T): T1: Hg≤0.53 µg/L, T2: 0.53<Hg≤1.15 µg/L, T3: Hg>1.15 µg/L. In the overall male sample, Hg was grouped into the following quintiles (Q) based on the frequency distribution to address dose-response: Q1: Hg ≤ 0.38 µg/L, Q2: 0.38<Hg≤0.65 µg/L, Q3: 0.65<Hg≤1.05 µg/L, Q4: 1.00<Hg≤1.73 µg/L, Q5:

Hg>1.73 µg/L. In the iodine-stratified subsamples, Hg was grouped into the following tertiles (T): T1: Hg≤0.54 µg/L, T2: 0.54<Hg≤1.24 µg/L, T3: Hg>1.24 µg/L.

Covariates and Modifiers

Dichotomous variables were created for race/ethnicity (non-Hispanic white relative to non-white), self-reported current hormone (including use of birth control pills and hormone therapy) and self-reported menopausal status. Age was measured in years as a continuous variable. Multicollinearity diagnostics indicated menopause and age were collinear; therefore, a categorical variable for age was created for 12-19 years, 20-49 years (referent), and 50 years and older, the latter was considered a reasonable proxy for menopausal status. For females, a categorical variable was created for NHANES-estimated 24 hour dietary intake of eicosapentaenoic acid (EPA) (CDC, 2012d,e) (none=referent, < median cutpoint=0.008 g, and ≥ median cutpoint=0.008g, and a category that included subjects for whom dietary intake was not ascertained; the latter was included to preserve sample size). For males, the median cut-point for EPA was 0.010 g. For females and males, a categorical variable was created to measure urine iodine (UI) status per the World Health Organization definitions of iodine deficient (UI <100 µg/L), iodine sufficient (100≤UI<300 µg/L) (referent), and iodine excess (UI≥300 µg/L) (WHO, 2006). In addition, for females, a dichotomous variable was created to stratify the analytic sample into women with urine iodine < and ≥ the sample median (143.25 µg/L), and for males, the median cut-point for urine iodine was 166.7 µg/L.

Statistical analysis

Statistical analysis was conducted using SAS version 9.3 (Cary, NC). Primary sampling units, strata, and medical examination sample weights including an environmental subsample weight for the one-third subsample with urine iodine measured in the NHANES 2009-2010 data file were incorporated in accordance with complex survey design recommendations for hypothesis testing and the generation of U.S. population-representative statistics (CDC, 2011c). Logistic regression analysis was used to evaluate the relationships between Hg and thyroid outcomes, in the overall sample and stratified by median cut urine iodine; the latter was supported by a marginally statistically significant interaction term for the hypothyroid risk factor outcome in the female subsample. Axtell et al. (2000) showed that use of a smoothed continuous measure of Hg can increase power to detect threshold effects that outweigh the beneficial effects of eicosapentaenoic acid (EPA) (Makino et al., 2001; Ergas et al., 2002); therefore, this study used both log-transformed and untransformed total blood Hg variables to evaluate departures from linearity. Odds ratios and 95% confidence intervals were reported as measures of estimated effects, both unadjusted and adjusted for covariates. Simple and multiple logistic regression were used to evaluate associations between the binary thyroid outcomes and Hg; the latter as both continuous log-transformed exposure measures and categorical exposure measures, with P-values reported for trend tests to evaluate dose-response relationships. A sensitivity analysis was conducted using unweighted measures of association by omitting weights from the model but incorporating sample strata to account for the design effects of sample stratification and primary sampling units to account for the design effects of clustering (CDC, 2011c). Additionally, a sensitivity analysis using the continuous log-transformed Hg variable in models stratified by World Health Organization iodine status (2007) was conducted, and findings are presented for

iodine deficient and excess subsamples. Adequacy of model fit was evaluated using the convergence criterion. Alpha significance levels were set a priori at 5 percent.

Results for Females

Descriptive statistics

The overall sample was comprised of 3976 females, 8% were thyroglobulin antibody positive, 14% were thyroid peroxidase antibody positive, 7% with thyrotropin levels > 4 μIU/mL, 3% with the hypothyroid risk factor identified by Walsh et al. (2010), and 5% with dual thyroid antibody positivity (Table 1). Mean total blood Hg was greater for cases than controls for thyroid outcomes that included measures of autoantibodies; however, findings were not statistically significant. Mean thyroglobulin antibody concentration was significantly greater among cases for all outcomes except thyrotropin > 4 μIU/mL, and mean thyroid peroxidase antibody concentration was significantly greater among cases for all outcomes. Mean concentrations of each thyroid antibody was greatest among cases with dual thyroid antibody positivity, compared to cases for all other thyroid outcomes, consistent with findings of Pedersen et al. (2003). A greater percentage of cases were comprised of nonHispanic white females compared to nonwhite females, for each of the thyroid outcomes, and this finding was statistically significant with the exception of the dual thyroid antibody positivity outcome. The differential distribution of cases and noncases by WHO iodine status subgroups was statistically significant only for the outcome of thyrotropin > 4 μIU/mL. For each of the thyroid outcomes, the distribution of cases and noncases by age group showed statistically significant differences, with a greater percentage of cases than controls comprised of females aged 50 years and older. The distribution of cases and noncases by hormone use was significantly different only for thyroglobulin antibody positivity

and thyroid peroxidase antibody positivity, with a greater percentage of noncases currently using birth control pills or hormone therapy. The distribution of cases and noncases was not significantly different by dietary eicosapentaenoic acid subgroups.

Nonstratified models

Statistically significant positive associations were observed in nonstratified, unadjusted models for the relationships between log-transformed continuous Hg and thyroid peroxidase antibodies (OR=1.32; 95% CI=1.05, 1.66) and elevated thyrotropin coincident with thyroid antibody positivity (OR=1.38; 95% CI=1.01, 1.88); however, estimates were attenuated and lost statistical significance in the adjusted models (OR=1.26; 95% CI=0.99, 1.62) and (OR=1.30; 95% CI=0.94, 1.81), respectively (Table 2). A departure from our previous findings is that total blood Hg was not significantly associated with thyroglobulin antibody positivity, although multivariable-adjusted odds were 49% higher for the highest Hg quintile compared to the lowest (OR=1.49) and the 95% confidence interval of 0.61, 2.77 included the original estimate of OR=2.24. This null finding was confirmed in a sample that evaluated women aged 20 and older, as in the original study design. Nonsignificantly elevated odds for the highest versus lowest Hg quintiles were also observed in nonstratified multivariable models for thyroid peroxidase antibody positivity (OR=1.15; 95% CI=0.80, 1.65); elevated thyrotropin coincident with thyroid antibody positivity, or hypothyroid risk factor, (OR=1.43; 95% CI=0.67, 3.08); and dual thyroid antibody positivity (OR=1.56; 95% CI=0.79, 3.09) (Table 2). Sensitivity analysis using unweighted models that incorporated complex survey strata and clusters showed similar results (not shown in tables). A term for the interaction between log-transformed, continuous total blood Hg and median-cut iodine status indicated marginal statistical significance in the model for the

hypothyroid risk factor (P=0.07). In light of this statistical finding, and in consideration of both iodine excess (Baskin, 2002) and deficiency (WHO, 2007) as risk factors for autoimmune thyroid disease, the sample was further stratified by iodine status.

Urine iodine < sample median

Among females with urine iodine below the sample median (Table 3), for the outcomes of thyroid peroxidase antibody positivity and the hypothyroidism risk factor, associations with the continuous, log-transformed Hg variable were statistically significant in both unadjusted (OR=1.66; 95% CI=1.17, 2.34 and OR=1.88; 95% CI=1.25, 2.85, respectively) and adjusted models (OR=1.66; 95% CI=1.19, 2.32 and OR=1.76; 95% CI=1.18, 2.62, respectively). In multivariable models, the highest relative to the lowest Hg tertile was elevated for all thyroid outcomes; however, only the model for the hypothyroid risk factor was statistically significant (OR=2.37; 95% CI=1.20, 4.71; P_{trend}=0.01).

Urine iodine ≥ sample median

Among women with urine iodine levels above the sample median (Table 4), no statistically significant associations between total blood Hg and thyroid outcomes were observed. Compared to women with urine iodine levels below the sample median, there was a change of direction for the relationship between the continuous Hg variable and the two outcomes that included measures of thyrotropin. With the exception of the hypothyroid risk factor, the relationship between all other thyroid outcomes and the highest Hg tertile relative to the lowest tertile also changed direction in multivariable models.

Stratification by WHO (2007) iodine categories

Sensitivity analysis of subsets by WHO iodine deficient and excess categories (Table 5) revealed statistically significant positive associations between continuous Hg and thyroid peroxidase antibody positivity among iodine deficient females (OR=1.69; 95% CI=1.15, 2.49), the hypothyroidism risk factor among iodine deficient females (OR=2.03; 95% CI=1.24, 3.32), and dual antibody positivity among females with iodine excess (OR=2.88; 95% CI=1.28, 6.49). Elevated thyrotropin was inversely associated with continuous Hg among females with excess urine iodine (OR=0.39, 95% CI=0.20, 0.78). With the exception of the hypothyroid risk factor among females with excess urine iodine, the remaining relationships were positive, albeit nonsignificant.

Results for Males

Descriptive statistics

The overall sample was comprised of 4110 males, 5% were thyroglobulin antibody positive, 7% were thyroid peroxidase antibody positive, 5% with thyrotropin levels >4 μIU/mL, 1% with the hypothyroid risk factor identified by Walsh et al. (2010), and 3% with dual thyroid autoantibody positivity. Mean total blood Hg levels were nonsignificantly higher in cases for the outcomes of thyroid peroxidase antibody positivity, thyrotropin > 4 μIU/mL, and the hypothyroid risk factor, and were nonsignificantly lower for the outcomes of thyroglobulin autoantibody positivity and dual autoantibody positivity (Table 6). Mean thyroglobulin autoantibody concentrations were highest among males with dual thyroid autoantibody positivity (171.64 IU/mL) and mean thyroid peroxidase autoantibody concentrations were highest among males with the hypothyroid risk factor (183.25 IU/mL). A greater percentage of cases were comprised of nonHispanic white males compared to nonwhite males, and this finding was

statistically significant with the exception of the thyroglobulin autoantibody positivity outcome. The differential distribution of cases and noncases by WHO iodine status subgroups was statistically significant only for the outcome of thyrotropin > 4 μIU/mL. For each of the thyroid outcomes, the distribution of cases and noncases by age group showed statistically significant differences, with a greater percentage of cases than controls comprised of males aged 50 years and older. The distribution of cases and noncases was significantly different by eicosapentaenoic acid subgroups for the outcomes of thyroglobulin autoantibody positivity and dual autoantibody positivity, but not for the other thyroid outcomes.

Nonstratified models

Statistically significant inverse associations were observed in nonstratified, multivariable models for the outcomes of thyroglobulin autoantibody positivity (OR, continuous Hg=0.62; 95% CI=0.42, 0.91; OR, highest Hg quintile relative to the lowest Hg quintile=0.40; 95% CI=0.21, 0.76; $P_{trend}<0.01$) and dual thyroid autoantibody positivity (OR, continuous Hg=0.41; 95% CI=0.18, 0.90; OR, highest Hg quintile relative to the lowest Hg quintile=0.25; 95% CI=0.10, 0.61; $P_{trend}<0.01$) (Table 7). A term for the interaction between log-transformed, continuous total blood Hg and median-cut iodine status did not indicate statistical significance for any of the outcomes; the lowest P-values were for the interaction between Hg and thyroglobulin autoantibody positivity (P=0.09) and for the hypothyroid risk factor (P=0.11).

Urine iodine < sample median

Among males with urine iodine below the sample median (Table 8), there were no significant findings in multivariable models.

Iodine ≥ sample median

Among males with urine iodine ≥ the sample median (Table 9), in multivariable models, significantly inverse associations were observed between thyroglobulin autoantibody positivity and continuous Hg (OR=0.29; 95% CI=0.11, 0.76) and categorical Hg tertiles 2 (T2) and 3 (T3) relative to the lowest tertile (T2 OR=0.49; 95% CI=0.25, 0.96; T3 OR=0.24; 95% CI=0.10, 0.57; $P_{trend}<0.01$), and between dual thyroid autoantibody positivity and continuous Hg (OR=0.20; 95% CI=0.05, 0.77) and categorical Hg tertiles (T2 OR=0.34; 95% CI=0.14, 0.83; T3 OR=0.21; 95% CI=0.09, 0.49; $P_{trend}<0.01$). A general pattern of nonsignificant inverse associations was otherwise observed, although the continuous Hg measure in the thyroid peroxidase autoantibody model showed virtually a null odds ratio.

Stratification by WHO (2007) iodine categories

Sensitivity analysis of subsets by WHO iodine deficient and excess categories using multivariable models (Table 10) showed a significantly positive association between continuous Hg and thyrotropin > 4 μIU/mL among iodine-deficient males (OR=1.89; 95% CI=1.05, 3.42) and a significantly inverse association between continuous Hg and thyroglobulin autoantibody positivity among males with excessive iodine levels (OR=0.21; 95% CI=0.05, 0.94).

Discussion

We report significant positive associations between total blood Hg and a risk factor for hypothyroidism, i.e., elevated thyrotropin coincident with thyroid autoantibody positivity (Walsh et al., 2010), among females, particularly those with lower iodine levels, with evidence of increasing dose-response. Sensitivity analysis evaluating this relationship in females who are

iodine deficient per the WHO (2007) definition supports this finding, with robust dose-response findings (OR for the highest Hg tertile=2.75; 95% CI=1.03, 7.34; P_{trend}=0.03), and thus, provides evidence to suggest that females with iodine deficiency represent a susceptible subgroup. Females with iodine deficiency also represented a susceptible subgroup for increased risk for thyroid peroxidase antibody positivity associated with Hg exposure, and females with excessive iodine levels may represent a susceptible subgroup with respect to Hg and dual thyroid antibody positivity. Further, by removing the influence of iodine sufficiency, sensitivity analysis revealed the relevance of the thyrotropin > 4 µIU/mL outcome. Specifically, the unique finding of an inverse relationship between Hg and thyrotropin > 4 µIU/mL among females with excess iodine, together with findings of an elevated, albeit nonsignificant, risk among females with iodine deficiency, raises the question of a possible synergistic effect of Hg with iodine deficiency on thyrotropin, and consequently, the elevated risk for the hypothyroid risk factor.

The potential influence of the essential trace element selenium on thyroid autoimmunity (Duntas, 2006) was also considered. A variable for estimated 24 hour selenium intake showed a weak positive correlation with total blood Hg, and was significantly positively associated with the elevated thyrotropin outcome measure, only. Inclusion of the median-cut selenium measure in the models for the relationship between total blood Hg and thyrotropin > 4 µIU/mL slightly strengthened effect estimates; however, results did not change substantially. Inclusion of the selenium covariate in the models for the hypothyroid risk factor also yielded similar results.

In males, a contrasting pattern was evident for outcome measures that included thyroid autoantibodies. Findings showed statistically significant inverse associations between total blood Hg and dual thyroid autoantibody positivity and thyroglobulin autoantibody positivity; both of

these findings were robust in the subset with urine iodine levels above the sample median, and Hg's inverse association with thyroglobulin autoantibody positivity was also robust in the subset with excessive iodine per the WHO designation (2007). Among iodine-deficient males, however, thyrotropin > 4 μIU/mL was positively associated with total blood Hg, a finding that parallels the weaker relationship observed among iodine-deficient females (OR=1.34; 95% CI=0.85, 2.13), and suggests a positive relationship between Hg and elevated thyrotropin regardless of sex. Another parallel finding is that inclusion of a median-cut selenium covariate in the models for elevated thyrotropin and the hypothyroid risk factor did not appreciably alter findings.

Further comparisons of these relationships with those observed in females reveals several interesting contrasts. First, in the overall samples, among females, Hg showed positive, albeit nonsignificant, associations with thyroid measures that included autoantibodies, whereas an inverse pattern was evident among males. Second, among females, positive associations between Hg and measures that included thyroid autoantibodies were evident in those with lower and deficient iodine levels, whereas among males, inverse associations between Hg and measures that included thyroid autoantibodies were evident in those with higher and excessive iodine levels. Thus, sexual dimorphism was suggested by both the direction of the association and the iodine subgroup of influence. Finally, an important difference was the finding that Hg was positively associated with a risk factor for hypothyroidism, i.e., elevated thyrotropin coincident with thyroid autoantibody positivity (Walsh et al., 2010), in females, but not in males. Therefore, Hg exposure may be a risk factor for autoimmune hypothyroidism in females, particularly those with lower iodine levels; however, at the population level, males do not appear to share this potentially synergistic environmental susceptibility.

Epidemiologic Consistency/Biological Coherence

Compared to our previous findings regarding associations between Hg and thyroglobulin autoantibody positivity (OR, continuous Hg: 1.83; 95% CI=1.21, 2.78 and OR, highest quintile Hg: 2.24; 95% CI=1.22, 4.12; P_{trend}=0.03) in women aged 20 and older (Gallagher and Meliker, 2012), findings in the current study regarding the association between the highest Hg quintile and thyroglobulin antibody positivity among females aged 12 and older (OR=1.49; 95% CI=0.61, 2.77), as well as in females aged 20 and older (OR=1.24; 95% CI=0.75, 2.03),were attenuated and did not reach statistical significance; therefore, our earlier findings may have been due to chance. A different sample weighting method may have also influenced results. In a supplemental analysis using an NHANES sample expanded to include survey year 2009/2010, as well as 2007/2008, but limited as in the first study to women aged 20 and older with dietary data and using dietary weights, the association between the highest Hg quintile and TgAb was statistically significant, albeit attenuated, and the 95% CI included the original estimate (OR=1.58; 95% CI=1.02, 2.46). Regardless, the association between TgAb and Hg is not interpreted as robust. In the current nonstratified analysis using the 2007-2010 sample not limited to those with dietary data, however, among females aged 12 years and older, a general pattern of increased odds for thyroid autoimmune-related outcomes nonsignificantly associated with elevated Hg levels was observed, particularly at the highest Hg quintile, ranging from 15% to 56% higher odds compared to the lowest Hg quintile in nonstratified analysis. Of note, in a sensitivity analysis that applied the dietary weight set to the subset of this 2007/2010 nonstratified sample with dietary data, statistically significant positive associations were observed for the relationships between Hg and thyroid peroxidase autoantibodies and the

hypothyroid risk factor; however, this finding was not robust in unweighted analyses of the subsample limited to subjects with dietary data, as well as the unrestricted sample. Similarly, the main study findings of associations between Hg and both thyroid peroxidase autoantibody positivity and the hypothyroid risk factor in females with lower iodine levels were robust using dietary subsample weights, yet attenuated in unweighted models. Sample weights were selected and combined across survey years in accordance with NHANES analytic guidelines (CDC, 2011d); however, given the differences observed between unweighted, dietary-weighted, and laboratory-weighted results, the effects of using different sample weights merit considerable future investigation. Although we cannot rule out chance findings or attribute a mechanism of action, in light of the original finding of a significant positive association between Hg and TgAb, together with the current finding of significant positive associations between Hg and TPO and the hypothyroid risk factor among the lower iodine subgroup, it is reasonable to consider these findings consistent with Pedersen and colleagues' (2003) interpretation that thyroid autoimmune positivity develops secondary to general alterations in the immune system, rather than specific antigenic mechanisms; the authors noted that both antibody concentrations were higher when dual antibody positivity was present, consistent with our findings. Further, this interpretation is coherent with mechanistic evidence of polyclonal IgG activation by Hg (Pusey et al., 1990; Abedi-Valugerdi, 2009). Several human observational studies have reported higher TgAb and TPOAb prevalence among subjects with moderate relative to mild iodine deficiency (Fenzi et al., 1986; Laurberg et al., 1998; Pedersen et al., 2003), and Li et al. (2008) found that high iodine intake was a risk factor for hypothyroidism among thyroid antibody positive subjects; therefore, both iodine deficiency and excess may represent susceptibility cofactors. Kawada et al. (1980) observed that Hg-exposed mice showed reduced uptake of iodine by the

thyroid gland. Iodine bound to thyroglobulin, a protein synthesized by thyroid follicular cells, forms monoiodotyrosine (MIT) and diiodotyrosine (DIT) which are combined by thyroperoxidase, a "coupling" enzyme, to form the thyroid hormones T4 (thyroxine) and T3 (triiodothyronine) (Klassen, 2008). In the hypothalamic-pituitary-thyroid axis, T4 and T3 provide negative feedback to the hypothalamus for the inhibition of thyrotropin release (Klaassen, 2008). Mercury inhibited this coupling process in mice, reducing iodine uptake and altering thyroid hormone levels (Kawada et al., 1980; Nishida et al., 1986). Using U.S. population-based data, Christensen et al. (2012) reported an inverse association between total blood Hg and the thyroid hormones T3 and free T3 in females and males with urinary iodine below the sample median. This supports the current study findings of elevated thyrotropin in subjects with lower iodine levels, as its release is inhibited by these hormones; however, definitive biological explanations for interactions with iodine status in humans are lacking.

Differing associations between Hg and thyroid immune markers by sex might be expected based on several lines of reasoning. Mechanistic studies indicate that sex hormones interact with cells of the immune system that are also affected by Hg. Estrogens induce Th2 cell development to activate antibody-producing B-cells, whereas testosterone can affect Th1 cell development leading to local inflammatory response (Lee and Chiang, 2012). Mercury's sulf-hydryl binding capacity facilitates its binding to glutathione to promote Th2 cells, and thus, similar to estrogen, activates antibody-producing B-cells (Shiraldi and Monestier, 2009). Androgens suppress B-cell immune responses, whereas estrogens (Da Silva, 1999) and Hg (Pusey et al., 1990; Abedi-Valgerdi,2009) activate B-cells to stimulate antibody production. Mechanistic, as well as epidemiological, evidence is lacking, however, of estrogen-Hg synergy in autoimmune disease.

Sexually dimorphic effects on the immune system may also act through the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis shows differential hormonal response to stress; for example, glucocorticoid response to stress, such as immune challenge, is inhibited by androgens and enhanced by estrogens (Da Silva, 1999). Animal studies showed that Hg accumulates in the adrenal gland, which produces glucocorticoids; however, how Hg might interact with the HPA axis in humans is poorly understood (Zhu et al., 2000). Recent research, however, observed that levels of cortisol, a prime glucocorticoid in humans, were inversely associated with blood levels of Hg in children, both male and female (Gump et al., 2012).

Within the hypothalamic-pituitary-thyroid axis, Hg-iodine interaction may influence thyroid function via inhibition coupling enzyme function with consequent disruption of the uptake of iodine (Kawada et al., 1980; Nishida et al., 1986), as previously described. Although Hg accumulates in the human pituitary and thyroid glands, epidemiological evidence fails to show adverse effects on endocrine function; therefore Zhu et al. (2000) suggest that Hg's toxicity is attributable to its sulf-hydryl binding property, and thus, has broader effects as an enzyme inhibitor. Questions are raised as to whether this process underlies the positive association between Hg and thyrotropin among iodine-deficient males and females by inhibiting negative feedback for thyrotropin release.

Strengths and Limitations

An overall advantage of using NHANES data is generalizability of findings to the U.S. population regarding associations between concurrently collected and rigorously measured biological markers of possible risk factors and immune indicators. In addition, Gonzales et al. (2002) observed a low within-subject variability of TgAb and TPOAb measurements overtime.

Repeated measurements, however, are needed to evaluate associations that account for intra-individual variability in blood Hg measurements over time, as recently shown by Tsuchiya et al., 2012. Further, in light of the small number of cases with the hypothyroid risk factor, as well as the inconsistent results using different sample weight sets, we cannot rule out chance findings. Therefore, the cross-sectional study design precludes causal determinations, and our findings merit cautious interpretation as a snapshot in time of the possible physiological relationship between Hg exposure and autoimmune thyroid outcomes in U.S. females and males aged 12 years and older.

Conclusion

We present novel evidence to suggest an interaction between sex and Hg-associated thyroid autoimmunity in humans. Mercury is a ubiquitous contaminant, and higher exposures may be a potential risk factor for thyroid autoimmune-related outcomes in nutritionally susceptible females, specifically, those with either deficient or excessive iodine levels. Autoimmune diseases are among the leading causes of death among young and middle-aged women (Walsh and Rau, 2000; NIH, 2005). Therefore, longitudinal studies with larger sample sizes are merited to validate our findings, which may be used to guide study design and shed light on public health knowledge for the protection of females, as well as vulnerable males, with respect to both Hg exposure and iodine nutrient status.

Table 1. Weighted[a] sample descriptive statistics, non-pregnant, non-lactating females aged 12 and older, NHANES 2007-2010 (n=3976)

	Thyroglobulin antibodies >4 IU/mL	Thyroid peroxidase antibodies >9 IU/mL	Thyrotropin > 4 µIU/mL	Thyrotropin > 4 µIU/mL coincident with thyroid antibody positivity[b] (hypothyroidism risk factor[c])	Both thyroglobulin antibody positivity and thyroid peroxidase antibody positivity
# cases (%) # noncases	321 (8%) 3655	551 (14%) 3425	268 (7%) 3708	124 (3%) 3852	183 (5%) 3793
Mean total blood mercury {µg/L} (SE): Cases Noncases	 1.54 (0.16) 1.45 (0.08)	 1.73 (0.19) 1.41 (0.07)	 1.44 (0.17) 1.46 (0.08)	 1.61 (0.15) 1.46 (0.08)	 1.66 (0.18) 1.45 (0.07)
Mean thyroglobulin antibody concentration {IU/mL} (SE): Cases Noncases	 135.32 (22.26)[d] 0.72 (0.01)	 46.33 (10.55)[d] 6.38 (2.16)	 56.25 (23.64) 9.45 (1.81)	 106.93 (38.28)[d] 9.17 (1.75)	 145.90 (29.16)[d] 5.81 (1.93)
Mean thyroid peroxidase antibody concentration {IU/mL} (SE): Cases Noncases	 118.82 (15.29)[d] 19.72 (2.32)	 181.08 (12.60)[d] 1.12 (0.05)	 113.25 (15.10)[d] 22.57 (2.50)	 215.47 (23.61)[d] 21.90 (2.39)	 216.69 (23.22)[d] 19.00
% cases/noncases comprised by nonHispanic white females	75%/68%[d]	77%/67%[d]	82%/67%[d]	85%/68%[d]	75%/68%
% cases/noncases by Iodine status[e]: Sufficient Deficient Excess	 44%/48% 36%/35% 21%/17%	 49%/47% 35%/35% 16%/18%	[d] 45%/47% 30%/36% 25%/17%	 46%/47% 33%/35% 21%/17%	 43%/47% 40%/35% 17%/17%
% cases/noncases by agegroup: 12-19 years 20-49 years 50+years	[d] 9%/13% 35%/49% 55%/38%	[d] 7%/13% 46%/49% 47%/38%	[d] 6%/13% 41%/49% 53%/39%	[d] 4%/13% 44%/48% 53%/39%	[d] 10%/12% 40%/49% 50%/39%
% cases/noncases currently using birth control pills or hormone therapy	5%/12%[d]	8%/12%[d]	12%/11%	9%/12%	4%/12%
% cases/noncases by estimated 24 hr EPA[f] intake: None Below median Above median Not ascertained	 19%/18% 36%/38% 40%/41% 5%/3%	 18%/18% 37%/38% 42%/41% 2%/3%	 21%/18% 38%/38% 39%/41% 2%/3%	 16%/18% 37%/38% 45%/41% 2%/3%	 18%/18% 33%/38% 43%/41% 5%/3%

a. Weighted for NHANES complex survey design.
b. Either thyroglobulin antibodies > 4 IU/mL or thyroid peroxidase antibodies > 9 IU/mL
c. Walsh et al. 2010
d. Significant difference between cases/noncases at P<0.05 using complex survey design
e. World Health Organization (2007) definitions for iodine sufficient based upon urine iodine concentrations (UI): sufficient: 100≤UI<300 μ/L; deficient: UI<100 μg/L; excess: UI≥300 μg/L
f. Eicosapentaenoic acid dietary intake estimated by NHANES, median=0.008 g

Table 2. Logistic regression results for the relationship between total blood mercury (Hg) and thyroid outcome measures; non-pregnant, non-lactating females aged 12 years and older; NHANES 2007-2010 (n=3976)

Outcome measure	No. cases	Unadjusted odds ratio (95% CI)	Adjusted[a] odds ratio (95% CI)
Thyroglobulin antibodies >4 IU/mL:			
(n= 321 cases and 3655 non-cases)			
Continuous, log-transformed Hg (μg/L)		1.20 (0.89, 1.60)	1.13 (0.82, 1.55)
Hg quintiles:			
Q1: Hg ≤ 0.37 μg/L	60	1.00	1.00
Q2: 0.37<Hg≤ 0.62 μg/L	67	1.32 (0.87, 2.00)	1.31 (0.84, 2.03)
Q3: 0.62<Hg≤ 1.00 μg/L	69	1.64 (1.05, 2.54)	1.58 (0.99, 2.53)
Q4: 1.00<Hg≤ 1.73 μg/L	51	1.01 (0.68, 1.50)	0.97 (0.61, 1.54)
Q5: Hg>1.73	74	1.60 (0.93, 2.76)	1.49 (0.61, 2.77)
{P-value for trend}		{0.25}	{0.46}
Thyroid peroxidase antibodies >9 IU/mL:			
(n= 551 cases and 3425 non-cases)			
Continuous, log-transformed Hg (μg/L)		1.32 (1.05, 1.66)	1.26 (0.99, 1.62)
Hg quintiles:			
Q1: Hg ≤ 0.37 μg/L	111	1.00	1.00
Q2: 0.37<Hg≤ 0.62 μg/L	104	0.84 (0.53, 1.33)	0.80 (0.50, 1.27)
Q3: 0.62<Hg≤ 1.00 μg/L	116	1.20 (0.80, 1.82)	1.11 (0.74, 1.69)
Q4: 1.00<Hg≤ 1.73 μg/L	97	0.85 (0.57, 1.27)	0.78 (0.50, 1.22)
Q5: Hg>1.73	123	1.28 (0.93, 1.78)	1.15 (0.80, 1.65)
{P-value for trend}		{0.15}	{0.46}
Thyrotropin > 4 μIU/mL:			
(n= 268 cases and 3708 non-cases)			
Continuous, log-transformed Hg (μg/L)		1.05 (0.74, 1.48)	1.02 (0.72, 1.44)
Hg quintiles:			
Q1: Hg ≤ 0.37 μg/L	57	1.00	1.00
Q2: 0.37<Hg≤ 0.62 μg/L	59	1.04 (0.62, 1.75)	1.01 (0.61, 1.66)
Q3: 0.62<Hg≤ 1.00 μg/L	50	1.06 (0.60, 1.86)	0.97 (0.57, 1.67)
Q4: 1.00<Hg≤ 1.73 μg/L	57	1.45 (0.89, 2.35)	1.35 (0.81, 2.26)
Q5: Hg>1.73	45	1.10 (0.57, 2.14)	1.02 (0.54, 1.96)
{P-value for trend}		{0.45}	{0.62}
Thyrotropin > 4 μIU/mL coincident with thyroid antibody positivity[b]			
(hypothyroidism risk factor[c]):			
(n= 124 cases and 3852 non-cases)			
Continuous, log-transformed Hg (μg/L)		1.38 (1.01, 1.88)	1.30 (0.94, 1.81)
Hg quintiles:			
Q1: Hg ≤ 0.37 μg/L	26	1.00	1.00
Q2: 0.37<Hg≤ 0.62 μg/L	22	0.79 (0.41, 1.53)	0.72 (0.37, 1.39)
Q3: 0.62<Hg≤ 1.00 μg/L	21	1.12 (0.54, 2.34)	0.97 (0.48, 1.95)
Q4: 1.00<Hg≤ 1.73 μg/L	29	1.43 (0.91, 2.25)	1.25 (0.79, 1.96)
Q5: Hg>1.73	26	1.66 (0.78, 3.52)	1.43 (0.67, 3.08)
{P-value for trend}		{0.06}	{0.12}
Both thyroglobulin antibody positivity and thyroid peroxidase antibody positivity			
(n=183 cases and 3793 non-cases)			
Continuous, log-transformed Hg (μg/L)		1.39 (0.98, 1.84)	1.27 (0.90, 1.80)
Hg quintiles:			
Q1: Hg ≤ 0.37 μg/L	33	1.00	1.00
Q2: 0.37<Hg≤ 0.62 μg/L	36	1.06 (0.63, 1.78)	1.06 (0.63, 1.77)
Q3: 0.62<Hg≤ 1.00 μg/L	41	1.67 (0.84, 3.30)	1.63 (0.81, 3.26)
Q4: 1.00<Hg≤ 1.73 μg/L	28	0.92 (0.52, 1.62)	0.90 (0.49, 1.64)
Q5: Hg>1.73	45	1.67 (0.89, 3.13)	1.56 (0.79, 3.09)
{P-value for trend}		{0.18}	{0.32}

a. Statistically adjusted for race/ethnicity (nonwhite=referent), urine iodine status (WHO definition of sufficient=referent), age group (20-49 years=referent; 12-19 years; 50 + years), hormone use (not currently using hormones=referent), and NHANES-estimated 24 hr dietary intake of eicosapentaenoic acid (referent=none; below sample median; above sample median; not ascertained).

b. Either thyroglobulin antibodies > 4 IU/mL or thyroid peroxidase antibodies > 9 IU/mL

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Table 3. Logistic regression results for the relationship between total blood mercury (Hg) and thyroid outcome measures; non-pregnant, non-lactating females aged 12 years and older with urine iodine levels below sample median (143.25 µg/L); NHANES 2007-2010 (n=1988)

Outcome measure	No. cases	Unadjusted odds ratio (95% CI)	Adjusted[a] odds ratio (95% CI)
Thyroglobulin antibodies >4 IU/mL:			
(n=145 cases and 1843 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.23 (0.86, 1.76)	1.20 (0.81, 1.77)
Hg tertiles:			
T1: Hg ≤0.53 µg/L	45	1.00	1.00
T2: 0.53<Hg≤1.15µg/L	42	0.95 (0.52, 1.73)	0.98 (0.55, 1.75)
T3: Hg>1.15 µg/L	58	1.38 (0.80, 2.38)	1.37 (0.77, 2.44)
{P-value for trend}		{0.23}	{0.26}
Thyroid peroxidase antibodies >9 IU/mL:			
(n= 252 cases and 1736 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.66 (1.17, 2.34)	1.66 (1.19, 2.32)
Hg tertiles:			
T1: Hg ≤0.53 µg/L	75	1.00	1.00
T2: 0.53<Hg≤1.15µg/L	73	0.77 (0.53, 1.12)	0.75 (0.52, 1.08)
T3: Hg>1.15 µg/L	104	1.34 (0.95, 1.89)	1.25 (0.85, 1.83)
{P-value for trend}		{0.09}	{0.19}
Thyrotropin > 4 µIU/mL:			
(n= 112 cases and 1876 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.30 (0.89)	1.22 (0.85, 1.75)
Hg tertiles:			
T1: Hg ≤0.53 µg/L	38	1.00	1.00
T2: 0.53<Hg≤1.15µg/L	36	1.23 (0.71, 2.13)	1.19 (0.68, 2.08)
T3: Hg>1.15 µg/L	38	1.58 (0.83, 3.01)	1.39 (0.74, 2.62)
{P-value for trend}		{0.18}	{0.32}
Thyrotropin > 4 µIU/mL coincident with thyroid antibody positivity[b] (hypothyroidism risk factor[c]):			
(n= 52 cases and 1936 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.88 (1.25, 2.85)	1.76 (1.18, 2.62)
Hg tertiles:			
T1: Hg ≤0.53 µg/L	13	1.00	1.00
T2: 0.53<Hg≤1.15µg/L	14	1.11 (0.45, 2.76)	1.13 (0.48, 2.63)
T3: Hg>1.15 µg/L	25	2.71 (1.20, 6.10)	2.37 (1.20, 4.71)
{P-value for trend}		{0.01}	{0.01}
Both thyroglobulin antibody positivity and thyroid peroxidase antibody positivity			
(n= 86 cases and 1902 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.42 (0.96, 2.09)	1.46 (0.91, 2.33)
Hg tertiles:			
T1: Hg ≤0.53 µg/L	26	1.00	1.00
T2: 0.53<Hg≤1.15µg/L	24	0.96 (0.45, 2.06)	1.02 (0.48, 2.15)
T3: Hg>1.15 µg/L	36	1.55 (0.76, 3.18)	1.60 (0.72, 3.56)
{P-value for trend}		{0.21}	{0.23}

a. Statistically adjusted for race/ethnicity (nonwhite=referent), age group (20-49 years=referent; 12-19 years; 50 + years), hormone use (not currently using hormones=referent), and NHANES-estimated 24 hr dietary intake of eicosapentaenoic acid (referent=none; below sample median; above sample median; not ascertained).
b. Either thyroglobulin antibodies > 4 IU/mL or thyroid peroxidase antibodies > 9 IU/mL
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Table 4. Logistic regression results for the relationship between total blood mercury (Hg) and thyroid outcome measures; non-pregnant, non-lactating females aged 12 years and older with urine iodine levels above sample median (143.25 µg/L); NHANES 2007-2010 (n=1988)

Outcome measure	No. cases	Unadjusted odds ratio (95% CI)	Adjusted[a] odds ratio (95% CI)
Thyroglobulin antibodies >4 IU/mL:			
(n= 176 cases and 1812 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.18 (0.78, 1.79)	1.11 (0.68, 1.80)
Hg tertiles:			
T1: Hg ≤0.53 µg/L	63	1.00	1.00
T2: 0.53<Hg≤1.15µg/L	56	1.29 (0.84, 1.98)	1.17 (0.70, 1.95)
T3: Hg>1.15 µg/L	57	1.06 (0.67, 1.69)	0.93 (0.53, 1.66)
{P-value for trend}		{0.82}	{0.76}
Thyroid peroxidase antibodies >9 IU/mL:			
(n= 299 cases and 1689 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.96 (0.61, 1.52)	0.89 (0.53, 1.51)
Hg tertiles:			
T1: Hg ≤0.53 µg/L	104	1.00	1.00
T2: 0.53<Hg≤1.15µg/L	103	1.26 (0.82, 1.92)	1.15 (0.75, 1.74)
T3: Hg>1.15 µg/L	92	0.96 (0.59, 1.58)	0.86 (0.51, 1.47)
{P-value for trend}		{0.85}	{0.54}
Thyrotropin > 4 µIU/mL:			
(n= 156 cases and 1832 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.88 (0.47, 1.65)	0.85 (0.43, 1.67)
Hg tertiles:			
T1: Hg ≤0.53 µg/L	60	1.00	1.00
T2: 0.53<Hg≤1.15µg/L	52	0.99 (0.65, 1.50)	0.93 (0.63, 1.38)
T3: Hg>1.15 µg/L	44	0.98 (0.53, 1.83)	0.92 (0.50, 1.71)
{P-value for trend}		{0.96}	{0.80}
Thyrotropin > 4 µIU/mL coincident with thyroid antibody positivity[b] (hypothyroidism risk factor[c]):			
(n= 72 cases and 1916 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.95 (0.54, 1.68)	0.89 (0.46, 1.72)
Hg tertiles:			
T1: Hg ≤0.53 µg/L	27	1.00	1.00
T2: 0.53<Hg≤1.15µg/L	23	1.07 (0.57, 2.01)	0.96 (0.49, 1.87)
T3: Hg>1.15 µg/L	22	1.19 (0.61, 2.33)	1.08 (0.53, 2.21)
{P-value for trend}		{0.61}	{0.83}
Both thyroglobulin antibody positivity and thyroid peroxidase antibody positivity			
(n= 97 cases and 1891 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.24 (0.74, 2.06)	1.12 (0.62, 2.02)
Hg tertiles:			
T1: Hg ≤0.53 µg/L	34	1.00	1.00
T2: 0.53<Hg≤1.15µg/L	31	1.29 (0.73, 2.28)	1.17 (0.61, 2.23)
T3: Hg>1.15 µg/L	32	1.02 (0.60, 1.73)	0.88 (0.48, 1.61)
{P-value for trend}		{0.96}	{0.61}

a. Statistically adjusted for race/ethnicity (nonwhite=referent), age group (20-49 years=referent; 12-19 years; 50 + years), hormone use (not currently using hormones=referent), and NHANES-estimated 24 hr dietary intake of eicosapentaenoic acid (referent=none; below sample median; above sample median; not ascertained).
b. Either thyroglobulin antibodies > 4 IU/mL or thyroid peroxidase antibodies > 9 IU/mL
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Table 5. Sensitivity analysis: Multivariable[a] logistic regression results for the relationship between continuous log-transformed total blood mercury (Hg) and thyroid outcome measures; non-pregnant, non-lactating females aged 12 years and older, NHANES 2007-2010, for iodine deficient[b] (n=1330) and iodine excess[c] subsamples (n=717)

	Iodine deficient: Urine iodine <100 µg/L	Iodine excess: Urine iodine ≥300 µg/L
	Odds ratio (95% CI) {# cases + # noncases}	Odds ratio (95% CI) {# cases + # noncases}
Thyroglobulin antibodies >4 IU/mL	1.10 (0.69, 1.75) {107 cases + 1223 noncases}	1.28 (0.61, 2.69) {58 cases + 659 noncases}
Thyroid peroxidase antibodies >9 IU/mL	1.69 (1.15, 2.49) {171 cases + 1159 noncases}	1.79 (0.68, 4.73) {101 cases + 616 noncases}
Thyrotropin > 4 µIU/mL	1.34 (0.85, 2.13) {67 cases + 1263 noncases}	0.39 (0.20, 0.78) {66 cases + 651 noncases}
Thyrotropin > 4 µIU/mL coincident with thyroid antibody positivity[d] (hypothyroidism risk factor[e])	2.03 (1.24, 3.32) {33 cases + 1297 noncases}	0.84 (0.47, 1.52) {23 cases + 694 noncases}
Both thyroglobulin antibody positivity and thyroid peroxidase antibody positivity	1.34 (0.74, 2.46) {63 cases + 1267 noncases}	2.88 (1.28, 6.49) {31 cases + 686 noncases}

a. Statistically adjusted for race/ethnicity (nonwhite=referent), age group (20-49 years=referent; 12-19 years; 50 + years), hormone use (not currently using hormones=referent), and NHANES-estimated 24 hr dietary intake of eicosapentaenoic acid (referent=none; below sample median; above sample median; not ascertained).

b. World Health Organization definition for iodine deficiency: urine iodine < 100 µg/L (WHO, 2007)

c. World Health Organization definition for iodine excess: urine iodine ≥ 300 µg/L (WHO, 2007)

d. Either thyroglobulin antibodies > 4 IU/mL or thyroid peroxidase antibodies > 9 IU/mL

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Table 6. Weighted[a] sample descriptive statistics, males aged 12 and older, NHANES 2007-2010 (n=4110)

	Thyroglobulin antibodies >4 IU/mL	Thyroid peroxidase antibodies >9 IU/mL	Thyrotropin > 4 µIU/mL	Thyrotropin > 4 µIU/mL coincident with thyroid antibody positivity[b] (hypothyroidism risk factor[c])	Both thyroglobulin antibody positivity and thyroid peroxidase antibody positivity
# cases (%)	199 (5%)	275 (7%)	206 (5%)	60 (1%)	104 (3%)
# noncases	3911	3835	3904	4050	4006
Mean total blood mercury {µg/L} (SE):					
Cases	1.51 (0.19)	1.77 (0.22)	2.24 (0.37)	2.10 (0.47)	1.22 (0.25)
Noncases	1.73 (0.11)	1.72 (0.10)	1.70 (0.10)	1.71 (0.10)	1.73 (0.11)
Mean thyroglobulin antibody concentration {IU/mL} (SE):					
Cases	115.82 (15.28)[d]	63.18 (12.94)[d]	50.47 (11.78)[d]	130.54 (33.12)[d]	171.64 (31.59)[d]
Noncases	0.67 (0.01)	1.70 (0.39)	4.19 (0.83)	4.09 (0.80)	1.66 (0.37)
Mean thyroid peroxidase antibody concentration {IU/mL} (SE):					
Cases	101.63 (18.20)[d]	142.74 (12.23)[d]	71.10 (16.79)[d]	183.25 (38.66)[d]	179.63 (24.59)[d]
Noncases	7.12 (1.22)	1.08 (0.04)	8.88 (1.28)	8.66 (1.24)	7.02 (1.18)
% cases/noncases comprised by nonHispanic white males	73%/68%	78%/67%[d]	78%/67%[d]	90%/67%[d]	82%/67%[d]
% cases/noncases by Iodine status[e]:					
Sufficient	44%/50%	47%/50%	43%/50%[d]	34%/50%	43%/50%
Deficient	30%/28%	27%/28%	23%/28%	30%/28%	29%/28%
Excess	25%/22%	26%/22%	33%/22%	36%/22%	27%/22%
% cases/noncases by agegroup:					
12-19 years	7%/13%[d]	7%/14%[d]	5%/13%[d]	2%/13%[d]	4%/13%[d]
20-49 years	41%/51%	46%/51%	43%/51%	48%/51%	40%/51%
50+years	52%/35%	48%/35%	51%/35%	50%/36%	55%/36%
% cases/noncases by estimated 24 hr EPA[f] intake:					
None	13%/17%[d]	15%/17%	22%/16%	19%/16%	12%/17%[d]
Below median	51%/35%	43%/35%	33%/36%	35%/36%	51%/36%
Above median	34%/44%	38%/44%	41%/44%	46%/44%	36%/44%
Not ascertained	2%/4%	3%/4%	3%/4%	<1%/4%	1%/4%

a. Weighted for NHANES complex survey design.

b. Either thyroglobulin antibodies > 4 IU/mL or thyroid peroxidase antibodies > 9 IU/mL

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d. Significant difference between cases/noncases at P<0.05 using complex survey design

e. World Health Organization (2007) definitions for iodine sufficient based upon urine iodine concentrations (UI): sufficient: 100≤UI<300 µg/L; deficient: UI<100 µg/L; excess: UI≥300 µg/L

f. Eicosapentaenoic acid dietary intake estimated by NHANES, median=0.010 g

Table 7. Logistic regression results for the relationship between total blood mercury (Hg) and thyroid outcome measures; males aged 12 years and older; NHANES 2007-2010 (n=4110)

Outcome measure	No. cases	Unadjusted odds ratio (95% CI)	Adjusted[a] odds ratio (95% CI)
Thyroglobulin antibodies >4 IU/mL:			
(n= 199 cases and 3911 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.68 (0.47, 1.00)	0.62 (0.42, 0.91)
Hg quintiles:			
Q1: Hg ≤ 0.38 µg/L	46	1.00	1.00
Q2: 0.38<Hg≤ 0.65 µg/L	45	1.22 (0.68, 2.20)	1.00 (0.56, 1.79)
Q3: 0.65<Hg≤ 1.05 µg/L	40	0.71 (0.41, 1.21)	0.58 (0.34, 1.00)
Q4: 1.05<Hg≤ 1.92 µg/L	37	0.79 (0.44, 1.43)	0.63 (0.34, 1.15)
Q5: Hg>1.92	31	0.51 (0.27, 0.97)	0.40 (0.21, 0.76)
{P-value for trend}		{<0.01}	{<0.01}
Thyroid peroxidase antibodies >9 IU/mL:			
(n= 275 cases and 3835 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.90 (0.64, 1.27)	0.86 (0.59, 1.25)
Hg quintiles:			
Q1: Hg ≤ 0.38 µg/L	62	1.00	1.00
Q2: 0.38<Hg≤ 0.65 µg/L	55	0.98 (0.55, 1.73)	0.85 (0.50, 1.44)
Q3: 0.65<Hg≤ 1.05 µg/L	47	0.75 (0.43, 1.31)	0.63 (0.34, 1.09)
Q4: 1.05<Hg≤ 1.92 µg/L	53	0.96 (0.65, 1.44)	0.80 (0.53, 1.23)
Q5: Hg>1.92	58	0.73 (0.40, 1.30)	0.61 (0.35, 1.06)
{P-value for trend}		{0.25}	{0.09}
Thyrotropin > 4 µIU/mL:			
(n= 206 cases and 3904 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.22 (0.90, 1.64)	1.18 (0.82, 1.68)
Hg quintiles:			
Q1: Hg ≤ 0.38 µg/L	56	1.00	1.00
Q2: 0.38<Hg≤ 0.65 µg/L	40	0.59 (0.33, 1.05)	0.53 (0.28, 0.98)
Q3: 0.65<Hg≤ 1.05 µg/L	30	0.54 (0.25, 1.17)	0.44 (0.20, 0.95)
Q4: 1.05<Hg≤ 1.92 µg/L	34	0.82 (0.47, 1.45)	0.67 (0.38, 1.17)
Q5: Hg>1.92	46	0.92 (0.56, 1.50)	0.76 (0.43, 1.32)
{P-value for trend}		{0.80}	{0.72}
Thyrotropin > 4 µIU/mL coincident with thyroid antibody positivity[b] (hypothyroidism risk factor[c]):			
(n= 60 cases and 4050 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.00 (0.59, 1.68)	0.91 (0.50, 1.67)
Hg quintiles:			
Q1: Hg ≤ 0.38 µg/L	16	1.00	1.00
Q2: 0.38<Hg≤ 0.65 µg/L	12	0.50 (0.17, 1.47)	0.41 (0.14, 1.25)
Q3: 0.65<Hg≤ 1.05 µg/L	5	0.12 (0.03, 0.47)	0.10 (0.03, 0.36)
Q4: 1.05<Hg≤ 1.92 µg/L	11	0.96 (0.38, 2.47)	0.72 (0.27, 1.90)
Q5: Hg>1.92	16	0.62 (0.23, 1.64)	0.46 (0.17, 1.25)
{P-value for trend}		{0.73}	{0.44}
Both thyroglobulin antibody positivity and thyroid peroxidase antibody positivity			
(n= 104 cases and 4006 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.48 (0.23, 1.03)	0.41 (0.18, 0.90)
Hg quintiles:			
Q1: Hg ≤ 0.38 µg/L	27	1.00	1.00
Q2: 0.38<Hg≤ 0.65 µg/L	22	0.95 (0.46, 1.97)	0.74 (0.35, 1.57)
Q3: 0.65<Hg≤ 1.05 µg/L	18	0.50 (0.21, 1.21)	0.39 (0.16, 0.92)
Q4: 1.05<Hg≤ 1.92 µg/L	19	0.53 (0.22, 1.24)	0.40 (0.17, 0.91)
Q5: Hg>1.92	18	0.34 (0.13, 0.87)	0.25 (0.10, 0.61)
{P-value for trend}		{0.01}	{<0.01}

a. Statistically adjusted for age, race/ethnicity (nonwhite=referent), urine iodine status (WHO definition of sufficient=referent), and NHANES-estimated 24 hr dietary intake of eicosapentaenoic acid (referent=none; below sample median; above sample median; not ascertained).

b. Either thyroglobulin antibodies > 4 IU/mL or thyroid peroxidase antibodies > 9 IU/mL

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Table 8. Logistic regression results for the relationship between total blood mercury (Hg) and thyroid outcome measures; males aged 12 years and older with urine iodine levels below sample median (166.7 µg/L); NHANES 2007-2010 (n=2054)

Outcome measure	No. cases	Unadjusted odds ratio (95% CI)	Adjusted[a] odds ratio (95% CI)
Thyroglobulin antibodies >4 IU/mL:			
(n= 92 cases and 1962 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.88 (0.56, 1.40)	0.84 (0.54, 1.31)
Hg tertiles:			
T1: Hg ≤0.54 µg/L	35	1.00	1.00
T2: 0.54<Hg≤1.24µg/L	30	0.74 (0.38, 1.42)	0.67 (0.35, 1.25)
T3: Hg>1.24 µg/L	27	0.66 (0.34, 1.25)	0.58 (0.32, 1.05)
{P-value for trend}		{0.21}	{0.08}
Thyroid peroxidase antibodies >9 IU/mL:			
(n= 124 cases and 1930 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.79 (0.51, 1.23)	0.75 (0.47, 1.18)
Hg tertiles:			
T1: Hg ≤0.54 µg/L	45	1.00	1.00
T2: 0.54<Hg≤1.24µg/L	38	0.83 (0.51, 1.37)	0.79 (0.46, 1.36)
T3: Hg>1.24 µg/L	41	0.71 (0.41, 1.21)	0.64 (0.37, 1.11)
{P-value for trend}		{0.21}	[0.11}
Thyrotropin > 4 µIU/mL:			
(n= 82 cases and 1972 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.56 (1.01, 2.42)	1.48 (0.90, 2.45)
Hg tertiles:			
T1: Hg ≤0.54 µg/L	29	1.00	1.00
T2: 0.54<Hg≤1.24µg/L	19	0.53 (0.24, 1.17)	0.48 (0.20, 1.11)
T3: Hg>1.24 µg/L	34	1.25 (0.71, 2.18)	1.08 (0.55, 2.09)
{P-value for trend}		{0.32}	{0.61}
Thyrotropin > 4 µIU/mL coincident with thyroid antibody positivity[b] (hypothyroidism risk factor[c]):			
(n=22 cases and 2032 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.60 (0.80, 3.19)	1.47 (0.61, 3.54)
Hg tertiles:			
T1: Hg ≤0.54 µg/L	5	1.00	1.00
T2: 0.54<Hg≤1.24µg/L	5	0.58 (0.15, 2.33)	0.48 (0.10, 2.26)
T3: Hg>1.24 µg/L	12	1.31 (0.59, 2.94)	0.99 (0.37, 2.67)
{P-value for trend}		{0.40}	{0.83}
Both thyroglobulin antibody positivity and thyroid peroxidase antibody positivity			
(n= 44 cases and 2010 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.68 (0.29, 1.59)	0.59 (0.25, 1.41)
Hg tertiles:			
T1: Hg ≤0.54 µg/L	16	1.00	1.00
T2: 0.54<Hg≤1.24µg/L	14	0.58 (0.20, 1.66)	0.53 (0.20, 1.43)
T3: Hg>1.24 µg/L	14	0.63 (0.25, 1.64)	0.53 (0.22, 1.28)
{P-value for trend}		{0.38}	{0.19}

a. Statistically adjusted for race/ethnicity (nonwhite=referent), age group (20-49 years=referent; 12-19 years; 50 + years), and NHANES-estimated 24 hr dietary intake of eicosapentaenoic acid (referent=none; below sample median; above sample median; not ascertained).

b. Either thyroglobulin antibodies > 4 IU/mL or thyroid peroxidase antibodies > 9 IU/mL
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Table 9. Logistic regression results for the relationship between total blood mercury (Hg) and thyroid outcome measures; males aged 12 years and older with urine iodine levels ≥ sample median (166.7 µg/L); NHANES 2007-2010 (n=2056)

Outcome measure	No. cases	Unadjusted odds ratio (95% CI)	Adjusted[a] odds ratio (95% CI)
Thyroglobulin antibodies >4 IU/mL:			
(n= 107 cases and 1949 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.38 (0.16, 0.88)	0.29 (0.11, 0.76)
Hg tertiles:			
T1: Hg ≤0.54 µg/L	41	1.00	1.00
T2: 0.54<Hg≤1.24µg/L	39	0.61 (0.30, 1.21)	0.49 (0.25, 0.96)
T3: Hg>1.24 µg/L	27	0.31 (0.14, 0.70)	0.24 (0.10, 0.57)
{P-value for trend}		{<0.01}	{<0.01}
Thyroid peroxidase antibodies >9 IU/mL:			
(n= 151 cases and 1905 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.08 (0.68, 1.70)	1.02 (0.60, 1.73)
Hg tertiles:			
T1: Hg ≤0.54 µg/L	51	1.00	1.00
T2: 0.54<Hg≤1.24µg/L	47	0.75 (0.41, 1.39)	0.60 (0.32, 1.14)
T3: Hg>1.24 µg/L	53	0.79 (0.44, 1.45)	0.66 (0.34, 1.28)
{P-value for trend}		{0.45}	{0.23}
Thyrotropin > 4 µIU/mL:			
(n= 124 cases and 1932 non-cases)			
Continuous, log-transformed Hg (µg/L)		1.02 (0.60, 1.72)	0.96 (0.53, 1.74)
Hg tertiles:			
T1: Hg ≤0.54 µg/L	57	1.00	1.00
T2: 0.54<Hg≤1.24µg/L	34	0.89 (0.40, 2.00)	0.76 (0.34, 1.69)
T3: Hg>1.24 µg/L	33	0.83 (0.46, 1.49)	0.70 (0.38, 1.27)
{P-value for trend}		{0.53}	{0.25}
Thyrotropin > 4 µIU/mL coincident with thyroid antibody positivity[b] (hypothyroidism risk factor[c]):			
(n= 38 cases and 2018 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.55 (0.18, 1.71)	0.46 (0.13, 1.59)
Hg tertiles:			
T1: Hg ≤0.54 µg/L	20	1.00	1.00
T2: 0.54<Hg≤1.24µg/L	10	0.72 (0.20, 2.66)	0.61 (0.17, 2.18)
T3: Hg>1.24 µg/L	8	0.41 (0.12, 1.45)	0.34 (0.10, 1.11)
{P-value for trend}		{0.16}	{0.08}
Both thyroglobulin antibody positivity and thyroid peroxidase antibody positivity			
(n= 60 cases and 1996 non-cases)			
Continuous, log-transformed Hg (µg/L)		0.29 (0.10, 0.88)	0.20 (0.05, 0.77)
Hg tertiles:			
T1: Hg ≤0.54 µg/L	25	1.00	1.00
T2: 0.54<Hg≤1.24µg/L	19	0.43 (0.18, 1.09)	0.34 (0.14, 0.83)
T3: Hg>1.24 µg/L	16	0.26 (0.11, 0.61)	0.21 (0.09, 0.49)
{P-value for trend}		{<0.01}	{<0.01}

a. Statistically adjusted for race/ethnicity (nonwhite=referent), age group (20-49 years=referent; 12-19 years; 50 + years), and NHANES-estimated 24 hr dietary intake of eicosapentaenoic acid (referent=none; below sample median; above sample median; not ascertained).
b. Either thyroglobulin antibodies > 4 IU/mL or thyroid peroxidase antibodies > 9 IU/mL
c. Walsh et al. 2010

Table 10. Sensitivity analysis: Multivariable[a] logistic regression results for the relationship between continuous log-transformed total blood mercury (Hg) and thyroid outcome measures; males aged 12 years and older, NHANES 2007-2010, for iodine deficient[b] (n=1090) and iodine excess[c] subsamples (n=945)

	Iodine deficient: Urine iodine <100 µg/L	Iodine excess: Urine iodine ≥300 µg/L
	Odds ratio (95% CI) {# cases + # noncases}	Odds ratio (95% CI) {# cases + # noncases}
Thyroglobulin antibodies >4 IU/mL	0.99 (0.50, 1.95) {56 cases + 1034 noncases}	0.21 (0.05, 0.94) {59 cases + 886 noncases}
Thyroid peroxidase antibodies >9 IU/mL	0.83 (0.42, 1.63) {73 cases + 1017 noncases}	1.09 (0.49, 2.41) {73 cases + 872 noncases}
Thyrotropin > 4 µIU/mL	1.89 (1.05, 3.42) {45 cases + 1045 noncases}	0.51 (0.18, 1.48) {71 cases + 874 noncases}
Thyrotropin > 4 µIU/mL coincident with thyroid antibody positivity[d] (hypothyroidism risk factor[e])	1.92 (0.78, 4.74) {16 cases + 1074 noncases}	0.22 (0.03, 1.87) {24 cases + 921 noncases}
Both thyroglobulin antibody positivity and thyroid peroxidase antibody positivity	0.75 (0.26, 2.21) {29 cases + 1061 noncases}	0.15 (0.01, 1.88) {33 cases + 912 noncases}

a. Statistically adjusted for race/ethnicity (nonwhite=referent), age group (20-49 years=referent; 12-19 years; 50 + years), and NHANES-estimated 24 hr dietary intake of eicosapentaenoic acid (referent=none; below sample median; above sample median; not ascertained).

b. World Health Organization definition for iodine deficiency: urine iodine < 100 µg/L (WHO, 2007)

c. World Health Organization definition for iodine excess: urine iodine ≥ 300 µg/L (WHO, 2007)

d. Either thyroglobulin antibodies > 4 IU/mL or thyroid peroxidase antibodies > 9 IU/mL

e. Walsh et al. 2010

Chapter 3

Total blood mercury and measles antibody concentrations in US children aged 6-11 years, NHANES 2003-2004

Abstract

Environmental toxicants, pathogens and host susceptibility cofactors may interact to contribute to disease. In vitro Hg exposure inhibited antiviral cytokines in human cells; however, little is known about the relationship between Hg and viruses in children. Children are vulnerable to Hg toxicity; lower vitamin B-12 and folate levels and higher homocysteine levels may represent susceptibility cofactors. This study aimed to evaluate associations between total blood Hg and measles antibodies in children, and the influence of these susceptibility cofactors.

Cross-sectional data on serum measles antibodies, Hg, homocysteine, methylmalonic acid (MMA, indicator of B-12 deficiency), and folate were obtained from the 2003-2004 NHANES for children aged 6-11 years with measles seropositivity (n=692). We used linear regression to evaluate relationships between measles antibodies and Hg, stratified by sex, MMA ≥, folate <, and homocysteine ≥ sample medians, adjusted for demographic, nutritional and environmental cofactors.

Hg (range: 0.10-19.10 μg/L) was inversely associated with measles antibodies (range: 1.00-28.24 units) in non-stratified analysis (n=692), yet positively associated among the subset of boys with higher MMA and lower folate (n=98). Among this subset with higher homocysteine levels (n=61), correlations were positive across all Hg quartiles relative to Q1 (Hg≤0.20 μg/L): Q2:β=6.60 (3.02, 10.19); Q3:β=8.49 (6.17, 10.81); Q4 (Hg>0.80 μg/L):β=4.90 (2.12, 7.67) (p_{trend}=0.077).

Stratification by susceptibility cofactors revealed opposing directionality for correlations between Hg and measles antibodies, with positive effect estimates at lowest exposures only among boys with higher MMA, lower folate and higher homocysteine levels.

Note: This chapter completely represents the original published study, with regression coefficients and additional methods details added to the residual graph captions:

Gallagher, CM, Smith, DM, Meliker, JR. Total blood mercury and serum measles antibodies in US children, NHANES 2003-2004. Sci Total Environ 2011a; 411:65-71.

Introduction

Environmental toxicants, microbial pathogens and host susceptibility are cofactors that may interact to contribute to disease risk, and therefore, it has recently been proposed that environmental epidemiological research integrate toxicological and infectious disease models to evaluate potential interactions (Feingold et al., 2010). *In vitro* exposures of human peripheral blood mononuclear cells (PBMCs) to organic Hg compounds inhibited type II interferon (IFN-γ) (de Vos et al., 2007; Gardner et al., 2010a), an antiviral cytokine that protects against persistent measles-virus infection of the central nervous system (CNS) (Finke et al., 1995; Patterson et al., 2002; Reuter and Schneider-Schaulies, 2010). Little is known, however, about the potential for Hg to interact with the measles virus in human *in vivo* studies.

Findings from experimental studies of the relationships between Hg and viruses are mixed. 2-Furylmercury chloride, an organic Hg derivative, was found to inhibit human rhinovirus (Verheyden et al. 2004). Mice infected with coxsackievirus B3 showed increased Hg in brain tissue and decreased Hg in serum relative to control mice (Ilback et al. 2007), heart viral titers were elevated in coxsackievirus B3 infected mice when first exposed to mercuric chloride (inorganic Hg) compared to unexposed infected mice (South et al., 2001), and liver viral titers were increased in herpes simplex virus type 2 infected mice when first exposed to mercuric chloride compared to unexposed infected mice (Christensen et al., 1996). The relationship between Hg exposures and serum viral antibody titers in humans, however, has not been previously investigated.

Feingold et al. (2010) specifically identified a gap in research using the US National Health and Nutrition Survey (NHANES) concerning interactions between environmental toxicants such

as Hg and viral pathogens. Mercury is a known neurotoxicant to which children are particularly susceptible (ATSDR 1999). As a sulfhydryl metal, Hg's general mechanism of toxicity is to bind with thiols of exposed cysteine residues on proteins (Klaassen, 2008), and has been shown to act as an immunotoxicant by dysregulating immune response (Gardner et al., 2010a). NHANES uses a cross-sectional study design to collect interview, medical examination and laboratory data from a probability sample of the U.S. population. Measles antibodies were measured in serum of children aged 6-11 years, along with total blood Hg levels for NHANES 2003-2004 (CDC, 2003-2004). Other years did not contain these data. The aim of the current study was to investigate whether there is a correlation between total blood Hg levels and measles antibody titers in children.

Our previous findings suggested that boys aged 3-5 with lower folate and higher methylmalonic acid (MMA, an indicator of B-12 deficiency) levels may be susceptible to Hg-associated alterations in amino acids, specifically, decreases in homocysteine levels (Gallagher and Meliker, 2011) potentially indicative of a metabolic disruption hypothesized to underlie cellular hypomethylation (Deth et al., 2008; Lee et al., 2009). Persistent infection of rat glioma cells with measles virus (subacute sclerosing panencephalitis, SSPE strain) has also been reported to induce hypomethylation (Munzel and Koschel, 1982). Therefore, to integrate possible host susceptibility cofactors with toxicant and pathogen exposures, our primary objective was to evaluate the relationship between total blood Hg and serum measles antibodies stratified by these same susceptibility factors, sex, MMA and folate levels. Because we previously found that boys with higher MMA and lower folate had higher homocysteine levels (Gallagher and Meliker, 2011) and experimental results suggested that homocysteine inhibits

metallothionein (Barbato et al., 2007), a protein that reduces Hg's bioavailability (Aschner, 1997, ATSDR, 1999) and cytotoxicity (Rising et al., 1995; Vitarella et al., 1996; Yao et al, 1999), a secondary objective was to evaluate the effect of additional stratification by homocysteine levels.

Methods

We conducted a secondary data analysis of cross-sectional data on serum measles antibodies, total whole blood Hg, plasma methylmalonic acid (MMA), serum folate, and plasma homocysteine from the 2003-2004 National Health and Nutrition Examination Surveys (NHANES) (CDC 2003-2004) for children aged 6-11 years who tested seropositive for measles antibodies (CDC 2006a) and whose parents dietary recall was categorized as reliable by NHANES (CDC 2007). NHANES uses a complex, multistage, probability sampling design to select participants representative of the civilian, non-institutionalized US population (CDC 2011d). Each sample person is assigned a sample weight that reflects adjustments for complex survey design (including oversampling), survey nonresponses (with adjustments for age and race), and post-stratification, in order to ensure that calculated estimates are US population representative (CDC 2010a). Whole blood Hg measurements were performed by the Division of Laboratory Sciences, National Center for Environmental Health, of the Centers for Disease Control and Prevention using inductively coupled plasma mass spectrometry (CDC 2006b). Although total blood Hg is a biomarker of both inorganic and organic Hg exposure over the past several weeks to months (ATSDR 1999), it is primarily a measure of organic Hg (CDC, 2010). Levels of measles IgG antibody were measured by enzyme immunoassay that used a lysate of measles virus-infected human fetal diploid lung cells (HFDL) as the viral antigen and a lysate of

uninfected HFDL as the control antigen, with a value of ≥ 1 representing seropositivity (McQuillan et al. 2007; CDC 2006a; CDC 2011e). Measles vaccination status was not recorded, however, it is likely that the measles titers for most children in this study represent vaccination rather than wild type virus exposures (CDC 2004a; 2005).

Increased MMA levels in serum are considered direct measures of vitamin B-12 tissue stores and the first indication of B-12 deficiency (Moelby et al. 1990). Blood samples from children were non-fasting samples (CDC 2004b). Because human and animal studies indicate that omega-3 polyunsaturated fatty acids (PUFAs) can have immunomodulatory effects (Ergas et al., 2002; Simopoulos, 2002), for example, eicosapentaenoic acid (EPA) (Makino et al., 2001; Ergas et al., 2002; Simopoulos, 2002) and docosahexaenoic acid (DHA) (Ergas et al., 2002; Simopoulos, 2002), and certain fish are high in both PUFAs and Hg (Oken et al., 2005; Mahaffey et al., 2008), it is important to control for possible confounding effects of PUFAs (Grotto et al., 2010). In consideration of scientific findings that DHA may be less specific to fish than EPA due to the presence of DHA in eggs (Beynen, 2004), we created a variable for EPA dietary intake using NHANES estimated 24-hour dietary EPA intake based upon reliable parental recall of their child's intake of specific foods over the past 24 hours (CDC, 2007), i.e., reference group=none; above and below the median of 0.007 gm for the sample subset of children aged 6-11 years. For this same sample subset, dichotomous variables were created using median cut-points for homocysteine ≥ 4.66 μmol/L, MMA ≥ 0.108 μmol/L and folate < 14.8 ng/mL. Additionally, the following median-cut dichotomous variables were created per scientific findings regarding associations with immune function: serum vitamin D ≥ 24 ng/mL (Arnson 2007), serum cotinine ≥ 0.080 ng/mL (Avanzini et al., 2006) and whole blood lead ≥ 1.4 μg/dL (Mishra, 2009).

Because cadmium has been associated with suppression of immediate hypersensitivity immune response in children (Ritz et al., 1998), we also created a dichotomous variable for whole blood cadmium; however, the smallest cutoff point between lowest and higher exposures was greater than the median, i.e., ≥0.20 μg/L, and therefore, this dichotomous variable represents a frequency distribution of 64% < 0.20 μg/L and 36% ≥ 0.20 μg/L. A dichotomous variable was created to adjust for below and above the poverty index ratio.

Our objective was to evaluate the relationship between Hg and measles virus antibodies in children with laboratory confirmed measles virus exposure, and therefore, the analytic sample domain was restricted to survey participants aged 6-11 years with measles seropositivity (≥1 unit) and without missing values for data on blood Hg, cadmium and lead, plasma homocysteine and methylmalonic acid, serum folate and cotinine, and the poverty index ratio. Subjects were further restricted to those with reliable status of parental 24-hour dietary recall (n=692). There were 702 observations with both Hg and measles antibody levels measured; of these, 10 observations were excluded due to measles seronegativity, for an overall sample of 692 children. Continuous blood Hg (μmol/L) measures were log-transformed, and scatterplots and residual plots visually inspected for linearity of the relationships between Hg and measles antibodies. Based upon weighted frequency distributions for the subsample of children aged 6-11 years, the following categorical variables were created for total blood Hg quartiles (Q): Q1: Hg≤0.20 μg/L; Q2: 0.20<Hg≤0.40 μg/L; Q3: 0.40<Hg≤ 0.80μg/L; Q4: Hg>0.80 μg/L.

We used linear regression analyses to evaluate the relationships between continuous and categorical Hg and measles antibodies, stratified by sex, MMA ≥ and folate < sample medians, as well as homocysteine ≥ the sample median, fully adjusted for continuous age, race (non-

Hispanic white compared to non-white), poverty, body mass index, serum vitamin D, and NHANES-estimated 24-hr dietary intake of EPA and selenium. Bivariate regression analysis was used to evaluate each covariate's relationship with measles antibody levels in each of the following subgroups: boys with high MMA and low folate, all other boys, girls with high MMA and low folate, and all other girls. Only the cotinine covariate showed no statistical evidence of association, so was omitted from all models. Variables determined a priori for model inclusion because of their potential relationships with measles antibody titers and Hg levels included age (CDC 2004a, 2005) and body weight (Ball et al. 2001), respectively; we used body mass index to proxy the latter as there was evidence of multicollinearity between body weight in kilograms and age in years. EPA was also determined a priori for model inclusion because of its relationship with Hg (Oken et al., 2005; Mahaffey et al., 2008; Grotto et al. 2010). A test for interaction between Hg, homocysteine and measles titers was conducted by entering a statistical interaction term for the interaction between homocysteine and Hg into the model with all covariates except cotinine. We used backwards elimination to identify covariates without evidence of a statistical correlation with measles. As a result, the fully-adjusted models presented do not include blood lead and cadmium. Statistical analysis was conducted using SAS version 9.2. Primary sampling units, strata and dietary intake survey subsample weights were incorporated to calculate sample means and 95% confidence intervals, to determine statistically significant differences among subset sample means and distributions of sample characteristics, and to perform linear regression procedures in accordance with complex survey design using the Taylor linearization procedure, which is robust against the likelihood of correlated errors and heteroscedasticity (Kott 1991). A separate analysis was also conducted using unweighted robust regression in order to evaluate robustness of results in unweighted analysis while maintaining stability against

heteroscedasticity (Carroll and Ruppert 1982). Additionally, the relationship between Hg, adjusted for covariates, and measles titers were graphically depicted by plotting a residualized Hg variable by measles titers. The plotted Hg variable was comprised of the residual values generated by modeling log-transformed Hg as the dependent variable and the remaining covariates as independent variables.The Rao-Scott Chi-square test used to determine statistically significant differences in proportions for weighted sample characteristics among subsets, and 95% confidence intervals were calculated for the difference between weighted means to determine statistical significance. To convert Hg reported as μmol/L to μg/L, μmol/L were divided by 4.99 (CDC, 2006b). Statistical significance was defined as an alpha level ≤ 0.05.

Results

We show in Table 1 that measles antibody concentrations for the overall sample ranged from 1.00-28.24 units, and that Hg ranged from 0.10-19.10 μg/L. Mean measles antibody levels were not statistically different between boys with high MMA and low folate (9.83; SD=0.88) and all other children (10.60; SD=0.33). Mean Hg levels were not statistically different between boys with high MMA and low folate (0.64 μg/L; SD=0.11) and all other children (0.69 μg/L; SD=0.08). Hg levels were similar for excluded observations. Boys with higher MMA and lower folate had significantly higher mean homocysteine (5.39 μmol/L; SD=0.24) compared to all other children (4.74 μmol/L; SD=0.04).

Table 2 presents linear regression results for the relationship between continuous log-transformed Hg (μmol/L) and measles virus antibody concentrations, for the overall sample (n=692), and for the following subsets: boys with high MMA and low folate (n=98), all other

boys (n=231), girls with high MMA and low folate (n=89), all other girls (n=274), and for the overall sample less boys with high MMA and low folate (all other children, n=594). There were no statistically significant associations between Hg and measles antibodies in unadjusted analysis; however, in multivariable analysis, boys with higher MMA and lower folate showed a positive association (β=1.62; 95% CI=0.06, 3.19), whereas the overall sample showed an inverse association (β=-0.87; 95% CI=-1.70, -0.03), as did the overall sample less boys with higher MMA and lower folate (β=-1.14; 95% CI=-1.88, -0.39), as well as all other boys (β=-1.39; 95% CI=-2.74, -0.04). Although continuous Hg was positively associated with measles antibodies among boys with higher MMA and lower folate, relationships were not statistically significant comparing higher Hg quartiles to the lowest Hg quartile.

We also examined the possibility of interaction between homocysteine and Hg. A covariate for the interaction between continuous Hg and homocysteine above/below the sample median was statistically significant in the multivariable model for the overall sample (p=0.043), thus supporting stratification by the dichotomous homocysteine covariate. Table 3 presents linear regression results for only those sample subsets with evidence of a statistically significant relationship between measles virus antibody concentrations and Hg, stratified by higher and lower homocysteine levels. Subsets shown include models for boys with higher MMA, lower folate and higher homocysteine (n=61), all other boys with lower homocysteine (n=119), and all other children, both boys and girls, with lower homocysteine (n=308). Using a continuous measure of Hg, in unadjusted models, none of the sample subsets showed a statistically significant relationship between measles antibody concentrations and Hg; however, multivariable regression results showed a positive association among boys with higher MMA, lower folate and

higher homocysteine (β=1.67; 95% CI=0.39, 2.95), whereas inverse associations were evident among all other boys with lower homocysteine (β=-1.87; -3.22, -0.52) and among all other children with lower homocysteine (β=-1.85; -3.06, -0.65). Figures 1, 2, and 3 depict these relationships graphically using a residualized Hg variable adjusted for covariates. Using Hg quartiles, in unadjusted analysis, the latter two subgroups showed inverse associations, with statistically significant relationships evident for Hg quartiles 2 and 4 (relative to the first quartile) among all other boys with lower homocysteine, and for the 4th Hg quartile among all other children with lower homocysteine; these inverse relationships were consistent with multivariable results (p-value for trend=0.001 and 0.015, respectively). Among boys with higher MMA, lower folate and higher homocysteine, although Hg quartiles did not show evidence of a statistically significant relationship with measles antibody concentrations in unadjusted analysis, in the statistically-adjusted model, relative to the lowest Hg quartile, each of the higher Hg quartiles showed a significantly positive association with measles antibody levels (p-value for trend=0.077).

In unweighted multivariable analysis, the positive associations between Hg and measles antibody concentrations were robust among boys with higher MMA, lower folate and higher homocysteine, as were the inverse associations between Hg and measles antibody titers among all other boys with lower homocysteine; however, results were not robust for all other children with lower homocysteine. Also in unweighted analysis, a statistically significant trend (p=0.006) was evident only for boys with higher MMA, lower folate and higher homocysteine.

Discussion

This research presents one of the first investigations of the relationship between Hg and measles antibodies in a human population. Overall sample findings suggest an inverse relationship between Hg and measles antibodies; however, among boys with higher MMA and lower folate a positive relationship was observed. Further, among boys with higher MMA, lower folate and higher homocysteine, this positive relationship was also evident using both continuous and categorical Hg measures, and showed stability in unweighted analysis. Unadjusted analyses did not consistently show significant associations between Hg and measles antibody concentrations. Including age, BMI and the potential confounding variable EPA in the model, however, resulted in stronger and more consistent associations between Hg and measles titers. Investigations such as this one into the relationship between Hg and viral antibodies are needed as we begin to learn how Hg can induce both suppressive and stimulating effects on the immune system (Havarinasab and Hultman 2005; Havarinasab et al. 2005; de Vos et al. 2007; Gardner et al. 2010a).

The findings of a positive association between continuous Hg and measles virus antibodies only among boys with lower folate and higher MMA, as well as an association present at very low Hg levels among the subset with higher homocysteine levels, suggests a subpopulation that may be uniquely susceptible to Hg immunomodulation by virtue of their MMA, folate and homocysteine levels; an interpretation further supported by similar mean Hg levels in these children compared to all other children. A biologically plausible mechanism of action underlying a positive association between Hg and measles antibodies among boys with higher MMA and lower folate might be suggested by in vitro findings in human PBMCs of the inhibitory effects of both subcytotoxic doses of ethylmercury (Gardner et al., 2010a) and higher doses of

methylmercury (de Vos et al., 2007) on IFN-γ, a cytokine required to overcome persistent measles virus-induced CNS infection (Reuter and Schneider-Schaulies, 2010). On the other hand, IFN-γ is also considered a proinflammatory cytokine, and in vitro studies indicate variable IFN-γ levels over time in ethylmercury-treated mice. Evidence is not currently available in support of or against these biological mechanisms in children. Further, in the current study, the strength of the positive Hg effect estimate was somewhat attenuated at the highest Hg quartile, and confidence intervals overlapped among mid- and highest quartiles, leaving open questions about dose-response effects among boys with higher MMA, lower folate and higher homocysteine.

Among all children with lower homocysteine, Hg was inversely associated with measles antibody levels, particularly at the highest Hg exposure. One interpretation is that the highest Hg levels inhibited measles virus replication, analogous to Verheyden and colleagues' (2004) experimental findings that 2-furylmercury, an organic Hg derivative, inhibited late, but not early, human rhinovirus RNA synthesis. Reverse causality may also play a role, as higher levels of measles antibodies may reduce levels of blood Hg via induction of metallothionein (MT). MT is known to bind with Hg to minimize its cellular bioavailability (Aschner, 1997; ATSDR, 1999) and protect against cytoxicity (Rising et al., 1995; Vitarella et al., 1996; Yao et al., 1999). Although it is unknown whether the measles virus induces MT production, findings from a study of HIV+ subjects during chronic viremic episodes indicated a positive relationship between sustained HIV virus replication and increased MT gene expression; the latter finding was absent in healthy, uninfected controls (Raymond et al. 2010). MT induction was found in mice infected with coxsackievirus B3, with subsequent decreased serum Hg and increased brain Hg (Ilback et

al., 2007). Thus, a biologically plausible mechanism for the inverse association between measles virus and total blood Hg may be MT induction and relatively unimpeded MT function with resultant decreased bioavailability of total blood Hg as measles antibody levels increase. Among all other boys with lower homocysteine, however, the 2nd Hg quartile also showed a statistically significant inverse association with measles antibody levels, but only the highest Hg quartile's inverse correlation with measles antibodies was robust in unweighted analysis, suggesting a possible threshold, rather than a dose-response. These hypothesized mechanisms notwithstanding, the cross-sectional study design limits interpretations of causality in either direction.

Additionally, a broader perspective that considers epigenetic effects is merited in light of Hg's inhibitory effects on methionine synthase (Waly et al., 2004) and hypothesized DNA hypomethylation (Deth et al., 2008) with consequent alterations in gene expression (Lee et al., 2009). For example, Toker and Huehn (2011) reviewed emerging scientific knowledge regarding the role of epigenetics in controlling expression of Foxp3 which is required for regulatory T cell (T_{reg}) development and immune function. Further, Sellin et al. (2009) showed that measles virus affects Foxp3T_{reg} homeostasis in mice, and speculated that the interplay between measles virus and T_{reg} cells may play a role in persistent measles virus infections in subacute sclerosing panencephalitis (SSPE). Future research regarding a potential relationship between Hg and Foxp3T_{reg} may yield additional insights.

This study is limited by lack of data on specific sources, forms, tissue distributions and timing of Hg exposures in the study children. Methylmercury is an organic Hg compound found in fish (Oken et al., 2005; Mahaffey et al., 2008) and ethylmercury is an organic Hg compound found in

the vaccine preservative thimerosal. Thimerosal has not been used in the measles vaccine, and has been removed from most vaccines (US FDA, 2010). The current study measured total blood Hg, which includes primarily organic Hg and, in the majority of the population, undetectable levels of inorganic Hg (CDC 2010b); however, this measure does not differentiate between different forms of organic mercurials, and consequently, interpretations are limited regarding sources of exposures. Because the clearance half-time of organic mercurials in the blood is relatively brief (< 3 months) (ATSDR 1999; Burbacher et al. 2005), Hg represents recent, but not historical exposures. Additional studies are needed to tease out the relative importance of different organo-mercurial exposures in relation to measles virus antibody levels.

The cross-sectional study design hinders our understanding of the dynamics between Hg and measles virus antibodies and how other factors, e.g., genetic, epigenetic, metallothionein, nutrients, amino acid metabolism, might influence this relationship. As in any epidemiologic study, the presence of additional unmeasured confounders or cofactors is a possibility; however, every effort was made to account for potential confounding variables. For example, in the adjusted model for boys with higher MMA, lower folate and higher homocysteine levels, adding estimated beneficial fish nutrient intake (EPA) into the model resulted in an increased effect estimate for each Hg quartile by more than 75% and each of these estimates was statistically significant, whereas without EPA-adjustment, the 2nd and 4th quartiles showed borderline significance. These results suggest that including even a crude estimate of EPA from a 24-hour dietary intake survey helped control for confounding of Hg by beneficial aspects of fish intake.

In summary, using a U.S. representative sample of children aged 6-11 years, we present early evidence of an association between levels of Hg in blood and measles antibodies. Novel findings

are reported of a positive association between very low levels of Hg exposure and measles antibodies among boys with lower folate, higher MMA and higher homocysteine, but inverse associations between higher levels of Hg exposure and measles antibodies among all other children. These findings highlight the importance of considering dynamics between toxicant exposures, pathogens and host susceptibility. Further research is warranted to discern sources and timing of concurrent Hg and measles virus exposures, biological interactions with nutritional and metabolic factors, related epigenetic processes, and correlations with immune and neurologic endpoints among susceptible infants and children.

Table 1. Sample subset weighted mean (standard error) values, frequency distributions and proportions for boys with lower folate and higher MMA (n=98) and for all other children (n=594), aged 6-11 years with seropositive measles viral antibody concentrations and reliable parental dietary recall report (n=692), NHANES 2003-2004.

	Boys with high MMA and low folate	All other children
Total blood mercury (μg/L)[a,b] Overall sample min, max: 0.10, 19.10	0.64 (0.11)	0.69 (0.08)
Measles antibody titer[a,c] Overall sample min, max: 1.00, 28.24 units	9.83 (0.88)	10.60 (0.33)
Homocysteine (μmol/L)[a,d] Overall sample min, max: 2.07, 24.51	5.39 (0.24)[e]	4.74 (0.04)
Methylmalonic acid (μmol/L)[a,d] Overall sample min, max: 0.04, 0.53	0.15 (0.007)[e]	0.12 (0.004)
Folate (ng/mL)[a,c] Overall sample min, max: 3.10, 58.10	11.99 (0.29)[e]	16.80 (0.32)
Vitamin D (ng/mL)[a,c] Overall sample min, max: 3.00, 44.00	26.87 (0.97)	26.32 (0.68)
Selenium[f] (mcg) Overall sample min, max: 10.90, 326.40	110.39 (6.22)	92.31 (3.22)
EPA[f] estimated dietary intake past 24 hrs: None ≤0.007 gm >0.007 gm Overall sample min, max: 0, 0.82	 29 (30%) 28 (29%) 41 (42%)	 199 (34%) 202 (34%) 193 (32%)
Age (years)	8.48 (0.23)	8.63 (0.12)
Body mass index (kg/m^2)	18.43 (0.43)	18.83 (0.25)
Non-Hispanic white	24 (24%)	154 (26%)
Below poverty index	26 (27%)	221 (37%)

a. Non-fasting blood sample.
b. Whole blood.
c. Serum.
d. Plasma.
e. Statistically significant difference at α=0.05 level, calculated based upon weighted complex survey design.
f. NHANES-estimated 24-hr dietary intake of selenium and eicosapentaenoic acid (EPA) based upon parental 24-hr recall.

Table 2. Weighted Linear regression coefficients (95% CI) for the relationship between continuous log-transformed total blood mercury (μmol/L) and measles virus antibody concentrations, for overall sample and sample subsets, children aged 6-11 years with seropositive measles viral antibody concentrations and reliable parental dietary recall report, NHANES 2003-2004.

	Overall sample (n=692)	Boys with high MMA and low folate (n=98)	All other boys (n=231)	Girls with high MMA and low folate (n=89)	All other girls (n=274)	Overall sample less boys with high MMA and low folate (n=594)
Unadjusted Model : continuous mercury	-0.55 (-1.47, 0.36) R^2<0.01	0.95 (-1.33, 3.24) R^2=0.01	-1.53 (-3.26, 0.20) R^2=0.03	-1.82 (-5.07, 1.43) R^2=0.03	0.01 (-1.70, 1.73) R^2<0.01	-0.84 (-1.78, 0.10) R^2<0.01
Fully Adjusted[c] Model: continuous mercury	-0.87[b] (-1.70, -0.03) R^2=0.12	1.62[b] (0.06, 3.19) R^2=0.21	-1.39[b] (-2.74, -0.04) R^2=0.30	-1.76 (-4.84, 1.32) R^2=0.20	-1.27 (-2.88, 0.62) R^2=0.13	-1.14[c] (-1.88, -0.39) R^2=0.16

a. Statistically adjusted for age, body mass index, NHANES-estimated dietary intake of eicosapentaenoic acid (EPA) and selenium, serum vitamin D, poverty, race/ethnicity, and plasma homocysteine. (Models for overall sample and overall sample less boys with high MMA and low folate also adjusted for sex, MMA and folate.)
b. $p \leq 0.05$
c. $p \leq 0.01$

Table 3. Weighted linear regression coefficients (95% CI) for the relationships between continuous total blood mercury (Hg), Hg quartiles (Q) and measles virus antibody concentrations, for boys with higher MMA, lower folate and higher homocysteine; all other boys with lower homocysteine; and all other children with lower homocysteine: children aged 6-11 years with seropositive measles viral antibody concentrations and reliable parental dietary recall report, NHANES 2003-2004.

	Boys with higher MMA, lower folate and higher homocysteine (n=61)	All other boys with lower homocysteine (n=119)	All other children with lower homocysteine (n=308)
Unadjusted, continuous log-transformed Hg (μg/L)	1.23 (-1.21, 3.68) R^2=0.03	-1.72 (-4.12, 0.67) R^2=0.06	-1.56 (-3.13, 0.02) R^2=0.03
Fully-adjusted[a] continuous log-transformed Hg (μg/L)	1.67[b] (0.39, 2.95) R^2=0.41	-1.87[c] (-3.22, -0.52) R^2=0.35	-1.85[c] (-3.06, -0.65) R^2=0.19
Unadjusted, Hg Quartiles: Q1: Hg≤0.20 μg/L Q2: 0.20<Hg≤0.40 μg/L Q3: 0.40<Hg≤0.80 μg/L Q4: Hg>0.80 μg/L R^2 (p value for trend)	 Reference 0.89 (-3.36, 5.14) 3.04 (-0.59, 6.66) 1.36 (-4.38, 7.10) 0.03 (0.586)	 Reference -5.20[c] (-8.98, -1.42) -1.89 (-5.86, 2.08) -4.81[b] (-9.57, -0.06) 0.20 (0.096)	 Reference -2.34 (-5.84, 1.17) -0.81 (-4.06, 2.43) -3.61[b] (-7.06, -0.17) 0.05 (0.093)
Fully-adjusted[a], Hg Quartiles: Q1: Hg≤0.20 μg/L Q2: 0.20<Hg≤0.40 μg/L Q3: 0.40<Hg≤0.80 μg/L Q4: Hg>0.80 μg/L R^2 (p value for trend)	 Reference 6.60[c] (3.02, 10.19) 8.49[d] (6.17, 10.81) 4.90[c] (2.12, 7.67) 0.56 (0.077)	 Reference -4.12[c] (-6.63, -1.61) -0.24 (-2.42, 1.94) -4.90[d] (-7.32, -2.49) 0.44 (0.001)	 Reference -1.87 (-5.02, 1.28) -0.37 (-3.10, 2.35) -3.83[c] (-6.30, -1.35) 0.20 (0.015)

a. Statistically adjusted for age, body mass index, NHANES-estimated dietary intake of eicosapentaenoic acid (EPA) and selenium, serum vitamin D, poverty, and race/ethnicity. (Models for all other boys and for all other children also adjusted for MMA and folate; and model for all other children also adjusted for sex.)

b. p≤0.05

c. p≤0.01

d. p≤0.001

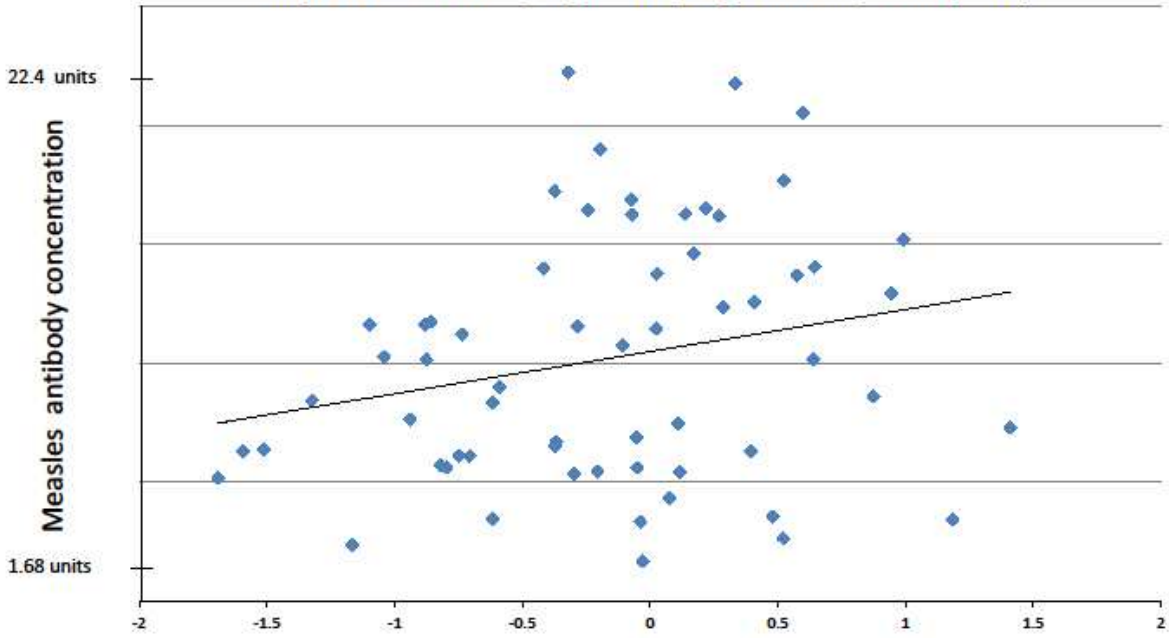
Note: Sample size by Hg quartiles (Q):

(i) Boys with higher MMA, lower folate and higher homocysteine- Q1, n=16; Q2, n=20; Q3, n=10; Q4, n=15

(ii) All other boys with lower homocysteine- Q1, n=32; Q2, n=40; Q3, n=30; Q4, n=17

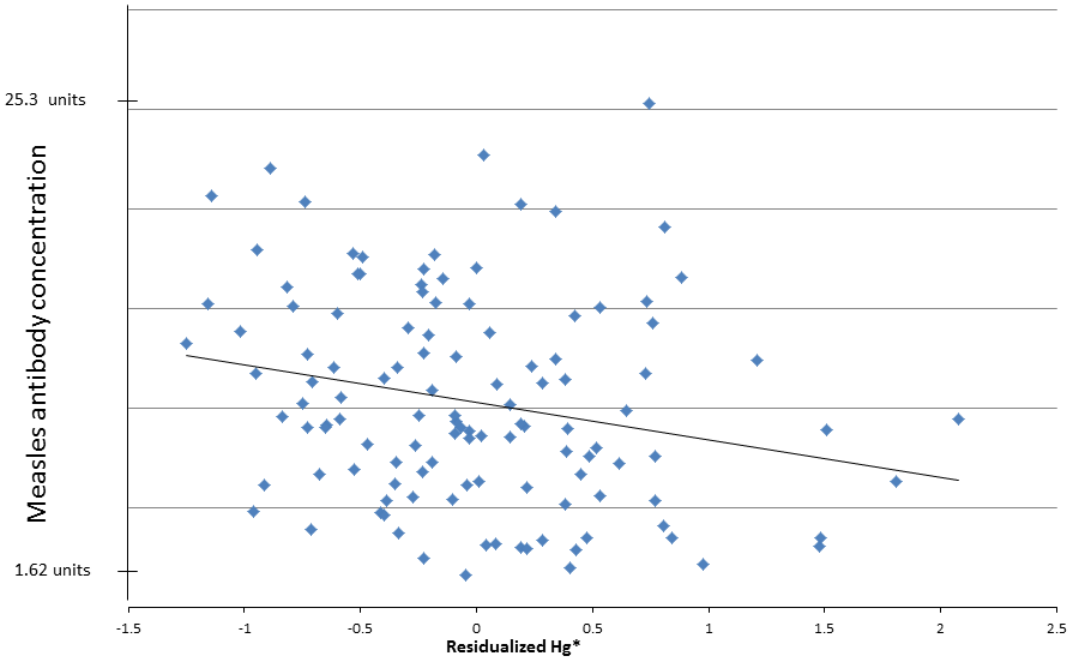
(iii) All other children with lower homocysteine- Q1, n=80; Q2, n=79; Q3, n=86; Q4, n=63

Figure 1. Residualized Hg vs. measles antibody concentration: boys with low folate, high MMA, high homocysteine (n=61)



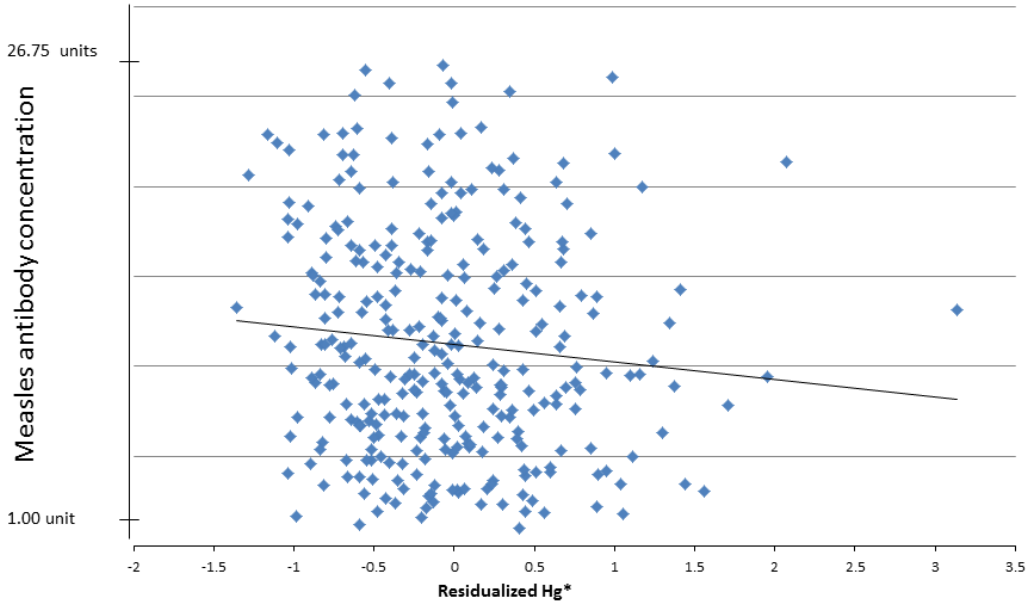
*β=1.78 (P=0.069); Log-transformed and adjusted for age, BMI, EPA, selenium, vitamin D, poverty, race/ethnicity; complex survey design not incorporated into residualized plot.

Figure 2. Residualized Hg vs. measles antibody concentration: boys with high folate, low MMA, low homocysteine (n=119)



*β=-1.89 (P=0.012); Log-transformed and adjusted for age, BMI, EPA, selenium, vitamin D, poverty, race/ethnicity.; Complex survey design not incorporated into residualized plot.

Figure 3. Residualized Hg vs. measles antibody concentration: all other children with high folate, low MMA, low homocysteine (n=308)



***β=-0.98 (P=0.080); Log-transformed and adjusted for age, BMI, EPA, selenium, vitamin D, poverty, race/ethnicity; Complex survey design not incorporated into residualized plot.**

Chapter 4

Total blood mercury and rubella antibody concentrations in US children aged 6-11 years, NHANES 2003-2004

Abstract

Children are susceptible to mercury (Hg) toxicity, and Hg has immunomodulatory effects. Lower folate and B-12, and higher homocysteine may represent susceptibility cofactors. A large proportion of variability in rubella immune response is attributable to environmental factors. This study aimed to evaluate the interaction between total blood Hg and nutritional and homocysteine status on rubella virus antibody concentrations. Cross-sectional data on rubella IgG antibody concentrations, Hg, homocysteine, methylmalonic acid (MMA, an indicator of B-12 deficiency), and folate were obtained from 2003-2004 NHANES for children aged 6-11 years with rubella seropositivity (n=690). Linear regression was used to evaluate relationships between log-transformed rubella concentrations and Hg, stratified by sex, MMA≥, folate <, and homocysteine ≥sample medians, adjusted for demographic and nutritional cofactors.

Hg was significantly positively associated with rubella antibody concentrations (β=0.24; 95% confidence interval (CI)=0.11, 0.38) in children with higher MMA, lower folate and higher homocysteine (n=110), yet inversely associated among all other children (β =-0.18; 95% CI=-0.34, -0.03) (n=580). Among the former, estimates (β) were positive across all Hg quartiles relative to the lowest (Q1) (Hg<0.30μg/L): Q2: β=0.23 (-.10, 0.56); Q3: β=0.35 (0.13, 0.57); Q4: β=0.53 (0.21, 0.84); P_{trend}<0.01. Conclusion: Findings are consistent with previously reported

associations between Hg and measles antibody concentrations, and highlight the importance of considering dynamics between toxicant exposures, pathogens and host susceptibility.

Note: This study completely represents the original study:

Gallagher CM, Smith DM, Golightly MG, Meliker JR. Total blood mercury and rubella antibody concentrations in US children aged 6-11 years, NHANES 2003-2004. Sci Total Environ 2012; 442C:48-55. doi: 10.1016/j.scitotenv.2012.09.041 [epub ahead of print]

Introduction

The integration of toxicological and infectious disease models to evaluate potential interactions is an emerging area of environmental epidemiological research need (Feingold et al., 2010). Serum vaccine antibody concentration is an increasingly recognized parameter of immune system response to environmental contaminants in population studies (Grandjean et al., 2012). Further, the U.S. Department of Health and Human Services prioritized the evaluation of relationships between environmental contaminants and immunologic outcomes in susceptible subpopulations (US DHHS, 2011a); however, epidemiologic studies stratified by susceptible subgroups are lacking.

Previously, we evaluated associations between Hg and measles antibody concentrations by nutritionally susceptible subpopulations in a US probability sample of measles IgG seropositive children aged 6-11 years using data from the 2003-2004 National Health and Nutrition Survey (NHANES) (Gallagher et al., 2011a). Similar to Heilmann and colleagues' finding of a nonsignificant inverse association between continuous measures of serum antibodies against tetanus and diphtheria vaccinations and hair Hg concentrations (2010), we observed significant inverse associations in the overall sample; however, when we stratified the analysis by nutritionally susceptible subgroups, we also observed that total blood Hg was positively associated with measles antibody concentrations among boys with higher methylmalonic acid, an indicator of B-12 deficiency, lower folate, and higher homocysteine levels, but was inversely associated with measles antibodies in all other children (Gallagher et al., 2011a). This preliminary evidence suggests that Hg exposure is associated with heterogeneity in

immunological responses in subsets of children differentiated by higher versus lower folate, vitamin B12 and homocysteine levels.

Scientific evidence supports the biological plausibility that folate, vitamin B12, and homocysteine may modify the relationship between Hg and immune function. In the methionine-methylation cycle, the enzyme methionine synthase interacts with folate and vitamin B12 to regenerate methionine, an essential amino acid and methyl-donor for DNA, RNA and other methylation reactions, from homocysteine, a non-essential amino acid (James, 2010). Methylation regulates gene expression that may be responsible for heterogeneity in immunological responses (Poland et al., 2007). Further, in vitro findings indicate that Hg inhibits the function of methionine synthase (Waly et al., 2004), a major driver of the functioning methionine-methylation cycle in conjunction with cofactors vitamin B12 and folate. Finally, homocysteine is integral to the methionine-methylation cycle, both a substrate for and by-product of methionine metabolism.

The most common cause of moderate hyperhomocysteinemia is folic acid and vitamin B12 deficiency (Schroecksnadel et al., 2003), and an inverse correlation between total plasma homocysteine and both folate and vitamin B12 levels in children has been consistently observed (Bjorke Monson and Ueland, 2003); however, much less is known about the relationship between homocysteine, folate, vitamin B12, and viral antibodies in children. Yet, research does suggest possible relationships between homocysteine and immune function. Plasma homocysteine was elevated in children with systemic lupus erythematosus (SLE) relative to healthy controls (Do Prado et al., 2006) and, in adult SLE patients, elevated plasma homocysteine was positively correlated with anti-N-homocysteine-albumin antibodies (Padjas et

al., 2007). Human cellular studies also indicate a possible role for T cell immune activation in the development of hyperhomocysteinemia (Schroecksnadel et al., 2003). Fuchs et al. (2001) describe a model in which immune activation drives oxidative stress with consequent folate and vitamin B12 depletion, leading to homocysteine accumulation. Further, mechanistic evidence suggests that homocysteine inhibits metallothionein (Barbato et al., 2007), a protein that reduces the bioavailability of Hg (Ascher, 1997; ATSDR, 1999), and that Hg also inhibits antiviral interferon (de Vos et al., 2007; Gardner et al., 2010a), cytokines that induce immunity to viruses by restricting viral spread (Stetson and Medzhitov, 2006). Homocysteine also serves as a bridge to the transsulfuration pathway for the synthesis of glutathione (GSH). Agrawal et al. (2007) showed that the organomercurial ethylmercury inhibited antiviral interferon by depleting glutathione. Another consequence of glutathione depletion is impaired methylation (Lertratanangkoon et al., 1997). The combined methylation cycle/transsulfuration pathway involving homocysteine, folate, and vitamin B-12 is emerging as a biologically plausible mediator of the association between environmental toxicants and immune-related disorders (Ji and Khurana Hershey, 2012).

Environmental factors might influence rubella antibody concentrations variability in children. Tan et al. (2001) compared post-vaccination measles and rubella antibody levels of monozygotic with dizygotic healthy twins in order to control for environmental factors, and therefore, estimate the heritability of immunogenicity. Results suggested that while measles had a heritability of 88.5%, only 45.7% of the variability in rubella antibody levels was attributed to genetic effects. Yet, despite Tan et al.'s (2001) finding that only 11.5% of measles titer variability was attributable to environmental factors in healthy children, our previous findings suggested an

association between Hg and measles concentrations in a subpopulation with possible B-12 and folate deficiencies and higher plasma homocysteine levels (Gallagher et al., 2011a). In light of the potentially greater environmental influence on rubella concentrations, the objective of the current study was to evaluate the interaction between Hg exposure and nutritional and homocysteine status on rubella virus antibody concentrations in U.S. children aged 6-11 years.

Methods

Study design

Cross-sectional data on serum rubella antibodies, total whole blood Hg, plasma methylmalonic acid (MMA), serum folate, plasma homocysteine, and covariates were obtained from the 2003-2004 National Health and Nutrition Examination Surveys (NHANES) for children aged 6-11 years whose parents dietary recall was categorized as reliable by NHANES (CDC, 2007). Blood samples were non-fasting samples (CDC, 2011f).

Outcome measure

Levels of rubella IgG antibody were measured by enzyme immunoassay and reported in Rubella International Units (IU), with a value of ≥ 10 considered seropositive for rubella (CDC, 2006a). Rubella vaccination status was not recorded; however, in the sample of children aged 6-11 years from the NHANES 2003-2004 birth cohort, greater than 10 units of rubella IgG antibodies most likely represent exposure to the vaccine-strain of the rubella virus and resultant vaccination-induced immunity, rather than past infection with the wild-type virus, given historical data on U.S. vaccine coverage pertinent to the study cohort (CDC, 2004a; CDC, 2005). In addition to “seronegative” and “seropositive” vaccine responses, a “serohyperpositive”

vaccination response was identified by Poland et al. (2002) in their study of measles titer variability among school children. Therefore, viral antibody concentrations may be meaningful across a continuum from lowest to highest levels. We used a continuous measure of rubella antibody concentrations as the study outcome measure, with log-transformation to address skewness.

Exposure measure

Whole blood Hg measurements were performed by the Division of Laboratory Sciences, National Center for Environmental Health, of the Centers for Disease Control and Prevention using inductively coupled plasma mass spectrometry (CDC, 2011g). Total blood Hg is primarily a biomarker of organic Hg exposure, and though it may also reflect exposure to inorganic Hg, total blood Hg is an established biomarker of exposure to methyl mercury over the past 1.5 to 2 months (EFSA, 2004). Mahaffey et al. (2004) showed a significant association between total self-reported fish intake over the past 30 days and total blood Hg in U.S. women of childbearing age (adjusted multiple correlation coefficient=.54; $p<0.0001$). Ethylmercury is an organomercurial metal previously found in unexpired childhood vaccines through January, 2003 (CDC, 2012f) and is currently found in small amounts in multi-dose vials of vaccines such as some influenza vaccines. Due to the greater frequency of dietary exposures relative to vaccine exposures, and the more rapid elimination of ethylmercury from the body compared to methylmercury (CDC, 2011b), total blood Hg more likely represents exposure to methylmercury in the current study. A continuous measure of total blood Hg (µmol/L) was log-transformed to address skewness. A categorical variable for total blood Hg quartiles (µg/L) was created based

upon the sample frequency distribution. To convert Hg reported as μmol/L to μg/L, μmol/L was divided by 4.99 (CDC, 2006b).

Covariates and Modifiers

The same covariates and modifiers as used in our previous study regarding associations between Hg and measles antibody concentrations (Gallagher et al., 2011a) were relevant to the current study. Therefore, the following covariates were evaluated for inclusion in multivariable models: age (years), body mass index, eicosapentaenoic acid (EPA), selenium, serum vitamin D, serum cotinine, poverty and race/ethnicity. Multivariable models were built based upon consideration of candidate covariate's relationships with the exposure and outcomes per the scientific literature, and consideration in multivariable models of stabililty of estimates. Because human and animal studies indicate that omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) can have immunomodulatory effects (Iwami et al., 2011), and certain fish are high in both PUFAs and Hg (Oken et al., 2005; Mahaffey et al., 2008), we controlled for the possible suppressive confounding influence of PUFAs (Grotto et al., 2010). We created a variable for EPA dietary intake using NHANES estimated 24-hour dietary EPA intake based upon reliable parental recall of their child's intake of specific foods over the past 24 hours (CDC, 2007), i.e., reference group=none; above and below the median for the sample subset of children aged 6-11 years. Estimated dietary intake of selenium and serum vitamin D measures was evaluated for linear relationships with rubella concentrations in order to determine assessment as either continuous or categorical measures. A dichotomous variable was also created to adjust for below and above the poverty index ratio. The rationale for stratified analyses by nutrient and homocysteine status built on our previous study of associations between measles

virus antibodies and total blood Hg (Gallagher et al., 2011a), and considered prior evidence in the literature, as well as results from statistical tests for interactions. Increased MMA levels in serum are considered direct measures of vitamin B-12 tissue stores and the first indication of B-12 deficiency (Moelby et al., 1990); serum folate is a reliable indicator of dietary folate intake and is a suitable biomarker of general folate status in population surveys (Green, 2011); and plasma homocysteine concentration remains relatively constant over a single month's duration (Garg et al., 1997), making these analytes useful biomarkers for cross-sectional analysis. To create covariates for stratified analyses, dichotomous variables were created using sample median cut-points for homocysteine, MMA and folate.

Analytic sample

Subjects were restricted to children aged 6-11 years seropositive for rubella antibodies, i.e., rubella antibody levels ≥ 10 IU, without missing values for Hg, covariates and modifiers. Trained NHANES interviewers conducted in person 24-hour dietary recall interviews for a subsample of participants. The current study sample was limited to this NHANES subsample (n=690).

Statistical analysis

All statistical analysis was conducted using SAS (Cary, N.C.) version 9.2. Bivariate linear regression and residual plots were visually inspected in order to determine whether linearity assumptions were met for linear regression, with re-examination using log-transformations. Multicollinearity was assessed using tolerance statistics (Allison, 2006).

Primary sampling units, strata, and dietary subsample weights were incorporated in accordance with complex survey design recommendations (CDC, 2011d) for hypothesis testing and the generation of U.S. population-representative statistics. Linear regression analysis was used to evaluate the relationships between continuous and categorical Hg and rubella antibodies, for the overall sample and stratified by sex, MMA ≥ and folate < sample medians, as well as homocysteine ≥ the sample median. To preserve power, and because findings were similar for boys and girls, statistical results are presented for these two subsets that each include both boys and girls. Subset 1 was comprised of children with lower folate, higher MMA, and higher homocysteine levels, a susceptible subset identified previously from investigation of measles concentrations (Gallagher et al., 2011a). Subset 2 was comprised of all other children. Linear regression coefficients and 95% confidence intervals were reported as measures of association, both unadjusted and adjusted for covariates. Simple and multiple linear regression were used to evaluate associations between the continuous outcome measure of log-transformed rubella concentrations and total blood Hg, as both a continuous log-transformed measure and categorical quartiles, with *P*-values reported for trend tests to evaluate dose-response relationships. Both weighted (NHANES dietary subsample weights) and unweighted models were evaluated and, since results were similar, weighted results are presented in the tables and results section in order to provide findings that are generalizable to the U.S. population of children aged 6-11 years. In the models using continuous natural log-transformed Hg, because the outcome is also natural log-transformed, linear regression coefficients are interpreted as the percentage increase or decrease in the average value of rubella antibodies per 1% increase in Hg, and in models using untransformed Hg quartiles, linear coefficients are interpreted as the percentage increase or decrease in the average value of rubella antibodies per unit increase of Hg (Vittinghoff et al.,

2005), i.e., change from first to fourth Hg quartile, as reported in the Discussion section. Adequacy of model fit was evaluated using the convergence criterion, and alpha significance levels were set a priori at 5 percent (2-sided).

The relationship between Hg, adjusted for covariates, and rubella antibody concentrations was graphically depicted by plotting a residualized Hg variable by untransformed rubella antibody concentrations; complex survey design weights, primary sampling units and strata were not incorporated into these plots. The plotted Hg variable was comprised of the residual values generated by modeling log-transformed Hg as the dependent variable and the remaining covariates as independent variables; sample weights were incorporated into this calculation, but not primary sampling units or strata. Figure captions present regression coefficients for both untransformed (depicted in figures) and log-transformed (not depicted in figures) rubella antibodies as the dependent variable.

Sensitivity analyses were conducted using alternative low folate cut-points of 12.9 ng/mL (bottom one-third of sample) and 13.8 ng/mL (midpoint percentile between bottom one-third and median-cut folate levels). In addition, separate models were run evaluating each combination of median-cut folate and homocysteine subset and each combination of median-cut MMA and homocysteine subset.

Results

In Subset 1 there were 110 children with lower folate, higher MMA and higher homocysteine, and in Subset 2, there were 580 children without these susceptibility cofactors (Table 1). Other than the modifier variables used to define these subsets, there were no statistically significant differences between subset means and proportions of covariates.

Among children with lower folate, higher MMA and higher homocysteine, in both unadjusted (β=0.25; 95% CI=0.12, 0.38) and adjusted (β=0.24; 95% CI=0.11, 0.38) models, log transformed continuous Hg (µmol/L) was significantly positively associated with log-transformed rubella virus antibody concentrations (IU) (Table 2). Among all other children, however, an inverse association was evident in both unadjusted (β=-0.12; 95% CI=-0.28, 0.04) and adjusted (β=-0.18; 95% CI=-0.34, -0.03) models. A similar pattern was evident using the following categorical Hg quartiles (µg/L) as the exposure variables: Quartile 1 (Q1, Referent): Hg<0.30 µg/L; Q2: 0.30≤Hg<0.50 µg/L; Q3: 0.50≤Hg<0.80 µg/L; Q4: Hg≥0.80 µg/L. In multivariable analysis, among children with lower folate, higher MMA and higher homocysteine, relative to Q1, increasing positive estimates were observed from the 2nd through the highest Hg quartiles (Q2: β=0.23; 95% CI=-0.10, 0.56; Q3: β=0.35; 95% CI=0.13, 0.57; Q4: β=0.53; 95% CI=0.21, 0.84) (P_{trend}<0.01). Among all other children, however, an inverse relationship was observed, particularly at the highest Hg quartile (Q2: β=-0.21; 95% CI=-0.58, 0.16; Q3: β=-0.15; 95% CI=-0.47, 0.18; Q4: β=-0.34; 95% CI=-0.67, -0.00) (P_{trend}=0.07).

Discussion

Interpretation of key findings

Children with nutritional susceptibility, i.e., lower folate and higher MMA levels, as well as higher homocysteine levels, showed a significant positive association between Hg and rubella antibody concentrations, whereas all other children showed an inverse, albeit variable, relationship. Among the susceptible subgroup, a 1% increase in total blood Hg was associated with a 0.24% increase in average rubella antibody concentration; whereas among all other children, a 1% increase in total blood Hg was associated with a 0.18% decrease in average

rubella antibody concentration. Further, among the susceptible subgroup, an increase in the total blood Hg level from the first to the fourth quartile was associated with a 70% increase in average rubella antibody concentration. On the other hand, among all other children, an increase in total blood Hg from the first to the fourth quartile was associated with a 40% decrease in average rubella antibody concentration. These associations were shown at levels of Hg exposure well below established safety levels. Findings are compatible with previously reported associations between Hg and measles antibody concentrations (Gallagher et al., 2011a) and therefore add weight to the likelihood that Hg may influence vaccine-type viral antibody levels among susceptible subgroups. Measles and rubella antibody concentrations were only moderately correlated (Spearman rho=0.40), so it is unlikely that associations between Hg and rubella antibodies were confounded by the coincident presence of measles antibodies. Further, sensitivity analysis (results not shown in tables) using alternative folate cut-points showed similar associations . In addition, analyses using separate combinations each of median-cut MMA and homocysteine subsets and median-cut folate and homocysteine subsets resulted in patterns similarly suggestive of positive relationships between Hg and rubella antibody concentrations among children with lower B vitamin levels coincident with higher homocysteine levels, but inverse relationships in the remaining subset combinations (results not shown in tables).

In contrast to our previous study regarding associations between Hg and measles antibody concentrations (Gallagher et al., 2011a), in the current study, positive associations between Hg and rubella antibody concentrations were not limited to boys. This disparity lends support to Poland and colleagues' conclusion that it is important to address sex-based differential immune

responses (2008). Umlauf et al. (2012) measured cytokines secreted by peripheral blood mononuclear cells following in vitro stimulation with live measles virus and showed that girls secreted significantly more of the antiviral interferon alpha than boys. On the other hand, Ovsyannikova et al. (2004) observed significantly higher antibody responses to rubella vaccine antigen among females. Studies using larger sample sizes are merited to evaluate sex-Hg interactions among susceptible subgroups.

Biologically plausible mechanisms of action

Mechanistic evidence supports the biological plausibility of nutrition-related effect modification of the relationship between Hg and immune response indicators. Organomercurial exposure, i.e., ethylmercury, in human neuronal cells has been shown to inhibit methioinine synthase, a driver of the methionine-methylation cycle, a metabolic process involving vitamins B-12 and folate (Waly et al., 2004). Further, experimental research found that homocysteine inhibited metallothionein (Barbato et al., 2007), a protein that reduces Hg's bioavailability (Aschner, 1997; ATSDR, 1999) and cytotoxicity (Rising et al., 1995; Vitarella et al., 1996; Yao et al., 1999). Therefore, nutritional cofactors and homocysteine may interact with organomercurials to disrupt essential human biological functioning. RNA methylation is one metabolic function supported by the methionine cycle (James, 2010) and is also an antiviral mechanism of interferons, cytokines with known antiviral actions (Mandell et al., 2009). For example, type II interferon (IFN-γ) protects against persistent measles-virus infection of the central nervous system (Reuter and Schneider-Schaulies, 2010). Gardner et al. (2010) showed that in vitro exposures of human peripheral blood mononuclear cells (PBMCs) to subcytotoxic doses (up to 200 nM) of the organic mercurial ethylmercury, but not methylmercury or

inorganic Hg, inhibited IFN- γ release (Gardner et al., 2010a). De Vos et al. (2007) exposed human PBMCs to increasing doses of methylmercury and inorganic Hg, and observed that methylmercury concentrations of 2 µM and inorganic Hg concentrations of 50 µM significantly decreased IFN-γ production. Interferon has also been shown to inhibit rubella virus replication in vitro (Wong et al., 1971), although it is unknown whether Hg disrupts this specific immune regulatory mechanism.

While minimum levels of viral antibody concentrations are established biomarkers of seropositivity, there is currently no standardized definition of excessive vaccine-type measles or rubella antibody levels in the general population. Taken together with findings of EEG abnormalities and elevated levels of wild-type measles and rubella antibodies in cerebrospinal fluid, however, elevated serum levels of wild-type measles and rubella antibodies are diagnostic indicators of rare viral infections of the central nervous system, i.e., subacute sclerosing panencephalitis (SSPE) and progressive rubella panencephalitis, respectively (Ziola et al., 1983). Although the etiology of these conditions is incompletely understood, a possible mechanism of action is inadequate clearance of viral infection (Ropper and Samuels, 2009), a mechanism consistent with the known protective influence of IFN-γ against SSPE (Reuter and Schneider-Schaulies, 2010). Ziola et al. (1983) found that viral clearance was prolonged in some measles and rubella patients, and suggested that whether acquired naturally or through administration of attenuated vaccines, viral persistence could have “subtle long term effects on human health”, e.g., persistent subclinical infections. Therefore, one possible interpretation of the higher rubella antibody levels associated with increasing Hg exposures among nutritionally susceptible children is lesser viral clearance and extended viral persistence. Marchant et al. (2006) showed that

environmental factors predominantly influence tetanus toxoid antibody persistence rather than initial vaccine response; therefore, among the seropositive children in the current study, a consistent interpretation is that relationships between Hg exposures and rubella IgG antibody concentrations similarly represent vaccine antibody persistence. Mechanistic and longitudinal studies are needed to build evidence-based scientific understanding, particularly with regard to developmental immunotoxicology and vulnerable periods of early immune development (Dietert, 2008).

Epidemiologic design considerations

Stern and Korn (2001) used simulation to show that large-scale epidemiologic research often fails to detect a dose-response relationship between Hg and neurodevelopmental endpoints if it only exists in sensitive subpopulations. Of note, in the current overall study sample (Subsets 1 and 2 combined) a non-significant inverse relationship between Hg and rubella antibody concentrations was observed (Table 3), lending support to the compromised sensitivity of epidemiologic studies absent the identification and analysis of susceptible subgroups. Figures 1 and 2 illustrate the opposing directionality of the linear relationships between Hg and rubella antibody concentrations comparing children with lower folate, higher MMA, and higher homocysteine to all other children, as also previously shown with regard to measles antibody concentrations in boys (Gallagher et al., 2011a). Such clearly divergent bimodal patterns are likely to mask statistical associations in epidemiologic research that fails to stratify by susceptible subpopulations. Children with lower nutrient levels may represent subpopulations susceptible to metal uptake (Gochfeld, 1997; Gallagher et al., 2011b), metabolic (Gallagher and Meliker, 2011) and immune influences (Gallagher et al., 2011a).

An important limitation of the current study is the cross-sectional study design and, consequently, our inability to establish a temporal sequence. Biotransformation of organic to inorganic Hg (Havarinasab and Hultman, 2005) and variable blood to tissue distributions of methyl- compared to ethyl-mercury (Burbacher et al., 2005) add to the challenges in using total blood Hg as a biomarker of exposure. The unavailability of data on immune-related conditions pertinent to determinations of adverse outcomes is another limitation, as is the lack of data on cell-mediated immune responses, such as antiviral interferons, for example, interferon-gamma, the principal cytokine produced after MMR immunization in infants (Pabst et al., 1997). Further, without data on individual immunization status and dates of immunization, interpretations of rubella antibody concentrations as immune response to rubella vaccination are limited, as wild-type exposures, although less likely, may have also occurred.

An overall advantage of using NHANES data is generalizability of findings to the U.S. population regarding associations between concurrently collected and rigorously measured biological markers of possible risk factors and immune indicators. Repeated measurements, however, are needed to evaluate associations that account for intra-individual variability over time, as recently shown with blood Hg (Tsuchiya et al., 2012). Therefore, our findings merit interpretation as a snapshot in time of the possible physiological relationship between organic Hg exposure and rubella antibody variability among nutritionally susceptible subgroups of children. Cohort studies are merited to evaluate temporal associations between exposures to environmental contaminants, humoral and cell-mediated immune responses to vaccination, susceptibility cofactors, and associated adverse and protective outcomes. Future studies that evaluate associations between Hg exposure and immune response to vaccinations among the susceptible

subgroups identified in this study may inform “adversomics: the emerging field of vaccine adverse event immunogenetics” (Poland et al., 2009), by incorporating both environmental and susceptibility cofactors. Although evidence on the safety and effectiveness of MMR vaccine supports current global public health immunization policies, the Cochrane Collaboration found no studies assessing the effectiveness of MMR in preventing rubella, determined that the design and reporting of safety outcomes were inadequate (Demicheli et al., 2012), and did not identify studies of susceptible subpopulations.

Public Health Significance

Healthy People 2020 targeted a 30 percent reduction in the level of total blood Hg in U.S. children and women of childbearing age (US DHHS, 2011b). Yet, historically, safe limits have been set based upon associations with neurodevelopmental endpoints, rather than immunologic indicators that mediate the cross-talk, or bi-directional interaction, between the brain and the immune system (Elenkov et al., 2000; Kerschensteiner et al., 2009; Buch, 2011). Our findings suggest immune variability at levels well below current safety thresholds. Environmental considerations may be particularly relevant for understanding biologically meaningful variability in response to vaccines among vulnerable subsets of children (US DHHS, 2011a; IOM, 2008). By investigating indicators of immune response among susceptible subpopulations, our findings shed light on the need for prospective studies to guide public health decision-making for the protection of the most vulnerable.

Table 1. Sample Subset Weighted Mean (Standard Error) Values, Frequency Distributions (#) and Weighted Proportions (%) for Subset 1: Children With Lower Folate, Higher MMA, and Higher Homocysteine Levels, and for Subset 2: All Other Children; Aged 6-11 Years With Seropositive Rubella Viral Antibody Titers and Reliable Parental Dietary Recall Report (n=690), NHANES 2003-2004.

	Subset 1: Children With Lower Folate, Higher MMA, and Higher Homocysteine (n=110)	Subset 2: All Other Children (n=580)
Total blood mercury (μg/L)[a,b]	0.72 (0.13)	0.68 (0.08)
Overall sample Min, max: 0.10, 19.10		
Rubella antibody level (IU)[a,c]	65.93 (4.01)	65.89 (4.68)
Overall sample Min, max: 10, 297		
Homocysteine (μmol/L)[a,d]	5.74* (0.13)	4.62 (0.04)
Overall sample Min, max: 2.07, 24.51		
Methylmalonic acid (μmol/L)[a,d]	0.16* (0.01)	0.12 (0.00)
Overall sample Min, max: 0.04, 0.53		
Folate (ng/mL)[a,c]	11.65* (0.20)	17.04 (0.38)
Overall sample Min, max: 3.10, 58.10		
Selenium[e] (mcg)	104.42 (7.79)	92.52 (3.31)
Overall sample Min, max:10.90, 326.40		
EPA[e] estimated dietary intake past 24 h # (%):		
None	31 (37%)	197 (45%)
≤0.007 g	29 (26%)	203 (31%)
>0.007 g	50 (37%)	180 (24%)
Overall sample Min, max: 0, 0.82		
Age (years)	8.86 (0.25)	8.57 (0.12)
Overall sample Min, max: 6,11		
Body mass index (kg/m^2)	19.23 (0.68)	18.75 (0.23)
Overall sample Min, max: 12.4, 45.13		
Males # (%)	59 (52%)	267 (50%)
NonHispanic white # (%)	34 (66%)	141 (60%)

MMA Methylmalonic acid * $P<0.05$

a. Non-fasting blood sample.
b. Whole blood.
c. Serum.
d. Plasma.
e. NHANES-estimated 24-h dietary intake of selenium and eicosapentaenoic acid (EPA) based upon parental 24-h recall

Table 2. Linear Regression Coefficients (95% CI) for the Relationships Between Continuous Log-transformed Rubella Virus Antibody Titers (IU) and Continuous Log-transformed Total Blood Mercury (μmol/L) and Total Blood Mercury Quartiles, for Subset 1: Children With Lower Folate, Higher MMA, and Higher Homocysteine Levels, and for Subset 2: All Other Children; Children Aged 6-11 Years With Seropositive Rubella Viral Antibody Titers and Reliable Parental Dietary Recall Report, NHANES 2003-2004.

	Subset 1: Children With Lower Folate, Higher MMA, and Higher Homocysteine (n=110)	Subset 2: All Other Children (n=580)
Unadjusted Model : Continuous mercury	R^2=0.09 0.25* (0.12, 0.38)	R^2=0.01 -0.12 (-0.28, 0.04)
Fully Adjusted[a] Model: Continuous mercury	R^2=0.21 0.24* (0.11, 0.38)	R^2=0.09 -0.18* (-0.34, -0.03)
Unadjusted Model, Mercury (Hg) quartiles (Q):	R^2=0.13	R^2=0.02
Hg <0.30 μg/L	Referent	Referent
0.30≤Hg<0.50	0.19 (-0.19, 0.57)	-0.17 (-0.53, 0.19)
0.50≤Hg<0.80	0.36* (0.10, 0.62)	-0.12 (-0.45, 0.21)
Hg≥0.80 μg/L	0.52* (0.22, 0.81)	-0.25 (-0.60, 0.11)
	P_{trend}<0.01	P_{trend}=0.18
Fully Adjusted[a] Model, Mercury (Hg) quartiles (Q):	R^2=0.25	R^2=0.09
Hg <0.30 μg/L	Referent	Referent
0.30≤Hg<0.50	0.23 (-0.10, 0.56)	-0.21 (-0.58, 0.16)
0.50≤Hg<0.80	0.35* (0.13, 0.57)	-0.15 (-0.47, 0.18)
Hg≥0.80 μg/L	0.53* (0.21, 0.84)	-0.34* (-0.67, -0.00)
	P_{trend}<0.01	P_{trend}=0.07

CI Confidence interval
Hg total blood mercury
MMA Methylmalonic acid
Q Quartile
*P<0.05

a. Statistically adjusted for age, body mass index, NHANES-estimated 24-hr dietary intake of eicosapentaenoic acid (EPA; no intake, above and below sample median) and selenium, race/ethnicity (nonHispanic white; referent=nonwhite), and sex.

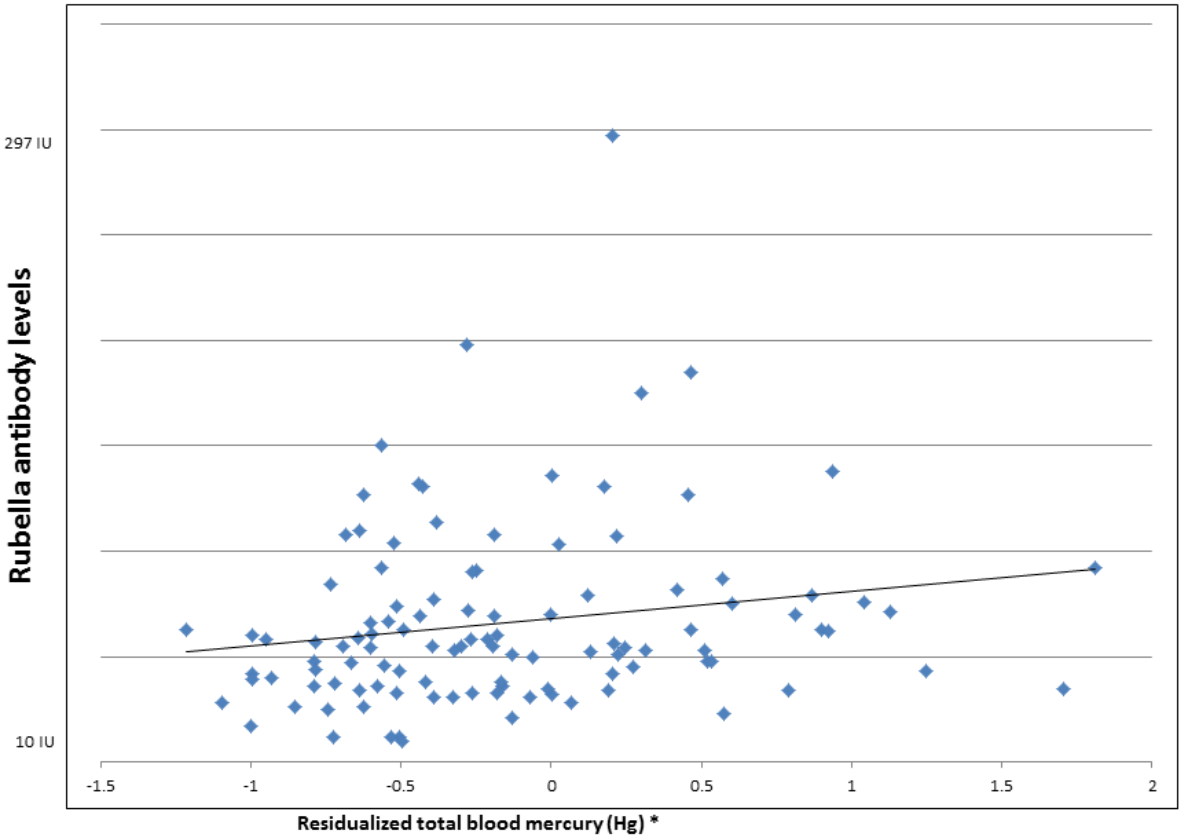
Table 3. Linear Regression Coefficients (95% CI) for the Relationships Between Continuous Log-transformed Rubella Virus Antibody Concentrations (IU) and Continuous Log-transformed Total Blood Mercury (μmol/L) and Total Blood Mercury Quartiles; Overall Sample: Children Aged 6-11 Years With Seropositive Rubella Viral Antibody Concentrations and Reliable Parental Dietary Recall Report, NHANES 2003-2004 (n=690).

Unadjusted Model :	
Continuous mercury	-0.05 (-0.21, 0.11)
Fully Adjusted[a] Model:	
Continuous mercury	-0.10 (-0.26, 0.06))
Unadjusted Model,	
Mercury (Hg) quartiles (Q):	
Hg <0.30 µg/L	Referent
0.30≤Hg<0.50	-0.12 (-0.41, 0.18)
0.50≤Hg<0.80	-0.05 (-0.35, 0.24)
Hg≥0.80 µg/L	-0.12 (-0.41, 0.18)
	P_{trend}=0.63
Fully Adjusted[a] Model,	
Mercury (Hg) quartiles (Q):	
Hg <0.30 µg/L	Referent
0.30≤Hg<0.50	-0.15 (-0.45, 0.16)
0.50≤Hg<0.80	-0.07 (-0.37, 0.22)
Hg≥0.80 µg/L	-0.18 (-0.48, 0.13)
	P_{trend}=0.32

CI Confidence interval
Hg total blood mercury
MMA Methylmalonic acid
Q Quartile
*P<0.05

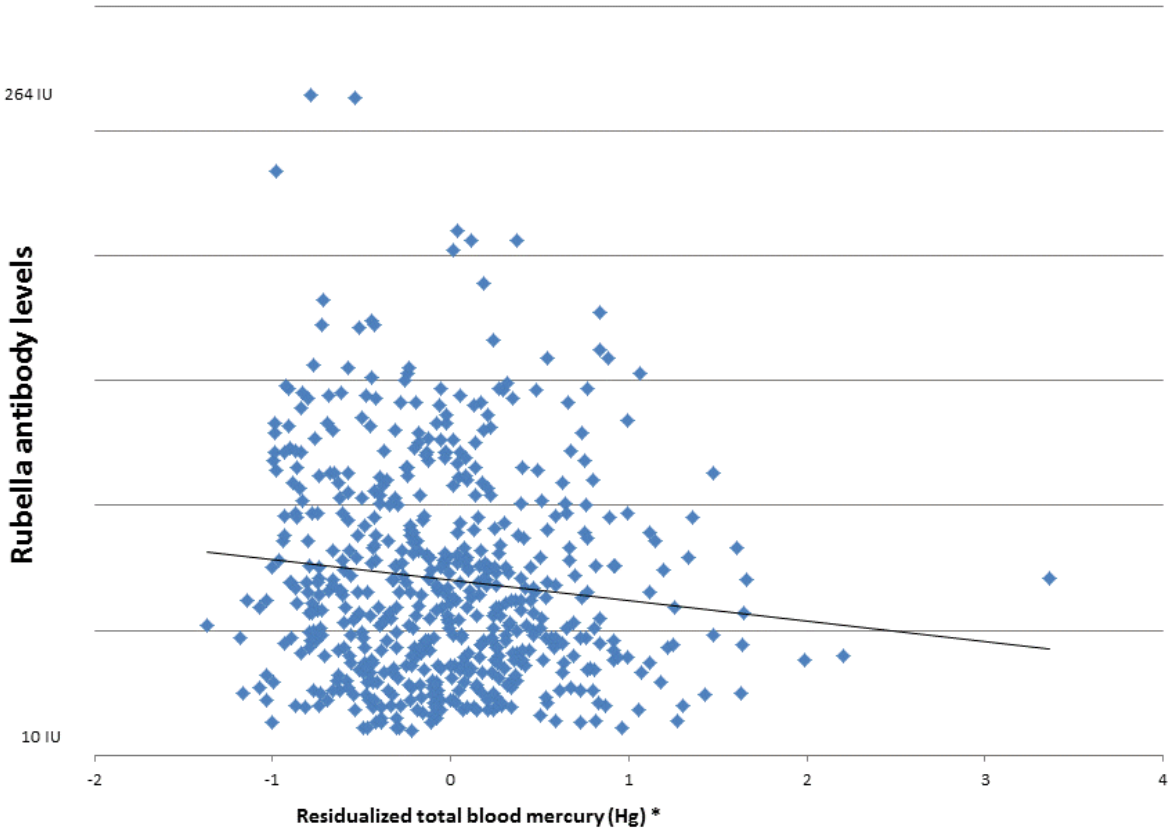
a. Statistically adjusted for age, body mass index, NHANES-estimated 24-hr dietary intake of eicosapentaenoic acid (EPA; no intake, above and below sample median) and selenium, race/ethnicity (nonHispanic white; referent=nonwhite), sex, and variables used for sample stratification in main analysis: median cut serum folate, MMA and plasma homocysteine levels.

Figure 1. Children With Lower Folate, Higher MMA, and Higher Homocysteine (n=110)



* Hg log-transformed and statistically adjusted for age, BMI, EPA, selenium, race, sex; complex survey design not incorporated into residualized plot; β = 12.75 (P=0.063) for untransformed rubella antibodies as dependent variable (shown); β = 0.22 (P=0.021) for log-transformed rubella antibodies as dependent variable (not shown).

Figure 2. All Other Children (n=580)



* Hg log-transformed and statistically adjusted for age, BMI, EPA, selenium, race, sex; complex survey design not incorporated into residualized plot. β=-8.12 (p=0.006) for untransformed rubella antibodies as dependent variable (shown); β=-0.10 (p=0.024) for log-transformed rubella antibodies as dependent variable (not shown).

Dissertation Research Conclusions

The investigations reported in this dissertation are among the first to examine associations between upstream biological indicators of human immune response and Hg and PCB exposures at lower environmental levels commonly found in the general population. This is also one of the first studies to examine environmental contaminant-immune associations in susceptible subpopulations. In U.S. females, ANA positivity was associated with serum PCB exposures at levels of exposure common in the U.S. population and substantially lower than exposure levels in another industrial-exposed populations (Cebecauer et al., 2009); in U.S. females with lower urinary iodine levels, a risk factor for hypothyroidism was associated with total blood mercury exposure at levels a fraction of the average blood mercury concentrations of riverine populations who showed associations between mercury and systemic autoimmune response (Nyland et al., 2011); and in nutritionally and metabolically susceptible U.S. children, measles and rubella IgG antibodies were positively associated with total blood mercury exposures at levels well below the 5.8 μg/L prenatal exposure level associated with adverse downstream neurodevelopmental endpoints (US EPA, 2012d). Further, by virtue of their potential to indicate early manifestations of biological disturbances, these upstream immune response indicators may represent more sensitive endpoints of the adverse effects of environmental toxicants than downstream endpoints such as autoimmune disease and other disorders characterized by immune dysregulation.

The novelty of the approach and the findings notwithstanding, as in any cross-sectional study, causal determinations are precluded and, as in any single observational study, chance findings and residual confounding cannot be ruled out. In addition, it is also important to note several specific study limitations. First, the outcomes and exposures studied are not a comprehensive

representation of pertinent human immune responses and exposures. Second, the outcome measures used in this dissertation research are not diagnostic of disease or direct measures of immune system response caused by Hg or PCBs subsequent to exposure; rather, they are considered as indicators of immune system responses associated with these contaminants. Third, to evaluate associations over time, repeated measures of exposure and outcome would be warranted. Finally, findings merit confirmation in studies with larger sample sizes. Because NHANES is intended for hypothesis testing to identify possible risk factors using a U.S. probability sample, any positive findings may shed important insights useful to guide further research. My synthesized interpretation of study findings will be presented in three sections pertinent to the type of immune response indicated by the outcome measure: (1) systemic autoimmune-related response; (2) thyroid autoimmune-related response; and (3) viral-related immune response.

(1) Systemic Autoimmune-related Response

The positive association between dioxin-like PCBs and ANA positivity in U.S. women suggests that exposure to environmental levels of this persistent pollutant may be sufficient to induce a systemic autoimmune response in this autoimmune-susceptible subpopulation, whereas Hg exposure levels in the U.S. are not. Null findings for an association between Hg and ANA were also observed using an expanded sample with additional NHANES survey years 1999-2000 and 2001-2002, including a model that stratified by median-cut homocysteine level in order to explore the potentially increased bioavailability of Hg associated with homocysteine's inhibition of metallothionein, as suggested by experimental results (Barbato et al., 2007). My null findings are inconsistent with findings in riverine populations in Amazonian Brazil (Nyland et al., 2011),

and might be explained by the lower Hg exposure levels found in the U.S. sample, e.g., levels that are insufficient to induce a systemic autoimmune response. In addition, although females are a susceptible subpopulation with regard to autoimmune disease, animal studies suggest that genetic susceptibility plays a role in Hg-induced autoimmunity; however, the current study did not subset the analysis by potential effect modifiers with previously shown genetic associations with Hg susceptibility in humans, such as glutathione and related enzymes known to bind with Hg (Gundacker et al., 2007; Goodrich et al., 2011). Dioxin-like PCBs, on the other hand, may interact with the AhR by mediating hormone-related susceptibility according to several lines of research. Specifically, mechanistic evidence supports AhR-mediated proinflammatory effects of dioxin-like PCBs (Hennig et al., 2002; Kim et al., 2012), the dioxin-like PCB congener 126 also exerts estrogenic effects (Mortenson and Arukwe, 2008), and the AhR mediates estrogen signalling through the ubiquitin system (Ohtake et al., 2011), which may be protective against systemic autoimmunity (Tavares et al., 2010; Kook et al., 2011), whereas its disruption has been linked to autoimmune diseases (Fierabracci, 2012). . Although further research is needed to elucidate AhR-mediated dioxin-like PCB immunotoxicity in females, findings of the current study add weight to previously reported associations between PCB exposure and ANA positivity (Cebecauer et al., 2009)and autoimmune morbidity (Lee et al., 2007) and mortality (Tsai et al., 2007) in humans.

(2) Thyroid Autoimmune-related Response

Among females with iodine deficiency, total blood Hg was positively associated with a risk factor for hypothyroidism (Walsh et al., 2010), a condition primarily caused by autoimmune thyroiditis (Baskin, 2002). One possible explanation for the association between Hg and target

organ autoimmune response, but not systemic autoimmune response, is thatHgaccumulates in the human thyroid gland (Falnoga et al., 2000; Nylander and Weiner, 1991) with consequent concentrated Hg exposures. Further, whereas the Hg and ANA study did not evaluate effect modification of a known susceptibility factor, the Hg and thyroid autoantibody study did stratify by iodine status. Zhu et al. (2000) suggest that Hg's toxicity is attributable to its sulf-hydryl-binding capacity, and thus, broad effects as an enzyme inhibitor. Consistent with this interpretation, Hg exposure in mice was shown to inhibit the coupling of iodine-containing thyroid proteins by the enzyme thyroperoxidase, reducing iodine uptake and altering thyroid hormones (Kawada et al., 1980; Nishida et al., 1986) known to have inhibitory effects on thyrotropin release in humans. This suggests iodine deficiency as an effect modifier, consistent with dissertation study findings of positive associations between Hg and the hypothyroid risk factor in iodine-deficient females, and between Hg and elevated thyrotropin among iodine-deficient females and males. Further, whereas Hg was positively associated with thyroglobulin autoantibodies in the NHANES 2007-2008 sample, the larger 2007-2010 sample showed instead positive associations between Hg and thyroid peroxidase autoantibodies among iodine deficient females, and between Hg and dual thyroid autoantibody positivity among females with excessive iodine levels. Although chance findings cannot be ruled out, these findings suggest that Hg-associated thyroid autoimmune response is not antigen-specific, but instead, may be polyclonally activated, consistent with mechanistic research. An important consideration is the inconsistent effect sizes and mixed findings of statistical significance as a consequence of the different weight sets used in the original compared to the current larger study. The NHANES analytic guidelines and tutorial (CDC, 2011d) recommend that researchers use the appropriate weights of the smallest subsample; an approach used in both the original and current thyroid studies; however,

given the unique 2009/2010 subsample weights for the smallest subset with urinary iodine measured, and the dietary sample weights used for the smallest subset with dietary data in NHANES 2007/2008 datafiles, sample weight subsets were not the same across these two studies. This poses a challenge to reconciliation and interpretation of findings across these studies, and methodologic studies are indicated to address this discrepancy. For the current study, however, our approach of using dietary subsample weights in a sensitivity analysis provides a useful point of comparison. Moreover, it may be most informative to consider overall patterns of association which displayed a consistent positive relationship between Hg and thyroid autoimmune response indicators in females although not always statistically significant across the weighted and unweighted analyses.

The contrasting pattern in males, particularly with regard to thyroid outcomes including autoantibody measures, but not for the elevated thyrotropin outcome, suggests a sexual dimorphism with regard to both the direction of the association and the iodine susceptibility group of influence. One possible interpretation is that, since androgens inhibit hypothalamic-pituitary-adrenal (HPA) glucocorticoid response to stress such as immune challenge (Da Silva, 1999), an immunosuppressive response is triggered by immune challenge by Hg exposure in males, but not in females, for whom estrogen enhances the HPA stress response (Da Silva, 1999). The potential for hormonal-immune synergism may further potentiate these effects, as estrogens induce Th2 cell development to activate antibody-producing B cells, and testosterone instead can affect Th1 cell development leading to local inflammatory response (Lee and Chiang, 2012).Whereas estrogens (DaSilva, 1999) and mercury (Pusey et al., 1990; Abedi-Valgerdi, 2009) polyclonally activate B-cells to stimulate autoantibody production, androgens suppress B-

cell immune responses (DaSilva, 1999; Lee and Chiang, 2012), and might contribute to the sexually dimorphic associations observed between mercury and thyroid autoimmune-related measures.

(3) Viral immune-related Response

Dissertation research findings of significant positive associations between total blood Hg and measles antibodies in boys with lower folate and B-12 and higher homocysteine, and between Hg and rubella antibodies in boys and girls, combined, with these same susceptibility cofactors, lend support to an interpretation consistent with Hg's affinity for thiols (organic compounds that contain sulfur and hydrogen, i.e., sulf-hydryl compounds), and consequently, broader enzyme effects. The combined methylation cycle/transsulfuration pathway involving homocysteine, folate, and vitamin B-12 is emerging as a biologically plausible modifier of the association between environmental toxicants and immune-related disorders (Figure 1; Ji and Khurana Hershey, 2012). Methylation regulates gene expression, an epigenetic effect that may be responsible for heterogeneity in immunological responses (Poland et al., 2007), and environmentally induced changes in DNA methylation are an epigenetic effect identified by a National Institute of Environmental Health Sciences expert panel workshop that may contribute to the induction of autoimmunity (Selmi et al., 2012). Mechanistic evidence supports Hg's interference with the methylation cycle/transsulfuration. First, the organomercurials methyl- (Smith and Smith, 1990) and ethyl- (Waly et al., 2004) Hg inhibit the function of the enzyme methionine synthase. Second, methyl- (Kaur et al., 2006) and ethyl- (James et al., 2005; Agrawal et al., 2007) Hg deplete the tripeptide glutathione, the most abundant intracellular source of thiols. Third, methyl- (de Vos et al., 2007) and ethyl- (Agrawal et al., 2007; Gardner et al.,

2010a) Hg inhibit antiviral interferon; specifically, interferon gamma, which is recognized as necessary to overcome persistent measles virus-induced central nervous system infection (Reuter and Schneider-Schaulies, 2010) and also as the principle cytokine produced after the MMR vaccination in infants (Pabst et al., 1997). Ethylmercury may inhibit interferon gamma by depleting glutathione (Agrawal et al., 2007), and glutathione depletion may lead to impaired methylation (Lertratanangkoon et al., 1997), a known antiviral mechanism of interferons (Mandell et al., 2009), thus highlighting the inter-relatedness of the methylation cycle/transsulfuration pathway (Figure 1).

Vaccine responses such as IgG antibodies are considered measures of T cell-dependent functional immunity (Jusko et al., 2010; Dietert and Holsapple, 2001), as well as indicators of persistence of viral-specific B cell immunity (Haralambieva et al., 2011). Therefore, one biologically plausible consideration is that organomercurials, shown to have immunosuppressive and immunostimulation effects on T cells and B cells (Havarainsab and Hultman, 2003), might also potentially alter immune responses via the methylation cycle/transsulfuration pathway by inhibiting antiviral interferon with consequent reduced viral clearance, increased viral antigen load, and viral persistence. Differential findings of an association between Hg and measles antibodies in susceptible boys, only, yet an association between Hg and rubella antibodies in susceptible boys and girls, may lend support to a virus- and sex-dependent antibody response; an interpretation that also may be indicated by other studies. For example, Umlauf et al. (2012) measured cytokines secreted by peripheral blood mononuclear cells following in vitro stimulation with live measles virus and showed that girls secreted significantly more of the antiviral interferon alpha than boys, and Ovsyannikova et al. (2004) observed significantly

higher antibody responses to rubella vaccine antigen among females. Accordingly, one possible interpretation is that, in children, associations between Hg and measles antibodies are only seen in boys, whereas associations with rubella antibodies are seen in both boys and girls.

The possibility of reverse causation also merits consideration, particularly with regard to the inverse relationships observed between Hg exposure and measles and rubella IgG antibodies in children with higher folate, higher vitamin B-12 and lower homocysteine levels. Metallothionein (MT) is known to bind with Hg to minimize its bioavailability (Aschner, 1997; ATSDR, 1999) and cytotoxicity (Rising et al., 1995; Vitarella et al., 1996; Yoa et al., 1999), and experimental research suggests that homocysteine inhibits MT function (Barbato et al., 2007). Further, viral induction of MT gene expression was observed among HIV+ subjects during chronic viremic episodes (Raymond et al., 2010). Therefore, it is biologically plausible that viruses induce MT with consequent reductions in Hg levels in the absence of MT inhibition by higher homocysteine concentrations; however, it is unknown whether measles and rubella viral antigens induce MT, and the findings of Barbato et al. (2007) require confirmation. Mechanistic studies may shed additional insights.

Recommendations for Future Research

Given the divergent findings by sex and nutritional susceptibility cofactors, an overarching recommendation is for environmental epidemiology to identify susceptible subpopulations pertinent to the outcomes and exposures of interest, and to conduct epidemiological research stratified by these vulnerability subsets. An important consideration is that stratification reduces sample size and power to detect statistically significant findings. Yet, without stratification, even a small sample might generate chance findings, either due to random noise, or as yet unidentified

and unmeasured influences on these upstream indicators of immune response endpoints. Therefore, prospective studies with sample sizes sufficiently large to stratify by susceptibility cofactors are merited to confirm these findings, given the potential implications for public health. In accordance with recommendations of an NIH- and EPA-funded workshop on risk assessment (Woodruff et al., 2008), prospective studies should also aim to identify periods of susceptibility by evaluating environmental chemical effects on biological upstream end points, as well as disease downstream end points (Figure 2). Future research should also include coincident measures of both autoimmune and immunosuppressive indicators in order to integrate knowledge of the relationships within the immunotoxicological continuum and the potential impacts on human disease. Longitudinal studies using larger sample sizes obtained from existing cohorts such as the National Children's Study (NIH, 2012), which follows children from before birth until aged 21 years recruited from multiple locations across the U.S., as well as new multi-center U.S. cohorts that include adults beyond age 20 years, are merited to validate the findings reported in this dissertation research and to establish temporality of associations. Additional information is warranted on sources and pathways of exposure in order to mitigate human risk. Further, experimental studies are needed to enhance scientific understanding of biological mechanisms of action.

The following specific recommendations for research are organized by the previously presented categories related to this dissertation research:

(1) Systemic Autoimmune-related Response

To evaluate longitudinal associations between Hg, PCBs and ANA positivity, panel studies that analyze Hg and dioxin-like and non-dioxin-like PCBs measured at multiple points in time,

separately for males and females, are warranted, with special consideration to multicollinearity in models that adjust for the potentially confounding influences of age, menopause, and nondioxin-like PCBs. Further, serum measures of beneficial fatty acids would enhance internal validity with regard to the potentially protective effects of eicosapentaenoic acid on autoimmune response (Iwami et al., 2011), possibly through DNA-protective, anti-inflammatory effects (Grotto et al., 2011). Pertinent disease outcomes such as systemic lupus erythematosis and rheumatoid arthritis should also be assessed, as well as more specific autoimmune indicators, e.g., cellular autoantibodies to ssDNA and dsDNA, and total IgG antibodies, and inflammatory/antiviral cytokines such as IFN-□. In addition, in light of recent findings that ANA positivity was associated with greater mortality risk among children and adolescents (Chou et al., 2011), future epidemiologic studies should oversample this susceptible population in order to conduct stratified analyses. Experimental studies are needed to elucidate the mechanisms of action for dioxin-like and nondioxin-like PCBs on immunosuppressive and immunostimulatory responses. In light of associations between Hg exposure and ANA positivity in higher exposure populations, possible susceptibility factors for Hg-induced cellular autoimmunity in lower exposure populations also merit investigation, for example, Hg-susceptible genes and/or related phenotypes (Gundacker et al., 2007; Goodrich et al., 2011).

(2) Thyroid Autoimmune-related Response

To evaluate longitudinal associations between Hg and thyroid autoimmunity, these same epidemiologic studies would also merit measurement of thyroid autoantibodies and thyrotropin, as well as thyroid hormones and related disease outcomes such as autoimmune thyroiditis, hypothyroidism and hyperthyroidism in order to assess pertinent health outcomes. To address

nutritionally susceptible subpopulations, urine iodine levels would be another important measure, with sufficient sample sizes to evaluate WHO iodine status. Additional mechanistic studies have the potential to shed insights on Hg-iodine interactions.

Recent research also addresses the potential relationships between prenatal Hg exposure (Boucher et al., 2012; Ursinyova et al., 2012; Sagiv et al., 2012), PCB exposures (Parham et al., 2012; Wise et al, 2012; Leijs et al., 2012; Sagiv et al., 2010)), thyroid hormone disruption (Ursinyova et al., 2012; Parham et al., 2012; Wise et al., 2012; Leijs et al., 2012) and childhood neurodevelopmental outcomes (Boucher et al., 2012; Wise et al., 2012; Sagiv et al., 2010, 2012), and therefore, it is important that the approach recommended above also include a cohort of pregnant women with follow-up of their children to evaluate these relationships. The current research finding of an association between Hg exposure and a risk factor for hypothyroidism among females, taken together with observations of neuropathological similarities between hypothyroidism and methylmercury toxicity and the implications for neurodevelopmental disorders (Soldin et al., 2008), lends support for maternal-child cohort studies to evaluate the effects of upstream Hg exposures to downstream thyroid and neurological disorders. The links between methylmercury exposure in utero and neurotoxicity (NRC, 2000) and between maternal hypothyroidism and adverse effects on infant neurodevelopment (Haddow et al., 1999; Pop et al., 2003) are widely recognized. Further, rat studies found that perinatal thimerosal exposure resulted in impaired motor learning and a significant decrease in cerebellar type 2 deiodinase (D2) necessary for thyroid hormone production in the brain (Sulkowski et al., 2012), as well as increased cerebellar expression of a gene negatively regulated by thyroid hormone in thimerosal-treated male rat pups (Khan et al., et al., 2012). Yet, studies that investigate Hg's upstream

effects on indicators of maternal thyroid function prospectively with downstream effects of infant neurodevelopment are lacking. The Generation R Study, a population-based cohort study conducted in Rotterdam, The Netherlands, sampled maternal blood at less than 18 weeks gestation, and reported 77% greater odds for attention deficit/hyperactivity problems in children at 3 years of age associated with TPOAb positivity (Ghassabian et al., 2012). Incorporation of assessments of the influences of environmental exposures into existing studies such as the Generation R study may shed important insights, particularly for attention deficit/hyperactivity disorder (ADHD), the most prevalent developmental disorder in the U.S. (Boyle et al., 2011), yet of unknown etiology. Of note, Boucher et al. (2012) and Sagiv et al. (2010) did not evaluate thyroid outcomes, but reported positive associations between prenatal methylmercury exposure (Boucher et al., 2010; Sagiv et al., 2012) and PCB exposure (Sagiv et al., 2010) and ADHD-related behavior. Further, Dorea et al. (2012) reported an inverse association between neurodevelopment at 6 months of age and postnatal ethylmercury exposure from vaccines, but not prenatal methylmercury exposure from fish consumption. Therefore, prospective cohort studies that assess both pre- and postnatal exposures and sources are important to inform scientific knowledge about susceptible windows of development.

(3) Viral immune-related Response

Along these lines of research, expansion of existing cohort studies such as the National Children's Study (NIH, 2012), as well as case-control studies of immune response to vaccination (Autism Speaks, 2012) are also merited to evaluate the relationships between Hg and other contaminant exposure and immunological responses to MMR vaccination in infants, e.g., serum measles and rubella IgG antibodies, IFN-□ , the principal cytokine produced after MMR

immunization in infants (Pabst et al., 1997; Ovsyannikova et al., 2003), as well as downstream neurological disorders and related immune response indicators. For example, higher levels of serum IgG4 antibodies were reported by Croonenbergs et al. (2002) and Enstrom et al. (2009) in children with autism compared to typically developing controls. Elevated IgG4 antibodies are an immune regulatory response to chronic exposure to an infectious agent (Enstrom et al., 2009) and may indicate an underlying autoimmune disorder or immunosuppression with consequent chronic viral infections (Croonenbergs et al., 2002). Additional autoimmune indicators observed in children with autism include brain-specific autoantibodies (Cabanlit et al., 2007; Wills et al., 2009; Goines et al., 2011). Children with autism also show altered T-cell responses indicative of immune dysregulation (Ashwood et al., 2011). Indicators of epigenetic-related heterogeneity in immunological response may also be important (Poland et al., 2007), particularly in light of findings that blood Hg levels were correlated with the expression of genes representing antigen presentation and amino acid metabolism in boys with autism but not in typically-developing boys (Stamova et al, 2011). Therefore, in the proposed studies, it would be informative to include these measures, as well as the outcome of autism, another developmental disability of unknown etiology, yet characterized by the most rapidly increasing prevalence in the U.S. (Boyle et al., 2011). It is important to note that a Cochrane Database Systematic Review concluded that exposure to the MMR vaccine was unlikely to be associated with autism, yet also described the design and reporting of safety outcomes as inadequate (Demicelli et al., 2012), and addressed neither co-exposures to immunotoxicants nor susceptible subpopulations. Children with lower folate and vitamin B-12 levels, and higher levels of homocysteine driven by these nutritional deficiencies (Schroecksnadel et al., 2004) may represent a subpopulation susceptible to disruptions in the methylation cycle/transsulfuration pathway, and thus, share a pathophysiology

relevant to autism. Specifically, researchers reported metabolic and genetic factors pertinent to alterations in the methylation cycle/transsulfuration pathway in children with autism (James et al., 2008); epigenetic alterations, i.e., DNA hypomethylation, were also observed in autistic children, but not in unaffected siblings or controls (Melnyk et al., 2012); and clinical trial findings demonstrated that treatment with folate and methylcobalamin (vitamin B-12) led to improved levels of metabolites of the methylation cycle/transsulfuration pathway (James et al., 2009). Findings of metabolic profiles indicative of reduced methylation capacity and DNA hypomethylation pertinent to folate metabolism in mothers of children with autism, but not in controls (James et al., 2010), suggest the potential importance of maternal-child epigenetic modifications, as well. Therefore, conducting the proposed research with stratification by the susceptibility cofactors identified in this dissertation research would be consistent with the following short-term objective of the U.S. Department of Health and Human Services, as stated in the 2011 Interagency Autism Coordinating Committee Strategic Plan for Autism Spectrum Disorder Research (US DHHS, 2011): "Support at least two studies to determine if there are subpopulations that are more susceptible to environmental exposures (e.g., immune challenges related to infections, vaccinations, or underlying autoimmune problems) by 2012." A pertinent line of scientific inquiry might be whether environmentally-related immunodeficiencies warrant contraindication of live vaccines in vulnerable children, with potential expansion of currently accepted categories of immune deficiency contraindications, e.g., "T-lymphocyte (cell-mediated and humoral)" (CDC, 2012g). A vulnerable group identified by an NIH- and EPA-funded toxicology workshop are newborns due to the immaturity of their immune systems (Woodruff et al., 2008); therefore, in consideration of ethyl mercury's inhibition of antiviral interferon (Gardner et al., 2010a), the Cochrane Collaboration's conclusion that MMR safety studies are

inadequate (Demicelli et al., 2012), and recent findings of an inverse association between infant neurodevelopment and postnatal ethylmercury exposure from vaccines (Dorea et al., 2012), vaccine safety studies of infants exposed to both live vaccines and ethylmercury-containing vaccines in countries still utilizing the latter are warranted. In addition, assessment of potential effect modification by DNA hypomethylation, for example, postnatally, as measured in children with autism by Melnyk et al. (2012), and prenatally, as measured in the mothers of autistic children by James et al. (2010), would be coherent with the National Institute of Environmental Health Sciences expert panel's determination that changes in DNA methylation are an important epigenetic effect that may contribute to the induction of autoimmunity (Selmi et al., 2012). Sex is another important potential effect modifier with regard to both the 4.68 times greater prevalence of autism in boys than girls (CDC, 2012h) and the continuum of developmental immunotoxicology, characterized by immunotoxicant-induced susceptibility to infections with subsequent autoimmunity (Dietert and Holsapple, 2007). Finally, emerging scientific knowledge of the bi-directional interaction between the brain and the immune system (Elenkov et al., 2000; Kerschensteiner et al., 2009; Buch, 2011) and the link between neuropsychiatric illness and autoimmunity (Kayser and Dalmav, 2011) highlight the need to evaluate the relationships between environmental exposures, biological upstream immune endpoints, and disease downstream endpoints that integrate physical with behavioral outcomes by including both immune and neurodevelopmental and neuropsychiatric disorders (Figure 2).

Concluding Statement

I present novel findings of positive associations between (A) dioxin-like PCBs and ANA positivity in females; (B) Hg exposure and a risk factor for hypothyroidism in women with lower

or deficient iodine levels; and (C) Hg exposure and elevated serum measles IgG antibodies in boys with lower folate and vitamin B-12 and higher homocysteine levels, as well as (D) elevated rubella IgG antibodies in boys and girls with this same nutritional deficiency. Taken together, these findings point to the possibility of associations between relatively low levels of environmental contaminants and immune response, and highlight the need for prospective studies that take an integrative approach to evaluate these relationships across the immunotoxicological continuum. Further research should also aim to enhance scientific understanding of the links between environmentally-induced early biological disruptions and downstream overt disease in order to address the physical and behavioral domains of health throughout the life course. This recommended research may help to guide early population risk assessment and public health decision-making for the protection of the most vulnerable, particularly during periods of susceptibility.

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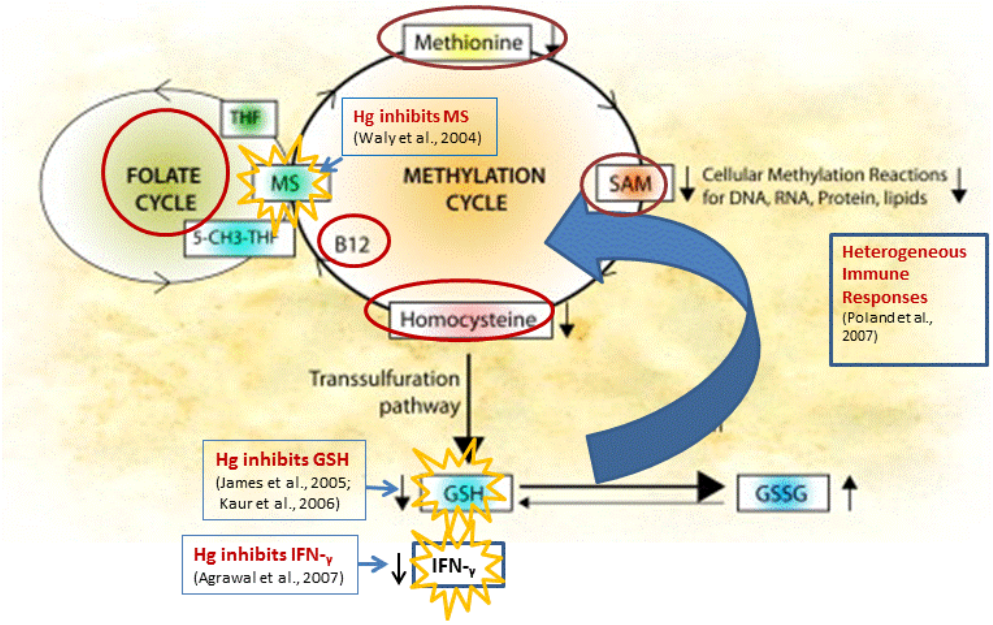
Figure 1

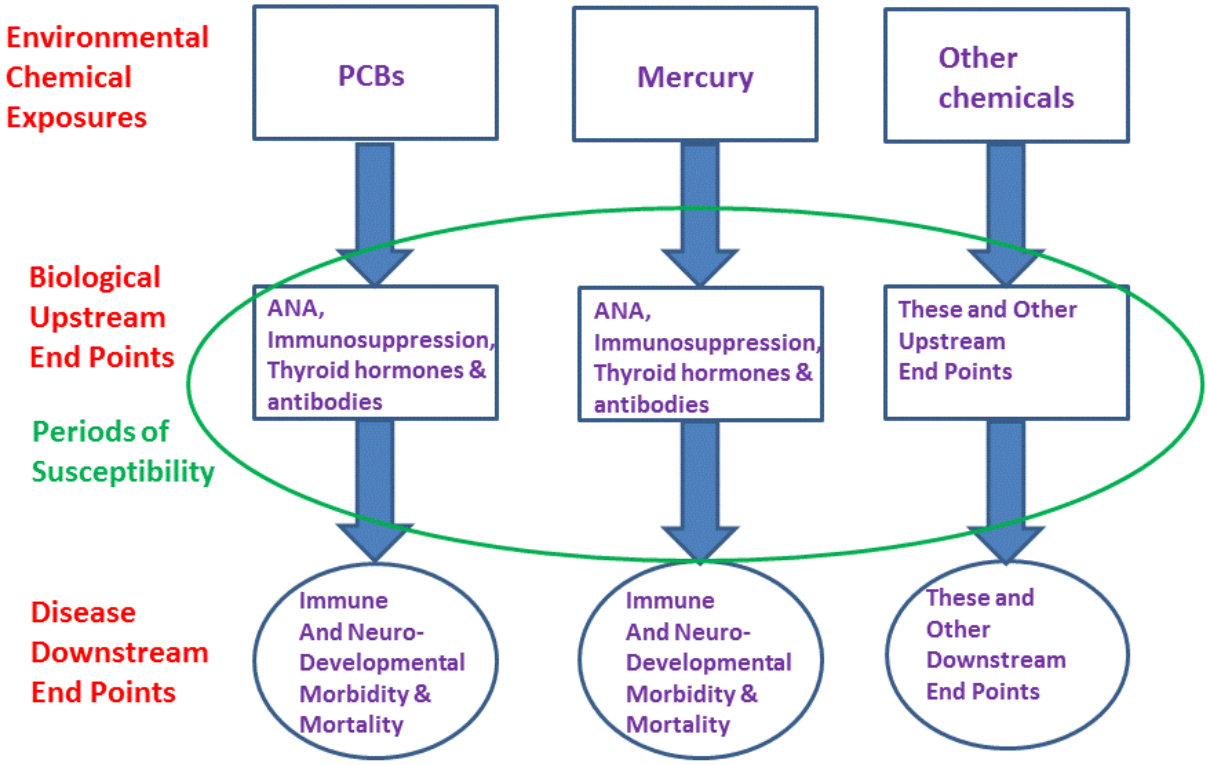
How might mercury interact with nutritional status to influence immune response?



Adapted with author's permission from: Ji H, Khurana Hershey GK. Genetic and epigenetic influence on the response to environmental particulate matter. J Allergy Clin Immunol 2012; 129: 33-41

Figure 1. How might mercury interact with nutritional status to influence immune response? The combined methylation cycle/transsulfuration pathway involving homocysteine, folate and vitamin B-12 is emerging as a biologically plausible modifier of the association between environmental toxicants and immune-related disorders. Folate and vitamin B-12 are cofactors needed for the biotransformation of homocysteine to methionine by methionine synthase. Methionine is needed to synthesize S-Adenosyl methionine (SAM), a key methyl donor for cellular methylation reactions. Methylation regulates gene expression that may be responsible for heterogeneity in immunological responses. Homocysteine also serves as a bridge to the transsulfuration pathway for the synthesis of glutathione. Mercury might affect this biological pathway in two ways pertinent to its broad enzyme- and thiol-binding capabilities. First, the organomercurials methyl- and ethyl-mercury inhibit the function of the enzyme methionine synthase. Second, these organomercurials deplete glutathione, the most abundant source of intracellular thiols, and inhibit antiviral interferon; specifically, interferon gamma, which is required to overcome persistent measles virus-induced central nervous system infection, and is also the principle cytokine produced after MMR vaccination in infants. Methylation is a known antiviral mechanism of interferons. Glutathione depletion impairs methylation, highlighting the inter-relatedness of the methylation cycle/transsulfuration pathway. Therefore, mercury may potentially alter immune responses via the methylation cycle/transsulfuration pathway by inhibiting antiviral interferon, with consequent immunosuppression, and there is biological plausibility that folate, vitamin B-12 and homocysteine may modify the association between mercury and immune responses.

Figure 2: Model for environmental chemical and early population risk assessment



"Toxicology assays identifying early biological changes from chemical exposure are increasing our ability to evaluate links between early biological disturbances and subsequent overt downstream effects." -Moving Upstream: A Workshop on Evaluating Adverse Upstream endpoints for Improved Decision Making and Risk Assessment, 2007

I propose this framework for research and highlight a model of environmental chemical and early population risk assessment that builds upon conclusions of an NIH- and EPA-funded workshop (Woodruff et al., 2008). Prospective studies are merited to identify periods of susceptibility for increased risk of upstream endpoints that represent early biological disturbances associated with chemical exposures, as well as downstream endpoints such as infections and neurodevelopmental morbidity. A prime example of a susceptibility period would be newborns with their unique vulnerability to chemical exposures due to an immature immune system. Examples of pertinent upstream endpoints are cytokines and IgG antibodies, as well as potential effect modifiers, such as susceptibility cofactors of the methylation cycle/transsulfuration pathway (Figure 1). The incorporation of autoimmune indicators into this model would be coherent with both the National Institute of Environmental Health Sciences' determination that epigenetic effects such as DNA hypomethylation may contribute to autoimmunity (Selmi et al., 2012) and with Dietert and Holsapple's (2007) concept of the continuum of developmental immunotoxicology, characterized by immunotoxicant-induced susceptibility to infections with subsequent autoimmunity. The inclusion of both upstream immune endpoints and downstream neurodevelopmental endpoints is important in light of emerging scientific knowledge about neuro-immune cross-talk within the central nervous system and comorbid immune dysregulation, both immunosuppression and immunostimulation, in children with neurodevelopmental disorders, e.g., autism.